THE BIFIDOBACTERIA AND RELATED ORGANISMS BIOLOGY, TAXONOMY, APPLICATIONS







Edited by PAOLA MATTARELLI, BRUNO BIAVATI, WILHELM H. HOLZAPFEL and BRIAN J.B. WOOD



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PAOLA MATTARELLI BRUNO BIAVATI WILHELM H. HOLZAPFEL BRIAN J.B. WOOD





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Preface

This book is dedicated to *Bifidobacterium*, one of the bacterial genera most agreeable to humans and, as evidence increasingly suggests, most agreeable for the majority, if not all, mammals.

What prompted the editors to submit the proposal for this new book was the awareness, supported by scientific evidence, of a growing interest in the role of beneficial microorganisms and, in particular, of bifidobacteria. This book presents authoritative reviews covering different aspects of bifidobacteria and other genera classified with them.

Chapters 1, 2, and 3 introduce the reader to some fundamental aspects of taxonomy, underlining the current status of the phylum Actinobacteria, genus *Bifidobacterium*, and family Bifidobacteriaceae. Chapter 1 introduces the reader to the great differences between bifidobacteria and lactic acid bacteria. Although long assumed to be closely related, modern evidence shows that they are phylogenetically far removed and, in fact, members of completely different phyla.

In recent years, the number of bifidobacterial species has greatly increased; currently 54 species and 10 subspecies are described. Lists of *Bifidobacterium* species and subspecies are available in Chapter 2. For each one a description and updated information from the literature are presented.

Chapter 3 examines the family Bifidobacteriaceae. It highlights the characteristics of the seven new taxa belonging to the so-called scardovial genera, describing the features of genus and species. The chapter also includes a short remembrance of Professor Vittorio Scardovi, as recognition for the scientific importance of his work.

Chapters 4–11 deal with physiological and biochemical aspects, providing essential information for a better understanding of bifidobacteria. Detailed advice is given for culture media and culture conditions for the detection of bifidobacteria in different environments, their cultivation, and their storage.

Microbial chemistry is an important tool for identifying many major structural components and for understanding their functional role in the physiology of bacterial cells. Bifidobacteria are saccharolytic and derive their energy by fermentation, mainly from carbohydrates, such as lactose, the most abundant solid constituent of breast milk, or from "indigestible" oligosaccharides of plant origin. Multiple bifidobacterial species in the infant gut could be explained by specific human milk oligosaccharide consumption strategies. The number of published bifidobacterial genome sequences continues to grow, bringing a better understanding of the characteristic metabolic traits and key functions of the various species. Three chapters are devoted to the nutritional requirements of bifidobacteria, bifidogenic effect of particular substrates, milk oligosaccharides, and carbohydrate metabolism.

Gut microbiota composition is an important health marker. Bifidobacteria are considered to be beneficial microorganisms. Studies on stress responses to oxygen and bile acid, the two major environmental stresses, provide information on their effects on growth and fermentation reactions of these anaerobes.

Humans depend on externally supplied folate, and folate-producing bifidobacteria can be an important source of this vitamin for the host. A growing use of bifidobacteria for probiotics can also help alleviate the global nutritionally important health problem of folate deficiency.

Exopolysaccharides are present on the surface of many bacteria, including *Bifidobacterium*. Their role in the colonization of their natural habitats and the cross-talk among bifidobacteria and host indicate their importance.

Bifidobacteria are believed to have coevolved through beneficially influencing the health of their human host. Studies on their ecological distribution and genetic adaptation are essential to verify the hypotheses of coevolution. Genetic manipulation technologies in bifidobacteria and applications of currently available systems represent a topic with extraordinary growth potential in the near future. Administration of bifidobacteria in clinical trials for therapeutic purposes points out the variability linked to the strains used, which should stimulate the isolation of new strains with possibly new potential applications. Bifidobacteria are presently applied for therapeutic purposes in treating some pathogenic infections; at the same time, they are considered a hope for the future as an additional resource for human health.

The immunological relevance of bifidobacteria shown by the immunomodulatory ability for the host is opening a new frontier for a rational modification of gut microbiota using specific bifidobacterial strains, to modify the immune responses not only in inflammatory or autoimmune disorders, but in other pathologies, such as cancer.

A growing field is the use of bifidobacteria as health support in animal nutrition. One of the best examples currently available shows that these beneficial symbiotic bacteria (present in the honey stomach) possess antimicrobial characteristics and produce bioactive metabolites that protect honeybees against pathogens and also explain the therapeutically significant properties of honey.

Production of probiotic bifidobacteria is more than just growing biomass. Many parameters affect their growth and stability, and the expression of their desired properties. These properties should therefore be considered early in the development of new probiotic strains.

The term "probiotic" is raising a worldwide debate. A chapter with an overview of the definitions of the three terms: prebiotics, probiotics, and synbiotics, from both a scientific and a regulatory point of view, was therefore considered a necessary part of this book.

As this preface reflects, the most important aspects of bifidobacteria were taken under consideration. The editors thank the publisher, who welcomed our proposal, as well as the authoritative international team of authors who made possible the realization of this book.

The Editors

1

The Phylum Actinobacteria

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Ac.ti.no.bac.te'ri.a. Gr. n. *actis -inos* a ray, beam, N.L. n. *bacter* a rod; suff. –*ia* ending denoting phylum; N.L. pl. neut. n. *Actinobacteria* actinomycete bacteria with diverse morphologies.

1.1 INTRODUCTION

The focus of this book is the genus *Bifidobacterium* and related organisms located within the family *Bifidobacteriaceae* that encompasses the genera: *Aeriscardovia, Alloscardovia, Bifidobacterium, Bombiscardovia, Gardnerella, Pseudoscardovia, Neoscardovia, Parascardovia,* and *Scardovia* (Biavati and Mattarelli, 2012; Downes et al., 2011; García-Aljaro et al., 2012; Huys et al., 2007; Jian and Dong, 2002; Killer et al., 2010, 2013; Simpson, 2004). These taxa will be covered in subsequent chapters; this chapter introduces the phylum *Actinobacteria* where the family *Bifidobacteriaceae* is phylogenetically located (Gao and Gupta, 2012; Ventura et al., 2007; Zhang et al., 2016). The phylum is comprised mainly of Gram-positive staining organisms with a high G + C DNA content (>55 mol.%) and constitutes one of the largest phyla within the domain *Bacteria* (Embley et al., 1994). A comprehensive review of all taxa within *Actinobacteria* is beyond the scope of this chapter and the reader is encouraged to review the primary literature where cited.

1.2 HISTORICAL BACKGROUND

Although the focus of this book is the genus *Bifidobacterium* and close relatives contained within the family *Bi*fidobacteriaceae, it would be remiss not to first discuss Lactobacillus and its history with Bifidobacterium. Both genera are routinely recovered from gastrointestinal (GI) and genital tracts of humans and animals, feces, and sewage; but it is their abundance and possible health-promoting effects in the human GI tract where their associations have been most studied (Bondarenko, 2006; Felis and Dellaglio, 2007; Kailasapathy and Chin, 2000; Paliy et al., 2009; Reuter, 2001; Turroni et al., 2008). Superficially, both genera physiologically resemble each other being saccharoclasitic and produce lactate and acetate as major end products of fermentation and are generally regarded as lactic acid bacteria (LAB) (Felis and Dellaglio, 2007; Holzapfel and Wood, 2014; Reuter, 2001). Therefore, it is not surprising that the members of these genera have been confused. However, several important traits can be used to differentiate between these taxa, for example, (1) the DNA mol.% G + C of bifidobacteria is much higher than lactobacilli (>60% compared to 33%–39%), (2) the hexose bifidum shunt and the presence of fructose-6-phosphate phosphoketolase (F6PPK), a key enzyme unique to the genus Bifidobacterium (Biavati and Mattarelli, 2006; Scardovi and Trovatelli, 1965). But it is worth noting that in the seventh edition of Bergey's Manual of Determinative Bacteriology (Breed et al., 1957) bifidobacteria were included in the genus Lactobacillus (Breed et al., 1957). It was not until the eighth edition of Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974), that bifidobacteria were classified as a separate genus Bifidobacterium (Biavati and Mattarelli, 2012; Sgorbati et al., 1995).

Furthermore, it was not until the application of molecular tools and in particular 16S rRNA gene sequencing that the true relationships were revealed and demonstrated the genera *Lactobacillus* and *Bifidobacterium* were phylogenetically far removed (Fox et al., 1980; Woese, 1987). Lactobacilli were shown to be related to low mol.% G + C Grampositive bacteria, such as clostridia and relatives while bifidobacteria were shown to be distantly related to this group being related to high mol.% G + C Actinomyces.



FIGURE 1.1 Phylogenetic classification system for *Actinobacteria* as proposed by Stackebrandt et al. (1997a,b). (A) Assignment of the ranks subclass, order, and family; (B) assignment of suborders and families of the order Actinomycetales.

This revelation resulted in a series of pivotal taxonomic proposals. First, Stackebrandt et al. (1997a) proposed a novel phylogenetic hierarchical system for "*Actinomyces*" solely based on 16S rDNA/rRNA sequences (Fig. 1.1A–B). The proposal included several taxonomic ranks between genus and the class *Actinobacteria*. In addition to the clustering of sequences, the delineation of taxa was accompanied with taxon-specific 16S rDNA/RNA signature nucleotides (Stackebrandt et al., 1997a). This provided a solid foundation for the taxonomy of this group and over the next decade many novel members were assigned to this class, leading Zhi et al. (2009) to update the signature nucleotides. In addition to these nucleotide signatures, several taxonomic ranks were also proposed with suborders



FIGURE 1.2 Phylogenetic classification system of Actinobacteria adopted by Bergey's Manual of Systematic Bacteriology (Garrity and Holt, 2001; Ludwig and Klenk, 2005; Ludwig et al., 2012).

Actinopolysporineae and Kineosporiineae being described that in turn encompassed novel families accommodating existing genera. In the same year Sorokin et al. (2009) also proposed the order Nitriliruptorales and the family Nitrilruptoraceae.

Second, in Volume 1 (The *Archaea* and the Deeply Branching and Phototrophic *Bacteria*) of the second edition of *Bergey's Manual of Systematic Bacteriology*, based on principle component analysis (PCA), Garrity and Holt (2001) demonstrated the phylogenetic depth of members of the class *Actinobacteria* was equivalent to that of existing phyla and that this group was clearly separate from the phylum *Firmicutes*. They therefore proposed elevating the class *Actinobacteria* to the rank of phylum thus confirming the separateness of the LAB (i.e., *Lactobacillus* and relatives) located in the phylum *Firmicutes* from the *Bifidobacteriaceae* (Garrity and Holt, 2001). Subsequently, phylogenomic studies using whole-genome-sequencing (WGS) and protein molecular signatures have made significant contributions to our understanding of the interrelationships found within the *Actinobacteria* and the *Bifidobacteriales*. The reader is encouraged to review the papers of Ventura et al. (2007), Gao and Gupta (2012), and Zhang et al. (2016).

The number and variety of identified species established the phylum Actinobacteria as one of the largest taxonomic groups of the domain Bacteria (Embley et al., 1994). Subsequently, the road map proposed by Garrity and Holt (2001) and amended by Ludwig and Klenk (2005) in the 2005 edition of Bergey's Manual of Systematic Bacteriology cumulated in the publication of a two-volume edition (The Actinobacteria, Parts A and B) of the manual (Garrity and Holt, 2001; Ludwig and Klenk, 2005; Ludwig et al., 2012). The classification system outlined in the second edition of *Bergey's Manual of Systematic Bacteriology* essentially simplified the hierarchical structure of Stackebrandt et al. (1997a,b); Subclass was elevated to the rank of Class and the rank of Suborder was eliminated (Fig. 1.2). However, the reader should be aware that the List of Prokaryotes Names with Standing in Nomenclature (LPSN) (http://www. bacterio.net/-classifphyla.html; http://www.bacterio.net/actinobacteria.html) still cites the taxonomic structure based on that of Stackebrandt et al. (1997a,b) and Zhi et al. (2009) (Gao and Gupta, 2012; Stackebrandt et al., 1997a; Zhi et al., 2009). As with any taxonomy, taxa continues to be added to the phylum Actinobacteria and readers should consult future editions of the Bergey's Manual of Systematics of Archaea and Bacteria (http://onlinelibrary.wiley.com/ book/10.1002/9781118960608), the International Journal of Systematic and Evolutionary Microbiology (http://ijs.microbiologyresearch.org/content/journal/ijsem/) and the LPSN (http://www.bacterio.net/) as organisms are described and names are validated. It is pertinent to note that the term *Actinobacteria* refers to all members of the phylum, whereas actinomyces should be reserved for strains belonging to the order *Actinomycetales*. The diversity of the *Acti*nobacteria based on 16S rRNA gene sequences is shown in Fig. 1.3.

4

1. THE PHYLUM ACTINOBACTERIA





1.2.1 Application of Genomics and Molecular Markers

Phenotypic characteristics that include morphology, physiology, and chemotaxonomy are useful for the assignment of organisms to species and genera; but the sheer diversity of taxa within the phylum Actinobacteria ensures that the level of congruence between phenotypic features is low (Embley et al., 1994; Stackebrandt and Schumann, 2006). The application of molecular methods allowed more precise insights into the taxonomy of this group of organisms and as stated earlier, led to the classification of the phylum Actinobacteria based on the 16S rRNA gene (Stackebrandt et al., 1997a). However, the diversity of this group of organisms makes it difficult to reliably determine the interrelationships or branching order of the higher taxonomic clades of this phylum (Stackebrandt et al., 1997b). The ranks above genus are distinguished by taxon-specific 16S rRNA signature nucleotides (Stackebrandt et al., 1997a), but with the frequent addition of novel taxa to the phylum these signature nucleotides require to be periodically revised and updated (Zhi et al., 2009). The resolution of 16S rRNA gene for the discrimination of novel taxa can be insufficient to separate very closely related organisms; gene sequences corresponding to *atpD*, *gyrB*, *recA*, *rpoB*, and sod have been extensively used as alternative chronometers (de Vos, 2011). Although molecular phylogenies based on single genes can lead to discrepancies, this can be reduced with the application of multiple housekeeping genes using multilocus sequence analysis (de Vos, 2011; Rokas et al., 2003). Indeed, the application of concatenated gene fragments significantly improved the phylogeny of members of the genera *Bifidobacterium*, *Kribbella*, and *Mycobacte*rium (Adékambi et al., 2011; Curtis and Meyers, 2012; Devulder et al., 2005; Ventura et al., 2006). Recently, Sen et al. (2014) used 100 completely sequenced genomes representing 35 families and 17 orders of the class Actinobacteria in a comprehensive study that employed a concatenate of 54 conserved proteins present in single copy in all these genomes. This study included phylogenetic trees based on 16S and 23S rRNA gene sequences or their concatenation, and a tree based on the concatenation of MLSA genes (encoding AtpI, GyrA, FtsZ, SecA, and DnaK). The outcome was several proposals; the order "Frankiales," which had an effectively but not validly published name, is split into several new orders, namely Frankiales, Geodermatophilales, Acidothermales, and Nakamurellales. In addition, the study strongly suggested that the order *Micrococcales* should be split into *Micrococcales*, *Cellulomonales*, and *Brachybacteriales*, but the authors did not formally propose these changes, citing the need to include additional genomes for a more robust analysis of this order (Sen et al., 2014).

The more recent application of whole genome sequencing (WGS) now adds yet another layer of discriminatory power and has been applied to members of the phylum Actinobacteria. Mycobacterium tuberculosis was the first actinobacterial genome to be sequenced in 1998, since then a multitude of genome sequences are now available. The first major investigation using advanced genomic methods was undertaken by Ventura et al. (2007) using the 20 actinobacterial genomes available at that time. An early observation from this study was that most genomes determined were circular, however, the genomes of Streptomyces and Actinobacterial taxa, such as Actinomyces, Amycolatopsis, Actinoplanes, Streptoverticillium, and Micromonospora were found to be linear. The study of Ventura et al. (2007) also demonstrated that the separation of the Actinobacteria from other bacteria is very ancient with the deepest branch separating bifidobacteria from all other families within the phylum Actinobacteria. Following this study, the number of actinobacterial genomes sequences has progressed at a tremendous rate. Comparative analyses of genome sequences have led to numerous molecular markers, which are providing powerful means for understanding microbial phylogeny and systematics (Gupta, 2014). Signature sequences in proteins are defined as regions in the alignments where a specific change is observed in the primary structure of a protein in all members of one or more taxa but not in other taxa. The changes in the genes/proteins sequences can be either the presence of particular amino acid substitutions or specific deletions or insertions defined as conserved signature indels (CSIs). The determination and use of CSIs have been pioneered by Gupta and coworkers (Ajawatanawong and Baldauf, 2013; Gao and Gupta, 2011; Gupta, 2014, 1998). Numerous CSIs have been identified for members of many different bacterial taxa and are able to resolve deeper-branching evolutionary relationships that those based on single genes or proteins (Brown et al., 2001; Gupta, 2014; Rokas et al., 2003). Different taxonomic ranks can now be clearly delineated in clear molecular terms based on multiple uniquely shared characteristics (synapomorphies). Inferences based on these CSIs are in excellent agreement with those based on phylogenetic approaches. A number of studies using WGS and concatenated protein sequences have been published, leading to number of phylogenetic trees, albeit based on a limited number of actinobacterial genomes (Adékambi et al., 2011; Alam et al., 2010). Gupta and coworkers have published a number of studies into Actinobacteria clade-specific CSIs (Gao, 2005; Gao et al., 2006). These studies culminated in the most comprehensive study to date that identified molecular signatures that are unique to most Actinobacteria, in addition, signatures to each order within the phylum were described (Gao and Gupta, 2012). It is beyond the scope of this chapter to comprehensively review all the findings of Gao and Gupta. Briefly, the phylogenetic tree based on concatenated sequences of 35 conserved proteins from 98 actinobacterial genomes is largely consistent with that generated

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from 16S rRNA genes sequences shown in Fig. 1.3. The majority of genera were found to cluster in the same clades but differences are found between the two methods, which may be resolved as additional genomes are sequenced.

1.3 PHENOTYPIC AND PHYSIOLOGICAL CHARACTERISTICS

The phylum *Actinobacteria* now represents one of the largest taxonomic units within the domain bacteria (Embley et al., 1994; Goodfellow, 2012b). Previous classifications of actinomycetes, based on morphology and physiology, did not reflect the natural phylogenetic relationships and so did not adequately define and differentiate between the different ranks within *Actinobacteria*. (Stackebrandt and Schumann, 2006). However, a consequence of circumscribing *Actinobacteria* purely on phylogenetic criteria is that the phylum exhibits an enormous range of morphologies (coccoid, rod-coccoid, hypal, and branched forms), physiological and metabolic capabilities (spore or nonspore-forming, production of extracellular enzymes, metabolic products, antibiotics) and chemotaxonomic features (fatty acids, menaquinones, peptidoglycan types) (Embley et al., 1994; Goodfellow, 2012a; Stach and Bull, 2005; Stackebrandt and Schumann, 2006; Ventura et al., 2007).

1.4 ECOLOGY

As one would expect from a large phylum, representatives of *Actinobacteria* are recovered from a wide range of sources including aquatic (freshwater and marine) and terrestrial environments; soil inhabitants, including nitrogenfixing symbionts and plant-associated commensals. Habitats also include more extreme locations, such as deep-sea sediments and hyperarid desert soils (Goodfellow, 2012a; Goodfellow and Fiedler, 2010; Stach and Bull, 2005; Stackebrandt and Schumann, 2006). *Actinobacteria* have also been found in the human body (from skin to mucosal surfaces) and are important members of a normal microbiota; indeed these organisms are significant members of the human GI tract and along with *Firmicutes, Bacteroidetes,* and *Proteobacteria* (Turnbaugh et al., 2007). In particular, *Corynebacterium, Propionibacterium, Rothia, Actinomyces,* and *Bifidobacterium* are the most important genera of *Actinobacteria* that are found in healthy individuals (Wu, 2013).

Well documented (but beyond the scope of this chapter) is the use of bifidobacteria in so-called functional foods with health-promoting or probiotic activities (Picard et al., 2005; Ventura et al., 2009, 2004). However, members of the phylum also include a number of prominent pathogens belonging to *Mycobacterium*, *Nocardia*, *Corynebacterium*, *Tropheryma*, and *Propionibacterium* (Berman, 2012).

1.5 NATURAL AND BIOACTIVE COMPOUNDS

Actinobacteria produce a huge range of extracellular enzymes and secondary metabolites of which antibiotics are a large and important group. Notably Actinomycetales and, in particular Streptomyces, have (and continue) to be the prime source of useful therapeutic agents. Unprecedented levels of antibiotic resistance in pathogens and the need to identify novel strategies to combat microbial infections have resulted in a renewed interest into the search for "natural or bioactive" compounds (Berman, 2012). In addition to microbial infection diseases, the "mining" or "bioprospecting" for active agents is also directed toward other life-threatening pathologies, such as cancer (Bull et al., 2005; Goodfellow and Fiedler, 2010; Talbot et al., 2006). In combination with classical culture-based strategies, the application of high-throughput methods for screening of active compounds now includes the mining of genomes for gene sequences corresponding to metabolic pathways and novel metabolites, this approach is now receiving much attention (Bull et al., 2005; Goodfellow and Fiedler, 2010; Talbot et al., 2010).

1.6 CONCLUDING REMARKS

Actinobacteria is one of the largest bacterial phyla with members recovered from a very wide range of aquatic and terrestrial habitats and the organisms display an equally diverse range of morphological, physiological, and chemotaxonomic properties. A significant observation using 16S rRNA gene sequence analysis was that *Lactobacillus* and *Bifidobacterium*, long assumed to be closely related, were phylogenetically far removed and in fact members of completely different phyla! Another important event was the application of nucleotide sequence and phylogenetic methods that resulted in a classification system of *Actinobacteria* provided by Stackebrandt and coworkers. This scheme was subsequently adopted and updated in the latest edition of *Bergey's Manual of Systematic Bacteriology*, largely regarded as the premier resource for microbial systematics, now commanding an impressive two volumes dedicated to *Actinobacteria*.

In addition to the wide range of environmental sources, of importance is the abundance of *Actinobacteria* in the human microbiome and their influence on health and disease processes that is driving a renewed search for novel bioactive compounds to combat major health issues. These factors strongly suggest that interest in *Actinobacteria* will continue with the identification of novel taxa and therapeutic compounds.

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2

Species in the Genus Bifidobacterium

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2.1 INTRODUCTION

Bifidobacteria were first described at the beginning of 1900 by the French paediatrician Tissier (Tissier, 1900), who observed a low number of bacteria characterized by a peculiar Y-shaped morphology in stools of infants with gastrointestinal disturbances when compared with those from healthy infants. He suggested that these bacteria could be administered to patients with diarrhea to help to restore a healthy gut microbiota. At the same time Metchnikoff, a Russian zoologist, pioneer researcher on immunology, correlated the potential life-lengthening properties of lactic acid bacteria with the longevity of Bulgarian peasants consuming large amounts of yogurt (Metchnikoff, 1908). This intuition has been the basis of the current concept that bifidobacteria are often associated with health-promoting activities, either as an endogenous member of the gut microbiota (immunomodulation, antagonistic activity toward pathogens, etc.) or as allochthonous probiotics species (restoring healthy gut microbiota). The intestinal microbiota studies started to reveal the great influence of bifidobacteria, which are considered helpful not only in the gastrointestinal apparatus but also in other systems, such as nervous [e.g., depression (Savignac et al., 2015)] and bone [e.g., arthritis reumatoides (Zamani et al., 2016)] systems. The current extensive genomic analyses will allow a deeper understanding of bifidobacterial diversity and will reveal host-bifidobacterial interactions in a more precise manner that could help in maintaining human and animal health. At the basis of all these studies, there is the knowledge of bifidobacterial species features and occurrence and the discovery of new species, obtaining new isolates that could be investigated for beneficial properties.

2.2 HISTORICAL BACKGROUND

The first *Bifidobacterium* strain, isolated by Tissier (1900) from the feces of a breast-fed infant, was named *Bacillus bifidus communis*. In the seventh edition of *Bergey's Manual of Determinative Bacteriology* (Breed et al., 1957), only *Lactobacillus bifidus* was reported, although in 1924 Orla-Jensen had already recognized the existence of genus *Bifidobacterium* as a separate taxon: "The different species of *Bacterium bifidum* doubtless constitute a separate genus, possibly forming a connecting link between lactic acid bacteria and propionic bacteria" (Orla-Jensen, 1924). In the United States, Gyorgy et al. (1954) described *L. bifidus* var. *pennsylvanicus*, a human milk-requiring variant of *L. bifidus*. Studies on bifidobacteria greatly increased in 1960 with the studies of Reuter in Germany (Reuter, 1963), Mitsuoka in Japan (Mitsuoka, 1969), and Scardovi in Italy (Scardovi et al., 1970, 1971). Reuter first arranged the previously described "biotypes" or "groups" in species, describing eight species in human feces: *B. bifidum* and *B. longum* in both adult and infant feces, *B. adolescentis* only in adults, and *B. parvulorum*, *B. liberorum*, *B. lactensis*, and *B. breve* only in infants.

Mitsuoka (1969) confirmed the work of Reuter (1963): he described *B. bifidum* biotype *a* and *B. longum* biotype *a* as present in adults, while in infants *B. bifidum* biotype *b*, *B. longum* biotype *b*, *B. adolescentis*, but *not B. paroulorum* and *B. lactensis*, were found. Mitsuoka's greatest contribution was the description of bifidobacterial species in animals: *B. thermophilum* (biotypes *a*, *b*, *c*, and *d* from hog and chicken), *B. pseudolongum* (biotypes *a*, *b*, *c*, and *d* from hog, chicken, cattle, calf, sheep, rat, mouse, and guinea pig), *B. longum* var. *animalis* (biotypes *a* and *b* from calf, sheep, mouse, and rat) (Mitsuoka, 1969). Scardovi and Trovatelli (1969) described *B. asteroides*, *B. coryneforme*, and *B. indicum* from honeybee and *B. ruminale* and *B. globosum* from bovine rumen. Researchers, in that period, utilized mostly phenotypical features (colony and cell morphology and fermentative patterns) for taxonomic purposes. Additional included criteria were: (1)

the measurement of DNA content of GC% introduced by Sebald et al. (1965) and (2) the presence of fructose-6-phosphate phosphoketolase (F6PPK) which splits the hexose phosphate into erythrose-4-phosphate and acetyl phosphate, introduced by Scardovi and Trovatelli (1965) and de Vries and Stouthamer (1967) as taxonomic markers for bifidobacteria. These additional criteria were essential to identify new species with features not classical for bifidobacteria, for example, unusual not-bifid morphology, such as in *B. coryneforme* or *B. asteroides* or the inability to ferment lactose, the presence of aldolase, and so on. As the bifidobacterial isolation from different sources increased in different countries, it became necessary to have unambiguous methods of identification both for known species and for new isolates. The DNA–DNA hybridization (DDH) method introduced by Scardovi et al. (1970) for bifidobacteria identification offered a direct measure of similarity of DNA. DDH provided a new dimension and rigorous systematic arrangement in bifidobacterial taxonomy. This method, recognized as important till date, has the advantage that it considers genetic relatedness of the entire chromosome. Uniqueness of several bifidobacterial species was confirmed and the existence of some other species was not justified: based on DDH, B. bifidum showed no similarity with any other species except with B. bifidum var. pennsylvanicus. Due to high genotypic similarity between B. liberorum, B. lactensis, and B. infantis, these species have been merged into one, B. infantis. A high similarity has been also found between B. parvulorum and B. breve, for which *B. breve* was maintained. In animal group, *B. longum* var. *animalis* type *b* appeared to be identical to *B. longum*, and B. longum var. animalis biotype a was found to be B. animalis. B. thermophilum and B. ruminale was the same species for which the *B. thermophilum* name has been retained. Poupard et al. (1973) reclassified bifidobacteria as a separate taxon and described the genus Bifidobacterium, consisting of 11 species, as it appears in the eighth edition of Bergey's Manual of Systematic Bacteriology (Buchanan and Gibbons, 1974); this genus was included in the family Actinomycetaceae of the order Actinomycetales. The description of 24 species has been recognized by the authoritative Bergey's Manual of Systematic Bacteriology (first edition) in 1986 (Scardovi, 1986). The Bifidobacterium species were described from different sources including dental caries, vaginal fluid and sewage, together with new species from infant and adult human feces and from chicken, rabbit, and bovine rumen. With the introduction of 16S rRNA/DNA sequence analysis, that, better than any other taxonomic method, places an organism in the framework of phylogenetic relationships, Stackebrandt et al. (1997) proposed a new hierarchical structure for the Actinobacteria phylum with six phylogenetically distinct lineages described as orders (Actinomycetales, Bifidobacteriales, Acidimicrobiales, Coriobacteriales, Sphaerobacteriales, and Rubrobacteriales): Bifidobacteriales order has been described with the type family Bifidobactenaceae and with the type genus Bifidobacterium. The description of 45 species has been recognized by Bergey's Manual of Systematic Bacteriology (second edition) in 2012 (Biavati and Mattarelli, 2012), and the number of species has constantly increased up to now (August 2016); currently 54 species and 10 subspecies are described with a total of 60 taxa (Table 2.1).

2.3 BRIEF GUIDELINE FOR NEW BIFIDOBACTERIAL SPECIES DESCRIPTION

The set of guidelines for the statement of a new species is clearly described in the minimal standard for description of new bifidobacterial species recently published by the "Subcommittee of Bifidobacterium, Lactobacillus, and related organisms" (Mattarelli et al., 2014). The approach to address a new isolate supposed to belong to bifidobacteria starts by the observation of bifidobacterial morphology; the other steps could be classical, evaluating the presence of F6PPK, or molecular, both utilizing a primer specific for bifidobacteria [e.g., xylulose-5-phosphate/fructose-6-phosphate phosphoketolase bifidobacterial gene (*xfp*) (Cleusix et al., 2010)] or analyzing the 16S rRNA gene sequence similarity. This last one can then provide the first insights into the organism's phylogenetic relationships to differentiate the new isolate from other bifidobacterial species. In the case of presence of value below 97% with other bifidobacteria, the isolate can possibly be a new species even if other genotypic analyses have to be performed (e.g., analysis of other housekeeping genes) (Mattarelli et al., 2014). When the value for 16S rRNA gene sequence similarity is above 97% (over full pairwise comparisons), DNA-DNA hybridizations or other techniques, such as housekeeping gene analysis are applied to individuate the most closely related species to establish whether separate species or genera are present. In both cases, the establishment of novel species or new genera (irrespective of the degree of sequence similarity) should be clearly and unambiguously documented (Tindall et al., 2010). Properties whose determination is compulsory for the description of new species include (1) phenotypic criteria (including information about ecological characteristics) and (2) genotypic criteria. The phenotypic description typically comprises parameters, such as cell shape, colony morphology, pH and temperature optima, biochemical and fermentative properties (API 50 CHL, RAPID ID32). It takes into account that the consideration of these characters has to be extended to chemotaxonomic characters, such as the structure of the peptidoglycan, to reflect the true scope of phenotypic characterization of Gram-positive microorganisms (Tindall et al., 2010). The genotypic description has to describe the 16S rRNA gene sequence of at least 1500 bp and construction of its phylogenetic tree. The need to use several different algorithms for constructing phylogenetic dendrograms and examining the reliability of branch points has been outlined. In addition, hsp60 and at least one housekeeping gene, chosen from among *clpC*, *rpoB*, *rpoC*, *dnaJ*, and dnaG, have to be described. In case of high similarity of 16S rRNA with other bifidobacterial species but in the presence of

TABLE 2.1	Bifidobacterium	Species	Undated	at January	2017
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	Species	Original label of type strains	Isolated from	References
1	B. actinocoloniiforme	LISLUC III-P2 ^T	Digestive tract content of Bombus lucorum	Killer et al. (2011)
2	B. aquikefiri	$R-54638^{T}$	Water kefir	Laureys et al. (2016)
3	B. adolescentis	E 194a ^T	Feces of human adults; bovine rumen; sewage	Reuter (1963)
4	B. aerophilum	TRE 17^{T}	Feces of Saguinus oedipus (red cotton tamarin)	Michelini et al. (2016a,b,c)
5	B. aesculapii	MRM $3/1^{T}$	Feces from baby <i>Callithrix jacchus</i> (common marmoset)	Modesto et al. (2014)
6	B. angulatum	В 677 ^т	Sewage; feces of human adults	Scardovi and Crociani (1974)
7	B. animalis			
	subsp. animalis	R 101-8 ^T	Feces of rats and guinea pigs	Masco et al. (2004); Scardovi and Trovatelli (1974)
	subsp. <i>lactis</i>	UR 1	Feces of chickens and rabbits; fermented milk (yogurt); and sewage	Masco et al. (2004); Meile et al. (1997)
8	B. asteroides	C 51	Intestine of <i>Apis mellifera</i> subsp. <i>caucasica, ligustica,</i> and <i>mellifera</i>	Scardovi and Trovatelli (1969)
9	B. avesanii	TRE C ^T	Feces of Saguinus oedipus (red cotton tamarin)	Michelini et al. (2016a)
10	B. biavatii	$AFB23-4^{T}$	Feces of Saguinus mida (red-handed tamarin)	Endo et al. (2012)
11	B. bohemicum	JEMLUC VII-4 ^T	Digestive tract content of Bombus lucorum	Killer et al. (2011)
12	B. bifidum	Ti ^T	Feces of human adults and infants and suckling calves; human vagina	Orla-Jensen (1924)
13	B. bombi	BLUCI/TP ^T	Digestive tract of bumblebees	Killer et al. (2009)
14	B. boum	RU 917 ^T	Bovine rumen; feces of piglets	Scardovi et al. (1979a)
15	B. breve	$S 1^{T}$	Feces of infants and suckling calves	Reuter (1963)
16	B. callitrichos	$AFB22-5^{T}$	Feces of Callithrix jacchus (common marmoset)	Endo et al. (2012)
17	B. catenulatum	B 669 ^T	Feces of infants and human adults; human vagina; sewage	Scardovi and Crociani (1974)
18	B. choerinum	SU 806 ^T	Feces of piglets; sewage	Scardovi et al. (1979a)
19	B. commune	LMG 28292 ^T	Bumblebee gut	Praet et al. (2015)
20	B. coryneforme	C 215 ^T	Intestine of Apis mellifera subsp. mellifera	Biavati et al. (1982); Scardovi and Trovatelli (1969)
21	B. crudilactis	$FR62/b/3^{T}$	Raw milk and raw milk cheeses	Delcenserie et al. (2007)
22	B. cuniculi	RA 93 ^T	Feces of rabbits	Scardovi et al. (1979b)
23	B. dentium	B 764 ^T	Human dental caries and oral cavity; feces of human adults; human vagina	Scardovi and Crociani (1974)
24	B. eulemuris	LMM_E3 ^T	Feces of Eulemuris macaco (black lemur)	Michelini et al. (2016b)
25	B. faecale	CU3-7	Feces of a 2-week-old baby	Choi et al. (2014)
26	B. gallicum	$P 6^{T}$	Human feces	Lauer (1990)
27	B. gallinarum	Ch 206-5 ^T	Chicken cecum	Watabe et al. (1983)
28	B. hapali	$MRM_8.14^{T}$	Feces of Callitrix jacchus (common marmosets)	Michelini et al. (2016c)
29	B. indicum	С 410 ^т	Intestine of Apis cerana	Scardovi and Trovatelli (1969)
30	B. kashiwanohense	HM2-2	Feces of a healthy infant (1.5 years old)	Morita et al. (2011)

(Continued)

TABLE 2.1	Bifidobacterium S	pecies Up	pdated at]	January 2017	(cont.)
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	Species	Original label of type strains	Isolated from	References
31	B. lemurum	LMC 13 ^T	Feces of an adult subject of the <i>Lemur catta</i> (ring-tailed lemur)	Michelini et al. (2016b)
32	B. longum			
	subsp. longum	E 194b ^T	Feces of human adults and infants and suckling calves; human vagina; sewage	Reuter (1963); Mattarelli et al. (2008)
	subsp. infantis	S 12 ^T	Feces of infants and suckling calves; human vagina	Reuter (1963); Mattarelli et al. (2008)
	subsp. <i>suis</i>	SU 859 ^T	Feces of piglets	Matteuzzi et al. (1971); Mattarelli et al. (2008)
	subsp. <i>suillum</i>	Su 851 ^T	Feces of piglets	Mattarelli et al. (2008); Reuter (1963); Yanokura et al. (2015)
33	B. magnum	$RA3^{T}$	Feces of rabbits	Scardovi and Zani (1974)
34	B. merycicum	RU 915B ^T	Bovine rumen	Biavati and Mattarelli (1991)
35	B. minimum	F 392 ^T	Sewage, pig cecum	Biavati et al. (1982)
36	B. myosotis	$MRM_{5.9^{T}}$	Feces of Callitrix jacchus (common marmosets)	Michelini et al. (2016c)
37	B. mongoliense	YIT10443 ^T	Fermented milk (airag)	Watanabe et al. (2009)
38	B. moukalabense	GG01 ^T	Feces of a wild lowland gorilla	Tsuchida et al. (2013)
39	B. pseudocatenulatum	В 1279 ^т	Feces of infants and suckling calves; sewage	Scardovi et al. (1979b)
40	B. pseudolongum			
	subsp. pseudolongum	PNC-2-9G ^T	Feces of bulls, calves, chickens, dogs, guinea pigs, pigs, and rats	Mitsuoka (1969); Yaeshima et al. (1992a,b)
	subsp. globosum	$RU 224^{T}$	Feces of lambs, piglets, rabbits, rats, and suckling calves; bovine rumen; sewage	Biavati et al. (1982); Scardovi et al. (1969); Yaeshima et al. (1992a,b)
41	B. psychraerophilum	$T16^{T}$	Pig caecum (content and ephithelium)	Simpson et al. (2004)
42	B. pullorum	Р 145 ^т	Feces of chickens	Trovatelli et al. (1974)
43	B. ramosum	TRE M^{T}	Feces of Saguinus oedipus (cotton top tamarin)	Michelini et al. (2016a)
44	B. reuteri	$AFB22-1^{T}$	Feces of Callitrix jacchus (common marmoset)	Endo et al. (2012)
45	B. ruminantium	RU 687 ^T	Bovine rumen	Biavati and Mattarelli (1991)
46	B. saguini	$AFB23-1^{T}$	Feces of red-handed tamarin	Endo et al. (2012)
47	B. saeculare	RA 161 ^T	Feces of rabbit	Biavati et al. (1991)
48	B. scardovii	SBL0071/83 ^T	Human blood	Hoyles et al. (2002)
49	B. stellenboschense	AFB23-3 ^T	Feces of S. mida (red-handed tamarin)	Endo et al. (2012)
50	B. subtile	F 395 ^T	Sewage; human carious lesions	Biavati et al. (1982)
51	B. thermacidophilum			
	subsp. thermacidophilum	36 ^{cT}	Waste water, pig feces	Dong et al. (2000); Zhu et al. (2003)
	subsp. porcinum	$P 3-14V^T$	Piglet feces	Zhu et al. (2003)
52	B. thermophilum	P 2-91 ^T	Feces of chickens, pigs, and suckling calves; bovine rumen; sewage	Mitsuoka (1969)
53	B. tissieri	$MRM_5.18^{T}$	Feces of Callithrix jacchus (common marmosets)	Michelini et al. (2016c)
54	B. tsurumiense	OMB 115 ^T	Hamster dental plaque	Okamoto et al. (2008)

other different characteristics from closely related bifidobacterial species, DNA–DNA hybridization is necessary if MLST analysis with the concatenated tree does not clarify the exact phylogenetic position. In the near future whole-genomic features can be utilized to obtain the genotypic information about similarity between a hypothetical new taxon and the bifidobacterial species known, rather than focus on a few single molecular markers, as in the current practice.

The description of species with more than one strain is encouraged because with distinctive phenotypical features associated to each strain, more strains can guarantee the description of the variability within a species. However, it has been found that it is often a very difficult task to isolate new species of this growth-fastidious group, and also the fact that there is only one new strain corresponding to a new species that does not invalidate the identification of that species. In the bifidobacteria, 8 species out of 54 referred to one species with a single strain description. Other strains of species initially described with one strain have been added, thanks to successive studies. For example, *B. callithricos* was isolated (Endo et al., 2012) in South Africa from *Callithrix jacchus* (common marmoset) and the new species was originally described using the only strain that was then available. Subsequently we have identified other strains belonging to *B. callithricos* in material taken from *Saguinus oedipus* (red cotton tamarin) (work by the authors of this chapter and others, currently awaiting publication).

Readers interested in pursuing this matter in greater depth should refer to Mattarelli et al. (2014).

2.3.1 Deposit of Strains into Public Culture Collections: Importance and Rules (Nagoya Protocol Compliance)

A new type strain must be deposited in at least two public collections in at least two different countries to have the name (and therefore the species) validated (Lapage et al., 1992); it is also highly recommended that the primary biological materials upon which data in publications or in public databases are based are made available, and preserved as deposited, so that spurious or unusual findings can be further explored or to allow further work as new technologies arise (Stackebrandt et al., 2014).

European public collections request that strains deposited with them meet some requisites, such as the permission for strain isolation and so forth in compliance with the Nagoya protocol. Genetic resources (GRs) are any material of plant, animal, microbial, or other origin containing functional units of heredity over which states exercise sovereign rights and traditional knowledge associated with genetic resources that are accessed after the entry into force of the Nagoya Protocol in the European Union.

The Nagoya Protocol on access to genetic resources (GRs) and the fair and equitable sharing of benefits, also known as Nagoya Protocol on Access and Benefit Sharing (ABS), is a 2011 supplementary agreement to the Convention on Biological Diversity (CBD) (CBD, 1992; CBD Nagoya, 2011). The Protocol significantly implements the CBD's third objective by providing a strong basis for greater legal certainty and transparency for both providers and users of GRs. Specific obligations to support compliance with domestic legislation or regulatory requirements of the Party providing GRs and contractual obligations reflected in mutually agreed terms are a significant innovation of the Protocol. These compliance provisions, as well as provisions establishing more predictable conditions for access to genetic resources will contribute to ensuring the sharing of benefits when genetic resources leave a party providing genetic resources. In addition, the Protocol's provisions on access to traditional knowledge held by indigenous and local communities when it is associated with genetic resources will strengthen the ability of these communities to benefit from the use of their knowledge, innovations, and practices. The protocol was adopted on October 29, 2010 in the European Union (EU) and entered into force on October 12, 2014 (https://www.cbd.int/abs/); 78 parties adhere to this protocol. This regulation applies to all EU Member States, regardless of their individual ratification of the Nagoya Protocol.

Only a few countries in Europe have access laws, which comprise national laws on Prior Informed Consent (PIC) and Mutually Agreed Terms (MAT), national contract law, and international private law. Regarding PIC, it is not clear if there is a different requirement for commercial and noncommercial activities. It seems that PIC is mandatory for both even if in a simplified form for noncommercial activities. MAT (monetary and nonmonetary benefit sharing, written agreement with competent national authority, terms on change of intent) is always requested even if PIC is not required.

It has been suggested that in the absence of clear rules, a due diligence system for the user of GRs should be applied. The following points should be followed to substantiate due diligence:

- 1. An obligation to seek, keep, and transfer to subsequent users:
 - a. The internationally recognized certificate of compliance (IRCC) and content of MAT.
 - b. In cases where no IRCC is available, information and relevant documents on:
 - date and place of access;
 - description;
 - the source from which genetic resources were directly obtained and subsequent users;
 - rights and obligations related to Accession Benefit Sharing;

- access permits;
- MAT.
- 2. In case of insufficient information or uncertainties about legality of access and use:
 - a. obtain an access permit and establish MAT or
 - **b.** discontinue utilization.

2.4 NEW INSIGHTS INTO BIFIDOBACTERIAL SPECIES ECOLOGY

Bifidobacterial species are found in the intestinal tracts of animals in many branches of that kingdom. They have been found in the following different ecological niches: the intestine, oral cavity, and vagina of humans; other animal intestines, including mammals and insects; and also in sewage, blood, and fermented food. All these niches are directly or indirectly linked to the human/animal intestinal environment. Many animal sources have been investigated for the occurrence of bifidobacteria but many others have to be investigated in the future and probably our knowledge about bifidobacterial species distribution is not exhaustive. The overview of bifidobacteria ecology suggests a strict association between bifidobacterial species and animal niches. Many types of bifidobacteria have been found in the feces of rabbits, chickens, cattle, mice, and piglets, some of which seem to be "host specific": *B. magnum* and *B. cuniculi* only in rabbit feces, *B. pullorum* and *B. gallinarum* only in the intestines of chickens, and *B. longum* subsp. *suis* only in pig feces. Many types of bifidobacteria have been recently found in nonhuman primates describing a fantastic biodiversity in lemurs (*Lemur catta* and *L. macaco*) and in *Callitricidae* (*S. oedipus, S. imperator, S. mida*, and *C. jacchus*).

Except for the human species *B. adolescentis* and *B. dentium*, found respectively in orangutan and chimpanzee (D'Aimmo et al., 2014), all other bifidobacterial species described in nonhuman primates, such as in gorilla (Tsuchida et al., 2013), in Callitricidae [common marmoset and tamarins (Michelini et al., 2016a,c; Modesto et al., 2014)] and in Lemuridae [ring-tailed lemur and black lemur) (Michelini et al., 2016b; Modesto et al., 2015)] are never found in humans or other animals (Fig. 2.1). The hypothesis of coevolution of microorganisms present in the gut microbiota and their host seems to be strongly supported by bfidobacterial speciation as confirmed by genome sequence analysis of B. asteroides a species typically found in honeybee. Its genome in fact revealed its predicted capability for respiratory metabolism. Conservation of the latter gene clusters in various B. asteroides strains enforces the notion that respiration is a common metabolic feature of this ancient bifidobacterial species, which has been lost in currently known mammal-derived Bifidobacterium species (Bottacini et al., 2012). Phylogenomic-based analyses suggested an ancient origin of B. asteroides and indicates it as an ancestor of the modern genus Bifidobacterium. It can be hypothesized that honeybee and bumblebee intestine is a phylogenetically antique ecological niche which only B. asteroides, B. coryneforme, B. indicum, B. actinocolooniforme, B. bohemicum, and B. commune inhabit. Interestingly, evidence has been presented that most of the bacterial species present in Apis and Bombus have been found neither in solitary bees nor elsewhere in the environment and occur as a set of deep-branching phylogenetic lineages. Within these lineages, taxa isolated from honeybees and bumblebees seem to constitute distinct sister clades. These findings indicate longstanding relationships between these bacteria and their hosts, potentially reflecting long-term coevolution, and suggesting the existence of specific symbiotic interactions relevant for the characteristic lifestyle of these insects (Engel and Moran, 2013). Phylogenetic tree genus based on 16S rRNA gene sequences and based on the concatenate of housekeeping hsp60, rpoB, clpC, dnaG, and dnaJ gene sequences are shown in Figs. 2.2 and 2.3, respectively.

Bifidobacteria have been found also in the wider environment, not confined only to the interior of living hosts, for example, *B. minimum* and *B. subtile* in sewage, *B. mongoliense* in airag (or koumiss, a Mongolian alcoholic drink made from fermented mare's milk with added salt), *B. aquikefiry* in water kefir (a homemade fermented beverage based on a sucrose solution with different dried and fresh fruits), *B. crudilactis* in meat, and *B. animalis* subsp. *lactis* in commercially fermented milk. These findings could be explained by the possibility of survival of bifidobacteria derived from fecal contamination, in extrabody environment, and this is the cause of isolation of bifidobacteria in extrabody environments. Also in honey, after its production, bifidobacteria can be found for some days.

Regarding the distribution of members of the genus *Bifidobacterium*, it has been suggested that their offspring are raised by parental care (e.g., mammals, birds, social insects), and it may thus be that such an ecological distribution is the consequence of direct transmission of bifidobacterial cells from parent/carer to offspring (Turroni et al., 2011).

2.5 LIST OF THE SPECIES OF THE GENUS BIFIDOBACTERIUM

Bifidobacterial species share common phenotypical features: they are gram positive, nonmotile, asporogenous, nonhaemolytic, F6PPK-positive, catalase- and oxidase-negative, and indole-negative. The fermentative characteristics of the species are described in Table 2.2. All other relevant phenotypical and genotypical properties of all the



FIGURE 2.1 Bifidobacterial species: occurrence in different habitats and period of species description.

2. SPECIES IN THE GENUS BIFIDOBACTERIUM



FIGURE 2.2 Phylogenetic relationships of members of *Bifidobacterium* genus based on 16S rRNA gene sequences. The tree was constructed by the neighbor-joining method and rooted with *Micrococcus luteus* DSM 20030T. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Bootstrap percentages above 50 are given at branching points.

2.5 LIST OF THE SPECIES OF THE GENUS BIFIDOBACTERIUM



FIGURE 2.3 Phylogenetic trees based on the concatenate of housekeeping *hsp60, rpoB, clpC, dnaG,* and *dnaJ* gene sequences showed the relationship of the members of *Bifidobacterium* genus. The tree was constructed by the maximum-likehood method and the sequence of *Mycobacterium tuberculosis* H37Rv was used as an outgroup. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Bootstrap percentages above 50 are given at branching points.

Substrates	1. B. actinocolo- niiforme	2. B. aquikerfiri	3. B. adolescentis	4. B. aerophilum	5. B. aesculapii	6. B. angulatum	7. B. animalis	subsp. <i>animalis</i>	subsp. <i>lactis</i>	8. B. asteroides	9. B. avesanii	10. B. biavatii	11. B. bohemicum	
α-L-Fucose	nd	nd	_d	nd	nd	_		_	_	_	nd	nd	nd	•
Amylopectin	nd	nd	+ ^b	nd	nd	+ ^b		v	v	_d	nd	nd	nd	
Amylose	nd	nd	v	nd	nd	_		_	_	_d	nd	nd	nd	
Arabic gum	nd	nd	v	nd	nd	_		_	_	_	nd	nd	nd	
Arabinogalactan	nd	nd	_	nd	nd	_		_	_	_d	nd	nd	nd	
L-Arabinose	+	+	+	_	+	+		+	+	_	+	_	+	
D-Cellobiose	+ ^g	_	+	V	+	_		V	_	+	-	+ ^g	_	
D-Fructose	_	+	+	+ ^g	-	+		+	_	+	+	nd	+ ^g	
D-Galactosamine	nd	nd	-	nd	nd	v		+ ^b	+ ^b	d	nd	nd	nd	
D-galactose	+	+	+	-	-	+		+	_	V	+ ^g	_	+	
Ghatti gum	nd	nd	_	nd	nd	_		_	_	_	nd	nd	nd	
Gluconate	nd	nd	+	nd	nd	v		_	_	+	nd	nd	nd	
D-Glucosamine	nd	nd	_	nd	nd	v		v	v	_d	nd	nd	nd	
D-Glucuronate	nd	nd	_	nd	nd	-		_	_	_	nd	nd	nd	
Guar gum	nd	nd	_	nd	nd	_		_d	_	_	nd	nd	nd	
Inulin	nd	nd	v	_	+	+		_	_	_	_	_	nd	
D-Lactose	nd	_	+	+ ^g	nd	+		+	+	_	_	nd	nd	
Locust bean gum	nd	nd	-	nd	nd	-		v	v	-	nd	nd	nd	
D-Maltose	-	+	+	+	+	+		+	+	v	-	_	_	
D-Mannitol	nd	+ ^g	v	-	+	-		_	-	-	-	_		
D-Mannose	_	+	v	+	+	-		v	_	_d	+	+ ^g	+	
D-Melezitose	-	-	+	-	+	-		v	-	-	-	-	-	
Melibiose	+ ^g	+	+	v	+ ^g	+		+	+	+		+	+	
Pectin	nd	nd	-	nd	nd	-		v	v	_d	nd	nd	nd	
Porcine gastric mucin	nd	nd	-	nd	nd	-		-	-	-	nd	nd	nd	
D-Raffinose	-	+	+	+ ^g	+	+		+	+	+	+	+	+	
D-Ribose	nd	+	+	V	+	+		+	+	+	-	-		
Salicin	nd	-	+	v	+	+		+	-	+	-	-		
D-Sorbitol	nd	nd	v	-	-	v		-	-	-	-	+		
Starch	nd	nd	+	nd	nd	+		+	-	-	nd	nd	nd	
D-Sucrose	+	+	+	+	+	+		+	+	+	+	+	-	
Tragacanth gum	nd	nd	-	nd	nd	-		-	-	-	nd	nd	nd	
D-Trehalose	+	nd	v	-	+	-		v	-	-	-	-	-	
Xylan	nd	nd	-	nd	nd	-		_d	_d	_d	nd	nd	nd	
D-Xylose	+	_	+	_	+	+		+	+	+1	+	_	+	

TABLE 2.2	Fermentative	Characteristics	Distinguishing	Species of	f Genus B	ifidobacterium ^a
			() ()			3

12. B. bifidum	13. B. bombi	14. B. boum	15. B. breve	16. B. callitrichos	17. B. catenula- tum	18. B. choerinum	19. B. commune	20. B. coryneforme	21. B. crudilactis	22. B. cuniculi	23. B. dentium	24. B. eulemuris	25. B. faecale	26. B. gallicum	27. B. gallinarum	28. B. hapali	29. B. indicum ^k
-	nd	-	+	nd	-	-	nd	-	nd	-	-	nd	nd	-	-	nd	-
v	nd	+	v	nd	d	+	nd	d	nd	+	+	nd	nd	+	-	nd	-
V	nd	+ ^b	v	nd	v	+	nd	d	nd	+ ^b	+	nd	nd	+	-	nd	_d
-	nd	-	-	nd	-	-	nd	-	nd	-	-	nd	nd	-	-	nd	-
-	nd	-	-	nd	-	-	nd	d	nd	-	-	nd	nd	-	-	nd	v
-	-	-	-	+	+	-	-	-	-	+	+	+ ^g	+	+	+	+	-
-	-	_	-	+	+	-	_	+	+	_	+	-	+	-	v	+ ^g	+
+ ^b	+	+	+	nd	+	-	+	+		-	+	+ ^g		+	+	+	+
-	nd	+	v	nd	v	+ ^b	nd	+		V	d			-	-		+
+	-	$+^{i}$	+		+	+		nd		+	+	-		+	+	-	v
-		_	_		-	-		-		_	_d			-	-		-
-		_	_		v	-		+		_	+		+ ^g	-	nd	-	+
v		+	v		v	d		+		+ ^b	v			+	-		+
-		_			-	-		-		_	-			-	-		-
-		_d	_		-	v		-		_	+			-	-		-
-		+	v	_	v	-		nd		-	-		+ ^g	-	+		-
+		v	+		+	+		-	+	-	+	+ ^g		-	+	+ ^g	-
-		-	-		-	V		-		-	+			-	-		-
_d	-	+	+	+	+	+		+		+	+			+	+		v
-		-	v	+	V	-		-	+	-	+	+	+	-	-	-	-
-	+	-	+	+	-	-	-	-	-	-	+			-	v	V	v
-	-	-	-	+	-	-		-	-	-	+	-	-	-	v	v	-
V	+	+	+		+	+		+	+	+	+			-	+	-	+
_d		-	v		v	-		_d		-	V			-	-		v
+	nd	-	-	nd	-	-	nd	-	nd	-	-	nd	nd	-	-	nd	-
-	+	+	+	+	+	+		+		-	+			-	+	+	+
-		-	+	-	+	-		+		-	+			+	+	-	+
-		-	+	+	+	-		+	-	-	+			+ ^g	+	+ ^g	+
-		-	v	+	+	-		-		-	-	+	-	-	-	-	-
-		+	-	-	-	+	-	-		+	+			+	-	-	-
v ^c	-	+	+	+	+	+	+	+		+	+	nd	+	+	+	+ ^g	+
-	nd	-	-	nd	-	-	nd	-	nd	-	-	nd	nd	-	-	nd	v
-	-	-	v	+	V	-	nd	-	nd	-	+	+	+	-	+	-	-
_d	nd	-	-	nd	v	-	nd	-	nd	-	v	nd	nd	-	-		-
	_	_	_	+	+	_	_	+	_	+	+	+	+	+	+	+ ^g	

(Continued)

2. SPECIES IN THE GENUS BIFIDOBACTERIUM

Substrates	30. B. kashiwanohense	31. B. lemurum	32. B. longum	<i>unsp.longum</i>	subsp. <i>infantis</i>	subsp. suis	subsp. <i>suillum</i>	33. B. magnum	34. B. merycicum	35. B. minimum	36. B. myosotis	37B. mongoliense	38. B. moukalabense	39. B. pseudocatenu- latum	40. B. pseudolongum
α-L-Fucose	nd	nd		-	+ ^b	_	_	_	_	-	nd	nd	nd	v	
Amylopectin	nd	nd		_	-	d	d	_d	v	+	nd	_	nd	$+^{b}$	
Amylose	nd	nd		_	-	d	d	_d	v	+	nd	nd	nd	$+^{b}$	
Arabic gum	nd	nd		v	-	_	-	-	-	_	nd	nd	nd	-	
Arabinogalactan	nd	nd		+ ^b	-	d	d	-	-	_	nd	nd	nd	-	
L-Arabinose		_		+	-	+	+	+	+	_	+	_		+	
D-Cellobiose	+	+		_	v	_		_	v	_	v	+	_	v	
D-Fructose	+	$+^{g}$		+	+	$\mathbf{v}^{\mathbf{j}}$		+	$+^{i}$	+	v	+ ^b		+	
D-Galactosamine				v	-	_d		v	$+^{b}$	-		-		-	
D-galactose				+	+	+		+	+	-	v	nd		+	
Ghatti gum				v	_	_		_	_	_		+		v	
Gluconate				_	_	_		_	_	_	_	nd		v	
D-Glucosamine				v	v	_d		+	_	_		+ ^b		_d	
D-Glucuronate				_	+	_		_	_	_		_		_	
Guar gum				_	_	_		_	_	_		nd		-	
Inulin	_			_	v	_	_	_	_	_		nd		_	
D-Lactose				+	+	+		+	+	_	+	_		+	
Locust bean gum				_d	_	_		_	_	_		+		_	
D-Maltose				+	+	+		+	+	+		nd		+	
D-Mannitol	_	+ ^g		_	_	_		_	_	_	v	+	_	_	
D-Mannose	+	+		v	v	$\mathbf{v}^{\mathbf{j}}$	+	_	_	_	+ ^g	_		+	
D-Melezitose	_	+		+	v	_	_	_	_	_	v	_	+	_	
Melibiose				+	+	+		+	+	_	v	_		+	
Pectin				_d	_	_		_	_	_		+		_	
Porcine gastric mucin	nd	nd		_	_	_	nd	_	_	_	nd	nd	nd	_	
D-Raffinose				+	+	+		+	+	_	v	nd		+	
D-Ribose		+		+	+	_		+	+	_	_	+		+	
Salicin				_	_	_	_	_	$+^{i}$	_	+	+ ^b		+	
D-Sorbitol	+			_	_	_	+	_	_	_	_	+ ^b	_	v	
Starch	_			_	_	_		_	+	+	_	_		+	
D-Sucrose				+	+	+		+	+	+	+	+		+	
Tragacanth gum	nd	nd		v	_	_	nd	_	_	_	nd	+	nd	_	
D-Trehalose	_	+ ^g		_	_	_	_	_	_	_	v	nd	nd	v	
Xylan	nd	nd		_	_	_	nd	_	_	_	nd	_	nd	v	
D-Xylose		+		v	v	+	+	+	+	_	+	nd	nd	+	

TABLE 2.2 Fermentative Characteristics Distinguishing Species of Genus Bifidobacterium^a (cont.)

"Symbols: +, 90% or more strains positive; -, 90% or more strains negative; v, 11–89% of strains positive; nd, not determined. All the strains tested ferment glucose, but not alginate, bovine submaxillary mucin, chondroitin sulfate, dextran, α -D-fucose, D-galacturonate, glycerol, gum karaya, heparin, hyaluronate, lactate, laminarin, ovomucoid, polygalacturonate, or L-rhamnose.

 ^{b}A few strains do not ferment this sugar.

^cWhen positive it is fermented slowly.

^dSome strains ferment this sugar.

^ebut some are negative, especially those from rabbit and rat feces. ^fSome strains can ferment it weakly.

⁸Generally delayed or slight fermentation.

^hSome strains from sewage ferment this sugar.

ⁱSome strains are weak fermenters.

^{*j*}Reported as "sometimes not fermented" (Matteuzzi et al., 1971).

^kSugars indicated "v" mainly give erratic results.

¹A few strains do not ferment pentoses.

All fermentative data derive from original publication decribing the species for the first time. All data relating to the degradation of complex carbohydrates are from Crociani, F., Alessandrini, A., Mucci, M.M., Biavati, B., 1994. Degradation of complex carbohydrates by Bifidobacteriumspp. Int. J. Food Microbiol.24, 199–210.

subsp. <i>pseudolongum</i>	upsp. globosum	41. B. psychraerophi- lum	42. B. pullorum	43. B. ramosum	44. B. reuteri	45. B. ruminantium	46. B. saguini	47. B. saeculare	48. B. scardovii	49. B. stellenboschense	50. B. subtile	51. B. thermacidophi- lum	subsp. <i>porcinum</i>	subsp. thermacidophi- lum ^d	52. B. thermophilum	53. B. tissieri	54. B. tsurumiense
_	-	nd	_	nd	nd	_	nd	_	nd	nd	_		nd	nd	_	nd	nd
+	+	nd	-	nd	nd	_d	nd	-	nd	nd	$+^{b}$		nd	nd	$+^{b}$	nd	nd
+	v	nd	_	nd	nd	d	nd	-	nd	nd	$+^{b}$		nd	nd	$+^{b}$	nd	nd
-	-	nd	-	nd	nd	-	nd	-	nd	nd	-		nd	nd	-	nd	nd
-	_d	nd	-	nd	nd	-	nd	-	nd	nd	-		nd	nd	d	nd	nd
+	V		+	+	-	-	+	+	+	+	-		-	$+^{i}$	-	+ ^g	+
V	-	+	-	-	+ ^g	-	+	-		-	-			nd	v	+	+
+	+ ^e	-	+	+		+		+			+			+	+	+	+
-	V		+			+		+	nd		+			nd	$+^{b}$		nd
+	+		$+^{g}$	-	-	+	+	$+^{g}$		+	+			+	+	+ ^g	+
-	-		-			-		-	nd		-			nd	-		nd
-	-		-			-		-			+			_i	-	-	+
V	V		+			+ ^b		+	nd		+			nd	v		nd
-	-		-			-		-	nd		-			nd	_		nd
-	-		-			-		-	nd		-			nd	_d		nd
-	-		+	_		-	_	+		-	V		-	-	v		-
V	+	-	-	+	-	+		+ ^g	+		-			_d	v	+	+
-	-		-			-		-	nd		-			nd	_d		nd
+	+	+	+	-	+	+	+	$+^{b}$	+	+	+			nd	+		+
-	-	-	-	-	-	+	-	-	-	+	-			-	_	+ ^g	+
+ ^g	-	-	+	-	-	-	-	+ ^b	+	-	-		-	-	_h	+ ^g	+
V	-	+	-	-	+ ^g	-	-	+	V	+	+			v	v	+ ^g	-
+	+		+	_		+		+	+		+			+	+	_	+
-	-		-			-		-	nd		V			nd	v		nd
-	-	nd	-	nd	nd	-	nd	-	nd	nd	-		nd	nd	_	nd	nd
+	+		+	-	+	+	+	+	+	+	+			+	+	+	+
+	+		+	+	-	+	+	+	+	+	+			+1	-	-	+
-	_ ^I		+	-	+	$+^1$	+	V		+	V			_a	v	+	+
-	-		-	-	-	-	-	_ ^u	-	+	+			+	-	-	-
+	+		-		+	+	-	-		-	+			v	+	-	+
+	+		+	-		+		+	+		+			+	+	+	+
_u	-		-		~	-		-	nd		-			nd	-		nd
-	- d		+	-	+ ^g	-	-	+	+	-	v		+	-	V	+	+
-	_ ^u		+			-		+	nd		-			nd	V	~	nd
+	V	+	+	+	+	-	+	+		+	-			_a	-	+ ^g	+

species properly accredited up to August 2016 are shown later, using data drawn in all cases from the publications that established each species.

2.5.1 Bifidobacterium actinocoloniiforme (Killer et al., 2011)

This species was isolated from the digestive tracts of bumblebees. The name derives from the morphology of colonies shaped like ray-shaped ones. B. actinocoloniiforme with B. bohemicum, B. bombi, and B. commune share the bumblebee intestinal niche. Cells are irregularly shaped rods (0.3–0.6 mm wide and 0.4–1.2 mm long) with enlarged or tapered ends (Fig. 2.4, panel 1), singly or in short chains. Colonies on TPY agar under anaerobic conditions are cream in color, sometimes irregularly circular with entire edges and rigid cores, and reach 2.05–3.97 mm in diameter after 2 days of incubation. Some colonies have filamentous parts growing around the solid core when they are incubated for 72 h under anaerobic conditions. Colonies are also formed under microaerophilic conditions, when they reach 1.79–2.62 mm in diameter after 2 days of incubation. Growth in TPY broth occurs at 25 and 37°C, but not at 47°C (after 24–48 h). In TPY broth, the lowest pH attained is 4.5; minimum initial pH for growth is pH 5. Lactic and acetic acids are produced in a theoretical final ratio of 1.5:1.0. Cells contain relatively large amounts of palmitic, oleic and stearic acids. DNA G + C content is 52.7%. B. actinocoloniiforme, on the basis of 16S rRNA gene similarity, clustered in the "asteroides group," revealing 96.2, 96.0, and 95.9% sequence similarities with its closest relatives B. asteroides, YIT 11866^T, B. indicum JCM 1302^T, and B. coryneforme ATCC 25911^T, respectively. The complete genome sequence of the type strain has been described by Chen et al. (2015). The type strain, isolated from the digestive tract contents of a bumblebee (*Bombus lucorum*) sampled from Central Bohemia (Czech Republic) in 2006, is LISLUC III-P2¹ (=DSM 22766^T=CCM 7728^T).

2.5.2 Bifidobacterium aquikefiri (Laureys et al., 2016)

This species was isolated from a household water kefir fermentation process. This is an unusual habitat for bifidobacteria and particular attention should be devoted to understanding if this finding could be related to external contamination (for example, from honey used sometimes to grow Kefir) or, if this is not the case, to understand how this anaerobic bacterium could be propagated in this particular environment. Cells are short rods 0.5–1.0 µm thick and 1–2 µm long without bifurcations; some cells are club-shaped. After 6 days at 28°C on M144 agar medium, colonies are about 1 mm in diameter, circular, convex, smooth with smooth edges, translucent, and cream-colored. Growth occurs under anaerobic, microaerobic, and aerobic conditions. The temperature range for growth is 4–37°C; no growth occurs at 45°C. The optimum temperature for growth is 28°C. Grows at pH 4.0–8.0 but no growth at pH 3.5 or 9.0. When grown on glucose in M144 broth, no gas is produced and the main metabolites are acetic acid, lactic acid, and formic acid. The molar ratio of acetic to lactic acid is 4.8:1 and lactic acid is produced exclusively in L-isomer form. The DNA G + C content is 52.6%. The highest level of 16S rRNA gene sequence similarities has been shown with *B. crudilactis* and *B. psychraerophilum* (97.4 and 97.1%, respectively). The analysis of 16S rRNA showed the highest similarity with *B. crudilactis* and *B. psychraerophilum* (97.4 and 97.1%, respectively). The type strain, R54638^T (=LMG 28769^T=CCUG 67145^T) was isolated from a household water kefir fermentation process carried out in Brussels, Belgium, in 2014. This species has been described based on a single strain.

2.5.3 Bifidobacterium adolescentis (Reuter, 1963)

In 1963, Reuter isolated *B. adolescentis* strains from adult feces and grouped them in four biovars (*a*, *b*, *c*, *d*) based on serological reactions and differences in the fermentation of mannitol and sorbitol. The cellular morphology is common to that of many other *Bifidobacterium* species (Fig. 2.4, panel 2). A study of DNA–DNA homology by Scardovi et al., 1971) confirmed that biovar *a* (ATCC 15703^T), biovar *a* (ATCC 15704), biovar *c* (ATCC 15705), and biovar *d* (ATCC 15704) possess high genetic relatedness (>70%) despite their fermentative different pattern. Difficulties in distinguishing *B. adolescentis* on the basis of phenotypical characteristics from other bifidobacteria isolated from the feces of human adults are reported by Yaeshima et al. (1992a), who used DNA base compositions and DNA–DNA homologies to correctly assign isolates to *B. adolescentis*. The PAGE procedure can also be successfully employed to group *B. adolescentis* strains (Biavati et al., 1982). *B. adolescentis* is one of the dominant bifidobacterial species in adult human large intestine. Moreover, *B. adolescentis* strains have also been isolated from bovine rumen, human infant, and orangutang (D'Aimmo et al., 2014). Some recent studies underlined again the diversity between isolates of *B. adolescentis* from different sources (Yasui et al., 2009). One hypothesis



FIGURE 2.4 Cellular morphology in the genus *Bifidobacterium*. Cells of the type strains were grown in TPY medium stabs; phase-contrast photomicrographs. (1) *B. actinocolooniforme;* (2) *B. adolescentis;* (3) *B. aeriphilum;* (4) *B. aesculapii;* (5) *B. angulatum;* (6a) *B. animalis* subsp. *animalis*; (6b) *B. animalis* subsp. *Lactis;* (7) *B. asteroides;* (8) *B. avesanii;* (9) *B. biavatii;* (10) *B. bifidum;* (11) *B. bohemicum;* (12) *B. bombi;* (13) *B. boum;* (14) *B. Breve;* (15) *B. callithricos.* (16) *B. catenulatum;* (17) *B. Choerinum;* (18) *B. Coryneforme;* (19) *B. crudilactis;* (20) *B. cuniculi;* (21) *B. dentium;* (22) *B. eulemuris;* (23) *B. gallicum;* (24) *B. gallinarum;* (25) *B. hapali;* (26) *B. indicum;* (27) *B. lemurum;* (28a) *B. longum* subsp. *Longum;* (28b) *B. longum* subsp. *Infantis;* (28c) *B. longum* subsp. *Suils;* (28d) *B. longum* subsp. *Suilum.* (29) *B. magnum;* (30) *B. merycicum;* (31) *B. minimum;* (32) *B. mongoliense;* (33) *B. myositis;* (34) *B. pseudocatenulatum;* (35a) *B. pseudolongum* subsp. *Pseudolongum;* (35b) *B. pseudolongum* subsp. *globosum;* (36) *B. psycraerophilum;* (37) *B. pullorum;* (38) *B. ramosum;* (39) *B. reuteri;* (40) *B. ruminantium;* (41) *B. saguini;* (42) *B. saeculare;* (43) *B. scardovii.* (44) *B. stellenboshense;* (45) *B. subtile;* (46a) *B. thermacidophilum* subsp. *thermacidophilum;* (46b) *B. thermacidophilum* subsp. *porcinum;* (47) *B. tissieri;* (48) *B. thermophilum;* (49) *B. tsurumiense.*
is the presence of surface polymorphism at the strain level with differences in cell surface polysaccharides and cell surface proteins involved in the adherence to individual hosts' intestines. Moreover, *B. adolescentis* strains isolated from various environments, such as human milk, human feces, and bovine rumen, revealed a high level of genetic variability, resulting in an open pan-genome. Compared to other bifidobacterial taxa, such as *B. bifidum* and *B. breve*, the more extensive *B. adolescentis* pan-genome supports the hypothesis that the genetic arsenal of this taxon expanded so as to become more adaptable to the variable and changing ecological niche of the gut. These increased genetic capabilities are particularly evident for genes required for dietary glycans breakdown (Duranti et al., 2013; Lugli et al., 2014). Recently, *B. stercoris*, described as a new species by Kim et al. (2010), has been shown to be completely related to *B. adolescentis* and has been be united under the same name. The mol.% G + C of the DNA is: 59 mol.% (Tm). The type strain is E194a^T (=ATCC 15703^T=DSMZ 20083^T=JCM 1275^T=LMG 10502^T=NCIMB 702204^T) isolated from the feces of an adult human. Reference strains are ATCC 15704, ATCC 15705, and ATCC 15706 isolated from the intestine of adult humans; DSMZ 28529 and DSMZ 28530 isolated from orangutang feces.

2.5.4 Bifidobacterium aerophilum (Michelini et al., 2016a)

This species was recently isolated from feces of cotton-top tamarin (*S. oedipus* L.). In the same niche *B. avesanii* and *B. ramosum* have also been described. These species from nonhuman primates do not cluster with bifidobacterial species from human or from any other animals: the same observation has been performed by Mitsuoka (2014). Cells are rods of various shapes (Fig. 2.4, panel 3) and when grown in Tryptone-Phytone_Yeast extract (TPY) broth, form a branched structure with a "Y" on both sides. Well-isolated colonies growing on the surface of TPY agar under anaer-obic conditions are white, opaque, smooth, and circular with entire edges, while embedded colonies are lens-shaped or elliptical. Colonies reach 1.0–2.0 mm in diameter after 3 days incubation. Cells can also grow under aerophilic and microaerophilic conditions. The ability to survive and grow in aerobic conditions is an uncommon feature for bifidobacteria. Growth occurs in the range 25–50°C, but no growth occurs at 20 or 56°C. Strains grow at pH 4.0–7.5. Optimal conditions for growth occur at pH 6 and 40°C. The peptidoglycan type is A3α L-Lys-L-Thr-L-Ala. The DNA G + C content of the type strain is 63.3 mol.%. The highest level of 16S rRNA gene sequence similarities has been shown with *Bifidobacterium scardovii* DSM 13734^T (mean value 96.6%). The type strain TRE 17^T (=DSM 100689^T=JCM 30941^T) and the reference strain TRE 26 (=DSM 100690=JCM 30942) were isolated from the feces of an adult subject of the cotton-top tamarin.

2.5.5 Bifidobacterium aesculapii (Modesto et al., 2014)

This species was isolated from baby common marmoset. This is the first species isolated from babies of nonhuman primates and the only species isolated exclusively from infants of nonhuman origin. The name "aesculapii" derives from "Aesculapius," from the snake-like appearance of the bacterium, resembling the serpent-entwined rod wielded by the Roman god Aesculapius. B. aesculapii has been isolated from C. jacchus, a New World monkey belonging to the *Callitricidae* family. *B. aesculapii* produces high amounts of exopolysaccharides (EPSs) which can play an important role in the enhancement of antiinflammatory activity, antagonistic activity toward pathogen for adhesion to substrate; moreover, EPSs are a defense strategy for the bacterium protecting from external stresses. Cells grown in TPY broth are rods of various shapes, occasionally swollen, always coiled or ring shaped or forming a "Y" shape at both ends (Fig. 2.4, panel 4). There is no difference in growth under either anaerobic or microaerophilic conditions. Wellseparated colonies on the surface of TPY agar under anaerobic conditions are white, opaque, smooth, and circular with entire edges, while imbedded colonies are lens-shaped or elliptical. Colonies reach 1.7–2.5 mm in diameter after 3 days of incubation. The temperature range for growth is 25–42°C; no growth occurs at 20 or 47°C. The optimum temperature for growth is 35–37°C. Grows at pH 4.5–7.0 with optimum growth at pH 6.5–7.0. Can grow in milk, under aerobic, microaerophilic, and anaerobic conditions. Lactic and acetic acids are produced as end products of glucose fermentation in a variable ratio ranging from 1:2 to 1:1.5. The peptidoglycan type is A4 α L-Lys-D-Ser-D-Asp. The DNAG+C content of the type strain is 64.7 mol.%. Phylogenetic analysis of the 16S rRNA gene sequence places the species in the B. scardovii subgroup of the genus Bifidobacterium. The draft genome sequence of the type strain has been described by Toh et al. (2015). The type strain, MRM $3/1^{T}$ (=JCM 18761^{T} =DSM 26737^{T}), and the reference strain MRM 4/2 (=JCM 187625=DSM26738) were isolated from fresh fecal samples of infant common marmosets (C. *jacchus*).

2.5.6 Bifidobacterium angulatum (Scardovi and Crociani, 1974)

This species was first isolated from human adults (only the type strain) and found in sewage. Since no strains have been assigned to B. angulatum in many other studies concerning the presence of bifidobacteria in human feces, the assumption that this species is a member of human intestinal microbiota is questionable. Recently Ushida et al. (2010) described isolates belonging to *B. angulatum*-like organisms from feces of chimpanzee in the wild, suggesting the presence of this species in this habitat. Cells, 0.6–0.7 by 1.5–3.0 pm., are generally disposed in "V" or "palisade" arrangements, such as the corynebacteria, rarely enlarged at the extremities, and branching is absent. This morphological type is unique among the known species of the genus Bifidobacterium (Fig. 2.4, panel 5). Colonies are circular, pulvinate, smooth with entire margins, porcelain white, glistening, and of soft consistency. Anaerobic, but more sensitive to oxygen than most bifidobacteria (from the depth of growth in stabs); CO_2 does not affect the sensitivity to oxygen, but it strongly enhances anaerobic growth. Optimum temperature is from 39–40°C; maximum, 42°C; minimum, 28–29°C; and with no growth at 27 or 44°C. pH relationships: initial optimum, 6.5–6.9; delayed growth at 6.3 or 7.2; no growth after 2 days at 4.5 or 8.0. Lactic and acetic acids in a molar ratio of 1:2.2 are produced in TPY medium. Isomeric type of lactic acid produced: L(+). Propionic and butyric acids not produced. CO₂ is formed only in the fermentation of gluconate. The peptidoglycan type is L-Lys-D-Asp. The DNAG + C content of the type strain is 59%. The genome sequence of the type strain JCM 7096^T has been described by Morita et al. (2015b). The type strain, isolated from adult human feces, is B 677^T (=ATCC 27535^T=DSM 20098^T=JCM 7096^T). Reference strains, isolated from sewage, are ATCC 27669, ATCC 27670 (=DSM 20225=JCM 1252), and ATCC 27671.

2.5.7 Bifidobacterium animalis (Masco et al., 2004; Mitsuoka, 1969; Scardovi and Trovatelli, 1974)

In 1969 Mitsuoka isolated from the feces of various animals strains phenotypically very similar to B. longum and referred to them as *B. longum* subsp. *animalis*: two biovars *a* and *b* were described. Two strains, one for each biovar, used as reference were found to be related to B. animalis subps. animalis [biovar a R-101-8T (=DSM 20104) actually is the type strain of the species] and to B. longum subsp. longum [biovar b C10-45 (DSM 20097)]. In 2004, B. lactis, described as a new species by Meile et al. (1997), based on new genotypic evidence, has been shown to represent a junior synonym of B. animalis. However, on the basis of data from Ventura and Zink (2002), B. animalis and B. lactis have been reclassified into the two new subspecies, Bifidobacterium animalis subsp. animalis and Bifidobacterium animalis subsp. lactis (Masco et al., 2004). It can be observed that these subspecies are distributed specifically in different habitats: in fact, B. animalis subsp. animalis comprises strains isolated from rat, while B. animalis subsp. lactis comprises strains isolated from chicken and rabbit other than the type strain from fermented milk and other strains from human feces and sewage. Regarding sewage, fermented milk, and human feces, it is reasonable to consider that the finding of *B. animalis* subsp. *lactis* in sewage has been a consequence of animal or human fecal contamination of water. On the other hand, the finding of *B. animalis* subsp. *lactis* in human feces can be the result of consumption of fermented milk with this species added as a probiotic. Strains of both subspecies have shown phase variations in colony appearance and in cellular morphology (Biavati et al., 1992a). Most significant is the fact that the transition to colony morphotype transparent (T) and opaque (O) is accompanied by a dramatic change in cell dimension: minute and mostly spherical from T colonies while those from O colonies are large and show the species-specific shape, that is, the central portion slightly enlarged (Fig. 2.5).



FIGURE 2.5 *Bifidobacterium animalis* **subsp.** *lactis* **strain P 23 (=ATCC 27536).** (1) Transparent white (T) and opaque black (O) colonies; (2) T colonies with O papillae viewed under different illumination; (3) and (4) cells from O and T colonies, respectively; and (5) mixed type from TPY stab (phase-contrast photomicrographs).

2.5.7.1 Bifidobacterium animalis subsp. animalis (Masco et al., 2004; Mitsuoka, 1969; Scardovi and Trovatelli, 1974)

Mitsuoka described the strain R101-8T in feces of rat. Cells, grown on the optimal TPY medium, characteristically show that the central parts are slightly enlarged (Fig. 2.4, panel 6a). The optimum growth temperature is 39–41°C. No growth occurs in slants incubated in air or in air enriched with carbon dioxide. No growth occurs in milk or milk-based media. Lactate and acetic acids are produced in a molar ratio of 1:3.6 \pm 0.3. pH relationships: initial optimum, 6.4–7.0; delayed growth at 6.0 or 7.4; no growth at 5.0 or 8.0. The peptidoglycan structure is L-Lys(L-Orn)-L-Ser-(L-Ala)-L-Ala2. The DNA G + C content is 61.3 mol.%. The genome sequence of the type strain ATCC 25527T has been described by Loquasto et al. (2013). Type strain, isolated from rat feces, is R101-8T (ATCC 25527T = DSM 20104T = LMG 10508T = JCM 1190T). Reference strain is ATCC 27672, isolated from rat feces.

2.5.7.2 Bifidobacterium animalis subsp. lactis (Masco et al., 2004; Mitsuoka, 1969; Scardovi and Trovatelli, 1974)

The historical background of *B. animalis* subsp. lactis dates back to 1997, when *B. lactis* was described as a new species. The misidentification of this new species *B. lactis* by Meile et al. (1997) was clarified by Masco et al. (2004) who describe B. animalis subsp. lactis subsp. nov. B. animalis subsp. lactis (BB12 strain especially) is currently the most utilized probiotic species among bifidobacteria. Biavati et al. (1992b) and Masco et al. (2004) showed in more than 30 brands of European, American, and Asian fermented products the species *B. animalis* subsp. lactis even if, in most cases, no claim for this species was present in the label of the products. The complete sequence and analysis of the genome of many strains of *B. animalis* subsp. lactis have been described (Loquasto et al., 2013). There is a lack of diversity within *B. animalis* subsp. *lactis*, which can be due to the intense focus on commercially relevant strains and the likely reisolation of these strains and their assignment as new strains: in fact the genome analysis of the different strain ATCC 27673 isolated from sewage showed a genetically distinct strain within this genetically monomorphic subspecies with respect to other strains isolated from human feces and deriving by ingestion of probiotic *B. animalis* susbp. lactis (Loquasto et al., 2013). Cells grown on TPY characteristically resemble bones (Fig. 2.4, panel 6b). The optimum growth temperature is 39–42°C. No growth occurs on agar plates exposed to air, but 10% oxygen in the headspace atmosphere above liquid media is tolerated. Growth occurs in milk or milk-based media. The molar ratio of acetate to lactate from glucose metabolism is about 10:1 under anaerobic conditions. Strains have been isolated from rabbit and chicken feces, fermented milk samples, human and infant feces, and from sewage. The peptidoglycan structure is L-Lys(L-Orn)-L-Ser-(L-Ala)-L-Ala2. The DNA G + C content is 61.0 mol.%. The genome sequence of the type strain DSM 10140T has been described by Barrangou et al. (2009). The type strain, isolated from fermented milk, is UR1T (=LMG 18314T=DSM 10140T=JCM 10602T). Reference strains are ATCC 27536 from chicken feces, ATCC 27674 from rabbit feces, ATCC 27673 from sewage, and ATCC 700541 from yogurt.

2.5.8 Bifidobacterium asteroides (Scardovi and Trovatelli, 1969)

B. asteroides, one of three species isolated from honeybee gut, is the only one found in the intestine of *Apis mellifera* irrespective of geographical provenance (Scardovi and Trovatelli, 1969): in fact, *B. asteroides* has been found in *A. mellifera* from Europe (Italy, France, Norway, Bulgaria, Russia, Greece, etc.) and from Malaysia and the Philippines. This may suggest that genetic of the host species could control factors for *B. asteroides* colonization.

The significance and the origin of bifidobacteria in the insect gut is presently unknown. *B. asteroides* was the most frequently isolated *Bifidobacterium* species in honeybee (Killer et al., 2010). Cells grown anaerobically in fresh rich media are 2–2.5 µm long, pear-shaped or slightly curved, tend to have pointed ends and are usually arranged radially around a mass of common hold-fast material (Fig. 2.4, panel 7). Colonies are circular, smooth, and convex and feature a uniformly entire edge and glistening surface; their stickiness is such that a colony can be removed by needle and can hardly be dispersed in water. Growth in static fluid culture tends to adhere to glass walls and to leave the liquid clear. A study of the presence of isozymes of transaldolase and 6-phosphogluconate dehydrogenase (6-PGD) in 85 strains detected eight transaldolases and nine 6–PGDs (Scardovi et al., 1979a,b). Of the 224 strains tested for the presence of plasmids, 33% contained a large variety of extrachromosomal elements of varying molecular weight (Sgorbati et al., 1982). Preliminary data on the structural relatedness among plasmids were collected by means of blot hybridization using many selected unrestricted plasmic probes. Thirteen plasmids were found in *B. asteroides* and their frequencies and distribution reported (Sgorbati et al., 1986), although the functions coded are still unknown. The peptidoglycan structure is L-Lys-Gly. The DNA G + C content is 59. The highest 16S rRNA gene sequence similarity of LMG 10735^T was to *B. coryneforme* LMG 18911^T and *B. indicum* LMG 11587^T (98%)

(Lugli et al., 2014). The genome sequence of the type strain DSM 20089^{T} has been described by Sun et al. (2015). The type strain is C51^T (=ATCC 25910^T=DSM 20089^{T} =LMG 10735^{T}) from the hindgut of Italian honeybees. Reference strain is ATCC 25909 = DSM 20431 from Italian honeybee.

2.5.9 Bifidobacterium avesanii (Michelini et al., 2016a)

This is one of the recently described species from nonhuman primate intestinal tract, which was shown to be a very rich source of bifidobacteria both for total abundance and for bifidobacterial species diversity. *B. avesanii* was named after Doctor Alberto Avesani, the Founding Father of Natura Viva Garda Zoological Park S.r.l., (Verona, Italy). It was isolated from a fecal sample of *S. oedipus* (red cotton tamarin which belongs to the family *Callitricidae* of New World monkey). Cells grown in TPY in anaerobiosis are rods of various shapes that form a branched structure with a "Y" on both sides (Fig. 2.4, panel 8). Well-separated colonies on the surface of TPY agar plates have a diameter of approximately1.5–2.5 mm after 2 days incubation under anaerobic conditions, and are white, opaque, smooth, and circular with entire edges, whereas embedded colonies are lens-shaped or elliptical. Cells can also grow under aerophilic and microaerophilic conditions. Growth in TPY broth occurs in the range 25–50°C, but not at 20 or 56°C (after 24–48 h). Cells can grow in the pH range of 4.0–7.5. Optimal conditions for growth occur at pH 6 and 40°C. The peptidoglycan type is A4β L-Orn(Lys)-D-Ser-D-Glu.The DNA G + C content of the type strain is 65.9 mol.%. The highest level of 16S rRNA gene sequence similarities has been shown with *B. scardovii* DSM 13734^T (mean value 96.6%). The type strain TRE C^T (=DSM 106805^T=JCM 30943 ^T) was isolated from the feces of an adult subject of the cotton-top tamarin. This species has been described based on a single strain.

2.5.10 Bifidobacterium biavatii (Endo et al., 2012)

Endo for the first time described bifidobacterial species from nonhuman primates. *B. biavatii* was named after Professor Bruno Biavati, in honor of his research on bifidobacteria. It was isolated from *S. mida* belonging to the *Callitricidae* family of New World monkeys. Cells are irregularly shaped rods, usually swollen and branched, measuring $0.5-1.0 \times 3-8 \mu m$ (Fig. 2.4, panel 9). Colonies, after incubation for two days on MRS agar supplemented with 50 mg (I - 1) L-cysteine are white, smooth, and approximately 1–2 mm in diameter under anaerobic conditions. Strain grows well at 30 and 42°C but not at 26 or 45°C. Cells grow at pH 5.0–7.0. The peptidoglycan type is L-Lys-L-Ser. The DNA G + C content of the type strain is 63.1 mol.%. Phylogenetic analysis based on 16S rRNA gene forms a couple between the species and *B. bifidum*. The type strain is AFB23-4^T (=JCM 17299^T=DSM 23969^T) isolated from feces of red-handed tamarin, collected at Cape Town, South Africa, in 2009. This species has been described based on a single strain.

2.5.11 Bifidobacterium bifidum (Orla-Jensen, 1924; Tissier, 1900)

B. bifidum is a type species of the genus Bifidobacterium, being the first described species. B. bifidum has been isolated from the feces of a breast-fed infant in 1899 by Tissier of the Pasteur Institute. It is one of the most fastidious bifidobacterial species: it grows poorly in lab medium because it requires special growth factor. This special growth factor has been called "Bifidus Factor." Human milk and hog gastric mucin were especially rich in the bifidus factor, of which the active substances have been identified as oligosaccharides containing, for example, N-acetyl-D-glucosamine, lacto-N-tetraose, lacto-N-fucopentose, galactose-acetyl glucosaminide (Furukawa et al., 1968). Intensive studies have been devoted to human milk oligosaccharides (HMOs) composition and they have been classified into 13 core structures that consist of lactose, at the reducing end, elongated by β 1–3-linked lacto-N-biose I (Gal β 1–3GlcNAc, LNB, type 1 chain) and/or β 1–3/6-linked N-acetyl-lactosamine (Gal β 1-4GlcNAc, LacNAc, type 2 chain) (Kobata, 2010). These core structures are frequently modified by fucose and sialic acid residues via $\alpha 1-2/3/4$ and $\alpha 2-3/6$ linkages, respectively. The unique feature of HMOs is the predominance of type 1 chains, and such a composition has not been observed in milk oligosaccharides from other mammals, including anthropoids (Asakuma et al., 2011). A peculiarity of this species is the ability to degrade mucin, hydrolyzing the glycosidic bonds of mucin and utilizing it as the sole carbon source (Guglielmetti et al., 2009; Turroni et al., 2014). B. bifidum is one of the most utilized species in probiotic foods, supplements, and pharmaceutical preparations. By regulatory definition, microbial cells must be alive in a sufficient number to define a product as probiotic. Due to high sensitivity to stresses, such as acidity and, in particular, oxygen, the commercial use of *B. bifidum* as a probiotic is not straightforward. Strategies to preserve probiotic cell viability utilizing microencapsulation, and the addition of prebiotic compounds to the preparation

could be helpful to resolve this critical point. Moreover, the limited biomass yields generally obtained in industrial fermentations due to the intrinsic stress sensitivity of *B. bifidum* remains the principal problem. Some cells grown in TPY agar stabs have an "amphora-like" cell shape (Fig. 2.4, panel 10). They are anaerobic, dying rapidly in aerobic subcultures. DNA G + C content is 61% mol. The genome sequence of the type strain JCM 1255^T has been described by Morita et al. (2015d). Isolated from feces of human adults and infants, suckling calf and human vagina. The type strain, isolated from stool of breast-fed infant, is TI^T (=ATCC 29521^T=DSM 20456^T=JMC 1255^T). Reference strains are: ATCC 11863(=DSM 20239=JMC 1209) from stool of infants, ATCC 15696 (=JCM 7004) from intestine of infant, ATCC 35914 from human feces USA, DSM 20082 (=JCM 1254), and DSM 20215 from intestine of adult.

2.5.12 Bifidobacterium bohemicum (Killer et al., 2011)

B. bohemicum was named *bohemicum* from Bohemia, referring to the Czech Republic where the bacterium was first isolated. It belongs to the group of bifidobacteria isolated from bumblebees. Cells are very irregularly shaped rods (0.2–0.4 mm wide and 0.6–1.0 mm long) with frequent constrictions and deformities. They are organized in chains in filament forms (Fig. 2.4, panel 11) Strictly anaerobic colonies on TPY agar under anaerobic conditions are cream colored, circular in shape with sharp, entire edges, frequently with small irregular cores. They appear in variable shapes and sizes (0.13–2.28 mm in diameter after 2 days of incubation) (Fig. 2.4, panel 11). Optimum temperature for growth is 37°C, with a minimum of 10°C and a maximum of 40°C. No growth occurs at 5 or 45°C. In TPY broth, the minimum initial pH for growth is pH 5 within a period of 24–48 h (weak growth observed at pH 4.5, and no growth observed at lower pH). Cells contain relatively large amounts of palmitic, oleic, and stearic acids. DNA G + C content is 51.2%. The highest level of 16S rRNA gene sequence similarities have been shown with *B. asteroides* YIT 11866^T, *B. indicum* JCM 1302^T, and *B. coryneforme* ATCC 25911^T (96.2, 96.0, and 95.9%, respectively). The type strain, JEMLUCVIII-4^T (=DSM 22767^T=CCM7729^T), was isolated from the digestive tract contents of a bumblebee (*Bombus lucorum*) sampled from South Bohemia, Czech Republic, in 2007.

2.5.13 Bifidobacterium bombi (Killer et al., 2009)

B. bombi strains were isolated from the digestive tracts of different bumblebee species (Bombus lucorum, Bombus pascuorum, and Bombus lapidarius). New bifidobacterial species have been isolated from insects by Scardovi and Trovatelli (1969) and since then no further species have been described in insects. Recently Killer et al. (2009) described the new bifidobacterial species *B. bombi* from bumblebees, which are, after honeybees, the second most valuable pollinators which are affected by population decline due to infectious diseases (Koch et al., 2012). Considering the probiotic activity of bifidobacteria in maintaining gut microbiota homeostasis especially for defense against pathogens and parasites, the study of these strains could be important also for their possible future probiotic application. Cells of *B. bombi* are short and irregularly shaped rods (0.4–0.5 μm wide and 0.9–1.1 μm long) (Fig. 2.4, panel 12). In culture, the appearance resembles dividing bacilli-form cells, frequently forming filaments, with irregular contractions along the cells and bifurcations. Strictly anaerobic. Colonies on TPY agar under anaerobic conditions are white, circular and low convex with sharp, undulate edges. Colonies reach 1.0–1.7 mm in diameter after 3 days of incubation. Good growth occurs at 10–37°C (optimum 30°C; maximum 37°C); very limited growth at 47°C. Minimal initial pH for growth is 4.5. The dominant fatty acids were oleic (C18:1; 7.49%) and arachidic (C20:0; 7.18%) acids. The type strain BluCI/TP^T contained a relatively high level of linoleic acid (C18:2; 7.34%). Heneicosanoic acid (C21:0) was not present. Four other fatty acids were present: palmitic (C16:0; 7.14%), stearic (C18:0; 5.91%), behenic (C22:0; 5.87%) and tricosanoic (C23:0; 5.38%) acids. The content of odd-numbered fatty acids was high (41.2% w/v). Increased content of unsaturated fatty acids corresponded with the lower optimal temperature for growth of BluCI/TP^T. The DNAG + C content of the type strain is 47.3 mol.%. The highest 16S rRNA gene sequence similarity of BluCI/TP^T was to B. asteroides CCUG 24607^T (93%). The type strain is BluCI/ TP^{T} (=ATCC BAA-1567^T=DSM19703^T), isolated from the digestive tract contents of *Bombus lucorum* from central Bohemia, Czech Republic.

2.5.14 Bifidobacterium boum (Scardovi et al., 1979a)

From a large number of animal strains surveyed by DNA–DNA hybridization, 36 strains from the rumen and five from piglet feces were assigned to the new species *B. boum* (Fig. 2.4, panel 13) that can be distinguished between other bifidobacterial species by means of transaldolase and 6-phosphogluconic dehydrogenase electhrophoretic forms and by PAGE protein electrophoresis (Biavati et al., 1982; Scardovi et al., 1979b). Cells are very irregular in shape,

generally occurring in pairs ($0.6-0.7 \,\mu$ m wide and $2.0-5.0 \,\mu$ m long); the paired cells form wide angles, with protuberances, knobs, or branching near the junction. Clumps of branched cells are frequent. Under not strictly anaerobiosis, extensive and complicated branching is frequent on cells with large rounded extremities (Fig. 2.4, panel 13). The sediment in liquid TPY cultures is flaky or in mold-like pellets and is dispersed only with difficulty; the supernatant is clear. Surface colonies in TPY are smooth, convex, from cream-colored to white, glistening, and soft; entire edges. Rather aerotolerant; grows in air + CO_2 . CO_2 is not required for growth under anaerobic conditions. Good growth occurs at 39–40°C (minimum, about 25°C; maximum, about 45°C; no growth at 20 or 46°C). Initial optimum pH is 6.5–7.0; growth is retarded at 6.0 or 7.4; no growth at 5.0 or 8.0. The molar ratio of lactic to acetic acid from the fermentation of glucose is about 1:3. L(+) lactic acid is produced. Small amounts of formic acid are produced. Propionic and butyric acids are not produced. The interpeptide bridge of the peptidoglycan of the cell wall of the type strain is Lys-Ser-Glu. The G + C content of the DNA is 60.0 mol.%. The DNA of this species is 60%–70% related to that of B. thermophilum, but is essentially unrelated to any other species (or homology group) of Bifidobacterium. ANI value analysis also showed the high value of 94.9%, which is very close to the generally accepted threshold (95%) for species recognition, between B. thermophilum JCM 1207^T and B. boum LMG 10736^T. Isolated from rumen of cattle and feces of pigs; also found in sewage. The type strain is Ru 917^T (=ATCC 27917^T=DSM 20432^T=JCM 1211^T), isolated from bovine rumen.

2.5.15 Bifidobacterium breve (Reuter, 1963)

Strain S50 (ATCC 15698), first described as "B. parvulorum biovar a" and strain S17c (ATCC 15699) first described as "B. parvulorum biovar b" (Reuter, 1963) were considered as synonyms of B. breve (Scardovi et al., 1971). B. breve with B. longum subsp. infantis and B. bifidum have been frequently detected in the gut of human infants, and their monophyletic origin suggests that there might be a high degree of correlation between the genomic features of these three *Bifidobacterium* species and their adaptability to the intestine of infants (Kwak et al., 2016). B. breve, B. longum subsp. infantis, and B. bifidum have additional oligosaccharide-degrading genes especially related to HMO degradation: this finding and the frequent detection of *B. breve*, *B. longum* subsp. *infantis*, and *B. bifidum* in guts of breast-fed infants suggest that carbohydrate metabolism, specifically HMO utilization, could be a major driving force for the evolution of these three gut-adapted species. So far B. breve and B. longum subsp. longum between the bifidobacterial species of human habitat are the only species to harbor plasmid (Lee and O'Sullivan, 2010; Sgorbati et al., 1982). Among bifidobacteria, strains of *B. breve* produce high levels of CLA (conjugated linoleic acid) (Coakley et al., 2003). CLA has been shown to exert several health benefits and has been demonstrated to have potent antiinflammatory, immunomodulatory, antiobese, and anticarcinogenic activity, with the ability to improve biomarkers of cardiovascular health (Russell et al., 2011). The genome sequence of the type strain JCM 1192^T has been described by Morita et al. (2015c). Cells are short, slender, or thick and with or without bifurcations, a morphology that suggests the specific epithet (Fig. 2.4, panel 14). The peptidoglycan structure is L-Lys-Gly. The G + C content of the DNA is 58 mol.%. The representatives of *B. breve* have been isolated from the feces of infant and suckling calf, human vagina, and sewage. The type strain, isolated from infant intestine, is SI^T (=ATCC 15700^T=DSM 20213^T=JCM 1192^T). Reference strains are: ATCC 15698 (=DSM 20091=JCM 1273) and ATCC 15701 (=JCM 7016) from intestine of human infant.

2.5.16 Bifidobacterium callithricos (Endo et al., 2012)

B. callithricos together with *B. reuteri* have been isolated from *C. jacchus* (Common marmoset) kept in animal houses in Western Cape, South Africa. Based on DGGE (denaturing gel gradient electrophoresis) analysis, Endo et al. (2010) suggested that species may be a predominant *Bifidobacterium* species in feces of marmoset and specific interaction, for example, nutrient requirement or specific adhesion to GIT, between host and the species would occur. Cells are irregularly shaped rods with rounded or tapered end, usually swollen and branched, measuring $0.6-1.2 \times 3-8 \mu m$ (Fig. 2.4, panel 15). Cells usually occur singly, in pairs or in chains. Facultatively anaerobic. Colonies on MRS agar supplemented with 50 mg/L L-cysteine after incubation for 2 days are white, smooth, and approximately 1 mm in diameter under anaerobic conditions. Strain grows well at 30 and 42°C and weakly at 26°C, but not at 45°C. Cells grow at pH 5.0–8.0. The peptidoglycan type is L-Lys (L-Orn)-D-Asp. Phylogenetic analysis of 16S rRNA gene places the species in the *B. adolescentis* group. The G + C content of the DNA is 64.3 mol.% (Tm). The highest 16S rRNA gene sequence similarities were found with *B. catenulatum* and *B. pseudocatenulatum* (96.2 and 96.1%, respectively). The type strain is AFB22-5^T (=JCM 17296^T=DSM 23973^T) from feces of common marmoset, collected at Cape Town, South Africa, in 2009. This species has been described based on a single strain but recently other strains belonging to this species have been found in red cotton tamarin housed in Italy (personal communication, 2016).

2.5.17 Bifidobacterium catenulatum (Scardovi and Crociani, 1974)

This species is reported as one of the most frequently isolated from adult and infant feces (Biavati et al., 1984, 1986). Lauer and Kandler (1983) on the basis of DNA–DNA hybridization showed high genetic similarity values (72%–74%) between B. catenulatum and B. pseudocatenulatum. However, the G + C content of their DNA differs by 3 mol.%; none of the strains genetically identified as *B. catenulatum* ferments starch or mannose, whereas strains of *B. pseudoca*tenulatum ferment these compounds. The isozymes of transaldolase and 6PGD of the two species migrated very differently (Scardovi et al., 1979b); furthermore, B. catenulatum, differently from B. pseudocatenulatum, has never been isolated from feces of suckling calves. Cell are generally clustered in group of three or four globular elements arranged in chains 1.3–1.7 µm in diameter; when not fragmented, the chains are 0.6–0.8 µm by 2.0–4.0 µm. The distal ends of the chains are usually tapered. Distinct branchings, club-swellings, or spatula-like extremities are generally absent (Fig. 2.4, panel 16). Surface colonies are smooth, convex, entire edges, cream colored to white, glistening, and soft. Anaerobic; CO_2 is without effect upon oxygen sensitivity or anaerobic growth. Temperature relationships: good growth occurs at 39-41°C (minimum 28-28.5°C; maximum, about 42.5-43°C; no growth at 26 or 44°C). Initial optimum pH is 6.5–6.9; growth is retarded at 6.3 or 7.2; no growth at 4.5 or 8.0. Lactic and acetic acids in a molar ratio of 1:2.15 are produced in TPY medium. Isomeric type of lactic acid produced: L(+). CO₂ is formed only in the fermentation of gluconate. The G + C content of the DNA is 54.7%. The highest 16S rRNA gene sequence similarity of LMG 11043^T was to *B. pseudocatenulatum* LMG 10505^T (98.9%) (Lugli et al., 2014). Investigation of ANI (Average Nucleotide Identity) values based on bifidobacterial genomes showed the value of similarity of 96.9% between B. catenulatum and B. kashiwanohense DSM 21854^T. When two or more genomes show ANI values of \geq 94% they are considered closely related (high probability to belong to the same species) (Lugli et al., 2014). The genome sequence of the type strain JCM 1194^T has been described by Morita et al. (2015e). B. catenulatum has been isolated from feces of infant and adult human, human vagina, and sewage. The type strain, isolated from human feces, is B669^T (=ATCC 27539^T=DSM 16992^T=JCM 1194^T). Reference strains are ATCC 27675 from human feces; ATCC 27676, ATCC 27677, and DSM 20224 (=JCM 7130) from sewage.

2.5.18 Bifidobacterium choerinum (Biavati et al., 1982; Scardovi et al., 1979a)

B. choerinum was originally considered an autochthonous *Bifidobacterium* species of the pig that is well adapted to the gut of preweaned piglets (Scardovi et al., 1979a). However, *B. choerinum* has also been recently isolated from young ruminant, both lambs and calves, feces (Bunešová et al., 2012; Vlková et al., 2010). *B. choerinum* showed a developed repertoire of genes allowing for the utilization of plant polysaccharides (Kelly et al., 2016). Cells are spindle- or lemon-shaped, 1.5–3.5 μ m long when freshly isolated. Cells occur singly or in pairs; after subculture in TPY medium, the cells are distinctly longer (6–7 μ m), with irregular contours; very few cells have branches (Fig. 2.4, panel 17). Cells are generally more uniform in shape when grown in liquid TPY medium; they are elongated (10–12 μ m), slightly bent, and with rounded ends. Some cells with enlarged extremities occur in star-like aggregates. Surface colonies (TPY medium) are smooth, convex, cream colored to white, glistening, and soft; entire edges. Relationships to oxygen and CO₂. Slightly anaerobic. Effect of CO₂ not detectable. Optimum temperature for growth is 41–42.5°C, with a minimum of 27°C and a maximum of 44.5–45°C. No growth occurs at 25 or 46°C. Initial optimum pH at 6.5; growth is retarded at 6.2 or 7.6; no growth at 5.2 or 8.5 pH (after 2 days). The molar ratio of lactic to acetic acid ranges from 1:4 to 1:8. Small amounts of formic acid are produced. Propionic and butyric acids are not produced. The peptidoglycan structure is Orn(Lys)-Ser-Ala2. The G + C content of the DNA is 66.3%. Isolated from feces of piglet, lamb, calf, and sewage. Type strain: SU 806^T (=ATCC 27686^T=DSM 20434^T).

2.5.19 Bifidobacterium commune (Praet et al., 2015)

Recent losses of *A. mellifera* and bumblebee species, and the potential association of these declines with various infectious agents, call for a better understanding of the bees' microbiota. The gut microbiota may contribute to honeybee and bumblebee health and new knowledge about the presence of beneficial bacteria could help in individuating strategies, utilizing them to inhibit the bee pathogens, for example, *Melissococcus plutonius* and *Paenibacillus larvae* (Audisio et al., 2011) and promote the digestion of carbohydrates (Engel et al., 2012). The gut microbiota of honeybees and bumblebees consists of a distinctive set of bacterial species including lactic acid bacteria (e.g., *L. apis, L. bombi, L. kunkeei, L. johnsonii, Fructobacillus fructosus,* and *Enterococcus faecium*) and bifidobacteria (*B. asteroides, B. coryneforme, B. indicum,* and *B. actinocoloniiforme, B. bohemicum,* and *B. bombi*). Recently the new species *B. commune* has been described in the gut of multiple bumblebee species (e.g., *Bombus alpinus, Bombus caliginosus,* and *Bombus fraternus*). Cells

are bifurcated and elongated cocci (0.5–1 μ m wide and 2 μ m long). Growth is observed after 48 h on MTPY and MRS agar at 37°C in an anaerobic and microaerobic atmosphere. Colonies on MRS agar are white to beige, 1 mm, round, and shiny. Growth was observed in MRS broth at 10 and 15°C (although weak), 37°C, and pH 5 and pH 7, but not at 45°C, pH 3, and pH 9. Only the L-isomeric form of lactic acid is produced. The DNA G + C content is 54.3%. Analysis of the 16S rRNA gene sequence revealed that LMG 28292^T was most closely related to the *B. bohemicum* (94.1%) and *B. bombi* (93.3%) type strains, which also have been isolated from bumblebee gut specimens. The type strain LMG 28292^T (=DSM 28792^T), was isolated from the gut of a *Bo. lapidarius* bumblebee in 2013 in Ghent, Belgium. Reference strain is LMG 28626 from bumblebee gut.

2.5.20 Bifidobacterium coryneforme (Biavati et al., 1982; Scardovi and Trovatelli, 1969)

In 1969, Scardovi and Trovatelli isolated the three new species B. asteroides, B. coryneforme, and B. indicum from the intestine of honeybee. In particular these three species differed in geographical host habitat: B. asteroides was isolated from A. mellifera L. in Europe and Philippine; B. indicum was isolated from A. cerana indica L. from Malaysia, Japan, and the Philippines and from A. dorsata from India and the Philippines; B. coryneforme is represented by strains occasionally isolated from different honey-bee samples from A. mellifera (Giordani and Scardovi, 1970; Scardovi and Trovatelli, 1969). In 1980, when the amended edition of Approved Lists of Bacterial Names was edited, the new species B. coryneforme was inadvertently omitted and consequently it had no taxonomic standing. Therefore, in 1982 the reinstatement of the name B. coryneforme was proposed (Biavati et al., 1982). The type strains of B. coryneforme and B. indicum were shown to have identical profiles by DGGE and BOX-PCR, and the recently sequenced genome showed a very high identity (ANI = 98.6%) between B. coryneforme and B. indicum (Lugli et al., 2014), starting the discussion on the taxonomic status of both species. Most probably these two species will be unified in a single species with a description of two subspecies. Cells with morphology suggesting corynebacteria are coccoid, oval, or club-shaped rods that occur singly, in pairs, and in clumps 0.7–1.2 µm by 0.9–3.0 µm (Fig. 2.4, panel 18). Colonies on blood agar plates incubated anaerobically for 48 h at 37°C are punctiform to 1 mm in diameter, circular, entire, convex, translucent to semiopaque, grayish white, shiny, and smooth. Strict anaerobic. Optimum growth temperature is 37–38°C. Only the L-isomeric form of lactic acid is produced. The molar ratio of lactic to acetic acid ranges from 1:3.5. The peptidoglycan structure is L-Lys-D-Asp. The DNA G + C content is 62.4 mol.%. The type strain is $C215^{T}$ (=ATCC 25911^{T} =DSM 20216^{T} =JCM 5819^{T}) isolated from the intestine of honeybee, A. mellifera subsp. mellifera and subsp. caucasica from Norway.

2.5.21 Bifidobacterium crudilactis (Delcenserie et al., 2007)

This species was first isolated in raw milk and raw milk cheese in France. The raw milk samples from cows and goats used for cheese production were mixtures of milk samples received from different farms. In addition, Watanabe et al. (2009) isolated another new species, named *Bifidobacterium mongoliense*, from airag, a traditional Mongolian fermented product from mare milk. The species *B. crudilactis* and *B. mongoliense* were detected in raw milk cheeses from the Vercors region (Bunešová et al., 2012). The origin of these bacteria is not known, yet animal feces are likely the source because raw milk was collected from farms; however, their presence probably contributes to the specific organoleptic and technologic characteristics of those cheeses (Delcenserie et al., 2013). The cells are irregularly shaped rods, curved, arranged singly, with occasional bifurcations and spatulated extremities, and 0.5×1.5 –2.5 µm in size in 24 h TPY cultures (Fig. 2.4, panel 19). Colonies on TPY agar at 39°C under anaerobic conditions are cream, circular, and convex with whole edges. They reach a diameter of up to 1 mm within 48 h. They have a reduced diameter (less than 1 mm) under aerobic conditions within the same time. Optimum growth temperature for the 10 strains is 39°C, with a maximum of 45°C (within 8 days) and a minimum of 5°C (4°C for three strains, within 14 days). It is able to grow at 4°C. In TPY, the minimum initial pH for growth is 4.7 (within 48 h; weak growth at pH 4.4 within 15 days, and not at pH 3.8). Ten strains were isolated from raw milks and raw milk cheeses (Delcenserie et al., 2007). The mean DNA G + C content for 10 strains is 55.3 mol.%. The DNA G + C content of $FR62/b/3^{T}$ is 56.4 mol.%. Analysis of the 16S rRNA gene sequence revealed that FR62/b/3^T was most closely related to the *B. psychraerophilum* LMG 21775^T (99.8%) followed by *B. mini*mum (96.6%). The type strain is $FR62/b/3^{T}$ (=LMG 23609^T=CNCM I-3342^T) isolated from raw cow milk in France.

2.5.22 Bifidobacterium cuniculi (Scardovi et al., 1979a,b)

B. cuniculi inhabits rabbit intestinal tracts, it has been isolated from stomach, small and large intestine in rabbits: the amount is highest in the large intestine, second in the jejunum, and none is found in the duodenum (Niu

et al., 1996). Cells are stout rods, 2–4 µm in length, with irregular contours and small central swellings or knobs. Chains of cells, with apparently pedunculated symmetrical sprouts, occur rarely. Extensive branching occurs only in liquid cultures with fermentable sugars, such as starch or maltose. In TPY liquid medium, the cells are generally shorter and have nearly regular contours. This general morphology is very similar to that of *B. pseudolongum* subsp. *globosum*; however, the short knobs or protuberances, often at the middle of the cells, are characteristic of *B. cuniculi* cells grown in solid TPY medium (Fig. 2.4, panel 20). Surface colonies (TPY medium) are smooth, convex, cream colored to white, glistening, and soft, with entire edges. Highly anaerobic; CO₂ has no effect upon oxygen sensitivity and anaerobic growth. Optimum growth at 36–38°C; minimum, about 29°C; maximum, 42.5–43°C; no growth occurs at 28.5 or 44°C. Initial optimum pH is 6.7–7.1; growth is retarded at 6.4 and 7.6; no growth occurs at 5.0 or 8.5.The G + C content of the DNA is 64.1 mol.%. The peptidoglycan structure is Orn(Lys)-Ala(Ser)-Ala2. The type strain is RA93^T (=ATCC 27916^T=DSM 20435^T) from feces of rabbit.

2.5.23 Bdentium (Scardovi and Crociani, 1974)

B. dentium has been isolated from oral cavity and due to the fact that its presence is associated with dental caries, it is considered as a potential cariogenic microorganism. Recent studies showed that its numbers are under the detection limit or very low on caries-free tooth surfaces, but high in deep lesions, and data from studies of denture plaque provide further insight into its ecology (Henne et al., 2015; Mantzourani et al., 2010). Unlike mutans streptococci, B. dentium does not colonize hard surfaces per se, since denture plaque associated with denture stomatitis harbored high levels of mutans streptococci, lactobacilli, and yeasts, but not B. dentium (Kaur et al., 2013). This indicates that *B. dentium* does not simply colonize intact dental hard surfaces but instead suggests that lesions, after initiation by other species including *Streptococcus mutans*, facilitate the attachment and proliferation of *B. dentium*. In contrast to mutans-streptococci the presence of this species might therefore be more of a result than the cause of initial lesions. Thus, B. dentium and mutans streptococci in the mouth are significant independent factors (Kaur et al., 2013). ATCC 15423 and ATCC 15424, first described as "Actinomyces eriksonii" by Georg et al. (1965), were considered as synonyms of *B. dentium* (Biavati et al., 1982). Cells are slender rods 2–8 µm long, with the extremities often swollen, dumbbell shaped, or enlarged to give a spatula-like appearance; branchings occur at the distal ends of the cells; chains of globular elements are absent. The general morphology is similar to that of *B. longum* (Fig. 2.4, panel 21). Surface colonies are flat or umbonate when aged, with entire or undulate margins, often raised, glistening, cream colored to white, soft. Anaerobic; CO₂ does not affect sensitivity to oxygen or anaerobic growth. Optimum temperature is 39–41°C; minimum, 24–25°C; maximum 42°C; no growth at 23 or 44°C. Initial optimum pH is 6.5–6.9; delayed growth at 6.3 or 7.2; no growth after 2 days at 4.5 or 8.0. Lactic and acetic acids in a molar ratio of 1:2.5 are produced in TPY medium. Isomeric type of lactic acid produced: L(+). CO₂ is formed only in the fermentation of gluconate. The peptidoglycan structure is L-Lys(L-Orn)-D-Asp. The DNA G + C content is 61.2 mol.%. B. dentium has been isolated mostly from human dental caries and oral cavities but also from feces of human adult, human vagina, human clinical samples, and from feces of chimpanzee (D'Aimmo et al., 2014). Type strain: B 764^r(=ATCC 27534^r=DSM 20436^r=JCM 1195^r). Reference strains: ATCC 15423 (=DSM 20084) lung abscess in adult male; ATCC 15424 from pleural fluid from adult male; ATCC 27678 from human feces; ATCC 27679 from human vagina; ATCC 27680 from human oral cavity; DSM 20221 (=JCM 7135) from human dental caries; DSM 28535 from chimpanzee feces; DSM 28536 from chimpanzee feces.

2.5.24 Bifidobacterium eulemuris (Michelini et al., 2016b)

This species is part of a group of bifibidobaterial species isolated from nonhuman primates. It has been isolated from black lemur (*Eulemur macaco*) belonging to *Callitricidae* family of New World monkey. Its morphology is very similar to that of *B. lemurum* isolated from *L. catta* but the cells are considerably smaller. Phylogenetic analysis based on 16S rRNA gene sequences showed the highest levels of sequence similarity between the type strain of *B. eulemuris* and *B. lemurum* showed DSM 28807T (99.3%), but DNA–DNA relatedness with *B. lemurum* DSM 28807^T showed similarity in values of 65.4–67.2%. *B. pullorum* LMG 21816^T and *B. longum* subsp. *infantis* ATCC 15697^T (96.4 and 96.3%, respectively) are the next most similar strains. Cells grown in TPY broth are rod-shaped, frequently forming filaments, with irregular contractions along the cells and bifurcations (2–5 μ m) (Fig. 2.4, panel 22). Spatially well-separated colonies on the surface of TPY agar under anaerobic conditions are white, opaque, smooth, and circular with entire edges, while embedded colonies are lens-shaped or elliptical. Colonies reach 1.0–2.5 mm in diameter after 3 days of incubation. The temperature range is 35–42°C; no growth occurs at 30 or 47°C. The optimum temperature for growth is 37–42°C. Grows at pH 5.5–7.0, with an optimum at pH 6.5–7.0. Can grow in milk, under both microaerophilic and

anaerobic conditions. The peptidoglycan type is A3a L-Lys-L-Ser-L-Thr-L-Ala. The DNA G + C content of the type strain is 62.3 mol.%. Phylogenetic analysis of the 16S rRNA gene sequence places the species in the *B. longum* subgroup of the genus *Bifidobacterium*. The type strain, LMM_E3^T (=DSM 100216^T=JCM30801^T), was isolated in February 2014 from a fresh fecal sample of an adult black lemur (*E. macaco*) that was housed under seminatural conditions in the Natura Viva Garda Zoological Park S.r.l. (Verona, Italy). This species has been described based on a single strain.

2.5.25 Bifidobacterium fecale (Choi et al., 2014)

This species was isolated from feces of a 2-week old baby. Colonies on TOS-propionate agar plates are white, sleek, and slightly convex. Cells are Y-shaped short rods, 2.4–3.8 μ m long and 1.2–1.3 μ m wide. Growth occurs only under anaerobic conditions. Cells grow at 30–42°C (optimum 37°C) and in the pH range 5.0–7.0 (optimum pH 7.0). The predominant fatty acids are C16:0, C18:1 ω 9c, and C18:1 ω 9c dimethylacetate. The DNA G + C content of the type strain is 58.6 mol.%. Analysis of the 16S rRNA gene sequence revealed that CU3-7T^T was most closely related to the *B. adolescentis* KCTC 3216^T (98.4%), followed by *B. ruminantium* KCTC 3425^T (97.9%). The type strain is CU3-7^T (=KACC 17904^T=JCM 19861^T), isolated from healthy human feces.

2.5.26 Bifidobacterium gallicum (Lauer, 1990)

The description of this species is based on phenotypic and genotypic characterization of a strain found by Henri Beerens (Institut Pasteur de Lille, France) in 1967 as a human fecal isolate. However, its true habitat remains uncertain because no additional strains have been isolated up to now. Cells are short rods with rounded ends, generally 0.7–0.9 μ m by 1.5–3.0 μ m, mostly arranged in pairs or short chains when grown in liquid culture (Fig. 2.4, panel 23). Irregular elongations and swellings occur in cells obtained from colony growth. Slimy, capsule-like material is excreted in complex media containing meat extract, bacteriological peptone, salts, and fermentable carbohydrates. Colonies are whitish, opaque, round, and entire, and have a soft consistency. The main fermentation product is acetic acid, and its concentration is about 15 times that of L-(+)-lactic acid; in addition, variable amounts of ethanol (and certainly formic acid) are formed. No D-(–)-lactic acid or gas is produced. Metabolism is strictly anaerobic. Optimum growth occurs at 37–39°C; no growth occurs below 30 or at 45°C and above. Growth occurs in the range of pH 5.0–8.0; optimum growth occurs at pH 6.0–7.0. The cell wall polysaccharide comprises a galactan polymer of unknown structure; minor amounts of rhamnose may be found in addition to galactose after growth on less complex media. The peptidoglycan structure is Lys-Ala-Ser. The G + C content of the DNA is 61 mol.%. The type strain P 6^T (=DSM 20093^T=ATCC 49850^T=JCM 8224^T). This species has been described based on a single strain.

2.5.27 Bifidobacterium gallinarum (Watabe et al., 1983)

Barnes and Impey (1972) isolated unidentified *Bifidobacterium* strains from the ceca of chickens. A group of 10 of these strains has been described by Watabe et al. (1983) as *B. gallinarum*. *B. pullorum* LMG 21816^T, *B. saeculare* LMG 14934^T, and *B. gallinarum*. LMG 11586^T set have ANI values above 95% and DNA–DNA homology values of about 62%–68%, showing a high similarity at genomic levels of these species (Biavati et al., 1991; Lugli et al., 2014). Cells are slightly curved, short rods (0.5–1.0 μm wide by 1.0–2.5 μm long) with tapered ends, usually arranged singly or in short chains (Fig. 2.4, panel 24). Swellings or club-shaped cells are frequent. In liquid aged cultures, some cells have short branches. Surface colonies on BL agar after 2 days of anaerobic incubation are punctiform to 1 mm in diameter, circular, entire, flat to convex, gray to grayish white, transparent to translucent, and glistening. Strictly anaerobic. Final pH of glucose or fructose broth: 4.5–5.1. lactic acid and acetic acid in a molar ratio of 1:4.0 in EG broth containing these carbohydrates. Propionic acid and butyric acid are never formed. The peptidoglycan structure is L-Lys-L-Ala-L-Ser. The G + C content of the DNA is 65.7 mol.%. Type strain is Ch206-5^T (=ATCC 33777^T=DSM 20670^T), isolated from chicken cecum.

2.5.28 Bifidobacterium hapali (Michelini et al., 2016c)

This species belongs to the group of species recently isolated from nonhuman primates. It has been isolated from common marmoset (*C. jacchus*) belonging to *Callitricidae* of New World monkeys. Cells growing in TPY broth under anaerobic conditions are rods of various shapes, forming a branched structure with a "Y" at the end (Fig. 2.4, panel 25). Well-isolated colonies on the surface of TPY agar plates reach a diameter of about 1.5–2.5 mm after 2 days of incubation under anaerobic conditions. The colonies are white, opaque, smooth, and circular with entire edges, but when

embedded, the colonies are lens-shaped or elliptical. Cells are able to survive under microaerophilic conditions. The temperature for growth is 25–42°C; no growth at 20 or 44°C. Cells grow at pH 4.5–7.5. The optimal growth conditions are pH 6.5 and 37°C. The major cellular fatty acids are C16:0, C18:1ω9c, and C14:0. The peptidoglycan type is L-Lys (Orn)–L-Ala2-L-Ser. The DNA G + C content of the type strain is 56.4 mol.%. Analysis of the 16S rRNA gene sequence revealed that MRM_8.14T and MRM_9.14 showed 95.4% similarity to *B. stellenboschense* DSM 23968^T. The type strain MRM_8.14^T (=DSM 100202^T=JCM 30799^T) and the reference strain DSM 1002185(=JCM 30800) were isolated from the feces of common marmosets.

2.5.29 Bifidobacterium indicum (Scardovi and Trovatelli, 1969)

B. indicum, as well as B. asteroides and B. coryneforme, are the bifids inhabiting the intestine of honeybees (see B. coryneforme description). A phylogenomic analysis suggests that bifidobacteria associated with the honeybee is of ancient origin relative to bifidobacteria in mammals (Bottacini et al., 2012). Culture-based results and 454 amplicon sequencing demonstrate that bifidobacteria can be found throughout the alimentary tract but reside primarily in the hind gut of honeybees (Anderson et al., 2013; Moran et al., 2012). Cells grown in TPY agar stabs are 2–2.5 μm, generally in pairs, with slightly bifurcated ends to give bone-like appearance. Cells are never in star-like groups (Fig. 2.4, panel 26). Colonies are not distinguishable from those of *B. asteroides*. L(+)lactic and acetic acids are formed from glucose in ratio 1:3. Cell extracts are aldolase negative but possess the HMP path dehydrogenases. Catalase is not formed in anaerobiosis: only cells grown in hemin-containing media are catalase positive. Optimum growth temperature is 37–38°C. The peptidoglycan type is L-Lys-D-Asp. The DNA G + C content of the type strain is 60 mol.%. Sixty-seven percent of the 106 strains of *B. indicum* surveyed for the presence of plasmids were positive (Sgorbati et al., 1982) with three differing plasmids (Sgorbati et al., 1986). Isolated from intestine of A. cerana and A. dorsata (Scardovi and Trovatelli, 1969). Type strain: C 410^T (=ATCC 25912^T=DSM 20214^T=JCM 1302^T) isolated from A. cerana indica (Malaysia). Reference strain is ATCC 25913 from A. cerana indica (Japan). The taxonomic position is uncertain because it has high similarity values (16S rRNA gene, DNA–DNA homology value and ANI) with B. coryneforme, suggesting the unification of these two species (Lugli et al., 2014).

2.5.30 Bifidobacterium kashiwanohense (Morita et al., 2011)

The name of the species derives from Kashiwanoha in Japan, which is the name of the area surrounding the University of Tokyo, where it was originally isolated. This species has been isolated from the feces of a healthy infant in Japan in 2013. In 2015 (Vazquez-Gutierrez et al., 2015a,b) the *B. kashiwanohense* strain PV20-2 was isolated from the feces of an anaemic Kenyan infant obtained during an iron intervention study (Jaeggi et al., 2014): PV20-2 was functionally characterized and selected for its high siderophore activity and high iron internalization activity. Cells are rods measuring 1.0–1.3 μm. Colonies on BL agar after incubation under anaerobic conditions for 2 days at 37°C are beige, smooth, and approximately 1.0 mm in diameter. No growth is observed at 15 or 45°C. Produces L(+) lactic acid from D-glucose. The predominant cellular fatty acids of strains HM2-1 and HM2-2^T were 16:0 and 18:1ω9c, with proportions greater than 18% of the total. The peptidoglycan structure is Glu-Ala-Lys. The DNA G + C content is 56–59 mol.%. Analysis of the 16S rRNA gene sequence revealed that HM2-2^T showed high similarity with *B. catenulatum* JCM 1194^T, *B. pseudocatenulatum* JCM 1200^T, *B. dentium* ATCC 27534^T and *B. angulatum* ATCC 27535^T (97.4, 97.2, 96.7, and 96.5%, respectively). The genome of the type strain has been sequenced by Vazquez-Gutierrez et al. (2015a,b). The type strain is HM2-2^T (=JCM 15439^T=DSM 21854^T), isolated from feces of a healthy infant (1.5 years old) in Japan.

2.5.31 Bifidobacterium lemurum (Modesto et al., 2015)

This species has been isolated from nonhuman primates, particularly *L. catta* (ring-tailed lemur) which is originally from Madagascar. Ring-tailed lemur is an opportunistic omnivore with a wide dietary regime, which includes fruit, leaves, leaf stems, flowers, and so on (Gould, 2006). Dietary specialization in lemur species is always correlated with significant differences in gastrointestinal tract morphology. Indeed *L. catta* shows a somewhat enlarged haustrated cecum, a common adaptation to a herbivorous diet. This cecum harbors an intestinal symbiotic microbiota, and it has been assumed that this facilitates plant cell-wall breakdown and leaf fermentation (Campbell et al., 2000). Cells grown in TPY broth are rods, always coiled, ring-shaped, or having a "Y" shape at the end (Fig. 2.4, panel 27). They are microaerophilic. Well-separated colonies on the surface of TPY agar under anaerobic conditions are white, opaque, smooth, and circular with entire edges, while embedded colonies are lens-shaped or elliptical. Colonies reach 1.0–3.0 mm in diameter after 3 days of incubation. The temperature range for growth is 35–46°C; no growth

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occurs at 30 or 47°C. The optimum temperature for growth is 37–42°C. Grows at pH 5.5–7.0, with optimum growth at pH 6.5–7.0. Grows in milk under both microaerophilic and anaerobic conditions. The peptidoglycan of strain LMC 13T was A3 β L-Orn-L-Ser-L-Ala-L-Thr-L-Ala: this murein type is not unique among members of the genus *Bifidobacterium*, as it has also been found in *B. longum* species suggesting the relatedness of these species. Phylogenetic analysis of the 16S rRNA gene sequence places the species in the *B. longum* subgroup of the genus *Bifidobacterium*. Comparative analysis of 16S rRNA gene sequences showed that strain LMC 13^T showed the highest similarity to *B. longum* subsp. *suis* ATCC 27533^T (96.65%) and *B. saguini* DSM 23967^T (96.64%). The DNA G + C content of the type strain is 57.2 mol.%. The type strain LMC 13^T (=JCM 30168^T=DSM 28807^T) was isolated from fresh fecal samples of an adult subject of the ring-tailed lemur (*L. catta*), housed in February 2014 under semi-natural conditions in Natura Viva Garda Zoological Park S.r.l., Verona, Italy.

2.5.32 Bifidobacterium longum (Mattarelli et al., 2008; Reuter, 1963; Sakata et al., 2002; Yanokura et al., 2015)

B. longum may be considered the most common species of bifidobacteria, being found both in infant and adult feces (Biavati et al., 1984, 1986). In surveys of DNA–DNA relatedness, B. infantis, B. longum, and B. suis have been shown to have hybridization rates of about 70% and higher while possessing more than 97% 16S rRNA gene sequence identity, suggesting interrelationships at the species level. In addition, these species showed high average nucleotide identity (ANI) values ranging from 95.5% to 96.6% (Lugli et al., 2014), which were higher than the 95% cut-off value recommended for species demarcation. B. longum, B. infantis, and B. suis were unified into a single species B. longum by Sakata et al. (2002) and Mattarelli et al. (2008) subsequently described three subspecies: B. longum subsp. longum, B. longum subsp. Infantis, and B. longum subsp. suis. In 2015 the genetic methods MLSA (multilocus sequence analysis), MLST (multilocus sequence typing), and AFLP (amplified fragment length polymorphism), as rapid and simple tests, have been tested to verify the current subspeciation of 25 strains of *B. longum*. Unexpectedly the strains belonging to the subspecies suis on the basis of the genetic method utilized clusters in two groups also corresponding to the ability of strains to possess or not urease activity: the new subspecies *B. longum* subsp. *suillum* has been described (Yanokura et al., 2015). A similar ecological distribution is shared by *B. longum* subsp. *longum* and *B. longum* subsp. *infantis*, which are especially found in the gastrointestinal tracts of humans and infants, respectively; B. longum subsp. suis and B. *longum* subsp. *Suillum* is typically found in pig gastrointestinal tract. Among the species usually found in humans, B. longum subsp. longum and B. breve are apparently the only ones that possess a large variety of plasmids (Sgorbati et al., 1982). Among bifidobacterial species, B. longum subsp. longum and B. longum subsp. infantis are considered one of the most important contributors to host health and representative strains are frequently used as a probiotics fermented products and pharmaceutical preparations. The comparative genomic analysis of 33 B. longum representatives showed the possibility to the presence of a fourth phylogenetic subgroup represented a fifth subspecies, which may be more prevalent in the Chinese population. However, validation of such a fifth subspecies and its possible geographical delineation will require further genome sequencing of additional *B. longum* isolates (O'Callaghan et al., 2015).

2.5.32.1 Bifidobacterium longum subsp. longum (Mattarelli et al., 2008; Reuter, 1963; Sakata et al., 2002)

This species has been used in probiotic compounds for more than 20 years and is one of the most studied bifidobacterial species for beneficial properties. The genome of B. longum subsp. longum NCC 2705 was the first, among bifidobacterial strains, to be completely sequenced. Sequencing the DNA of this strain has revealed much about its metabolic diversity and generated insights into other members of the genus (Schell et al., 2002). The B. longum core-genome contains genes that are predicted to encode xylanases, arabinofuranosidases, and associated ATP-binding cassette (ABC) transporters, which were found to be absent in the *B. breve*, *B. bifidum*, *B. animalis*, and *B. adolescentis* core genomes. This indicates that the conserved ability to metabolize certain, possibly xylose/arabinose-containing plant-derived carbohydrates confers an adaptive advantage to the *B. longum* species possess various genetic adaptations and associated ecological fitness to suit life in the GIT. Moreover, comparative analysis of *B. longum* highlights the versatility of this species and its ability to move with us from infancy to adulthood (O'Callaghan et al., 2015). Cells are elongated (6-8 µm long) with rare terminal bifurcation or clubs (Fig. 2.4, panel 28a). The peptidoglycan structure is L-Orn-L-Ser-L-Ala-L-Thr-L-Ala. The DNAG + C content is 61 mol.%. The genome sequence of the type strain JCM 1217T has been described by Fukuda et al. (2011). Isolated from feces of human adult, infant and suckling calves, human vagina, and sewage. The type strain is E 194bT (variant a) (= ATCC 15707T = DSMZ 20219T = JCM 1217T = NCIMB 702259T = LMG 13197T) isolated from the feces of an adult human. Reference strains are: ATCC 15708 (= JCM 7054) from intestine of infant; DSMZ 20097 from calf feces; ATCC BAA-2753 from human feces (Beijing); ATCC51870 from human feces; ATCC35183 (clinical isolates); ATCC BAA-999 from feces, healthy human female baby, June, 1969; ATCC 55813 from human feces.

2.5.32.2 Bifidobacterium longum subsp. infantis (Mattarelli et al., 2008; Reuter, 1963; Sakata et al., 2002)

Reuter (1963) described B. infantis distinct strains isolated from infant feces basing on for biochemical and serological features. Bifidobacteria strains from the same habitat but fermenting D-xylose, were separated and classified into two additional species, namely B. liberorum [strain S76e (= ATCC 15702)] and B. lactentis [strain 659 (= ATCC 25962], based on differences in other fermented sugars (Reuter, 1963). DNA-data subsequently showed that these species were identical to *B. infantis* (Scardovi et al., 1971). The sequencing of genome of the type strain demonstrated a large number of genes involved in catabolism of complex carbohydrates (Sela et al., 2008). Comparison of the closely related subspecies *longum* and *infantis* demonstrated that the former encodes enzymes for the digestion of plant oligosaccharides, while the latter has evolved the capacity to digest HMOs. Most HMO structures contain either fucose or sialic acid; among species of Bifidobacterium, only B. longum subsp. infantis, B. breve, and B. bifidum produce fucosidases and sialidases, and only subsp. *infantis* is able to digest all HMO structures (Underwood et al., 2015). Cellular morphology is similar to that of many other species of the genus (Fig. 2.4, panel 28b). This species is predominant in the feces of breast-fed infants and is apparently host-specific given that no strains of *B. longum* subsp. *infantis* have been found in the feces of human adults (Biavati et al., 1986). It has been isolated from feces of infant and suckling calk and from human vagina. The peptidoglycan structure is L-Orn-L-Ser-L-Ala-L-Thr-L-Ala. The DNAG + C content is 60.5 mol.%. The genome sequence of the type strain ATCC 15697T has been described by Sela et al. (2008). Type strain is S12T (=ATCC 15697^T=DSM 20088^T=JCM 1222^T) isolated from intestine of infant. Reference strains are isolated from intestine of infants: ATCC 15702 (=DSM 20090=JCM 1272); ATCC 15708; ATCC 17930 (=DSM 20218=JCM 1260); ATCC 25962=JCM 1210.

2.5.32.3 Bifidobacterium longum subsp. suis (Mattarelli et al., 2008; Reuter, 1963; Sakata et al., 2002)

Cells are slender elongated (2–6 µm long) with rare terminal bifurcation or clubs (Fig. 2.4, panel 28c). Colonies are circular, soft, smooth, and white with entire margins. Liquid cultures are at first turbid, after 24–36 h became clear with sediment dispersable at agitation. Optimum temperature is 38–39°C; minimum is 19–20°C, maximum is 44.5–45°C. Skim milk is acidified followed by coagulation in 1–2 days. Strains possess a strong ureolytic activity. High levels of urease activity were present in the cells grown in the absence of urea, suggesting that this enzyme is not inducible (Crociani and Matteuzzi, 1982). The peptidoglycan structure is L-Orn-L-Ser-L-Ala-L-Thr-L-Ala. The DNA G + C content is 62 mol.%. Type strain is Su 859T (=ATCC 27533^T=DSM 20211^T=JCM 1269^T) from feces of a piglet. Reference strains, isolated from feces of piglet, are: ATCC 27531; ATCC 27532 (=JCM 7139).

2.5.32.4 Bifidobacterium longum subsp. suillum (Mattarelli et al., 2008; Reuter, 1963; Sakata et al., 2002; Yanokura et al., 2015)

This subspecies has been described in 2015 based on strains isolated in the 1970s (Matteuzzi and Crociani, 1973). Cells grown in modified Gifu Anaerobic Medium (GAM) broth are rods of various shapes (0.4–0.7 × 1.5–3 µm in size), with rounded or tapered ends, sometimes curved, swollen, and branched (Fig. 2.4, panel 28d). After anaerobic growth at 37°C for 2 days, colonies on modified GAM agar are 2–3 mm in diameter; they are convex, white, opaque, smooth, and circular with entire edges. The temperature range for growth is 20–45°C. The optimum temperature for growth is 25–37°C. Grows at pH 5.0–7.0 with an optimum but no growth at pH 9.5. Peptidoglycan type is A3 (L-Orn-L-Ser-L-Ala-L-Thr-L-Ala), with the presence of Ala, Glu, Ser, Thr, and Leu/Orn + Lys in ratio of 2:1:1:0.7:0.7. The DNA G + C content of the type strain is 61.8 mol.%. The type strain, Su 851^T (=DSM 28597^T=JCM 19995^T), and the reference strain Su 864 were isolated from the feces of piglets.

2.5.33 Bifidobacterium magnum (Scardovi and Zani, 1974)

This species has been isolated from rabbit feces and until now it is the only known niche of occurrence. This species is characterized by the unusually large dimension of its cell, especially when grown in the absence of Tween 80. Cells, in fact, are long, thick, irregularly contoured rods measuring 2 μ m by 10–20 μ m and occurring frequently in aggregates; branches are common; some cells have tapered ends or are spindle-shaped. Club-shaped or swollen rods are rare (Fig. 2.4, panel 29). Surface colonies (TPY medium) are circular, raised with somewhat elevated central area, whitish to gray, opaque, and soft. Highly anaerobic; CO₂ has no effect on oxygen sensitivity or anaerobic growth. Optimum temperature is 37–39°C; minimum is 28°C; maximum is 42.5–43°C; does not grow at 26 or 44.5°C. This species is the only acidophilic one within the genus with an initial optimum pH at 5.3–5.5; growth is retarded at pH 5.0 or 5.9; no growth (after 2 days) at pH 4.2 or 7.0. The lactic acid and acetic acid molar ratio is I:1.9 in MRS medium. Produces L(+) lactic acid. Formic, propionic, and butyric acids are not produced. Peptidoglycan structure

is L-Lys(L-Orn)-L-Ala2-L-Ser. The DNA G + C content of the type strain is 60 mol.%. Type strain is RA 3^{T} (=ATCC 27540^T=DSM 20222^T=JCM 1218^T) isolated from feces of adult rabbits. Reference strain is ATCC 27681 (=JCM 7120; ATCC 27682=DSM 20220=JCM 7132) from rabbit feces.

2.5.34 Bifidobacterium merycicum (Biavati and Mattarelli, 1991)

This species has been isolated from bovine rumen. The other bifidobacterial species found in the bovine rumen are: *B. boum, B. pseudolongum* subsp. *globosum, B. ruminantium,* and *B. thermophulum. B. adolescentis,* even if this species has been typically found in humans, has been also isolated from this niche. Cells are irregularly shaped rods that are 2.0–5.0 μ m long and are sometimes arranged in pairs, which form angles (Fig. 2.4, panel 30). Surface colonies on TPY agar are soft, smooth, convex, circular with entire margins, cream colored to white, and glistening. Anaerobic. Carbon dioxide has no effect upon oxygen sensitivity, but it enhances anaerobic growth. The optimum temperature for growth is 38–42°C. No growth occurs below 25 or at 45°C and above. The optimum initial pH is 6.5–6.9. Growth is delayed at pH 6.3 or 7.2. No growth occurs at pH 4.5 or 8. The molar ratio of lactic acid to acetic acid from the fermentation of glucose ranges from 1:2 to 1:5. No gas, propionic acid, or butyric acid is produced. Peptidoglycan structure is L-Lys(L-Orn)-D-Asp. The G + C content of the DNA is 60 mol.%. Analysis of the 16S rRNA gene sequence revealed that LMG 11341^T showed high similarity with the *B. angulatum* LMG 11039^T (98.2%) (Lugli et al., 2014). The type strain is strain Ru915B^T (=ATCC 49391^T=DSM 6492^T=JCM 8219^T), which was isolated from bovine rumen. Reference strains, isolated from rumen of cattle, are DSM 6493 (=JCM 8220) and DSM 6494.

2.5.35 Bifidobacterium minimum (Scardovi and Trovatelli, 1974; Biavati et al., 1982)

The specific epithet refers to the very small size of the cells, a morphology resembling that of *B. asteroides* without star-like aggregates. In addition to morphology, this species possesses the last anodal isozyme of transaldolase (Scardovi et al., 1979b). This taxon was called a "minimum" DNA homology group by Scardovi and Trovatelli (1974) and consisted of only two strains isolated from a single specimen of wastewater. Its natural habitat was unknown but probably could be related to cecum of pigs after its finding in this niche by Simpson et al. (2003). Cells are very small (0.5–0.6 µm by 0.5–2.8 µm, oval, club-shaped, or short rods that occur in short chains and in clumps (Fig. 2.4, panel 31). Surface colonies in prereduced anaerobically agar streak tubes are 1 mm in diameter, circular, entire, convex, translucent, gray, white, or buff; shiny and smooth. Cultures in broth with a fermentable carbohydrate produce dense turbidity with a flocculent sediment and a final pH of 4.1–4.4. Lactic and acetic acids are produced in a molar ratio of 1:3.3. L(+) lactic acid is formed. Propionic acid, butyric acids are not produced. Peptidoglycan structure is L-Lys-L-Ser, the same found in *B. biavatii*. The G + C content of the DNA is 61.5%. Lugli et al. (2014) basing on 16S rRNA and 23S rRNA gene sequences showed, in addition to the previously recognized groups *B. asteroides, B. boum*, *B. longum, B. pullorum, B. pseudolongum*, and *B. boum* groups (Ventura et al., 2006), a novel 16S/23S rRNA-based group which includes *B. minimum* LMG 11592^T, *B. mongoliense* DSM 21395^T, *B. psychraerophilum* LMG 21775^T, and *B. crudilac-tis* LMG 23609^T (Lugli et al., 2014). Type strain is F 392^T (=ATCC 27538^T=DSM 20102^T=JCM 5821^T).

2.5.36 Bifidobacterium mongoliense (Watanabe et al., 2009)

During a study on the diversity of lactic acid bacteria (LAB) in airag (fermented mare's milk) and tarag (fermented milk of cows, yaks, goats, or camels), traditional fermented milk products from Mongolia, bifidobacterial strains YIT 10443^T and YIT 10738 were isolated from two of 22 airag samples, which were collected in the Mongolian provinces of Umnugobi and Uburhangai, respectively (Watanabe et al., 2008). Cells grown in modified GAM broth are rods of various shapes, $0.4-0.6 \times 0.8-2 \mu m$, with rounded or tapered ends, sometimes curved, swollen, and branched (Fig. 2.4, panel 32). Facultatively anaerobic. Pinpoint colonies (0.2-0.4 mm in diameter) are formed under aerobic conditions after 2 days incubation at 30°C. After anaerobic growth at 30°C for 5–6 days, colonies on modified GAM agar are 2–3 mm in diameter; they are convex, white, opaque, smooth, and circular with entire edges. The temperature range for growth is 15–35°C; no growth occurs at 10 or 40°C. The optimum temperature for growth is 25–30°C. Grows at pH 4.5–8.0 with an optimum at pH 6.5–7.0. Peptidoglycan structure is L-Lys-D-Asp type with the presence of Lys, Glu, Ala, and Asp. The DNA G + C content of the type strain is 61.1 mol.%. Phylogenetic analysis of the 16S rRNA gene sequence places the species in the *B. minimum* subgroup of bifidobacteria. The type strain, YIT 10443^T (=JCM 15461^T=DSM 21395^T), and the reference strain YIT 10738 were isolated from airag, a traditional fermented mare's milk product, which was collected in the Mongolian provinces of Umnugobi and Uburhangai, respectively, in 2004.

2.5.37 Bifidobacterium moukalabense (Tsuchida et al., 2013)

It has been isolated from the feces of nonhuman great ape. *Bifidobacterium angulatum*-like bacteria, which are of human type, have been detected in wild chimpanzees in Bossou, Guinea (Ushida et al., 2010). However, from feces of wild chimpanzees in Mahale, Tanzania bifidobacterial 16S rRNA genes of nonhuman-type bifidobacteria were retrieved (Ushida, 2009). Unlike chimpanzees in Bossou, which live close to villages, chimpanzees in Mahale live in remote areas far from human activity. *B. moukalabense*, which possess 16S rRNA gene similarity values close to those of the chimpanzees in Mahale, has been isolated from the feces of a wild lowland gorilla (*Gorilla gorilla gorilla*) in Gabon. Cells on BL agar are rods of various shapes (0.6 μ m wide and of variable lengths, primarily 2–4 μ m) with rounded ends, often curved, swollen and branched. Obligately anaerobic. Colonies on BS agar are 1–3 mm in diameter, brownish-red, opaque, convex, and disc-shaped after 48 h of incubation at 37°C under anaerobic conditions. The optimum growth temperature is 37°C. The major fatty acids are C16:0, C18:1 ω 9c, and C14:0. The DNA G + C content of the type strain is 60.1 mol.%. The type strain GG01^T (=JCM 18751^T=DSM 27321^T) was isolated from feces of a wild lowland gorilla in Moukalaba-Daudou National Park, Gabon.

2.5.38 Bifidobacterium myosotis (Michelini et al., 2016c)

It has been isolated from common marmoset (*C. jacchus*). In the same niche *B. aesculapii*, *B. hapali*, *B. tissieri*, *B. callitrichos*, and *B. reuteri* have been isolated. Cells grown in TPY broth are rods of varying shapes, forming a branched structure with a "Y" at the end (Fig. 2.4, panel 33). The well-separated colonies grown on the surface of TPY agar under anaerobic conditions are white, opaque, smooth, and circular with entire edges, while the embedded colonies are lens-shaped or elliptical. The colonies reach a diameter of 1.0–2.0 mm after 3 days of incubation. Cells are able to survive under microaerophilic conditions. The temperature for growth is 25–46°C; no growth occurs 20 or 48°C. Cells grow at pH 4.5–8. The optimal conditions for growth are pH 7 and 40°C. The major cellular fatty acids are C14:0, C16:0, and C18:1ω9c dimethylacetal. The peptidoglycan type is L-Glu-L-Ala-L-Lys. The DNA G + C content of the type strain is 65.1 mol.%. Analysis of the 16S rRNA gene sequence revealed that MRM_5.9^T showed high similarity with the 96.4% to *B. callitrichos* DSM 23973^T. The type strain MRM_5.9^T (=DSM 100196^TJCM 30796^T) and the reference strain DSM 100217 (=JCM 30897) were isolated from the feces of common marmosets. This species has been recently found also in red cotton tamarin housed in Italy (personal communication, 2016).

2.5.39 Bifidobacterium pseudocatenulatum (Scardovi et al., 1979a)

B. pseudocatenulatum together with B. adolescentis, B. longum subsp. longum, B. bifidum and B. catenulatum are the typical species found in adults; in infants B. breve, B. longum subsp. infantis, and B. bifidum are the species most abundant, but also B. catenulatum, B. pseudocatenulatum can be found, with B. adolescentis mostly associated with formula fed infants. The occurrence of bifidobacterial species in human individuals varies from 1 to 5 species and high interindividual variability in numbers and species has consistently been reported (Delgado et al., 2006), so it is very difficult to assess the real occurrence of the different species in gut microbiota. According to Biavati et al. (1986), this species most frequently found in human adult feces. This fact may be influenced by difficulties in differentiating B. pseudocatenulatum from B. catenulatum due to their high genetic similarity (see B. catenulatum description). Abundant in the feces of breast- and bottle-fed infants and human adults, in the feces of suckling calves and in sewage. Cells are irregularly shaped rods, 0.6–0.7 µm by 2–5 µm, generally arranged in pairs, which form angles with protuberances, knobs, or short branches near the point of contact. Strains with much more variable and irregular shape are occasionally found, including bifid forms similar to those of *B. bifidum* or pairs of cells with swollen ends similar to those of *B. longum*. Some strains show distorted chains of small coccoid and irregular cells or long, branched, hyphal-like cells (Fig. 2.4, panel 34). Strain-to-strain variability in cell morphology is common in this species. Surface colonies in TPY medium are smooth, convex, cream colored to white, glistening, and soft; entire edges. Anaerobic: CO₂ is without effect upon oxygen sensitivity or anaerobic growth. Optimum temperature of growth is 39–41°C; minimum is 28–29°C; maximum, 42–43°C; no growth at 26 or 44°C. Initial optimum pH is 6.5–6.9; growth is retarded at 6.0 or 7.2; no growth at 4.5 or 8.0. The molar ratio of lactic to acetic acid from the fermentation of glucose is 1:2. L(+)-lactic acid is produced. Large quantities of formic acid are produced. Propionic and butyric acids are not produced. CO₂ is produced only in the fermentation of gluconate. The peptidoglycan structure is Lys-Ala2-Ser. The G + C content of the DNA is 57.5 mol.%. The complete genome sequence of the type strain has been described by Morita et al. (2015a). Abundant in sewage, in the feces of breast- and bottle-fed infants and human adults and in the feces of suckling calves. Type strain: B 1279^T=ATCC 27919=DSM 20438=JCM 1200. Reference strain is DSM 20439 isolated from sewage.

2.5.40 Bifidobacterium pseudolongum (Biavati et al., 1982; Mitsuoka, 1969; Yaeshima et al., 1992a,b)

The first description dated from Mistuoka who isolated different bifidobacteria from a variety of animals and recognized them as *B. pseudolongum* and the four biovars *a* (strain PNC-2-9G^T from pig), biovar *b* (strain 28T from chicken), biovar *c* (strain 29SRT from chicken), and biovar *d* (strain Mo2-10 from mouse) were proposed on the basis of differences in the fermentation of mannose, lactose, cellobiose and melezitose (Mitsuoka, 1969). Because of genetic and phenotypic similarity between *B. pseudolongum* (Mitsuoka, 1969) and *B. globosum* (Biavati et al., 1982), these two species are rearranged taxonomically as subspecies: *B. pseudolongum* subsp. *pseudolongum* and *B. pseudolongum* subsp. *globosum* (Yaeshima et al., 1992a,b). The two subspecies share a similar ecological distribution but *B. pseudolongum* subsp. *globosum* is mainly found in the rumen and intestine of ruminants, whereas no strain isolated from the rumen has been assigned to *B. pseudolongum* subsp. *pseudolongum*. This species showed a cosmopolitan lifestyle, being distributed in many hosts (chicken, rabbit, pig, calf and bovine rumen, rat). A study on the presence of bifidobacterial outer proteins (BIFOPs) in the cell wall of 150 strains of *B. pseudolongum* isolated from different animals was conducted by Mattarelli et al. (1993); 60% of the strains examined were apparently devoid of BIFOPs. BIFOP expression changes, caused by growth temperature, were observed in seventy strains. In general, BIFOP expression at low temperatures was considerably attenuated while at medium and high-growth temperatures it increased (Mattarelli et al., 1999).

2.5.40.1 Bifidobacterium pseudolongum subsp. pseudolongum (Scardovi and Trovatelli, 1974)

The first description dated from Mitsuoka (1969) who isolated different strains from pig, chicken, dog, bull, calf, rat, and guinea pig. *B. pseudolongum* has about 70% DNA–DNA homology with *B. globosum* and showed similar phenotypic features and identical peptidoglycan structure. The cell morphology is shown in Fig. 2.4, panel 35a. The peptidoglycan structure is L-Orn(L-Lys)-L-Ala2-3. The G + C content of the DNA is 64.8 mol.%. Type strain is PNC-2-9GT(=ATCC 25526^T=DSM 20099^TJCM 1205^T) from feces of pig. Reference strains are: DSM 20085 (=JCM 1264) from mouse feces; DSM 20094 (=JCM 1266) and DSM 20095 (=JCM 1267) from chicken feces.

2.5.40.2 Bifidobacterium pseudolongum subsp. globosum (Biavati et al., 1982; Scardovi et al., 1969)

B. globosum subsp. *globosum* is undoubtedly the predominant bifidobacterial species in a large number of animal digestive tracts. Isolated from feces of calves, chickens, lambs, piglets, rabbits, rats, in a single specimen of feces of the human infant, and in sewage (Scardovi et al., 1979b). The cell morphology is shown in Fig. 2.4, panel 35b. Large molecular weight plasmids (13.5, 24.5, and 46 MDa) were found in *B. pseudolongum* subsp. *globosum* strains (Sgorbati et al., 1982). The peptidoglycan structure is L-Orn(L-Lys)-L-Ala2-3. The G + C content of the DNA is 64.1 mol.%. Type strain is Ru 224T(=ATCC 25865^T=DSM 20092^T = JCM 5820^T) isolated from bovine rumen. Reference strains are ATCC 25864 (=JCM 7089) and JCM 7092 from bovine rumen.

2.5.41 Bifidobacterium psychraerophilum (Simpson et al., 2004)

Originally isolated in pig cecal samples from Simpson et al. (2003) and described as new species in 2004. Cells are short and irregularly shaped rods, approximately 0.7–1.0 μ m wide and 0.8–1.5 μ m long with occasional bifurcations, arranged singly or in pairs (Fig. 2.4, panel 36). Colonies on mMRS agar under anaerobic conditions are white, circular, and convex with smooth edges and reach a diameter of up to 3 mm after 3 days incubation at 37°C. Colonies are also formed under aerobic conditions, but attain a reduced diameter of ~1 mm after 3 days of incubation. Optimal temperature for growth is 37°C; maximum temperature for growth is 42°C, with no growth at 46°C. Growth occurs at 4°C, although it is considerably reduced. Lowest pH attained is 4.0, with a minimum initial pH for growth of 4.5. DNA G + C content is 59.2 mol.%. Phylogenetic analysis of the 16S rRNA gene sequence places the species in the *B. minimum* subgroup of bifidobacteria (Lugli et al., 2014). Type strain is T16^T (PFGE type F) (=LMG 21775^T=NCIMB 13940^T) isolated from a pig cecum (contents and epithelium) in Fermoy, Ireland. Previously termed *B. pyschroaerophilum*.

2.5.42 Bifidobacterium pullorum (Trovatelli et al., 1974)

Morphology is of help in recognizing this species, whose cells are mostly arranged in irregular chains often of great length (Fig. 2.4, panel 37). Cells are slender and slightly curved rods 2–8 µm long with tapered ends, mostly arranged in irregular chains. Rarely occurring branchings are mostly short and disposed at right angle. Often cells are poorly

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refractile and appear as empty or vacuolized. Some swellings or club-shaped cells appear at high temperatures of incubation (42–43°C). Colonies are convex, with entire edges, cream colored to white, glistening, and soft. Highly anaerobic (oxygen inhibition zone deeper than 5 mm); CO₂ without effect upon oxygen sensitivity or anaerobic growth. Optimum temperature growth is 37–39°C; minimum 30–30.5°C; maximum 44.5–45.0°C; no growth at 29 or 46.5°C. pH relationships: initial optimum 6.7–6.9; delayed growth at 6.5 and 7.3; no growth at 5.5 and 8.7. Lactic and acetic acid ratio 1:3.5 in TPY. Isomeric type of lactic acid: DL. Some formic acid is produced from glucose. Propionic and butyric acid not formed. DNA G + C content is 67.4 mol.%. For good growth, nicotinic acid, pyridoxin, thiamin, folic acid, *p*-aminobenzoic acid, and Tween 80 are required. The peptiglycan structure is L-Lys-D-Asp. The G + C content of the DNA is 67 mol.%, the highest value reported for bifidobacteria. The type strain, isolated from feces of chickens, is P 145^T (=ATCC 27685^T=DSM 20433^T).

2.5.43 Bifidobacterium ramosum (Michelini et al., 2016a)

This recently described species. In *S. oedipus*, a New World monkey belonging to *Callithricidae* family, besides *B. ramosum*, *B. avesanii*, and *B. aerophilum* have been described. In the fecal sample of *S. oedipus* another four new bifidobacterial taxa currently under investigation have been detected (personnel communication). Cells, when grown in TPY broth under anaerobic condition, are rods of various shapes that form a branched structure with a "Y" on both sides (Fig. 2.4, panel 38). On the surface of TPY agar, the colonies have a diameter of 1.0–2.5 mm after 2 days incubation and are white, opaque, smooth, and circular with entire edges, whereas embedded colonies are lens-shaped or elliptical. Cells can also grow under aerophilic and microaerophilic conditions. Growth in TPY broth occurs in the range 25–50°C but no growth occurs at 20 or 56°C (after 24–48 h). Strains grow in the pH range 4.0–7.5. Optimal conditions of growth occur at pH 6 and 40°C under either microaerophilic or anaerobic conditions. The peptidoglycan type is A3 β L-Orn-L-Ser-L-Ala. The DNA G + C content of the type strain is 63.0 mol.%. The type strain showed the highest level of 16S rRNA gene sequence similarities with *B. scardovii* DSM 13734^T (96.6%). The type strain is TRE M^T (=DSM 100688^T=JCM 30944^T) isolated from the feces of an adult subject of the cotton-top tamarin. This species has been described basing on a single strain.

2.5.44 Bifidobacterium reuteri (Endo et al., 2012)

B. reuteri belongs to common marmoset gut microbiota where *B. aesculapii*, *B. callitrichos*, *B. myosotis*, *B. hapali*, and *B. tissieri* have been described. Cells are irregularly shaped rods with rounded or tapered end, usually swollen and branched, measuring $0.3-1.0 \times 1.5-6 \mu m$ (Fig. 2.4, panel 39). Cells are nonmotile and usually occur singly or in pairs. Facultatively anaerobic. Colonies on MRS agar supplemented with 50 mg/L L-cysteine after incubation for 2 days are beige, smooth, and approximately 1–2 mm in diameter under anaerobic conditions. Strain grows at 37°C but not at 30 or 42°C. Cells grow at pH 5.0–7.0. The peptidoglycan type is L-Lys-Gly. The DNA G + C content is 61.3 mol.%. Phylogenetic analysis of 16S rRNA gene places the species in the *B. boum* group. The type strain is AFB22-1^T (=JCM 17295^T=DSM 23975^T) isolated from feces of common marmoset, collected at Cape Town, South Africa, in 2009. This species has been described based on a single strain.

2.5.45 Bifidobacterium ruminantium (Biavati and Mattarelli, 1991)

Bifidobacteria are present in rumen in large numbers, especially when animals are fed starch-rich diets. Beside this species, *B. pseudolongum* subsp. *globosum*, *B. thermophilum*, *B. boum*, and *B. merycicum* are commonly found in this habitat. Cells are 3.0–6.0 µm in length, are irregular in shape, rarely have terminal bifurcations, and often occur in pairs, which form wide angles (Fig. 2.4, panel 40). Surface colonies on TPY agar are soft, smooth, convex, circular with entire margins, cream to white, and glistening. Anaerobic. Carbon dioxide has no effect upon oxygen sensitivity, but it enhances anaerobic growth. The optimum temperature for growth is 38–42°C. No growth occurs below 25°C or at 45°C and above. The optimum initial pH is 6.5–6.9. Growth is delayed at pH 6.3 or 7.2. No growth occurs at pH 4.5 or 8. The molar ratio of lactic acid to acetic acid from the fermentation of glucose ranges from 1:2 to 1:3. No gas, propionic acid, or butyric acid is produced. The peptidoglycan structure is L-Lys(L-Orn)-L-Ser-(L-Ala)-L-Ala2. The G + C content of the DNA is 57 mol.%. Phylogenetic analysis of the 16S rRNA gene sequence places the species in the *B. adolescentis* subgroup of bifidobacteria with *B. adolescentis* as closest species (Lugli et al., 2014). The type strain, isolated from bovine rumen, is strain Ru 687^T (=ATCC 49390^T=DSM 6489^T=JCM 8222^T). Reference strains, isolated from rumen of cattle, are DSM 6490 (=JCM 8509) and DSM 6491 from rumen of cattle.

2.5.46 Bifidobacterium saguini (Endo et al., 2012)

B. saguini has been isolated from *S. mida* (golden-handed tamarin or Midas tamarin) belonging to *Callithricidae* family. *B. biavatii* and *B. stellenboshense* have been isolated from the same source while *B. avesanii*, *B. aerophilum*, and *B. ramosum* are from *S. oedipus* (red cotton tamarin). The tamarin gut microbiota showed a great biodiversity of bifidobacterial species. Cells are irregularly shaped rods with rounded or tapered ends, usually swollen and branched, measuring $0.4-1.0 \times 2-6 \,\mu$ m. Cells usually occur singly, in pairs or in chains (Fig. 2.4, panel 41). Facultatively anaerobic. Colonies on MRS agar supplemented with 50 mg/L L-cysteine after incubation for 2 days are beige, smooth, and approximately 1–2 mm in diameter under anaerobic conditions. Strain grows well at 37 and 42°C and weakly at 30°C, but not at 45°C. Cells grow at pH 5.0–7.0. The peptidoglycan type is L-Lys-Gly. The DNA G + C content is 57.3 mol.%. Phylogenetic analysis of 16S rRNA gene places the species in the *B. longum* group. The type strain is AFB23-1^T (=JCM 17297^T=DSM 23967^T) isolated from feces of red-handed tamarin, collected at Cape Town, South Africa, in 2009. This species has been described basing on a single strain.

2.5.47 Bifidobacterium saeculare (Biavati et al., 1991)

The specific epithet "saeculare" is in honor of the Ninth Centenary of the Foundation of Bologna University (Italy). Trovatelli et al. (1974) found that four strains of bifidobacteria isolated from feces of rabbit were characterized as "unassigned homology group I." Subsequently, on the basis of DNA–DNA hybridization, phenotypic characters and electrophoretic patterns of proteins and of isozymes the "unassigned homology group I" was described as a new species and named *B. saeculare*. Cells are short, slightly curved rods, 2.0–4.0 µm long, isolated or in pairs; longer, thicker or spindle-shaped cells are frequent in some successive transfers or reisolates (Fig. 2.4, panel 42); at low incubation temperatures in liquid cultures branching cells are numerous. Surface colonies on TPY agar are convex, with entire edges, cream colored or white, glistening and soft. Anaerobic. Carbon dioxide has no effect upon oxygen sensitivity or anaerobic growth. The peptidoglycan type is L-Lys-Gly. The DNA G + C content is 63 mol.%. Phylogenetic analysis of 16S rRNA gene places the species in the *B. pullorum* group, underlyng the high genetic similarity with *B. pullorum* and *B. gallinarum* (Lugli et al., 2014). The type strain is RA 161^T (=ATCC 49392^T=DSM 6531^T=JCM 8508^T). Reference strains, isolated from rabbit feces, are DSM 6532; DSM 6533.

2.5.48 Bifidobacterium scardovii (Hoyles et al., 2002)

Named after Vittorio Scardovi, in recognition of his contribution to our knowledge of the bifidobacteria, *B. scardovii* has been isolated from human clinical sources but habitat is not known. Cells are rod-shaped cells, some of which are curved (Fig. 2.4, panel 43). Facultatively anaerobic. Colonies are 2 ± 3 mm in diameter, convex, shiny, with a dense white center. Cell wall murein is of the A3 α type, L-Lys(L-Orn)–L-Ser(L-Ala)–L-Ala2. The DNA G-C content of the type strain is 60 mol.%. The complete genome sequence of the type strain has been described by (Morita et al., 2015a). The type strain is CCUG 13008^T (=DSM 13734^T) isolated from human blood.

2.5.49 Bifidobacterium stellenboshense (Endo et al., 2012)

This species belongs to nonhuman primates bifidobacterial species. It was isolated from feces of red-handed tamarin (*S. mida*) in animal houses in Cape Town, South Africa. Cells are irregularly shaped rods, usually swollen and branched, usually occur singly, in pairs or in chains measuring $0.5-1.0 \times 4-10 \mu m$ (Fig. 2.4, panel 44). Colonies on MRS agar supplemented with 50 mg/L L-cysteine after incubation for 2 days are white, smooth, and approximately 1–2 mm in diameter under anaerobic conditions. Strain grows well at 26 and 42°C and weakly at 20°C but not at 15 or 45°C. Cells grow at pH 5.0–8.0. The peptidoglycan type is L-Lys (L-Orn)-D-Asp. The DNA G + C content is 66.3 mol.%. Phylogenetic analysis based on 16S rRNA gene forms a couple between the species and *B. scardovii*. The type strain is AFB23-3^T (=JCM 17298^T=DSM 23968^T) isolated from feces of red-handed tamarin, collected at Cape Town, South Africa, in 2009. This species has been described basing on single strain.

2.5.50 Bifidobacterium subtile (Biavati et al., 1982; Scardovi and Trovatelli, 1974)

This taxon, previously described and referred to as the *subtile* DNA homology group with five strains isolated from sewage (Scardovi and Trovatelli, 1974), has been described in 1982. Its natural habitat was unknown. Recently *Scardovia inopinata* and *B. subtile* were both isolated from the infected dentine of the leathery and soft lesions:

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these data suggest that nonoral bifidobacteria may transiently colonize the oral cavity. Although B. subtile has been isolated from lesion tooths, indicating colonization of carious root lesions, this species is considered nonoral bifidobacteria and may suggest that nonoral bifidobacteria including bifidobacteria used as probiotics, may colonize carious teeth (Mantzourani et al., 2009). Cells in TPY are 0.8 μm by 2.1–4.5 μm, straight or bent rods that occur singly or in pairs (Fig. 2.4, panel 45). Longer, branching, bifid, knobbed, and club-shaped forms in tangled masses are produced in semisolid thioglycolate medium. Smaller coccoid and rod-shaped cells that occur in short chains are produced in broth media that do not contain fermentable carbohydrates. Colonies on the surface of blood agar incubated anaerobically are punctiform to 1 mm in diameter, circular, entire, slightly raised, translucent, colorless, shiny, and smooth. Similar (but smaller) colonies are produced on blood agar incubated in a candle jar. Dense turbidity with a granular or flocculent sediment and a final pH of 4.1–4.8 are produced in peptone-yeast extract broth containing fermentable carbohydrates. Slight to moderate turbidity is produced in peptone-yeast extract broth containing carbohydrates that are not fermented. The optimum temperature for growth (34–35°C) is the lowest among bifidobacteria (37–41°C range). The peptidoglycan type is L-Lys-D-Asp. The DNA G + C content is 61.5 mol.%. Phylogenetic analysis of the 16S rRNA gene sequence places the species in the B. minimum subgroup of bifidobacteria (Lugli et al., 2014). The type strain and the reference strains, isolated from sewage, are F 395^{11} (=ATCC 27537^T=DSM 20096^T=JCM 5822^T); ATCC 27683 (=JCM 7109); ATCC 27684.

2.5.51 Bifidobacterium thermoacidophilum (Dong et al., 2000; Zhu et al., 2003)

In 2001 this species was isolated from an anaerobic digester for the treatment of wastewater from a bean-curd farm. In 2003 the species has been taxonomically reclassified in the two subspecies *B. thermacidophilum* subsp. *thermo-acidophilum* and *B. thermacidophilum* subsp. *porcinum*. von Ah et al. (2007) showed that DNA–DNA hybridization data revealed a very close relationship between *B. thermophilum* species and *B. thermacidophilum* species and questioned the current classification of *B. thermacidophilum* as a discrete species.

2.5.51.1 Bifidobacterium thermacidophilum subsp. thermoacidophilum

This species differs from other bifidobaterial species in its phenotypic characteristics, such as its ability to grow at relatively high temperatures (49.5°C). Cells are irregular rods, arranged singly or occasionally in a "V" shape, are $0.5-1.5-2.5 \mu m$ in size after 16–24 h in TPYG liquid at 37°C (Fig. 2.4, panel 46a). Liquid cultures of some strains can form a homogenous sediment, but it is readily dispersed by shaking. Anaerobic growth also occurs in standing culture fewer than 90% air plus 10% CO₂. Colonies on TPYG agar are circular, convex with entire edges, white, and reach 1.5-2 mm in diameter after 24 h cultivation. The optimum temperature for growth is 37–41°C while the temperature range for growth is $30-49.5^{\circ}$ C. The optimum initial pH is $7 \pm 0-7 \pm 2$; growth at pH 4 ± 5 is quite good but is delayed at pH 4.0. Litmus milk is not acidified and coagulated by most of the strains. The fermentation products from glucose are acetic and lactic acid at a molar ratio of 2.46–2.72:1. Final pH values of 3.6 can be reached. The G–C content of the DNA is 57.7 mol.%. Type strain, isolated from the wastewater of a bean-curd farm in Beijing, is 36T (=CGMCC AS 11.2282^{T} =DSMZ 15837^{T} =JCM 11165^{T} =LMG 21395^{T}).

2.5.51.2 Bifidobacterium thermoacidophilum subsp. porcinum (Zhu et al., 2003)

Isolated from feces of piglet. It has been detected also from feces of newborn infants after 1–2 days after the birth (Kheadr et al., 2007). The phenotypic features of this subspecies meet the type subspecies description. The cell morphology is shown in Fig. 2.4, panel 46b. Fermentation products from glucose are acetic and lactic acids at a molar ratio of 4.9:1. Maximum temperature for growth is 46.5°C. DNA G + C content is 61.2 mol.%. The type strain showed the highest level of 16S rRNA gene sequence similarities with *B. boum* (97.7%), *B. thermacidophilum* (97.2%), and *B. thermophilum* (97.0%). The type strain, isolated from the feces of a piglet, is P3-14T (=AS 1.3009^{T} =LMG 21689^{T}).

2.5.52 Bifidobacterium thermophilum (Mitsuoka, 1969)

Mitsuoka (1969) proposed the specific epithet *thermophilum* for strains of bifidobacteria that have been isolated from the feces of chickens and swine, owing to their ability to grow at 46.5° C and to resist heating at 60° C for 30 min; the four biovars are all from feces of pig corresponding to biovar *a* [strain P2-91^T (ATCC 25525^T)], biovar *b* (strain 14-44), biovar *c* (strain P16-6), and biovar *d* (strain Nissin) were distinguished according to differences in the fermentation of melezitose and lactose. Scardovi et al. (1969) named strains that fermented neither pentoses nor lactose, which he isolated from bovine rumens, as *B. ruminale*. Subsequent comparative data have shown that these species are identical (Scardovi et al., 1971, 1979b; Biavati et al., 1982). *B. thermophilum* strains were related to ruminants and

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pigs, but in 2007 strains of *B. thermophilum* have been isolated from infant feces (von Ah et al., 2007). Based on this finding Gavini et al. (1991) disapproved that *B. thermophilum*, previously only isolated from animal sources and therefore suggested to be used as differential species between animal and human contamination, should be used to discriminate human and nonhuman pollution. *B. thermophilum*, such as *B. pseudolongum* subsp. *globosum*, can grow in 90% air + 10% CO₂ without the cells becoming catalase- or pseudo-catalase (hemin) positive. A few strains genetically assigned to *B. thermophilum*, which, in contrast to other strains of the species, fermented arabinose and xylose, have been found in sewage and in piglets (Scardovi et al., 1979a,b). Cells are slender, slightly curved, often with tapered ends, protuberances or irregularities near the junction of paired cells, rare branchings, and arranged singly or in pairs never in clumps or in angular disposition ((Fig. 2.4, panel 47). The peptidoglycan structure is L-Orn(L-Lys)-D-Glu. DNA G + C content is 60 mol.%. Phylogenetic analysis of the 16S rRNA gene sequence places the species in the *B. boum* subgroup of bifidobacteria (Lugli et al., 2014). Isolated from feces of pig, chicken, and suckling calf, bovine rumen and sewage. Type strain is P2-91^T (=ATCC 25525^T=DSM 20210^T=JCM 1207^T). Reference strains are: ATCC 25866 (=DSM 20212=JCM 1268) and ATCC 25867 from bovine rumen and DSM 20209 from rumen.

2.5.53 Bifidobacterium tissieri (Michelini et al., 2016c)

This species belongs to nonhuman primate bifidobacterial species. It has been isolated from *C. jacchus* (common marmoset). Cells grown in TPY broth under anaerobic conditions are rods of varying shape that form a branched structure with a "Y" at the end (Fig. 2.4, panel 48). Colonies on the surface of TPY agar reach a diameter of 1.0–2.5 mm after 2 days of incubation and are white, opaque, smooth, and circular with entire edges; any embedded colonies show lens or elliptical shapes. Cells are able to survive under aerophilic and microaerophilic conditions. The temperature for growth is 25–40°C; no growth at 20 or 44°C. Grows at pH 5.5–7.5. The optimal growth conditions are pH 6.5 and 35°C. The major cellular fatty acids are C16:0, C18:1ω9c, and C14:0. The peptidoglycan type is L-Glu-L-Ala-L-Lys. The DNA G + C content of the type strain is 63.7 mol.%. Analysis of the 16S rRNA gene sequence revealed that MRM_5.9^T showed high similarity with the 96.4% to *B. callitrichos* DSM 23973^TThe type strain is MRM_5.18^T (=DSM 100201^T=JCM 30798^T), and the reference strain MRM_9.02 (=DSM 100342=JCM 30803) were isolated from the feces of common marmosets.

2.5.54 Bifidobacterium tsurumiense (Okamoto et al., 2008)

Isolated from dental plaque of hamsters. Colonies on Brucella HK blood agar plates are white, rough, and slightly convex. Cells are short rods or coccoid when grown on Brucella HK blood agar under anaerobic conditions for 24 h (Fig. 2.4, panel 49). Facultatively anaerobic: it can grow also in aerobic conditions. Growth occurs at 25 and 45°C; optimal temperature for growth is 37°C. Growth also occurs on LBS and CFAT agars. End products of glucose fermentation are acetic and lactic acids at a molar ratio of 3:2. The cell-wall amino acids consist of Glu-Lys-Asp-(Ala)2. The DNA G + C content is 53 mol.%. The levels of similarity for the partial 16S rRNA gene sequence of strain OMB115^T in relation to the type strains of *B. boum, B. thermophilum, B. indicum,* and *B. thermacidophilum* were 96.1, 95.2, 94.9, and 94.2%, respectively. Type strain, isolated from dental plaque from hamsters fed with a high-carbohydrate diet, is OMB115^T (=DSM 17777^T=JCM 13495^T).

In recent years, the number of bifidobacterial species described has greatly increased. Investigating new habitats and new hosts will certainly provide new discoveries for enthusiastic taxonomists together with deeper understanding of beneficial functionalities of this important bacterial group.

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2. SPECIES IN THE GENUS BIFIDOBACTERIUM

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3

Related Genera Within the Family *Bifidobacteriaceae*

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3.1 INTRODUCTION

Within the class of Actinobacteria, Stackebrandt et al. (1997) proposed the new order Bifidobacteriales with the family Bifidobacteriaceae as type family of the order. At that time only two genera were described to be members of the *Bifidobacteriaceae* (i.e., the genus *Bifidobacterium* as a type genus and the genus *Gardnerella*). Following the new classification system, phylogenetically closely related genera are clustered into the family Bifidobacteriaceae on the basis of 16S rRNA/DNA sequences analysis; this is of paramount importance in the new taxonomic vision if compared to the more traditional chemotaxonomic, morphological, and physiological properties. In the following years the family Bifidobacteriaceae was expanded so that at present in addition to Bifidobacterium (Orla-Jensen, 1924) with 54 validated species and ten subspecies, and Gardnerella (Greenwood and Pickett, 1980) with the species Gardnerella vaginalis, another seven genera were allocated in the family. This chapter will be devoted to the so-called scardovial genera, since the genus *Bifidobacterium* will be treated in a separate chapter while the genus *Gardnerella* is marginal for the scope of the present book and is already well represented in the literature. The new taxa belonging to the scardovial genera were isolated from a variety of habitats: Aeriscardovia aeriphila from a porcine cecum, Alloscardovia omnicolens from human clinical samples, Alloscardovia macacae from the milk of a macaque, Alloscardovia criceti from dental plaque of golden hamsters, Bombiscardovia coagulans from the digestive tract of bumblebees, Neoscardovia arbecensis from porcine slurries, Parascardovia denticolens from human dental caries, Pseudoscardovia suis and P. radai from the digestive tract of wild pigs, Scardovia inopinata from human dental caries, and S. wiggsiae from a wound infection in the arm of an intravenous drug user. Considering the use of beneficial bacteria in different applications, one aspect of great importance refers to their classification into risk groups. Most countries use a four-level risk group system according to their virulence and potential danger to humans. Almost all the species belonging to scardovial genera are classified in risk group 1 that refers to organisms that generally do not cause disease in healthy adult humans. Only Alloscardovia omnicolens is classified into risk group 2, which refers to microorganisms with a moderate risk for humans. The data on the risk level were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen and are partially based on the list from Technical Rules for Biological Agents. Within the family Bifidobacteriaceae the major genera include Bifidobacterium and Gardnerella. Bifidobacteria are playing an important role in the development of new concepts related to beneficial bacteria found in human and animal intestinal tracts. Their use in food and pharmaceutical preparations as probiotics is a fast-growing business. Gardnerella vaginalis is the only species of this genus. Isolated from the human genital and urinary tracts, it is classified in risk group 2. For a long time it has been considered as the etiological agent of bacterial vaginosis. Nowadays it is assumed that bacterial vaginosis is associated with a large spectrum of anaerobic bacteria. The role of *G. vaginalis* in this disease is controversial, as demonstrated in recent studies that demonstrated a high rate of detection of G. vaginalis in subjects without bacterial vaginosis (Fredricks et al., 2007).

The present book is devoted to the genus *Bifidobacterium*. The editors have considered it worthy, for a complete picture of the family, to dedicate a short chapter to the so-called scardovial genera, although in most cases both biodiversity and practical importance are largely unknown. The last edition of *Bergey's Manual of Systematic Bacteriology*



FIGURE 3.1 Vittorio Scardovi. Source: From Turtura, G.C., Crociani F., 1996. In commemoration of Vittorio Scardovi (1918–1995). Ann. Microbiol. Enzimol. 46, 157–170.

(Biavati and Mattarelli, 2012) report the description of the family *Bifidobacteriaceae*, but this overview lacks descriptions of several new genera and species that have been discovered after its publication.

To conclude the introduction, we would like to offer some words in memory of Professor Vittorio Scardovi (Fig. 3.1). His invaluable contributions to the taxonomy and ecology of the genus *Bifidobacterium* were a basis for successive descriptions of seven new genera of the family *Bifidobacteriaceae*. The international recognition of the great scientific importance of his work is documented by the nomenclature of seven genera of the family *Bifidobacteriaceae*, in addition to *Bifidobacterium scardovii*, named after him.

Vittorio Scardovi was born in Bologna, Italy, on October 26, 1918, second to last of six brothers. In 1941 he completed his study, obtaining a master's degree in agricultural science from the University of Bologna with full marks and honor. At that time the PhD wasn't part of the national education system. In 1962 he was named full professor of agricultural microbiology at the Agricultural Faculty in Piacenza, where he stayed for several years. In 1968 he returned to Bologna University as director of the Institute of Agricultural Microbiology, a position that he held until 1993. He was a member of numerous national and international organizations. He received a gold medal from the President of the Italian Republic for his great contributions to the sciences and education. On August 11, 1995, he suddenly passed away. Until the end of his life he devoted a large part of his time to research, which was his true passion. The authors of this chapter have had the privilege to know how kind and sensitive he was to people of all ages and of different backgrounds. The scientific work was largely dedicated to the study of the genus *Bifidobacterium*, establishing the bases for the application of many of the species described as health-promoting bacteria for humans and animals.

3.2 PHENOTYPIC CHARACTERISTICS

One important trait for the classification of a strain as a member of the family *Bifidobacteriaceae* is the fructose-6-phosphate phosphoketolase (F6PPK) test (Biavati and Mattarelli, 2006).

A modification of the phosphoketolase assay was proposed by Orban and Patterson (2000) for a more rapid process of cell disruption by pretreating cells with the detergent hexadecyltrimethylammonium bromide (cetrimonium bromide), which was proposed as an alternative to the traditional cell disruption procedures. Lactic and acetic acids are produced from glucose fermentation. After isolation the characterization of strains is a key element not only for taxonomic purposes but also for its possible application. With reference to the family *Bifidobacteriaceae*, useful information can be found on the "minimal standard requirements for the description of new species of the genera *Bifidobacterium*, *Lactobacillus*, and related genera" (Mattarelli et al., 2014b).

Differential phenotypic characteristics among the scardovial species are shown in Table 3.1.

Some phenotypic characteristics, mainly carbohydrate fermentation and enzyme patterns, are known to vary between strains within a single species. In this sense the reliability of the data reported in the description of a species is largely dependent on the methods used, on the number of samples studied, and on the tested strains. For this reason we have preferred to avoid the insertion of these data in the species description, limiting the information to more robust characteristics.

We insert in this section the additional data available that can be used, taking into consideration the previous observation.

3.2.1 Carbohydrate Fermentation and Enzyme Patterns

3.2.1.1 Aeriscardovia aeriphila

All strains ferment glucose, salicin, arabinose, and raffinose. None ferment rhamnose, trehalose, sorbitol, lactose, mannitol, or glycerol. All strains are positive for α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, α -arabinosidase, arginine arylamidase, proline arylamidase, phenylalanine arlyamidase, leucine arylamidase, tyrosine arylamidase, glycine arylamidase, histidine arylamidase, and serine arylamidase activities. All strains are negative for β -galactosidase-6-phosphate, β -glucuronidase, *N*-acetyl- β -glucosaminidase, glutamic acid decarboxylase, alkaline phosphatase, leucyl glycine arylamidase, pyroglutamic acid arylamidase, alanine arylamidase, glutamyl glutamic acid arylamidase, and urease activities not reduced to nitrite. Biochemical profiles of the four strains tested were obtained with the API Rapid ID32A and 20A test strips (bioMérieux). Data are from Simpson et al. (2004).

3.2.1.2 Alloscardovia omnicolens

All strains ferment D-glucose, sucrose, D-maltose, salicin, D-xylose, L-arabinose, and raffinose.

None ferments rhamnose (except one strain) sorbitol (except two strains) mannitol (except two strains), or glycerol (except one strain). All strains exhibit α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, α -arabinosidase (except one strain), arginine arylamidase, proline arylamidase, phenylalanine arylamidase, leucine arylamidase, tyrosine arylamidase, glycine arylamidase, histidine arylamidase, and serine arylamidase activities. None of the strains exhibits arginine dihydrolase, β -galactosidase-6-phosphate, β -glucuronidase, *N*-acetyl- β -glucosaminidase, glutamic acid decarboxylase, α -fucosidase, alkaline phosphatase, leucyl glycine arylamidase, pyroglutamic acid arylamidase, alanine arylamidase, glutamyl glutamic acid arylamidase, or urease activities or indole production. The carbohydrate-fermentation patterns of 12 isolates were determined using API Rapid ID 32A and API 20A test strips (bioMérieux). Data are from Huys et al. (2007).

3.2.1.3 Alloscardovia macacae

The carbohydrate fermentation and enzyme production patterns of the type strain M8 were determined using API 50 CHL, Rapid ID 32A, and API ZYM test strips (bioMérieux). The available results from Killer et al. (2013b) are reported in Table 3.1.

3.2.1.4 Alloscardovia criceti

L-Arabinose, ribose, D-xylose, galactose, glucose, fructose, mannose, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, melezitose, raffinose, gentiobiose, D-turanose, and 2 keto-gluconate are fermented. Glycerol, erythritol, D-arabinose, L-xylose, adonitol, β -methyl-D-xylopyranoside, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α -methyl-D-mannopyranoside, *N*-acetyl-glucosamine, inulin, starch, glycogen, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate and 5 keto-gluconate are not fermented. Activities of α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase, α -arabinosidase, arginine arylamidase, proline arylamidase, phenylalanine arylamidase, leucine arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, serine arylamidase, esterase, esterase lipase, acid phosphatase, α -galactosidase, β -galactosidase, α -glucosidase, α -phosphoamidase, and β -glucosidase are observed. No activity is observed for catalase, urease, arginine dihydrorase, β -galactosidase, -6-phosphate, β -glucuronidase, *N*-acetyl- β -glucosaminidase, glutamic acid decarboxylase, α -fucosidase, nitrate reduction, indole production, alkaline phosphatase, pyroglutamine arylamidase, glutamyl glutamic acid arylamidase, trypsin and chymotrypsin, α -fucosidase, leucylglycine arylamidase, lipase, valine arylamidase, cysteine arylamidase, and α -mannosidase. The

Characteristics	Alloscardovia macacae M8 ^T	Alloscardovia omnicolens DSM 21503 ^T	Alloscardovia criceti DSM 17774 ^T	Aeriscardovia aeriphila DSM 22365 ^T	Bombiscardovia coagulans DSM 19704 ^T	Neoscardovia arbecensis PG10 ^T	Pseudoscardovia suis DPTE4 ^T	Pseudoscardovia radai DPVI-TET3 ^T	Parascardovia denticolens DSM 10105 ^T	Scardovia inopinata DSM 10107 ^T	Scardovia wiggsiae C1A_55 ^T
DNA G + C content (mol.%)	50.1	47.3–48.3	53.0	54.7	46.6	57.0	51.8-52.0	53.4	55.0	54.0	55.0
Peptidoglycan structure	A4α L-Lys- D-Asp	A4α L-Lys- D-Asp	A4α L -Lys- Ser-Glu	nd	A4α L-Lys- D-Asp	A1γ meso- Dpm-direct	A3β L-Orn(L- Lys)-L-Ser(L- Ala)-L-Ala ₂	A4β L-Orn(L- Lys)-D-Ser-D-Glu	nd	A4α L-(Lys/ Orn)-(Ser/ Thr)-Glu	A4α L- (Lys/Orn)- Thr-Glu
Polar lipids ^a	$2 \times PL,$ $5 \times GL, DPG$	nd	$3 \times PL,$ $6 \times GL, 5 \times L,$ DPG	nd	GLs, DPG, PL, PGLs	GLs, DPG, $2 \times PL$, PG	$2 \times PGL$, PLs, GL, DPG	PG, DPG, 5 GL, 3 PGL, PL	nd	GLs, DPG, PL, PGL	GLs, DPG, PL, PGL
Tolerance to oxygen	_	+	+	+	+	_	_		_	(+)	_
Growth at low pH	≥5.0	≥4.5	≥4.5	≥4.5	≥4.5	≥4.4	≥4.0	≥5.0	≥4.9	≥4.9	nd
Temperature range for growth (°C)	30-40	28–45	25–45	30-46	5–42	15–42	10–46	5–46	27-44	27–44	nd
Carbohydrate metabolism (API 50 CHL)											
L-Arabinose	+	+	+	+	+	+	_	_	+	_	nd
D-Xylose	+	+	+	_	_	+	_	_	_	+	_
D-Galactose	+	+	+	+	_	+	_	_	+	_	+
D-Mannose	+	-	+	_	_	_	_	-	_	_	(+)
Amygdalin	+	_	+	-	+	+	+	-	-	+	nd
Cellobiose	+	+	+	-	_	+	_	-	+	_	_
Lactose	(+)	+	+	-	_	+	_	_	+	+	+
Melibiose	+	+	+	+	-	_	_	-	+	+	+
Trehalose	_	_	+	-	+	_	_	-	-	_	_
Melezitose	+	_	+	+	-	_	(+)	-	-	_	nd
Starch	-	+	-	+	-	+	+	+	+	-	nd
Glycogen	_	+	_	+	_	+	+	+	+	+	nd
Gentiobiose	(+)	_	+	_	+	_	_	-	_	+	nd
D-Turanose	+	+	+	_	_	(+)	_	_	_	+	nd

TABLE 3.1 Differential Phenotypic Characteristics and DNA G + C Content Among All Known Species Belonging to the Scardovial Genera

	Potassium gluconate	-	(+)	_	_	_	+	-	_	_	_	nd
E (1 Z	nzyme activities apid ID 32A, API YM)											
	Phenylalanine arylamidase	+	_	+	+	+	nd	-	+	(+)	(+)	+
	Arginine arylamidase	+	+	+	+	+	nd	-	+	+	+	nd
	Leucylglycine arylamidase	_	_	_	(+)	+	nd	-	-	(+)	_	nd
	Glycine arylamidase	_	+	+	+	+	nd	-	+	+	_	nd
	α-Arabinosidase	(+)	+	+	+	+	nd	_	_	+	_	nd
	Naphtol-AS-BI- phosphohydrolase	+	nd	nd	nd	nd	+	-	-	nd	nd	nd
	Histidine arylamidase	+	+	+	+	+	nd	_	+	+	(+)	nd
	Alanine arylamidase	+	_	+	_	_	nd	_	_	(+)	+	nd
	Serine arylamidase	+	+	+	+	-	nd	_	+	+	+	+

All data are from the following studies: Crociani et al. (1996); Jian and Dong (2002); Okamoto et al. (2007); Killer et al. (2010); Downes et al. (2011); Garcia-Aljaro et al. (2012); Killer et al. (2013a,b, 2014).

All strains ferment D-glucose, D-fructose, maltose (except *Bombiscardovia coagulans*), raffinose, and sucrose (except *Aeriscardovia aeriphila*). All strains do not produce acid from glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, methyl β -D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α -D-glucopyranoside (except *Bombiscardovia coagulans*), *N*-acetylglucosamine, arbutin, trehalose, inulin, xylitol, D-lyxose, D-tagatose, D-fucose (except *Neoscardovia arbecensis*), L-fucose, D-arabitol, L-arabitol, potassium 2-ketogluconate (except *Metascardovia criceti*), or potassium 5-ketogluconate. All strains are positive for α -galactosidase (except *Pseudoscardovia radai*), β -galactosidase (except *Pseudoscardovia radai*), α -glucosidase, β -glucosidase, β -glucosidase, and tyrosine arylamidase (nd in *Neoscardovia arbecensis*), but negative for urease, β -galactosidase (-f-phosphate, β -glucosidase, *N*-acetyl- β -glucosidase (weak reaction in *Pseudoscardovia radai*), glutamic acid arylamidase (weak reaction in *Aeriscardovia aeriphila*), indole production, gelatin hydrolysis, nitrate reduction, alkaline phosphatase, proglutamic acid arylamidase, glutamyl glutamic acid arylamidase (weakly positive reaction in *Alloscardovia macacae*), catalase, and oxidase.

+, Positive; (+), weakly positive; -, negative; nd, no data available.

^a Polar lipid abbreviations: DPG, diphosphatidylglycerol; GL, glycolipid; L, lipid; PG, phosphatidylglycerol; PGL, phosphoglycolipid; PL, phospholipid.

phenotypic characteristics of three isolates were determined using API 50CHL, API Rapid ID 32A, and API-ZYM (bioMérieux). Data are from Killer et al. (2013b) and Okamoto et al. (2007).

3.2.1.5 Bombiscardovia coagulans

Various biochemical characteristics observed by Killer et al. (2010) are listed in Table 3.1.

3.2.1.6 Neoscardovia arbecensis

All three tested strains of *N. arbecensis* form a homogenous group able to ferment L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, salicin, D-cellobiose, D-maltose, D-lactose, D-saccharose, D-raffinose, starch, glycogen, D-turanose, L-fucose, and potassium gluconate. All three strains have the following activities: F6PPK, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α - and β -galactosidase, and α - and β -glucosidase. The phenotypic characteristics of three strains were determined using API 20A, API 50CHL, and API ZYM (bioMérieux). Data are from Garcia-Aljaro et al. (2012).

3.2.1.7 Parascardovia denticolens

D-Ribose, galactose, glucose, fructose, lactose, cellobiose, sucrose, maltose, melibiose, raffinose, dextrin, starch, amylose, amylopectin, inulin, and salicin are fermented. D-Xylose, D-glucuronate, L-rhamnose, mannose, α -D-galacturonate, D-fucose, L-fucose, D-galactosamine, melezitose, gluconate, lactate, xylan, polygalacturonate, alginate, arabinogalactan, pectin, laminarin, D-mannitol, D-glucitol, glycerol, hyaluronate, heparin, chondroitin sulfate, ovomucoid, bovine submaxillary mucin, porcine gastric mucin, gum arabic, gum ghatti, gum guar, gum locust bean, gum karaya, and gum tragacanth are not fermented. Fermentation of L-arabinose, D-glucosamine, and trehalose is variable. Dextran is fermented by 75% of the 16 strains. To determine sugar fermentation patterns, TPY broth was used without glucose but was supplemented with bromocresol purple (30 mg/L) as a pH indicator. Most of the substrates tested were added at a concentration of 0.5% (wt./vol.); growth, color changes, and pH values were recorded after 7 days of anaerobic incubation in GasPak jars at 37°C. Sixteen strains were tested. Data are from Crociani et al. (1996) and Jian and Dong (2002).

3.2.1.8 Pseudoscardovia suis

The carbohydrate fermentation and enzyme production patterns of the type strain M8 were determined using API 50 CHL, Rapid ID 32A, and API ZYM test strips (bioMérieux). The available results from Killer et al. (2013a) are reported in Table 3.1.

3.2.1.9 Pseudoscardovia radai

The following carbon sources are able to be utilized: D-glucose, D-fructose, starch, and glycogen. Negative for production of acids from glycerol, erythritol, D- and L-arabinose, D-ribose, D- and L-xylose, D-adonitol, methyl β -D-xylopyranoside, D-galactose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α -D-mannopyranoside, methyl α -D-glucopyranoside-*N*-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, lactose, melibiose, aesculin, raffinose, trehalose, inulin, melezitose, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D- and L-fucose, D- and L-arabitol, potassium gluconate, potassium 2-ketogluconate, and potassium 5-ketogluconate. Activities of α -glucosidase, β -glucosidase, proline arylamidase, leucine arylamidase, tryosine arylamidase, arginine arylamidase, phenylalanine arylamidase, glycine arylamidase, histidine arylamidase, trypsin, *N*-acetyl- β -glucosaminidase, and esterase lipase are detected. Production of α -galactosidase, β -galactosidase, urease, arginine dihydrolase, β -galactosidase-6-phosphate, α -arabinosidase, β -glucuronidase, glutamic acid decarboxylase, α -fucosidase, alkaline phosphatase, leucyl glycine arylamidase, pyroglutamic acid arylamidase, cystine arylamidase, alkaline phosphatase, alkaline phosphatase, esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, α -chymotrypsin, naphthol-AS-BI-phosphohydrolase, α -mannosidase, catalase, and oxidase is not observed. The biochemical characteristics of the type strain were determined using API 50 CH, RAPID ID 32A, API ZYM, and API 20A (bioMérieux). Data are from Killer et al. (2014).

3.2.1.10 Scardovia inopinata

D-Xylose, D-ribose, glucose, fructose, sucrose, maltose, dextrin, starch, amylose, and amylopectin are fermented. L-Arabinose, D-glucosamine, L-rhamnose, mannose, α-D-galacturonate, D-fucose, L-fucose, D-galactosamine, trehalose, cellobiose, gluconate, lactate, xylan, polygalacturonate, alginate, arabinogalactan, pectin, laminarin, D-mannitol, D-glucitol, glycerol, hyaluronate, heparin, chondroitin sulfate, ovomucoid, bovine submaxillary mucin, porcine gastric mucin, gum arabic, gum ghatti, gum guar, gum locust bean, gum karaya, and gum tragacanth are not fermented.

3.3 PHYLOGENETIC RELATIONSHIPS

Galactose is characteristically not fermented by this species. Fermentation of melibiose, lactose, salicin, D-glucuronate, melezitose, raffinose, and inulin is variable. Only 1 of 31 strains does not ferment dextran. Produces acetylmethylcarbinol and hydrolyzes asparagine. Nitrate reduction and indole, hydrogen sulfide, and catalase or pseudocatalase (hemin) production are negative. Gelatin is not liquefied; ammonia is not produced from urea or arginine. To determine sugar fermentation patterns, TPY broth was used without glucose but was supplemented with bromcresol purple (30 mg/L) as a pH indicator. Most of the substrates tested were added at a concentration of 0.5% (wt./vol.); growth, color changes, and pH values were recorded after 7 days of anaerobic incubation in GasPak jars at 37°C. Thirty-two strains were tested. Data are from Crociani et al. (1996), Jian and Dong (2002), and Downes et al. (2011).

3.2.1.11 Scardovia wiggsiae

Ferments D-arabinose, fructose, galactose, glucose, maltose, raffinose, ribose, and sucrose without production of gas; weakly ferments mannose; does not ferment cellobiose, dextran, mannitol, melezitose, rhamnose, sorbitol, trehalose, or xylose. Fermentation of lactose, melibiose, and salicin is variable. The production of acid from sugars was determined in peptone–yeast extract broth prepared in an anaerobic workstation with the addition of prereduced anaerobically sterilized sugars. Five isolates were tested. Data are from Downes et al. (2011).

3.3 PHYLOGENETIC RELATIONSHIPS

The 16S rRNA gene sequences of all the members of *Bifidobacteriaceae* actually validated retrieved from the DDBJ/GenBank/EMBL databases were aligned using Clustal Omega in CLC Sequence Viewer. A phylogenetic tree was reconstructed with the neighbor-joining method (Saitou and Nei, 1987) and the evolutionary distances were computed by the Kimura 2-parameter method (Kimura, 1980) using MEGA VERSION 6.0 (Tamura et al., 2013). The tree was rooted using *Micrococcus luteus* DSM 20030^T. The phylogenetic relationships are shown in Fig. 3.2.



0.02

FIGURE 3.2 Phylogenetic tree reconstruction of the family *Bifidobacteriaceae*, by means of their 16S rRNA gene sequences. The evolutionary history was inferred using the neighbor-joining method. The evolutionary distances were computed using the Tajima–Nei method and are in the units of the number of base substitutions per site. The bootstrap calculations were based on 1000 random reconstructed trees. GenBank accession numbers are in parentheses. The taxa included in the *Bifidobacterium* group are listed in Table 3.2. Bootstrap values (>50%) are shown at branch nodes. Bar, 0.02 substitutions per nucleotide site. The tree was rooted using *Micrococcus luteus* DSM 20030^T as outgroup. Evolutionary analyses were conducted in MEGA6.

3. RELATED GENERA WITHIN THE FAMILY BIFIDOBACTERIACEAE

TABLE 3.2 Taxa Included in the Bifidobacterium Group

Species	Collection no.	GenBank accession no.
Bifidobacterium adolescentis	DSM 20089	AB437355
Bifidobacterium angulatum	ATCC 27535	D86182
Bifidobacterium animalis subsp. animalis	JCM 1190	D86185
Bifidobacterium asteroides	DSM 20089	EF187235
Bifidobacterium bifidum	DSM 20456	AB437356
Bifidobacterium boum	JCM 1211	D86190
Bifidobacterium breve	ATCC 15700	AB006658
Bifidobacterium catenulatum	DSM 16992	AB437357
Bifidobacterium choerinum	ATCC 27686	D86186
Bifidobacterium coryneforme	DSM 20216	AB437358
Bifidobacterium cuniculi	DSM 20435	AB438223
Bifidobacterium dentium	ATCC 27534	D86183
Bifidobacterium gallicum	JCM 8224	D86189
Bifidobacterium gallinarum	JCM 6291	D86191
Bifidobacterium indicum	JCM 1302	D86188
Bifidobacterium longum subsp. infantis	ATCC 15697	D86184
Bifidobacterium longum subsp. longum	ATCC 55813	DB437359
Bifidobacterium longum subsp. suis	ATCC 27533	M58743
Bifidobacterium magnum	JCM 1218	D86193
Bifidobacterium merycicum	JCM 8219	D86192
Bifidobacterium minimum	DSM 20102	AB437350
Bifidobacterium moukalabense	JCM 18751	AB821293
Bifidobacterium pseudocatenulatum	JCM 1200	D86187
Bifidobacterium pseudolongum subsp. pseudolongum	JCM 1205	D86195
Bifidobacterium pullorum	JCM 1214	D86196
Bifidobacterium reuteri	DSM 23975	AB613259
Bifidobacterium ruminantium	JCM 8222	D86197
Bifidobacterium saguini	DSM 23967	AB559504
Bifidobacterium scardovii	DSM 13734	N180852
Bifidobacterium subtile	DSM 20096	D89378
Bifidobacterium thermacidophilum subsp. porcinum	DSM 17755	AB437361
Bifidobacterium thermacidophilum subsp. thermacidophilum	DSM 15837	AB437362
Bifidobacterium thermophilum	DSM 20210	AB437364

3.4 DESCRIPTION OF THE MINOR GENERA OF THE BIFIDOBACTERIACEAE FAMILY AND LIST OF THE SPECIES

3.4.1 The Genus Aeriscardovia

In a taxonomic study of 160 bifidobacteria strains recovered from different fractions of a porcine cecum, two groups were not assigned to any known *Bifidobacterium* species. The strains were identified and provisionally assigned to two new species, termed *Bifidobacterium aerophilum* and *Bifidobacterium psychroaerophilum* (Simpson et al., 2003). At a

later time *Bifidobacterium psychroaerophilum* was effectively described as new species while *Bifidobacterium aerophilum* on the bases of 16S rDNA and HSP60 gene sequences was reclassified within the family *Bifidobacteriaceae* as *Aeriscardovia aeriphila* (Simpson et al., 2004).

Gram-stain-positive, catalase- and oxidase-negative nonmotile, nonspore forming, and appearing as irregularly shaped rods, *Aeriscardovia* is isolated from pig cecum. The optimal growth is obtained under anaerobic conditions; because of the high tolerance to oxygen, *A. aeriphila* also grows under aerobic conditions, yielding elongated cells possibly due to incomplete cell division. Only one species belongs to the genus. The DNA G + C content of the type species is 54.7 mol.%. Data are from Simpson et al. (2004).

3.4.1.1 Aeriscardovia aeriphila

Short and irregularly shaped rods, approximately 0.6–0.9 μ m wide and 0.8–1.5 μ m long, arranged singly or in pairs, but not in chains, characterize *A. aeriphila*. A high tolerance to oxygen is shown. Under aerobic conditions, cell morphology includes elongated cells of approximately 4–6 μ m in length. Colonies on modified mMRS agar medium supplemented with 0.05% (wt./vol.) cysteine-hydrochloride under anaerobic conditions are gray–white, circular, and flat to convex with entire edges, and reach a diameter of up to 3 mm after 3 days' incubation at 37°C. Colonies are also formed under aerobic conditions, but a diameter of only ~1 mm is attained after 5 days' incubation. Optimum temperature for growth is 37°C, with a maximum of 46°C and minimum of 30°C. No growth occurs at 48 or 25°C. The lowest pH value recorded after growth is 4.2; minimum initial pH for growth is 4.5. DNA G + C content is 54.7 mol.%.

Originally isolated from a pig cecum, it is also reported by Shin et al. (2010) as a dominant species in a two-stage anaerobic digestion system treating food waste and recycling wastewater when the pH value is between 4.2 and 4.4. Type strains: T6, LGM21773, NCIMB13939. Data are from Simpson et al. (2004).

3.4.1.1.1 Additional Information

The *A. aeriphila* type strain was included in a study devoted to the tolerance of *Bifidobacterium* species to heat and oxygen survival following spray drying and storage (Simpson et al., 2005). Spray drying can be a good technology to manufacture powders containing high numbers of viable beneficial bacteria.

Within bifidobacteria nine species were considered to be relatively tolerant to both heat and oxygen, four species were tolerant to oxygen, and 12 species were considered sensitive to oxygen and heat. *A. aeriphila* was found to be tolerant to oxygen but sensitive to heat. These kinds of studies are of paramount importance for a possible application of *A. aeriphila* as a probiotic integrator in feed for pigs to enhance the number of beneficial bacteria in the cecal microbiota. The data were obtained from the type strain. A study on the variability within strains belonging to the species may be helpful to find a best candidate for this kind of application.

3.4.2 The Genus Alloscardovia

The taxonomic position of 12 isolates collected between 1978 and 2005 in Belgium, Sweden, and Norway, and originating from various human clinical samples, including urine, blood, urethra, oral cavity, tonsil, and abscesses of lung and aortic valve, was defined by Huys et al. (2007). Analysis of 16S rRNA gene sequence similarities revealed that the isolates were most closely affiliated to *Parascardovia denticolens* (93.0%–93.2%), *Scardovia inopinata* (92.9%–93.1%), and other members of the *Bifidobacteriaceae*.

The results of partial sequencing of the heat-shock protein 60 gene (hsp60) and determination of the DNA G + C contents (47.3–48.3 mol.%) indicated that the isolates belong to a novel genus within that family.

The values for DNA–DNA binding among four members of the novel genus were in the range of 89%–100%, indicating that the strains should be considered as a single novel species of a novel genus, for which the name *Alloscardovia omnicolens* was proposed (Huys et al., 2007).

So far three species have been described: *Alloscardovia omnicolens* in 2007 (Huys et al., 2007), *Alloscardovia macacae* in 2013 (Killer et al., 2013b), and *Alloscardovia criceti* in 2013 (Killer et al., 2013b), first described in 2007 as *Metascardovia criceti* (Okamoto et al., 2007).

The genus *Alloscardovia* comprises Gram-stain-positive, catalase- and oxidase-negative, nonmotile, nonsporeforming bacteria. Cells are coccoid, short, irregularly shaped, and slightly curved rods, which are sometime constricted at termination rods, preferentially anaerobic. The major fatty acids are C18:1, C14:0, and/or C16:0. Two species of the genus (*A. omnicolens* and *A. macacae*) display the peptidoglycan type A4α L-Lys-D-Asp; *A. criceti* displays the peptidoglycan type A4α L-Lys-Ser-Glu. The peptidoglycan contains the amino acids alanine, glutamic acid, and lysine. The DNA G + C content is in the range of 47.3–53.0 mol.%. The type species is *Alloscardovia omnicolens*. Data are from Huys et al. (2007), Okamoto et al. (2007), and Killer et al. (2013b).

3.4.2.1 Alloscardovia omnicolens

Short, irregularly shaped rods are arranged singly or in pairs. Optimal growth occurs under anaerobic conditions on modified Columbia agar after 24 h at 37°C, whereas growth under aerobic conditions occurs slowly, resulting in pinpoint-sized colonies after 72 h. Considering different temperatures, anaerobic growth occurs at 28°C (after 72 h), 37–40°C (after 24 h), and 45°C (after 96 h), but not at 50°C (after 96 h).

Likely commensal in the gastrointestinal tract and the oral cavity of humans, the specific epithet "omnicolens" (dwelling everywhere in the human body) is derived from the isolates obtained from various human clinical samples, including urine, blood, urethra, oral cavity, tonsil, and abscesses of lung and aortic valve. The actual clinical significance of *A. omnicolens* isolation in clinical specimens remains poorly documented. The type strain was isolated from the tonsil of a 25-year-old woman from Kristianstad, Sweden, in 1993. DNA G + C content within the species (mol.%): 47.8 \pm 0.5. Type strains CCUG 31649 (=LMG 23792). Data are from Huys et al. (2007).

3.4.2.1.1 Additional Information

In a study of in vitro antimicrobial susceptibility of *Alloscardovia omnicolens* and molecular mechanisms of acquired resistance, isolates were recovered from urine (n = 20), bronchoalveolar lavage (n = 3), abscesses (n = 2), genital (n = 2), blood (n = 1), superficial wound (n = 1), and urinary catheter (n = 1) specimens. All 30 strains were accurately identified as *A. omnicolens* by partial 16S rRNA gene sequencing as previously described. The type strain *A. omnicolens* was also included. All the 31 strains exhibited low MICs for β -lactams, glycopeptides, linezolid, tetracyclines, and cotrimoxazole. One strain showed MICs $\geq 256 \ \mu g/mL$ for both erythromycin and clindamycin with a single-point mutation in 23S rRNA. One strain likely had acquired fluoroquinolone resistance associated with a unique mutation in ParC (Isnard et al., 2015).

Within *Bifidobacteriaceae* the predominant isolates from saliva and infected dentine were *Bifidobacterium dentium*, *B. longum, Scardovia inopinata, Parascardovia denticolens*, and *Alloscardovia omnicolens*. *Bifidobacteriaceae* were present at high levels in the saliva of adults, and their numbers were significantly correlated with those of caries-associated organisms. The study suggests a deep investigation of the oral ecology of *Bifidobacteriaceae* to determine the utility of these organisms as markers of caries risk (Beighton et al., 2008).

A study on the effect of regular and moderate red wine consumption on the diversity and occurrence of different groups of bacteria that are represented in human saliva was done by Barroso et al. (2015). The results showed that daily consumption of 250 mL red wine during 4 weeks does not change the overall diversity and stability of representative bacterial groups found in human saliva. *Bifidobacterium dentium, Bifidobacterium* spp., and *Alloscardovia omnicolens* were the most representative bifidobacterial species.

Fifteen clinical strains fit into only four species: *A. omnicolens* (five isolates), *Bifidobacterium scardovii* (four isolates), *B. longum* (two isolates), and *B. breve* (four isolates). All five *A. omnicolens* isolates, one of the *B. breve* isolates, and three of the four *B. scardovii* isolates were cultured from urine. One *B. scardovii* isolate was from a patient with a genitourinary tract wound infection, two *B. longum* isolates were from abdominal wounds, and three *B. breve* isolates were from blood cultures. The study enlarges the spectrum of diseases and clinical sources associated with *A. omnicolens* and *Bifidobacterium* spp. The difficulties in distinguishing these organisms from other genera, such as *Actinomyces* suggest the use of 16S rRNA gene sequencing. *Bifidobacterium* species and *A. omnicolens* are difficult to identify and may be missed in specimens by many laboratories, thus producing incomplete information about the pathogenic potential of these organisms (Mahlen and Clarridge, 2009).

Following the result of Chaban et al. (2014), the dominant taxa of the vaginal microbiota were shown to be *Alloscardovia omnicolens*, *Bifidobacterium longum*, and *Streptococcus agalactiae*. In addition, Bertholom (2014) suggests *Alloscardovia omnicolens* as an emergent pathogen of the urogenital system.

3.4.2.2 Alloscardovia macacae

From one milk sample of a female macaque (*Macaca mulatta*) bred in captivity in the Central Bohemia region of the Czech Republic, bacterial isolates were characterized by sequencing of 16S rRNA. Ten isolates were identified as *Bifidobacterium adolescentis*.

One strain, designated M8, had 96.6% and 96.4% 16S rRNA gene sequence similarity to *Metascardovia criceti* and *Alloscardovia omnicolens*, suggesting the description of a new species (Killer et al., 2013b)

The M8 cells are Gram positive and catalase and oxidase negative. The morphology ranges from small coccoid cells to a mixture of longer, slightly curved, and occasionally irregular cells. Arranged singularly, they are sometimes constricted at termination. Colonies after 3 days' cultivation on TPY are very variable in shape and size. Optimum temperature for growth is 37°C, with a maximum of 40°C and minimum of 30°C. Minimum initial pH for growth is 5. DNA G + C content is 50.1 mol.%. Cells contain relatively high amounts of oleic, palmitoleic, and myristic acids.

Strain M8 is susceptible to cephalosporins (except cefixime), macrolides, penicillin-derived antibiotics (except oxacillin), vancomycin, rifampicin, clindamycin, chloramphenicol, tetracycline, and meropenem, and resistant to aminoglycosides, fluoroquinolones, sulfonamides, and the chemotherapeutics mupirocine and metronidazole.

The peptidoglycan structure is of the type A4 α L-Lys-D-Asp. The peptidoglycan contains the amino acids alanine, aspartic acid, glutamic acid, and lysine in a molar ratio of 1.0:0.7:1.0:0.5. The type strain, M8 (=DSM 24762 = CCM 7944), was isolated from the milk of a female macaque bred in captivity in the Central Bohemia region (Czech Republic). Data are from Killer et al. (2013b).

3.4.2.3 Alloscardovia criceti

The results obtained by Killer et al. (2013b) revealed that *Alloscardovia omnicolens* and *Metascardovia criceti* belong to a single genus within the family *Bifidobacteriaceae*. These bacterial genera were described simultaneously in 2007 (Huys et al., 2007; Okamoto et al., 2007). However, the genus *Alloscardovia* was published in July and the genus *Metascardovia* later in November. Therefore *Metascardovia criceti* was reclassified as *Alloscardovia criceti* comb. nov. Basonym: *Metascardovia criceti* (Killer et al., 2013b; Okamoto et al., 2007).

The following description refers to data obtained by Okamoto et al. (2007) and Killer et al. (2013b).

Brownish-gray, rough, slightly convex colonies are formed on *Brucella* HK blood agar. When grown under anaerobic conditions for 24 h, the cells are irregular rods, with occasional bifurcated extremities and tapered ends. Longer incubation under similar conditions resulted in changes of Gram reactions and the formation of shorter rods. Cells are tolerant to aerobic condition. Optimum temperature for growth is 37°C, with a maximum of 45°C and minimum of 25°C. Minimum initial pH for growth is 4.5. DNA G + C content is 53 mol.%. The peptidoglycan structure reveals type A4 α L-Lys-Ser-Glu. Completely hydrolyzed peptides comprise the amino acids alanine, serine, glutamic acid, and lysine with the following quantitative ratio: 2.8:1.3:1.4:1.0. Cellular polar lipids are represented by diphosphatidylglycerol, phospholipids, glycolipids, and lipids. The cells are isolated from golden hamster dental plaque fed with a high-carbohydrate diet. The type strain is OMB105 (=JCM 13493 = DSM 17774).

3.4.2.3.1 Additional Information

The type strains of the three species belonging to *Alloscardovia* were tested by Killer et al. (2013b) to establish susceptibility and resistance to 33 different antibiotics and two chemotherapeutics. The strains were susceptible to cephalosporins (except cefixime), macrolides, penicillin-derived antibiotics (except oxacillin), vancomycin, rifampicin, clindamycin, chloramphenicol, tetracycline, and meropenem, but were resistant to aminoglycosides, fluoroquinolones, sulfonamides, mupirocine, and metronidazole. The three strains differed from each other in moderate susceptibility to nine antibiotics.

3.4.3 The Genus Bombiscardovia

To date, three *Bifidobacterium* species have been isolated from bees' digestive tracts. Of the three species, two, *B. asteroides* and *B. coryneforme*, are found in the *Apis mellifera* intestine, whereas *B. indicum* is found in the intestine of *Apis cerana* and *Apis dorsata* from the Philippines and Malaysia, respectively (Scardovi and Trovatelli, 1969). A fourth species, *Bifidobacterium bombi*, known to be isolated only from the digestive tract of bumblebees, has been characterized (Killer et al., 2009).

The isolation and identification of new bacterial strains related to the family *Bifidobacteriaceae* is described by Killer et al. (2010).

Of the 43 F6PPK positive strains isolated from the digestive tracts of 3 different bumblebee species, 2 were taken as representatives of the new isolates. Analyses of the partial 16S rRNA gene sequences of the representative strains showed only 92.8% and 92.5% similarity to *Bifidobacterium coryneforme* and *Bifidobacterium indicum*, 92.2% similarity to *Alloscardovia omnicolens*, and slightly reduced similarity of 91% to other members of the family *Bifidobacteriaceae*.

Additional analyses of the partial heat-shock protein 60 (Hsp60) gene sequence, definition of the peptidoglycan type, characteristics of the polar lipids, definition of the major fatty acids, and other analyses indicated that the isolates represented a new genus within the family *Bifidobacteriaceae*.

On the basis of the obtained results, a novel taxon, *B. coagulans* gen. nov., sp. nov., was proposed (Killer et al., 2010).

3.4.3.1 Additional Information

Pollination by honeybees and bumblebees is widely recognized as an important service for wild plant communities, as well as for the agricultural ecosystems. According to a US Department of Agriculture report, honeybees pollinate about one-third of the human diet, and pollination is responsible for \$15 billion in added crop value. Commensal
gut *Bifidobacteriaceae* can modulate the innate immunosystem and strengthen the epithelium barrier, limiting pathogens' contact with the epithelium by the secretion of antimicrobial compound or chemical competition. The fundamental role of honeybees and bumblebees has attracted the interest of researchers for studies connected to their gut microbiota with particular attention to beneficial bacteria like some of the *Bifidobacteriaceae* species.

Honeybees and bumblebees are also important as social insects. As suggested by Billiet et al. (2017), colony contact contributes to the diversity of gut bacteria in bumblebees. *Bifidobacteriaceae* require contact with nest mates to colonize the gut of adult bumblebees (*Bombus terrestris*) and a significantly lower bacterial diversity was observed in bumblebees that were completely excluded from colony contact during their adult lives.

Very little is known about the diversity and ecology of bacteria occurring in nondomesticated bees like bumblebees, which are of similar importance to honeybees for pollination. In a study done by Koch and Schmid-Hempel (2011) of the most common bumblebee species in Central Europe, bees from three locations in Germany and Switzerland were sampled for their bacterial communities. The gut microbiota of bumblebees is apparently composed of relatively few highly specialized bacteria, indicating a strong interaction with and possibly important functions for their hosts.

Koch et al. (2012) present a study on the ecological dynamics of gut microbiota in bumblebees and identify parasite infections, colony identity, and colony age as important factors influencing the diversity and composition of the bacterial communities, suggesting a remarkable ability of the host to maintain a homeostasis in this community under widely different environments.

The diet of bumblebees consists of pollen and nectar. Diet composition could be used to influence the bacterial community in the gut to improve the health of mass-produced bumblebees used for biological pollination. Billiet et al. (2015) showed the influence of diet on the microbial composition of indoor-reared bumblebees (*Bombus terrestris*). Three sugar syrups and sterilized pollen were tested. A high-fructose concentration can prevent the growth of *Bifidobacteriaceae*, but once the *Bifidobacteriaceae* colonized the gut, they could maintain their prevalence even under high-fructose concentrations.

To understand the role of the gut microbial composition in protecting bees against pathogens, *Bifidobacteriaceae* species were used as dietary supplements. The obtained results show a positive effect in the reduction of *Nosema ceranae* in *Apis mellifera* (Baffoni et al., 2015).

3.4.3.2 Bombiscardovia coagulans

Cells growing in liquid anaerobic media in the form of large coagulates are nonspore-forming and Gram-positive, and appear as short and occasionally irregularly shaped rods. They were arranged singly or in pairs, but not in chains. Colonies on TPY agar under anaerobic conditions were found to be very rigid, circular, and convex in shape, with whole edges looking gray-white and often with small indentations. Colonies formed under aerobic conditions were smaller and of different shapes.

Optimum temperature for growth is 37°C, peaking at 42°C with a maximum of 45°C and minimum of 5°C. The ability of bacteria found in bumblebees to grow even at temperatures as low as 5°C could be related to the adaptation of these bacteria to unfavorable conditions in the digestive tract of bumblebee queens during the winter hibernation. Minimum initial pH for growth is 5. DNA G + C content is 46.6 mol.%. The peptidoglycan structure reveals type A4 α L-Lys-D-Asp. Murein contains the amino acids alanine, aspartic acid, glutamic acid, and lysine in a molar ratio of 1.6:0.9:1:1. The type strain produced acetic and lactic acids from glucose at a molar ratio of 3.3:1. Major fatty acids are C18:1, C20:0, and C18:0. *Bombiscardovia coagulans* can be isolated from the digestive tract of various bumblebee species.

The type strain, BLAPIII-AGV (=ATCC BAA-1568 = DSM 22924), is isolated from the digestive tract contents of *Bombus lapidarius* from Central Bohemia, Czech Republic. Data are from Killer et al. (2010).

3.4.4 The Genus Neoscardovia

Among five strains isolated from pig slurries, three were selected for taxonomic studies.

Analysis of the 16S rRNA gene sequence showed a maximum identity of 94% to various species of the family *Bifidobacteriaceae*. However, phylogenetic analyses of 16S rRNA and HSP60 gene sequences revealed a closer relationship of these strains to members of the recently described *Aeriscardovia*, *Parascardovia*, and *Scardovia* genera, than to other *Bifidobacterium* species. On the bases of phylogenetic results a new genus belonging to the family *Bifidobacteriaceae* was proposed (Garcia-Aljaro et al., 2012). Only recently the new genus and species were validated, appearing on the list of new names and new combinations previously effectively, but not validly, published (Oren

and Garrity, 2015). The genus *Neoscardovia* comprises anaerobic, Gram-stain-positive, catalase- and oxidase-negative, nonmotile, nonspore-forming, irregularly shaped rods occasionally forming a "Y" shape. Only *N. arbecensis*, type species, belongs to the genus. The DNA G + C content of the type species is 57 mol.%. The peptidoglycan structure is A1 γ meso-Dpm-direct. The prevailing cellular fatty acids are C16:0 and C18:1 ω 9c, and the major polar lipids consist of a variety of glycolipids, diphosphatidyl glycerol, two unidentified phospholipids, and phosphatidyl glycerol. Data are from Garcia-Aljaro et al. (2012).

3.4.4.1 Neoscardovia arbecensis

The name of the species came from the village of Arbeca in Catalonia. It is characterized by Gram-positive bacilli showing pleomorphic cells with occasional bifurcations, sometimes arranged in a "Y" form, obligate anaerobe, non-spore-forming. Colonies growing on Columbia blood agar with the addition of L-cysteine hydrochloride at 0.5 g/L and glucose at 5 g/L, are white, approximately 1 mm in diameter after 72 h incubation at 37°C, smooth, glistening, convex, and circular with entire edges. The growth temperature is in the range of 15–42°C. Colonies are able to grow between pH 5 and pH 8 at 37°C. The lowest pH attained after growth was 4.39. The G + C content of the type strain PG10^T is 57 mol.%. It is isolated from pig slurries while searching for host-specific bifidobacteria to track the source of fecal pollution in water. This species might be useful to establish the source of fecal contamination of porcine origin. The type strain is PG10 (=CECT 8111 = DSM 25737). Data are from Garcia-Aljaro et al. (2012).

3.4.5 The Genus Parascardovia

In a taxonomic study, 70 strains isolated from dental caries were classified in three different taxa. One of these taxa was identified as *Bifidobacterium dentium*. The other two were described as new *Bifidobacterium* species: *Bifidobacterium inopinatum* and *Bifidobacterium denticolens* (Crociani et al., 1996). On the basis of the results obtained from the phylogenetic analysis of both 16S rRNA and HSP60 sequences, the DNA base compositions, and some phenotypic characteristics, Jian and Dong (2002) proposed that these two new *Bifidobacterium* species should be transferred to two new genera as *Scardovia inopinata* and *Parascardovia denticolens*.

The genus *Parascardovia* is described as anaerobic Gram-stain-positive, nonacid-fast, nonmotile, and nonsporeforming, with small, slender rods of variable shape. It is found in human dental caries. Only the *Parascardovia denticolens* type species belongs to the genus. The DNA G + C content is 55 mol.%. The peptidoglycan structure is not available.

3.4.5.1 Parascardovia denticolens

The description of Parascardovia denticolens (Jian and Dong, 2002) refers to the work done by Crociani et al. (1996) for *Bifidobacterium denticolens*. The results of the phenotypic characterization of the 32 strains obtained from eight specimens show, as happens in many cases, a variability within the species. Characteristics are Gram-positive, nonmotile, nonspore-forming, small, slender rods that occasionally are arranged in a V shape ($0.8-1.5 \mu m \log p$). Morphology is variable depending on the cultural conditions. In the presence of lactose, the cells of arabinose-negative strains are very long (4–5 μm), are sometimes branched, and have wide ends; the cells of arabinose-positive strains are smaller and coccoid (0.4–0.6 μ m long). At an initial pH of 7.7 or 8.0 the cells are very small (0.4–0.6 μ m); at an initial pH of 4.9 or 5.5 the cells are longer and branched or short (0.8–2.5 µm) and frequently club shaped. In liquid cultures incubated in air/ CO_2 (1/10) at 37°C, the cells are much longer rods with swollen ends; however, the cells become very long (3–10 µm) and branched when cultures are incubated at 30°C, whereas the cells are unbranched and flexuous with protuberances if they are grown at 42°C. Liquid cultures of this species always have clear supernatants with compact sediment in clumps that cannot be dispersed with vigorous swirling. Colonies on TPY agar are soft, smooth, circular, and convex with irregular margins, glistening and cream to white. They are anaerobic, and CO_2 enhances anaerobic growth. The optimum temperature for growth is 36.5–38.5°C, the minimum temperature is 27.0–30.5°C, and the maximum temperature is 42–44°C. No growth occurs at 25 or 46°C. The optimum initial pH is 6.7–7.3, growth is delayed at pH 4.9 or 8.0, and no growth occurs at pH 4.4 or 8.5. The G + C content of the DNA is 55 ± 1 mol.% (as determined by the thermal denaturation method). They are found in human dental caries. The type strain is strain B3028 (=DSM 10105 = CCUG35728 = LMG18312 = NCTC12936).

3.4.5.1.1 Additional Information

The human stomach, when healthy, is not a suitable host for microorganisms, but in pathological conditions, such as autoimmune atrophic gastritis or omeprazole-treated gastritis, microbial overgrowth can be observed. In a study

on the occurrence of *Bifidobacteriaceae* in the human hypochlorhydria stomach, a total of 55 isolates were assigned to the *Bifidobacteriaceae* family.

These 55 *Bifidobacteriaceae* isolates were found to belong to three prevalent species: *Bifidobacterium dentium* (5 isolates), *Scardovia inopinata* (14 isolates), and *Parascardovia denticolens* (34 isolates). This study suggests that the *Bifidobacteriaceae* species typically found in the oral cavity readily colonize the hypochlorhydria stomach (Mattarelli et al., 2014a).

A study on the microbiota from caries and vital coronal pulps was performed in nine children (age range: 2.6–5.0 years). The presence in coronal pulps of *Parascardovia* in addition to another few species suggests that this taxon may play direct or supporting roles in pulp pathology in children (Chalmers et al., 2015).

P. denticolens might contribute to the initial establishment of the microbiota in the newborn since it was found in breast milk (Solís et al., 2010). The only strain isolated was used in a subsequent study, to establish the genome sequence (Gueimonde et al., 2012). *Parascardovia denticolens* was also detected in the feces of one of the 12 South African horses studied by Endo et al. (2009).

3.4.6 The Genus Pseudoscardovia

Several studies have been devoted to the microbiota of the digestive tract of domestic pigs. Within the family *Bifidobacteraceae* several species of bifidobacteria were found: *Bifidobacterium thermophilum*, *Bifidobacterium psy-chraerophilum*, *Bifidobacterium choerinum*, *Bifidobacterium boum*, and the subspecies *Bifidobacterium thermacidophilum* subsp. *porcinum* and *Bifidobacterium longum* subsp. *suis*. Furthermore, *Aeriscardovia aeriphila* was isolated from a porcine cecum and *Neoscardovia arbecensis* from pig slurries.

From the digestive tract of a wild boar (*Sus scrofa*), Killer et al. (2013a) isolated two strains identified by genotypic, phylogenetic, and phenotypic analyses. The results suggest the classification as members of the family *Bifidobacteriaceae*. Nevertheless, they differ from the described genera in many characteristics. Therefore, a new genus *Pseudoscardovia* was proposed with a specific epithet of *P. suis*. In a successive paper Killer et al. (2014) described a strain isolated from a subspecies of wild boar (*Sus scrofa scrofa*) as a new species belonging to the genus *Pseudoscardovia* and named *P. radai*.

Pseudoscardovia are Gram-stain-positive, catalase- and oxidase-negative, nonmotile, nonspore forming, and strictly anaerobic. Growth occurs at a relatively wide range of temperatures (10–46°C) and pH (4.0–9.5). Two species have been described, the type species *Pseudoscardovia suis*, along with *Pseudoscardovia radai*.

3.4.6.1 Pseudoscardovia suis

Cells appear as regularly shaped and sometimes slightly curved rods occurring mainly in pairs, an unusual morphology, not observed in bifidobacteria and other *Scardovia* spp. Colonies on TPY agar under anaerobic conditions after 72 h are white or cream in color, opaque, forming irregular burls of various sizes. Most of the colonies reach only 0.2–0.3 mm in diameter. They are strictly anaerobic. Optimum temperature for growth is 37°C. Peptidoglycan structure is A3 β L-Orn(L-Lys)-L-Ser(L-Ala)-L-Ala2. Major fatty acids in cells are C16:0, C18:1, and C14:0. Polar lipids consist of a variety of phosphoglycolipids and phospholipids, a glycolipid and diphosphatidylglycerol. DNA G + C content of the type strain is 51.8 mol.%. The type strain, DPTE4 (=DSM 24744 = CCM 7942), was isolated from the digestive tract contents of wild boar (*Sus scrofa*) originated from the Central Bohemia region (Czech Republic). Data are from Killer et al. (2013a).

3.4.6.2 Pseudoscardovia radai

Cells growing in anaerobic TPY broth are irregular club-shaped rods with no branching, occurring mostly singly, 0.6–1.2 µm wide, and 1.1–4.3 µm long. Colonies on TPY agar under anaerobic conditions after 72 h have a soft consistency and are circular and convex with entire edges and cream in color. Most colonies reach a maximum of 2.1 mm in diameter. However, some can reach 6.0 mm in diameter. Optimum temperature for growth is 37°C; they are strictly anaerobic. They are peptidoglycan type A4 β L-Orn(L-Lys)-D-Ser-D-Glu, thus different from that identified in *P. suis*. The resulting structure of the peptidoglycan has not been identified in any other scardovial species or species of bifidobacteria. The DNA G + C content of the type strain is 53.4 mol.%. Relatively large proportions of C_{14:0}, C_{18:1} ω 9c, and C_{16:0} fatty acids are present. Principal polar lipids in cells are phosphatidylglycerol and diphosphatidylglycerol and a number of unidentified glycolipids and phosphoglycolipids. The type strain, DPVI-TET3 (=DSM 24742 = CCM7943), was isolated from the small intestine content of a male of the wild boar subspecies (*Sus scrofa scrofa*) caught in the Central Bohemia region. Data are from Killer et al. (2014).

3.4.7 The Genus Scardovia

Jian and Dong (2002) proposed that two *Bifidobacterium* species, *Bifidobacterium inopinatum* and *Bifidobacterium denticolens* described by Crociani et al. (1996), should be transferred to two new genera as *Scardovia inopinata* and *Parascardovia denticolens*.

The first description of the genus *Scardovia* by Jian and Dong (2002) was emended at a later time by Downes et al. (2011). The following information was taken from the two articles. Cell morphology ranges from small coccoid cells to a mixture of longer and straight, curved, and branched cells in diphtheroidal arrangements. They are Gram-positive nonacid-fast, nonspore-forming, nonmotile, and anaerobic. The major fatty acids are $C_{16:0}$ and $C_{18:1}$ ω 9c. Polar lipids consist of a variety of glycolipids together with diphosphatidylglycerol, an unidentified phospholipid.

Two species have been described, the type species *Scardovia inopinata* and *Scardovia wiggsiae*, both present in the oral cavity.

3.4.7.1 Scardovia inopinata

The description of Scardovia inopinata (Jian and Dong, 2002) refers to the one by Crociani et al. (1996) for Bifidobacterium denticolens. The description was emended later on by Downes et al. (2011). The description is based on 16 isolates obtained from 5 specimens of dental caries. They are characterized by Gram-positive, nonmotile, nonsporeforming, small, slender rods that occasionally are arranged in a V shape and are $0.8-1.5 \mu m$ long. Morphology is variable depending on the cultural conditions. In the presence of lactose, the cells of arabinose-negative strains are very long (4–5 μ m), are sometimes branched, and have wide ends; the cells of arabinose-positive strains are smaller and coccoid (length, 0.4–0.6 µm). At an initial pH of 7.7 or 8.0 the cells are very small (length, 0.4–0.6 µm); at an initial pH of 4.9 or 5.5 the cells are longer and branched (length, 0.8–2.5 µm) and frequently club shaped. In liquid cultures incubated in air/CO₂ (1/10) at 37°C the cells are much longer rods with swollen ends; however, the cells become very long (length, 3–10 μm) and branched when cultures are incubated at 30°C, whereas the cells are unbranched and flexuous with protuberances if they are grown at 42°C. Colonies on TPY agar are soft, smooth, circular, convex with irregular margins, glistening, and cream to white. Anaerobic CO_2 enhances anaerobic growth. The optimum temperature for growth is 36.5–38.5°C, the minimum temperature is 27.0–30.5°C, and the maximum temperature is 42–44°C. No growth occurs at 25 or 46°C. The optimum initial pH is 6.7–7.3, growth is delayed at pH 4.9 or 8.0, and no growth occurs at pH 4.4 or 8.5. Downes et al. (2011) added the following data. The major fatty acids are $C_{16:0}$, C_{181} ω 9c, and C_{140} . The cellular polar lipids include a variety of glycolipids together with diphosphatidylglycerol, an unidentified phospholipid, and an unidentified phosphoglycolipid. The peptidoglycan structure is A4 α L-(Lys/ Orn)-(Ser/Thr)-Glu. The G + C content of the DNA is 54 mol.%. They are found in human dental caries. The type strain is strain B3028 (=DSM 10105).

3.4.7.1.1 Additional Information

Within the family *Bifidobacteriaceae* three species, *Bifidobacterium dentium*, *Parascardovia denticolens*, and *Scardovia inopinata*, are present as an abundant population on the oral cavity. More recently a new species, *Scardovia wiggsie* (Downes et al., 2011), was isolated from human carious dentine.

Oral *Bifidobacteriaceae*, having both acidogenic and aciduric characteristics, can play a relevant role in the progression of dental caries, including root caries. These organisms can routinely be isolated from lesions associated with dental root caries using appropriate cultural methods. Additional studies improving the number of samples of lesions from the enamel surfaces of the deciduous or permanent dentitions and root caries lesions can clarify if there is a characteristic microbiota associated with each type of carious lesion (Mantzourani et al., 2009).

An interesting characteristic shared by *P. denticolens* and *S. inopinata* is the ability to degrade complex carbohydrates, including dextran, that can potentiate the production of demineralizing acid within oral biofilm in the absence of dietary fermentable carbohydrates (Mantzourani et al., 2009; Modesto et al., 2003). It is interesting to note that *B. dentium* is routinely present in samples from caries, whereas only one of either *S. inopinata* or *P. denticolens* will be present in any one sample, suggesting that there is a kind of mutual exclusion operating here (Modesto et al., 2006). In an edentulous mouth, where there is gingivitis or some other inflammation, other nonoral bifidobacteria are mostly present, and the most representative species are *B. breve*, *B. longum* subsp. *longum*, and *B. scardovii* (Mantzourani et al., 2010). Ling et al. (2010) found 200 genera belonging to 10 phyla present as members of the oral microbiota in children. The study did not detect any *Bifidobacteriaceae*.

The complete genome sequence of the type strain of *S. inopinata* is available in a paper published by Oshima et al. (2015).

3.4.7.2 Scardovia wiggsiae

The description by Downes et al. (2011) is based on five strains isolated from the human oral cavity and one strain isolated from an infected arm wound. These strains are gram-positive, anaerobic, nonspore-forming, and nonmotile bacilli (0.6–0.7 μ m by 1.6–4 μ m). Cells are pleomorphic with straight, slightly curved, and some club-shaped forms arranged singly, in pairs, or in short chains, with some branched and diphtheroidal arrangements. After 7 days on fastidious anaerobe agar supplemented with 5% horse blood (FAA), colonies are pleomorphic, 0.4–1.2 mm in diameter, circular, irregular or molar tooth in shape, entire or undulate, gray, off-white or cream, convex, and opaque. The major fatty acids are C_{16:0} and C_{18:1} ω 9c. Cellular polar lipids include a variety of glycolipids together with diphosphatidylglycerol, an unidentified phospholipid, and an unidentified phosphoglycolipid. Peptidoglycan is of the type A4 α L-(Lys/Orn)-Thr-Glu. The G + C content of the DNA is 55 mol.%. The type strain is C1A_55 (=DSM 22547 = CCUG58090), isolated from the human oral cavity and an arm wound from an intravenous drug user.

3.4.7.2.1 Additional Information

Several studies claim a role for *S. wiggsiae* in a wide variety of oral disease conditions. It is important that new methods for the detection of *S. wiggsiae* be developed. Examples of detection are by PCR of *Scardovia wiggsiae* in combination with *Streptococcus mutans*, for the accurate prediction of caries risk in children (Vacharaksa et al., 2015).

In a study by Tanner et al. (2011), *S. wiggsiae*, detected by PCR, showed a strong association with severe early childhood caries, even stronger than *S. mutans*.

Tian et al. (2014) developed a disposable, paper-based PCR-dipstick DNA chromatography method. Parallel oligonucleotides were immobilized on a dipstick strip for multiplex analysis of target DNA sequences of the cariesassociated bacteria, *Streptococcus mutans, Streptococcus sobrinus, Scardovia wiggsiae, Actinomyces* species, and *Veillonella parvula*, to specifically recognize target DNA sequences.

The acidogenic–aciduric species *Scardovia wiggsiae* correlated with advanced cariogenic activity (Henne et al., 2015). On the other hand, niches of the oral cavity with no caries activity also harbor *S. wiggsiae*. The authors suggest *S. wiggsiae* as a potential new marker species for caries activity if the relative abundance is considered.

3.5 CONCLUDING REMARKS

Bacterial relationships based almost exclusively on a single molecule type, namely 16S rRNA, have enabled the classification of some new isolates closely related to the genus *Bifidobacterium* into new genera within the family *Bifidobacteriaceae*. Starting in 2002, 7 new genera (the so-called scardovial genera) and 11 species have been described. The growing attention devoted to the genus *Bifidobacterium*, of which this book is also an expression, is mainly connected to its species' role as beneficial bacteria as commensals in humans and animals, influencing the physiology of the host and maintaining a healthy gastrointestinal tract.

As it appears in the literature, the new scardovial genera have received, up to now, scarce attention, although they are considered closely related to bifidobacteria, and so are then potentially important for applications as beneficial bacteria. In most cases, both biodiversity and practical importance are largely unknown. It is desirable that scientists will consider aspects of application, collecting a larger number of strains and performing new studies connected to the requirement for specific characterization related to an application.

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4

Isolation, Cultivation, and Storage of Bifidobacteria

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4.1 INTRODUCTION

The main topics of this chapter are isolation, cultivation, and storage of members of the genus *Bifidobacterium*. Discovered at the end of the 19th century, bifidobacteria are considered as key commensals in host-microbe interactions, and are believed to play a crucial role in nutrition, immunomodulation, and resistance to infection. This has fostered numerous studies focusing on their role in gastrointestinal ecology and on possible health promoting aspects related to their presence in the gut. Today, bifidobacteria have become economically important: several species have been assessed by the European Food Safety Authority (EFSA) as qualified presumption of safety and are currently added in high numbers as probiotics in numerous food preparations (EFSA, 2016; Rios-Covián et al., 2016). However, the ability of bifidobacteria to exert beneficial effects on human health is a species- and strain-specific feature (Ramos et al., 2013). This explains the need for a continuous search for novel strains with probiotic potential (Awasti et al., 2016) which in turn requires a culture-dependent method with the ability to effectively elect and select them. Isolation and cultivation in appropriate selective and culture media requires detailed knowledge of the nutrition and the physical conditions for growth of the given organisms (Stolp and Starr, 2013).

The lack of an accurate picture of the bifidobacterial species diversity in different hosts and natural environments is partly due to a lack in technical advances in the selection field.

Over the past three decades, molecular biology was an enormous driving force in uncovering the microbial diversity in gut, feces, breast milk, and fermented products (Allen-Blevins et al., 2015; Cabrera-Rubio et al., 2016; De Filippo et al., 2010; Donaldson et al., 2015; Frese et al., 2015; Gomez et al., 2016; Hunt et al., 2011; Jost et al., 2013; Mazidi et al., 2016; Rodríguez et al., 2015; Thompson et al., 2015; Zhang et al., 2016). Despite more traditional methods indicating that bifidobacteria constituted an important part of the core gut microbiota in human and nonhuman hosts, metagenomic studies have revealed their low abundance or even apparent absence (McKenney et al., 2015; Xu et al., 2013, 2010). The reason for such a low detection of *Bifidobacterium* spp. by culture-independent investigations is most likely due to technical biases, in particular those related to DNA extraction protocols and/or the PCR primers used. Thus, even though a vast amount of microbial diversity has been revealed since the advent of omics-based approaches, huge knowledge gaps remain between the known phyla and those possessing cultured representatives (Alain and Querellou, 2009). On the other hand, it is remarkable how much we have learned about bifidobacterial species distribution in different hosts and environments by studying only a small fraction of its diversity with culture dependent techniques (Angelakis and Lagier, 2016; Endo et al., 2012; Kesmen et al., 2012; Lagier et al., 2015; Michelini et al., 2015a,b, 2016a; Modesto et al., 2014, 2015; Ramos et al., 2010). When used in combination, molecular and culture methods provide an efficient approach to the enumeration and identification of bifidobacteria from different biological materials. Cultivation and purification also provide new genome sequences that assist in designing better primers and probes for the refinement of molecular detection methods. History shows that the majority of advances in basic and applied microbial science, including physiology, biochemistry, genetics, medicine, diagnostics, veterinary, agriculture, and biotechnology are founded in studies of pure cultures (Prakash et al., 2013b).

The high demand for new strains with validated probiotic properties has motivated a large numbers of studies focusing on the isolation and selection of new bifidobacterial strains from their natural sources. Samples of gastrointestinal tract (GIT) contents, such as feces, samples of oral swabs, breast milk, and vaginal swabs are rich and well-used natural sources for isolation of bifidobacteria in human and nonhuman hosts. In order to allow the selective enumeration of bifidobacteria on the agar medium, certain aspects have been considered, such as the provision of special growth factors, fortification of the medium with blood or elective carbohydrates, and antimicrobial substances that can inhibit the growth of related bacteria [in particular, lactobacilli and other lactic acid bacteria (LAB), propionibacteria, clostridia, and *Actinomyces* spp.].

However, the choice of a selective medium is a challenging current task as several factors may have a negative impact on the final result.

At the time of writing, none of the selective media developed for *Bifidobacterium* isolation and enumeration is satisfactory for each different type of sample.

Each medium has its own merits and demerits: for example, the inability to detect some species, reduced sensitivity for stored samples, inadequate inhibition of the growth of LAB or clostridia, or complex composition and preparation procedure.

The present chapter will deal with the elective and selective media used to cultivate and to enumerate bifidobacteria in their different niches, highlighting that the bifidobacteria selective media should be chosen in respect of origin of the sample tested. Moreover, the industrial exploitation of bifidobacteria as probiotic cultures and their claimed effects on host health have also resulted in an increased interest in techniques for their maintenance and long-term preservation, which are required to guarantee long-term delivery of stable cultures in terms of viability and activity. Indeed, it is evident that the work on isolation and characterization seems incomplete until the obtained axenic cultures are adequately preserved. Long-term storage of the isolated strains without changes in their phenotypic and genotypic features is mandatory for future reference, industrial applications, research, and new discoveries in their microbiology (Prakash et al., 2013b). Therefore, the final section of the chapter will be devoted to the appropriate preservation protocol(s) suitable for *Bifidobacterium*.

4.2 CULTIVATION

Bifidobacteria are widely distributed in nature and in their environment they have adapted to the habitats most suitable for their needs (Kwak et al., 2016; Milani et al., 2015a, 2016). In the laboratory, however, bifidobacteria are generally known to be difficult to grow and their cultivation is often related to the presence of various growth factors in the media (Biavati and Mattarelli, 2012).

Bifidobacteria are not acid tolerant, they are anaerobes and consequently they cannot grow under oxygencontaining air or in a medium with high oxidative potential. However, sensitivity to oxygen is different among different strains and species (Scardovi, 1986) and some species are reported to show O₂ tolerance only in the presence of CO₂ (Scardovi and Crociani, 1974; Scardovi and Trovatelli, 1969; Scardovi et al., 1979, 1969). Moreover, CO₂ seems to be essential for colony development (Kawasaki et al., 2007).

Bifidobacteria can grow well in a medium containing appropriate reducing agents, such as cysteine, cysteinehydrochloride, thioglycolate, sodium sulfite, and ascorbic acid. Cysteine and cystine are also considered essential amino acids for growth as they provide a nitrogenous source (Hoover, 2014; Roy, 2001; Scardovi, 1986; Tamura, 1983). Additionally, ammonium salts can serve as a source of nitrogen (Hoover, 2014). Iron (both oxidation forms), magnesium, and manganese are necessary trace elements (Bezkorovainy et al., 1996).

Concerning requirement for vitamins, bifidobacteria usually need a full complement of the B vitamins for optimal growth, even though human strains of bifidobacteria can synthesize relatively large amounts of vitamins B_6 (pyridoxin), B_7 (biotin) B_9 (folic acid), B_{12} (cyanocobalamin) (D'Aimmo et al., 2014, 2012; Deguchi et al., 1985; Hoover, 2014; Pompei et al., 2007; Rossi et al., 2011). Pantothenic acid (or frequently pantetheine) and riboflavin were reported to be required by almost all strains of bifidobacteria (Modesto et al., 2003). Biotin seems to be essential for bifidobacteria of infant origin (Tamura, 1983). B vitamin requirements can be easily supplied by yeast extract, considered to be an excellent growth promoter (Ibrahim and Bezkorovainy, 1994).

Bifidobacteria also require carbohydrates: most strains can utilize glucose, galactose, lactose, lactulose, oligosaccharides, and products of starch hydrolysis, and bicarbonates as carbon sources (Hoover, 2014; Tamura, 1983).

Bifidobacteria may also convert CO_2 in bicarbonate, by carbonic anhydrase, whose presence has been reported in their genomes (Van der Meulen et al., 2006). Lauer and Kandler (1976) have shown that small amounts of succinic acid are probably produced through a CO_2 fixation reaction. However, Chiappini (1996) showed that although external CO_2 can be fixed by bifidobacteria and seems to stimulate growth, it is not incorporated during the conversion of phosphoenolpyruvate to oxaloacetate.

Oligosaccharides are also used as bifidogenic (prebiotics) factors to stimulate the growth of bifidobacteria. It is assumed that these carbohydrates are selectively fermented by bifidobacteria. Oligosaccharides with bifidogenic activity

are: lactulose, *trans*-galactosyl oligosaccharides (TOS), fructo-oligosaccharides (FOS), isomalto-oligosaccharides, raffinose, and soybean oligosaccharides.

Hence, bifidobacteria are normally defined as nutritionally fastidious anaerobic microorganisms and their complex growth requirements are usually satisfied only in rich culture media. For this reason, substrates with a complex composition, containing products, such as liver or meat extracts, a wide range of peptones, bovine casein digest, bovine milk whey, hog gastric mucin, yeast extract, tomato juice, horse blood, or human milk, which supply nurturing substances for growth, have been described and are used for routine culturing and for optimal propagation of *Bifidobacterium* spp.

Furthermore, the growth conditions of bifidobacteria in a culture medium should be improved using suitable techniques. The liquid media or liquefied agar in tubes should be held in a boiling water bath or steamer for at least 20 min to remove dissolved oxygen, then cooled and inoculated immediately.

There are several optimal (basal) culture media (fresh and/or dehydrated) recommended for the cultivation of bifidobacteria. According to Rasić and Kurmann (1983), these media may be divided into complex, selective, semisynthetic, and synthetic, as well as commercial dehydrated culture media.

Blood liver (BL) agar medium is considered to be an optimal culture medium for maintenance and detection of bifidobacteria. It was first described by Ochi et al. (1964), by Mitsuoka (2014) and Mitsuoka et al. (1965), and finally modified by Teraguchi et al. (1978). It is occasionally called the *Mitsuoka* culture medium (Rasić and Kurmann, 1983). However, BL is a self-prepared culture medium with a complicated composition, not recommended for routine work as it is time consuming to prepare and contains a lot of ingredients, some of which must be filter sterilized (e.g., the preparation of a liver infusion) (Roy, 2001). In 1995, Lim et al. proposed the use of BL agar, which is available as commercial dehydrated medium.

In 1986, Scardovi indicated that bifidobacteria vary widely in their physiological requirements for growth, suggesting the use of a complex, nonselective medium TPY (Trypticas–Phytone–yeast extract), containing Trypticase and Phytone that permits satisfactory growth of all *Bifidobacterium* species. TPY (Scardovi, 1986) is considered as an elective medium and it is commonly used for the routine culturing of bifidobacteria from all known habitats, as well as for their isolation. In this latter case, however, it allows the growth of other bacteria, especially LAB from all habitats. It requires no blood supplements or components that are difficult to prepare, and is now available commercially (Barrett et al., 2011; Petr and Rada, 2001).

de Man, Rogosa, Sharpe medium (MRS) was originally designed for isolating and counting of a specific group within the genus *Lactobacillius*. The acetate concentration inhibits the growth of some brewery contaminants and the original paper reporting its formulation specifies that the carbohydrate(s) used in its preparation should be matched to the substrate from which the bacteria are to be isolated. When modified with the addition of 0.5 g/L-cysteine–HCl, MRS is considered an optimal liquid medium and it can provide overall growth factors for bifidobacteria. L-cysteine is added to lower the oxido/reduction potential in culture media and provides better anaerobic conditions for the growth of bifidobacteria (Roy, 2001). MRS is also used with an added 1% ascorbic acid and 0.02% cysteine hydrochloride for mass cultures (Rasić and Kurmann, 1983).

According to Pacher and Kneifel (1996), the supplementation of modified MRS with water-soluble vitamins (thiamin, riboflavin, and pantothenic acid) and lactulose syrup in addition to glucose may improve the growth of certain *Bifidobacterium* species. Several growth promoting substances derived from human and bovine whey might also be added to MRS because both α -lactalbumin and β -lactoglobulin are good bifidobacterial promoters (Ibrahim and Bezkorovainy, 1994). Indeed, the supplementation of the modified MRS of Pacher and Kneifel (1996) with human whey was very effective for growth of bifidobacteria.

The modified Wilchins–Chalgren (WC) medium supplemented with the soya peptone (5 g/L), L-cysteine (0.5 g/L), and Tween 80 (1 mL/L) (Bunešová et al., 2012) can provide optimal conditions for propagation of bifidobacteria. WC medium is designed for anaerobic microorganisms, it contains glucose as the sole carbon source, which might limit bifidobacterial growth, since some species do not utilize this sugar (Rada and Petr, 2000). With respect to the nutritional requirements of bifidobacteria, the soya peptone contains raffinose-series oligosaccharides. Therefore, supplementation of WC medium with soya peptone (5 g/L) seems to be an optimal way to meet the nutritional requirements of bifidobacterial species, because raffinose-series oligosaccharides can serve as a good carbon source (Rada et al., 2008). Comparable growth of bifidobacteria has been demonstrated in both TPY broth and WC broth supplemented with 5 g/L soya peptone (Bunesova et al., 2015).

Other complex culture media suitable for propagation of bifidobacteria and available in commercial trade are brain infusion broth (BHI), Gifu anaerobic medium (GAM), chopped meat medium, Columbia agar base (CAB), Eggert and Gagnon medium, and reinforced clostridial medium (RCM). To these media are added 0.5 g/L cysteine hydrochloride, if needed, to lower the redox potential (Barrett et al., 2011; Roy, 2001; Scardovi, 1986).

Semisynthetic and complete chemically defined media (Kongo et al., 2003; Modesto et al., 2003) have been developed for bifidobacteria for different purposes, including investigations of the nutritional requirements of bifidobacteria, obtaining auxotrophic mutants for different compounds (Sakaguchi et al., 2013; Veda et al., 1983), and isolation of bioactive molecules (e.g., folic acid) (D'Aimmo et al., 2014, 2012; Pompei et al., 2007; Rossi et al., 2011) mimicking natural environments (Bezkorovainy et al., 1996).

Examples of semisynthetic media are Tomarelli's and Norris' media, whereas Petuley's medium, Poch's medium, and Bezkorovian's medium are entirely synthetic culture media (Rasić and Kurmann, 1983).

4.3 ISOLATION

Bifidobacteria were originally isolated and described in the feces of breast-fed infants by Henry Tissier, who observed an abundance of an irregular Y-shaped bacterium and named it Bacillus bifidus communis because of its supposed tendency to bifurcate. In fact, the term bifidus in Latin means "forked in two parts." Tissier used 1% deep glucose-agar tubes inoculated with varying dilutions of fecal emulsion as the primary culture medium. Higher dilutions were investigated under a microscope to detect *Bifidobacterium bifidum* (branched forms). Positive findings were followed by transferring the same type of colonies to dextrose agar to obtain pure cultures (Rasić and Kurmann, 1983). Later interest in the isolation and growth requirements of this organism was stimulated by its apparently unique occurrence in the stools of nurslings since it was surmised that its presence in the intestine was of physiologic significance and might prevent intestinal disorders caused by other bacteria (Norris et al., 1950). However, since the early days of its discovery a further investigation was thought desirable into methods of isolating and cultivating bifidobacteria. Other authors tried to change or improve upon this Tissier's technique but essential advances were not obtained. Indeed, one of the early obstacles encountered was the difficulty of recovering this organism in pure culture despite its dominance in the suckling infant intestine. Isolation of *Bifidobacterium* strains in axenic culture, their cultivation and storage under laboratory conditions are and have been a prerequisite for thorough, in-depth, and unambiguous studies of the physiology, genomics, and ecology of this group of prokaryotes. Actually, the number and variety of methods devised for the isolation and cultivation of bifidobacteria are proportional to the difficulties encountered in obtaining them.

Bifidobacteria are currently represented by 60 validated taxa, 52 of which have been isolated mainly from the GIT contents of a wide variety of human and nonhuman hosts (e.g., human and nonhuman-primates, birds, ungulates, lagomorphs, and rodents) and insect pollinators (Biavati and Mattarelli, 2012; Killer et al., 2011, 2009; Kopecný et al., 2010; Michelini et al., 2015a,b, 2016a; Modesto et al., 2014, 2015; Praet et al., 2015).

The currently recognized species, their origins, the media for isolating and conditions for storing representatives are listed in Table 4.1.

Though bifidobacteria have been isolated and are commonly found in the gut, these microorganisms have also been found in other ecological niches, some of which are atypical: oral cavity (e.g., *B. dentium*, *B. tsurumiense*) (Okamoto et al., 2008; Scardovi and Crociani, 1974), human blood and urine (e.g., *B. scardovii*) (Hoyles et al., 2002), sewage (e.g., *B. minimum*, *B. subtile*, and *B. thermacidophilum*) (Scardovi and Trovatelli, 1974), human breast milk (e.g., *B. longum* susbp. *longum*, *B. breve*) (Arboleya et al., 2011; Gueimonde et al., 2007; Jost et al., 2013; Martín et al., 2003), nonhuman breast milk (*B. adolescentis*) (Killer et al., 2013), raw milk and raw milk cheese (*B. crudilactis*) (Delcenserie et al., 2007; Gulitz et al., 2013; Hsieh et al., 2012; Laureys and De Vuyst, 2014; Laureys et al., 2016; Meile et al., 1997; Watanabe et al., 2009) surface waters and meat samples (*B. pseudolongum* subsp. *pseudolongum*, *B. thermophilum*, *B. choerinum*) (Delcenserie et al., 2011, 2008; Mattarelli and Biavati, 2014). Bifidobacteria are also added to food, dairy products, and beverages (Biavati and Mattarelli, 2012; Laureys et al., 2016; Tojo et al., 2014; Ventura et al., 2014).

All these (natural and artificial) niches are very complex microbial ecosystems, in which many kinds of bacterial species live side-by-side and bifidobacteria are not always among the numerically dominant groups. Detection and (or) enumeration of bifidobacteria as a single group will always involve elimination of most of the other bacteria. It is thus obvious that it would be necessary to have a medium that selectively promotes (or favors) the growth of bifidobacteria, while suppressing (or disfavoring) the other bacteria.

Selection may be based on nutrition (metabolism) of the desired organism, on inhibition (counter selection) of the undesired organism, or on combined nutritional and toxicological action.

Different (selective) strategies have been employed and many efforts have been directed toward development of different standard effective media with selectivity for bifidobacteria in different environments.

Most of the selective media reported to date are based on a basic complex medium suitable for the growth of anaerobic bacteria, as well as bifidobacteria, which is supplemented with one or more selective or elective substances

Species	Type strains	Origins	Selective mediums	Storages	References
Bifidobacterium actinocoloni- iforme	DSM 22766	Bumblebee digestive tract	mTPY (mupirocin, 100 mg/L and glacial acetic acid 1 mL/L) and pollen medium [pollen (30 g), glucose (20 g), agar (15 g), yeast extract (4 g), soybean peptone (3 g), sodium acetate (3 g), ammonium citrate-primary (2 g), Na ₂ HPO ₄ (2 g), Tween 80 (1 g), glacial acetic acid (1 g), cysteine hydrochloride (0.5 g) and mupirocin (0.1 g). The medium pH was 6.5 ± 0.2. The pollen extract (Pleva Ltd., Potštejn, Czech Republic) was initially boiled for 50 min and then, after centrifugation, added to the medium (100 mL/L)]	ND	Killer et al. (2011)
Bifidobacterium aquikefiri	CCUG 67145	A household water kefir fermentation process	mTPY agar medium Gulitz et al. (2013), supplemented with cycloheximide (final concentration of 0.1 g/L; Sigma-Aldrich, Saint Louis, MO, USA), kanamycin sulfate (final concentration of 0.05 g/L; Sigma-Aldrich), mupirocin (final concentration of 0.05 g/L; AppliChem GmbH, Darmstadt, Germany), and amphotericin B (final concentration of 0.005 g/L; Sigma-Aldrich)	ND	Laureys et al. (2016)
Bifidobacterium adolescentis	ATCC 15705	Intestine of human adult	Bifidus blood agar	ND	Reuter (1971)
Bifidobacterium aerophilum	DSM 100689	Cotton-top tamarin (<i>Saguinus</i> <i>oedipus</i>) fecal samples	mTPY (mupirocin, 100 mg/L)	Frozen (-135° C) and freeze- dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%)	Michelini et al. (2016a)
Bifidobacterium aesculapii	DSM 26737	Baby marmoset (<i>Callithrix</i> <i>jacchus</i>) fecal samples	mTPY (mupirocin, 100 mg/L)	Frozen (-135° C) and freeze-dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%), (0.3%)	Modesto et al. (2014)
Bifidobacterium angulatum	ATCC 27535	Human adult fecal samples	TPY without Tween 80	Frozen (-135° C) and freeze-dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%), (0.3%)	Scardovi and Crociani (1974)
Bifidobacterium animalis subsp. animalis	ATCC 25527	Rat fecal samples	BS MITSOUKA	ND	Mitsuoka (1969)
Bifidobacterium animalis subsp. lactis	DSM 10140	Fermented milk	Modified agar of LAPIERRE containing double amounts of sodium propionate (6 g/L; Fluka) and additionally cysteine hydrochloride (Fluka) at 0.5 g/L, nalidixic acid (Sigma) at 30 mg/L and 0.001 g/L resazurine, respectively (medium B)	ND	Meile et al. (1997)

TABLE 4.1 Currently Recognized *Bifidobacterium* Species, Their Origins, the Media for Isolating, and Conditions for Storing Representatives

(Continued)

Species	Type strains	Origins	Selective mediums	Storages	References
Bifidobacterium asteroides	ATCC 25910	Bee intestine	TPY agar initially formulated without Tween 80	Frozen (-135° C) and freeze-dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%), (0.3%)	Scardovi and Trovatelli (1969)
Bifidobacterium avesanii	DSM 100685	Cotton-top tamarin (<i>Saguinus oedipus</i>) fecal samples	mTPY (mupirocin 100 mg/L) agar	Frozen (-135° C) and freeze-dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%), (0.3%)	Michelini et al. (2016a)
Bifidobacterium biavatii	DSM 23969	Red-handed tamarin (<i>Saguinus midas</i>) fecal samples	BSM agar [MRS + cysteine + lithium mupirocin (100 mg/L)]	Frozen stocks at -80° C in nutrient broth supplemented with 20% (v/v) glycerol	Endo et al. (2012)
Bifidobacterium bifidum	ATCC 29521	Infant feces	Deep glucose-agar tubes inoculated with varying dilutions of fecal emulsion as the primary culture medium	ND	Tissier (1900)
Bifidobacterium bohemicum	DSM 22767	Bumblebee digestive tract	mTPY (mupirocin, 100 mg/L and glacial acetic acid 1 mL/L) agar	ND	Killer et al. (2011)
Bifidobacterium bombi	DSM 19703	Bumblebee digestive tract	mTPY (mupirocin, 100 mg/L) agar	ND	Killer et al. (2009)
Bifidobacterium boum	ATCC 27917	Bovine rumen	TPY agar	Frozen (-135° C) and freeze-dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%), (0.3%)	Scardovi et al. (1979)
Bifidobacterium breve	ATCC 15700	Infant feces	Bifidus blood agar	ND	Reuter (1963)
Bifidobacterium callitrichos	DSM 23973	Adult common marmoset (<i>Callithrix</i> <i>jacchus</i>) feces	BSM agar [MRS + cysteine-HCl (0.05%) + lithium mupirocine (100 mg/L)]	At –80°C in nutrient broth supplemented with 20% (v/v) glycerol	Endo et al. (2012)
Bifidobacterium catenulatum	ATCC 27539	Human adult fecal samples	TPY agar	Frozen (-135° C) and freeze-dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%), (0.3%)	Scardovi and Crociani (1974)
Bifidobacterium choerinum	ATCC 27686	Piglet feces	TPY agar	Frozen (-135° C) and freeze-dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%), (0.3%)	Scardovi et al. (1979)
Bifidobacterium commune	DSM 28792	Bumble bee gut	mTPY (mupirocin, 100 mg/L and glacial acetic acid 1 mL/L)	ND	Praet et al. (2015)
Bifidobacterium coryneforme	ATCC 25911	Bee intestine	TPY agar initially formulated without Tween 80	Frozen (-135° C) and freeze-dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%). (0.3%)	Scardovi and Trovatelli (1969)

TABLE 4.1	Currently Recognized Bifidobacterium Species, Their Origins, the Media for Isolating, and Conditions for Storing
	Representatives (cont.)

Species	Type strains	Origins	Selective mediums	Storages	References
Bifidobacterium crudilactis	LMG 23609	Raw milk cheese	ND	ND	Delcenserie et al. (2007)
Bifidobacterium cuniculi	ATCC 27916	Rabbit feces	TPY agar initially formulated without Tween 80	Frozen (-135°C) and freeze-dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%), (0.3%)	Scardovi et al. (1979)
Bifidobacterium dentium	ATCC 27534	Oral cavity	TPY agar initially formulated without Tween 80	Frozen (-135° C) and freeze-dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%), (0.3%)	Scardovi and Crociani (1974)
Bifidobacterium faecale	JCM 19861	Human feces	TOS + lithium mupirocine	Maintained as a glycerol suspension (30%, w/v) at 280°C	Choi et al. (2014)
Bifidobacterium gallicum	ATCC 49850	Human feces	VL agar (meat-yeast-agar)	ND	Lauer (1990); Rasić and Kurmann (1983)
Bifidobacterium gallinarum	ATCC 33777	Chicken cecum	BL agar medium l21 (Nissui), 50 mL defibrinated horse blood l21, 15 mg sodium propionate l21 (Wako), 50 mg paromomycin sulfate l21 (Sigma-Aldrich), 200 mg fradiomycin sulfate l21 (Wako), and 3 g lithium chloride l21 (Wako)	ND	Watabe et al. (1983)
Bifidobacterium hapali	DSM 100202	Baby marmoset (<i>Callithrix</i> <i>jacchus</i>) fecal samples	mTPY agar (mupirocin, 100 mg/L)	Frozen (-135° C) and freeze-dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%), (0.3%)	Michelini et al. (2015a,b)
Bifidobacterium indicum	ATCC 25912	Bee intestine	TPY agar initially formulated without Tween 80	Frozen (-135° C) and freeze-dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%), (0.3%)	Scardovi and Trovatelli (1969)
Bifidobacterium kashiwanohense	DSM 21854	Infant feces	Blood liver agar plates (Eiken Chemical)	ND	Morita et al. (2011)
Bifidobacterium lemurum	DSM 28807	Adult ring tailed lemur (<i>Lemur catta</i>) fecal samples	TOS agar	Frozen (-135° C) and freeze-dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%), (0.3%)	Modesto et al. (2015)
Bifidobacterium longum subsp. infantis	ATCC 15697	Infant feces	Bifidus blood agar	ND	Reuter (1963)
Bifidobacterium longum subsp. longum	ATCC 15707	Adult feces	Bifidus blood agar	ND	Reuter (1963)
Bifidobacterium longum subsp. suillum	DSM 28597	Piglet feces	TPY agar	Frozen in 10% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%)	Yanokura et al. (2015)

TABLE 4.1	Currently Recognized Bifdobacterium Species, Their Origins, the Media for Isolating, and Conditions for Storing
	Representatives (cont.)

(Continued)

Species	Type strains	Origins	Selective mediums	Storages	References
Bifidobacterium longum subsp. suis	ATCC 27533	Piglet feces	TPY medium initially formulated without Tween 80	Frozen (–135°C) and freeze-dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%), (0.3%)	Matteuzzi et al. (1971)
Bifidobacterium magnum	ATCC 27540	Rabbit feces	TPY medium initially formulated without Tween 80	Frozen (-135° C) and freeze-dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%), (0.3%)	Scardovi and Zani (1974)
Bifidobacterium eulemuris	DSM 100216	Adult lemur macaco (<i>Eulemur macaco</i>) fecal samples	TOS agar	Frozen (-135° C) and freeze-dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%), (0.3%)	Michelini et al. (2016b)
Bifidobacterium merycicum	ATCC 49391	Bovine rumen	TPY agar	Frozen (-135° C) and freeze-dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%), (0.3%)	Biavati and Mattarelli (1991)
Bifidobacterium minimum	ATCC 27538	Sewage	TPY agar	Frozen (-135° C) and freeze-dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%), (0.3%)	Biavati et al. (1982)
Bifidobacterium mongoliense	DSM 21395	Fermented milk	MRS agar (glucose replaced with 1% lactose) supplemented with 0.001% sodium azide and 0.001% cycloheximide	Nutrient broth (BD Difco) containing 10% (v/v) DMSO and stored at -80°C	Watanabe et al. (2009)
Bifidobacterium moukalabense	JCM 18751	Gorilla feces	BL agar medium (Nissui), 50 mL defibrinated horse blood, 15 mg sodium propionate (Wako), 50 mg paromomycin sulfate (Sigma-Aldrich), 200 mg fradiomycin sulfate (Wako), and 3 g lithium chloride (Wako)	ND	Tsuchida et al. (2013)
Bifidobacterium myosotis	DSM 100196	Baby common marmoset (<i>Callithrix</i> <i>jacchus</i>) feces	TPY + mupirocin (100 mg/L)	Frozen (-135° C) and freeze-dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%), (0.3%)	Michelini et al. (2015b)
Bifidobacterium pseudocatenu- latum	ATCC 27919	Infant feces	TPY agar	Frozen (-135° C) and freeze-dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%), (0.3%)	Scardovi et al. (1979)
Bifidobacterium pseudolongum subsp. globosum	ATCC 25865	Bovine rumen	TPY agar initially formulated without Tween 80	Frozen (-135° C) and freeze-dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%), (0.3%)	Scardovi et al. (1969)
Bifidobacterium pseudolongum subsp. pseudolongum	ATCC 25526	Pig s	BS MITSOUKA: BL agar as the basal medium supplemented with BS solution containing sodium propionate (15 g/L), paromomycin sulfate (0.05 g/L), neomycin (0.1 g/L) and lithium chloride (3 g/L)	ND	Mitsuoka (1969)

TABLE 4.1	Currently Recognized Bifidobacterium Species, Their Origir	ns, the Media for Isolating, and Conditions for Storing
	Representatives (cont.)	

Species	Type strains	Origins	Selective mediums	Storages	References
Bifidobacterium psychraerophilum	LMG 21775	Porcine cecum	MRS + cysteine (0.05%) + mupirocin (50 mg/L)	ND	Simpson et al. (2004a,b)
Bifidobacterium pullorum	ATCC 27685	Chicken feces	TPY agar	Frozen (-135° C) and freeze-dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%), (0.3%)	Trovatelli et al. (1974)
Bifidobacterium ramosum	DSM 100688	Cotton-top tamarin (<i>Saguinus</i> <i>oedipus</i>) fecal samples	mTPY agar (mupirocin, 100 mg/L)	Frozen (-135° C) and freeze-dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%), (0.3%)	Michelini et al. (2016a)
Bifidobacterium reuteri	DSM 23975	Adult common marmoset (<i>Cal- lithrix jacchus</i>) feces	BSM agar [MRS + cysteine + lithium mupirocin (100 mg/L)]	At –80°C in nutrient broth supplemented with 20% (v/v) glycerol	Endo et al. (2012)
Bifidobacterium ruminantium	ATCC 49390	Bovine rumen	TPY agar	Frozen (-135° C) and freeze-dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%), (0.3%)	Biavati and Mattarelli (1991)
Bifidobacterium saeculare	ATCC 49392	Rabbit feces	TPY agar	Frozen (-135° C) and freeze-dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%), (0.3%)	Biavati et al. (1991)
Bifidobacterium sanguini	DSM 23967	Red-handed tamarin (<i>Saguinus midas</i>) fecal samples	BSM agar [MRS + cysteine + lithium mupirocin (100 mg/L)]	At –80°C in nutrient broth supplemented with 20% (v/v) glycerol	Endo et al. (2012)
Bifidobacterium scardovii	DSM 13734	Human sources	Chocolate blood agar	ND	Hoyles et al. (2002)
Bifidobacterium stellenboschense	DSM 23968	Red-handed tamarin (<i>Saguinus midas</i>) fecal samples	BSM agar [MRS + cysteine + lithium mupirocin (100 mg/L)]	At -80°C in nutrient broth supplemented with 20% (v/v) glycerol	Endo et al. (2012)
Bifidobacterium subtile	ATCC 27537	Sewage	TPY agar	Frozen (-135° C) and freeze-dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%), (0.3%)	Biavati et al. (1982)
Bifidobacterium thermacidophilum subsp. porcinum	DSM 17755	Piglet feces	Hungate anaerobic roll-tube technique with TPY	ND	Zhu et al. (2003)
Bifidobacterium thermacidophilum subsp. thermoaci- dophilum	DSM 15837	Anaerobic digester	Hungate anaerobic roll-tube technique with TPY	ND	Dong et al. (2000)
Bifidobacterium thermophilum	ATCC 25525	Piglet feces	BS MITSOUKA	ND	Mitsuoka (1969)
Bifidobacterium tissieri	DSM 100201	Baby common marmoset (<i>Cal-</i> <i>lithrix jacchus</i>) fecal samples	mTPY agar (mupirocin, 100 mg/L)	Frozen (-135°C) and freeze-dried in 20% (w/v) sterile skimmed milk solution supplemented with lactose (3%) and yeast extract (0.3%), (0.3%)	Michelini et al. (2015b)

TABLE 4.1	Currently Recognized Bifidobacterium Species, Their Origins, the Media for Isolating, and Conditions for Storing
	Representatives (cont.)

(Continued)

Species	Type strains	Origins	Selective mediums	Storages	References
Bifidobacterium tsurumiense	JCM 13495	Hamster dental plaque	CFAT agar. Composition (L ⁻¹) follows: trypticase soy broth (BBL Microbiology Systems), 30 g; glucose, 5 g; agar, 15 g; cadmium sulfate, 13 mg; sodium fluoride, 85 mg; neutral acriflavin, 1.2 mg; potassium tellurite, 2.5 mg; basic fuchsin, 1.3 mg; defibrinated sheep blood, 50 mL	ND	Okamoto et al. (2008)

 TABLE 4.1
 Currently Recognized Bifidobacterium Species, Their Origins, the Media for Isolating, and Conditions for Storing Representatives (cont.)

BSM, Bifidobacterium selective medium; BL, blood liver; MRS, de Man, Rogosa, Sharpe medium; ND, not described; TOS, trans-galactosyl oligosaccharides; TPY, trypticas-phytone-yeast extract.

that suppress the growth of other bacteria and only permit bifidobacteria to grow. Common basic media are MRS, TPY, WC broth (WCB), CAB, RCM, glucose BL medium, Eggert & Gagnon medium, TOS.

Common elective and selective substances are carbohydrates (raffinose, lactose, galactose, FOS, TOS), antibiotics (norfloxacin, aztreonam, netilmicin, neomycin, paromomycin sulfate, nalidixic acid, kanamycin, mupirocin, dicloxacillin) short chain fatty acids (propionate and butyrate), bile, iodoacetic acid, sodium azide, sodium propionate, 2,3,5 triphenyl-tetrazolium chloride (TTC), ascorbic acid, and lithium chloride.

Differential and chromogenic media are also reported: Raffinose agar, X_Gal, reinforced clostridial prussian blue agar (RCPB), tryptose proteose peptone yeast extract + prussian blue agar (TPPY + PB) (Roy, 2001).

An ideal medium should be easy to prepare and inexpensive, allowing selective and differential isolation of bifidobacteria from their original samples.

At time of writing there is no standard medium for the detection of bifidobacteria (Ashraf and Shah, 2011; Novakova et al., 2016; Vlkova et al., 2015): several media are not truly selective for bifidobacteria, or especially suitable for the analysis of (fecal) samples with low bifidobacterial count and some of them are time consuming to be prepared. In addition, some of the selective media do not allow the growth of all the species of the genus *Bifidobacterium*. This fact is of concern since several studies have shown that medium composition affects the total number of bifidobacteria recovered (Apajalaht et al., 2003).

Moreover, no one of the available selective media allow for the ready identification of *Bifidobacterium* colonies. All media rely on subculturing colonies and further testing of morphology and physiological profiles to obtain a final estimation of the number of bifidobacteria. All of the isolated colonies must be studied further in order to differentiate *Bifidobacterium* spp. from other microorganisms.

The ability of a medium to elect and select for bifidobacteria is often judged by the relative frequency of isolates, which have typical bifidobacterial morphology (Barrett et al., 2011).

However, cellular morphology should not be a confirmatory criterion for the assigning of an organism to the genus *Bifidobacterium*, since not all bifidobacteria readily exhibit the characteristic and eponymous branching morphology (Biavati et al., 1992) and conversely there exist branched cells that do not belong to bifidobacteria. The F6PPK assay appeared to be inevitable for an accurate evaluation of the bifidobacteria selective media. Indeed, the positive F6PPK reaction is the most direct and reliable characteristic distinguishing bifidobacteria from lactobacilli, propionibacteria, and other related bacteria (Biavati et al., 1992; Scardovi, 1986), especially among bacteria that had bifid-like morphology, but lacked F6PPK. However, as F6PPK is also present in all the other genera of *Bifidobacteriaceae*, to correctly attribute newly isolated strains to the genus *Bifidobacterium*, analysis of 16S rRNA sequence is mandatory.

Therefore, final identification of bifidobacteria can be, and frequently is, long and tedious since it required skilled personnel and several additional tests for reliable results.

The range of media developed for the recovery and enumeration of bifidobacteria will be reviewed in the following sections in respect of the origin of the samples tested.

4.3.1 Fecal Samples

The development of media to detect bifidobacteria from their natural habitat, that is, the GIT of humans and animals, has focused primarily on fecal material. Several hundred species of bacteria have been detected in the feces

4.3 ISOLATION

and colon of humans, but a few major obligately anaerobic groups, including the *Bifidobacterium* genus, dominate (Barrett et al., 2011). In upper GIT regions, such as the jejunum, ileum, and cecum, bifidobacteria are not dominant and coexist with facultative anaerobes and anaerobs, including the Gram-positive LAB, *Lactobacillus, Streptococcus*, and *Enterococcus*, and the Gram-negative *Enterobacteriaceae* and *Bacteroidaceae* family (Barrett et al., 2011).

To isolate bifidobacteria from fecal and intestinal contents, the samples are usually collected with sterile spoon or spatula and transferred into sterile tubes containing anaerobic transport media used to keep viability of anaerobic bacteria. The samples are brought to the laboratory at 4°C to stop the bacterial growth and immediately analyzed or stored at low temperature until processing for isolation.

Stool samples may also be collected in sterile cryovials or tubes containing preservation media prepared with 20% glycerol and 0.05% (final volume) cysteine hydrochloride. These preservation media may be either phosphate buffered saline (containing 150 nM NaCl, 10 mM NaH₂PO₄, 20 nM NaH₂PO₄, pH 7.4), or the saline (0.9% NaCl), or modified PBS (0.1% peptone, 0.25% yeast extract) (Endo and Gueimonde, 2015). The samples should be analyzed within 6 h or they should be frozen at -80° C or lower until processing for isolation.

For analysis the samples are serially diluted in buffers or prereduced buffers or broth. Prereduced frequently used buffers are: anaerobic dilution buffer, quarter strength Ringer solution supplemented with cysteine (0.05%), WCB, saline supplemented with cysteine (0.2%), reduced physiological salts solution, reduced medium (BHI: 0.5% glucose, 0.5% yeast extract, 0.25% cysteine, 0.001% vitamin k₁, and 0.002% hemin), and peptone water with cysteine-HCl (0.05%) (Endo and Gueimonde, 2015). Nonprereduced buffers, for example, saline and PBS, are also used sometimes (Ferraris et al., 2010). The serially diluted samples are then spread or pour plated onto a selective or nonselective medium for bifidobacteria, and usually cultured at 37°C for 48–72 h under anaerobic conditions, using gas generating systems or anaerobic workstations.

To detect bifidobacteria in fecal or intestinal content samples, many different nonselective and selective media have been used and described (Beerens, 1991; Bunesova et al., 2014; Mikkelsen et al., 2003; Thitaram et al., 2005). The solid nonselective media indicated for detection of bifidobacteria are RCM and RCM plus lactose and sheep blood (mRCM); BL agar, BL agar without blood, TPY, modified MRS agar, TOS.

One of the first useful media for the enumeration of bifidobacteria was *Lactobacillus* agar modified from the medium of Reuter by Klupsch (Roy, 2001).

Several authors isolated bifidobacteria by means of plating serial dilutions of fecal samples in nonselective media, such as TPY agar, deep glucose agar, or Bifidus blood agar, TOS (Modesto et al., 2015; Rasić and Kurmann, 1983; Reuter, 1963; Scardovi, 1986).

In 1953, Mitsuoka developed BL agar and suggested its use for routine culture, differentiation, and isolation of various intestinal bacteria (Mitsuoka, 2014). This procedure has been described in more detail by Laroia and Martin (1991); however, the described formulation is incomplete as the defibrinated blood component (50 mL/L of horse or sheep blood of cooled medium) was inadvertently omitted from the original formulation of Teraguchi et al. (1978). Modified BL agar is also used because this medium has been proven in several laboratories to provide accurate counting of bifidobacteria (Roy, 2001).

Mitsuoka et al. (1965) developed *Bifidobacterium* selective (BS)-agar I and II both based on BL agar supplemented with BS solution containing the antibiotics neomycin sulfate (200 mg/L) and paromomycin sulfate (50 mg/L for BS_I and 20 mg/L for BS_II) together with other bacteriostatic agents, such as lithium chloride (3 mg/L) and sodium propionate (1.5% of a 40% solution in BS_I and 1.0% in BS_II) (Mitsuoka, 2014; Rasić and Kurmann, 1983). Later, Teraguchi et al. (1978) developed an improved selective medium NPNL agar, which was based on BL-agar modified by the exclusion of blood and containing the same selective substances as BS-agar but with the addition of nalidixic acid (Section 4.3.2).

In 1988, Muñoa and Pares developed BIM-25 agar, utilizing RCA as the basal medium, supplemented with nalidixic acid (20 mg/L), polymyxin B sulfate (8.5 mg/L), kanamycin (50 mg/L), sodium iodoacetate (25 mg/L), and triphenyltetrazolium chloride (TTC, 25 mg/L). It was both selective and differential: TTC significantly differentiated between *Bifidobacterium* spp. and other bacterial colonies (Muñoa and Pares, 1988). Bifidobacteria (confirmed by F-6-PPK detection) appeared as large (2 mm) white colonies, while pink colonies were either cocci, bifidobacteria, or other rods and red colonies were always Gram-positive cocci. However, a number of subsequent studies have reported short-comings with regard to the elective and selective properties of BIM-25 (Arroyo et al., 1995; Ingham, 1999; Silvi et al., 1996) and it appears that BIM-25 is not suitable for the enumeration of bifidobacteria from natural habitats (Barrett et al., 2011).

In 1990, Beerens described the development of a selective culture medium for *Bifidobacterium* spp. based on Columbia agar, containing 5 mL of propionic acid per liter and supplemented with glucose (5 g/L) cysteine hydrochloride (0.5 g/L) and adjusted to pH 5.0 by using 1 N NaOH. The medium was defined as elective for bifidobacteria since

Bereens stated that the addition of propionic acid enhanced the growth of bifidobacteria, although increasing the propionic acid from 5 to 10 mL/L reduced counts and caused an uncharacteristic coccoidal morphology for many *Bifidobacterium* strains (Beerens, 1990). Beerens medium cannot be stored because it cannot be autoclaved and therefore needs to be used within 48 h after preparation.

Several studies have since debated the elective and selective properties of Beerens medium (Apajalaht et al., 2003; Barrett et al., 2011; Hartemink and Rombouts, 1999; Pacher and Kneifel, 1996; Payne et al., 1999; Rada et al., 1999; Silvi et al., 1996). Beerens agar is reported as a suitable medium for the isolation and enumeration of bifidobacteria from gut microbiota and has often been used to count bifidobacterial populations in fecal and intestinal samples (Mikkelsen et al., 2003).

Silvi et al. (1996), described Beerens medium as the best medium for the determination of bifidobacteria but they also stated that the total bifidobacterial counts were significantly lower on Beerens than on the other media tested. Similarly Favier et al. (1997) emphasized that Beerens underestimated bifidobacteria in several of their human fecal samples. A poor recovery of *B. longum* subsp. *longum* and no growth of *B. bifidum* were reported on this medium and therefore it does not appear to be suitable for the enumeration of bifidobacteria from all their natural habitats (Roy, 2001).

In 1996, Hartemink and Rombouts developed a new selective and differential medium for the detection of bifidobacteria in intestinal and fecal samples, Raffinose-Bifidobacterium (RB) agar. The medium owes its selectivity to the presence of propionate (15 g/L) and lithium chloride (3 g/L) as inhibitory agents, and raffinose (7.5 g/L) as a selective carbon source. In addition, casein (5 g/L) is used as a protein source, which results in a zone of precipitation around the colonies of bifidobacteria. The substrate is free of antibiotics and easy to prepare. Bifidobacteria growing on RB agar show a yellow colony with a yellow halo and a precipitation zone around the colony. All human and dairy bifidobacteria grow well on RB agar, except for some *B. bifidum* strains. Some uncommon or animal species, B. gallicum, B. asteroides, B. animalis subsp. animalis, B. pullorum, and some B. bifidum either did not grow or did not show the characteristic reactions. In 1999, Hartemink and Rombouts tested the medium with fresh human feces, ileal samples of pigs (for small intestinal microbiota) and fecal samples of cats (a carnivorous animal), and compared it with Bereens and NPNL agar. They stated that RB agar and Beerens medium showed comparable results when used to quantify bifidobacteria in human feces, but none of the media tested gave results suitable for reliably counting bifidobacteria from pig and cat samples. Indeed, only 10% of the colonies on Beerens agar and 30% of distinctive colonies on RB agar could be identified as bifidobacteria when pig ileal content was used as the sample. Accordingly, Mikkelsen et al. (2003) reported that bifidobacteria appeared on RB agar as distinctive yellow colonies with a yellow halo and a surrounding precipitation zone, but similar characteristics have been shown by lactobacilli from pigs. They stated that Beerens and RB agar media are not adequate for the enumeration of bifidobacteria when LAB dominated, as in porcine intestinal samples.

Therefore, in samples where there is high occurrence of lactobacilli as in pig and in poultry ceca (Sarra et al., 1992), it is necessary to inhibit the growth of these bacteria on selective medium for isolation and enumeration of bifidobacteria. Rada (1997) found that the resistance to mupirocin should be used as an aid in the separation of *Bifidobacterium* sp. from lactobacilli. Whereas lactobacilli are susceptible to this compound, bifidobacteria are resistant. However, mupirocin-resistant bacteria include both some Gram-positive and most Gram-negative anaerobes. For this reason, Rada et al. introduced the use of WC medium modified with the addition of mupirocin (100 mg/L) and acetic acid (1 mL/L) as selective agents (mWC) (1999). They evaluated five media to determine their selectivity for *Bifidobacterium* sp. in hen and rabbit cecal samples. Rogosa agar (RMS) modified by the addition of cysteine-hydrochloride (0.05% w/v), Beeren agar, BS 2 agar containing (per litre) sodium propionate 15 g, lithium chloride 3 g, paromomycin sulfate 50 mg and neomycin sulfate 200 mg, and modified WC agar (mWC) modified by the addition of acetic acid (1 mL/L) and mupirocin (100 mg/L). All proved to be selective for *Bifidobacterium* spp. from rabbit cecal samples. On the other hand, only mWC was suitable for the isolation and enumeration of bifidobacteria in both kinds of the samples tested. Suppression of lactobacilli and growth of bifidobacteria was realized by the addition of mupirocin. Addition of mupirocin together with acetic acid to the complex medium resulted in a preparation suitable for the isolation and enumeration of bifidobacteria.

Mupirocin is an antibiotic produced by *Pseudomonas fluorescens* and inhibits the growth of *Lactobacillus* spp., *Lactococcus lactis*, and *Bacillus* spp., but not *Bifidobacterium* spp. by blocking bacterial isoleucyl-tRNA synthetase (Gao et al., 2014). Since its introduction, mupirocin has been widely used as a selective agent in other basal media. In another subsequent study, Rada and Petr (2000) compared mWC and mTPY both modified with the addition of glacial acetic acid (1 mL/L) and mupirocin (100 mg/L) and evaluated their sensitivity and selectivity for bifidobacteria in the hen cecal samples. Both agars were shown to be selective for bifidobacteria, however, modified mTPY agar showed higher CFU/g than mWC agar. The authors stated that the media for selective enumeration and isolation

of bifidobacteria in poultry cecal samples should not contain glucose as the sole carbon source and concluded that mTPY medium is highly selective and permits the growth of both glucose fermenting and glucose nonfermenting bifidobacteria (Rada and Petr, 2000). They also suggested that the bifidobacteria selective media should be chosen in respect of the animal species origin of the samples tested.

Thitaram et al. (2005) incorporated mupirocin and acetic acid into TOS propionate agar for the enumeration of bifidobacteria from chicken ceca. The authors noted that mupirocin (100 mg/L) suppressed the growth of presumptive bifidobacteria by 1.6–1.8 log₁₀ CFU from chicken cecal contents and therefore recommended the addition of half dose (50 mg/L) of mupirocin and 1% (v/v) glacial acetic acid (TOS-AM50) for the enumeration of *Bifidobacterium* spp. from such letter samples.

However, mupirocin media with acetic acid are reliable only for intestinal samples in which bifidobacteria predominate since their selectivity is often reported as limited for those complex samples containing more diverse microbiota. Many anaerobic bacteria, especially clostridia, have demonstrated resistance to mupirocin. Together with coliforms, lactobacilli and *Bacteroides* spp., clostridia have often been reported to be the predominant fecal bacteria in bifidobacteria-deficient samples (Edwards and Parrett, 2002; Novakova et al., 2016).

Vlková et al. (2005) found modified TPY medium supplemented with mupirocin (100 mg/L) and glacial acetic acid (1 mL/L), as not effective for the enumeration of bifidobacteria in infant stool samples with high numbers of clostridia, which were able to grow in the presence of mupirocin. Bifidobacteria-deficient intestinal microbiota are usually observed in infants delivered in large hospitals, born with low weight, by cesarean section or those who are fed with infant formulas. These features may increase the incidence of clostridia in the gut microbiota of these infants (Vlková et al., 2005). In animals, dogs (Handl et al., 2011) and pigs (Lu et al., 2007) are examples of species with characteristic clostridia-dominated microbiota (Novakova et al., 2016).

Similar results were presented by Rada and Petr (2000), who analyzed hen ceca samples using the same medium and showed that about 5% of the colonies that grew were nonbifidobacterial. In addition, Lakshminarayanan et al. (2013) reported that *Clostridium perfringens* was not inhibited by mupirocin in the dose 100 mg/L in MRS agar when the medium was used for the enumeration of bifidobacteria in fecal samples from elderly volunteers. Ferraris et al. (2010) tested different media for the detection of bifidobacteria in fecal samples, and although WC agar supplemented with mupirocin (50 mg/L) was shown to be the most selective medium, clostridia were isolated from 8 of the 15 samples tested.

Therefore, a selective medium supplemented with an anticlostridial agent would be advantageous for uncovering low abundance of bifidobacteria that may be masked by the growth of clostridia during fecal sample analyses, especially in individuals with suspected misbalanced microbiota.

In 2015, Vlkova et al., in an attempt to identify an antibiotic that inhibited the growth of clostridia while allowing the growth of bifidobacteria, tested the susceptibility of bifidobacteria and clostridia to 12 antibiotics and then used the identified substance to develop a selective cultivation medium for bifidobacteria. From their results only norfloxacin inhibited the growth of clostridia and did not affect the growth of bifidobacteria. Using pure cultures and fecal samples from infants, adults, calves, lambs, and piglets, the optimal concentration of norfloxacin in solid cultivation media was determined to be 200 mg/L. The final medium was WC modified by the addition of soya peptone (5 g/L), L-cysteine (0.5 g/L), and Tween 80 (1 mL/L) and supplemented with mupirocin (100 mg/L), glacial acetic acid (1 mL/L), and norfloxacin (200 mg/L) (mupirocin, acetic acid, norfloxacin agar, MAN) as selective agents. A stock solution of the selective agents for addition to the MAN agar was prepared by diluting 1000 mg of norfloxacin and achieve the desired final acidity of the media, 5 mL/L glacial acetic acid was added. The stock solution was filter sterilized and added in appropriate amounts to the medium containing all the remaining components. The medium was autoclaved and cooled to 48°C prior to adding the selective solution.

Although norfloxacin did not affect the growth of pure cultures of bifidobacteria, clostridial growth was inhibited on MAN agar, on which no visible colonies were detected. Similar results were obtained in the analysis of fecal samples. Although MAN agar was not completely selective, clostridial growth was suppressed, and all isolated F6PPK-negative bacteria were identified as Gram-positive cocci or Gram-negative rods. In the fecal samples from six infants delivered by cesarean section, bacterial counts greater than 10⁸ CFU/g were observed on MWCHmup medium and all the bacteria isolated were identified as clostridia. In contrast, no visible colonies were detected on MAN medium. These clostridia were reliably suppressed by norfloxacin, and no colonies of bacteria, even bifidobacteria, were detected on MAN. Vlkova et al. indicated that solid medium containing norfloxacin (200 mg/L) in combination with mupirocin (100 mg/L) and glacial acetic acid (1 mL/L) is suitable for the enumeration and isolation of bifidobacteria from fecal samples of different origins. In 2016, Novakova et al. tried to improve the "modified WC agar with mupirocin" (mWM), previously developed by Rada and Petr (2000) for the isolation of glucose nonfermenting bifidobacteria from hen ceca, by adding a newly identified anticlostridial agent, 8-hydroxy-quinoline (8HQ) (Novakova et al., 2013, 2014). They observed that the addition of 8HQ at 90 mg/L significantly improved bifidobacterial selectivity and specificity. However, the medium may underestimate bifidobacteria as a slightly lower recovery was shown with pure strains. When mWM agar was compared with mWMQ, viz. WC agar supplemented with mupirocin and 8HQ, results suggested that both selective media could be successfully used for enumeration and isolation of bifidobacteria if they are the dominant bacterial group in the sample. However, mWMQ is advantageous in bifidobacteria-poor samples, because it reduces the number of nonbifidobacterial isolates compared to MWM. Therefore, the authors stated that the newly composed mWMQ containing the anticlostridial agent 8HQ might be preferably recommended for bifidobacterial isolation from fecal samples of human and animal origin.

Taking into account all the different characteristics of the media so far analyzed, mupirocin medium is considered the best for enumeration of bifidobacteria from natural habitats (Barrett et al., 2011). Even more importantly, an accurate representation of the viable count of bifidobacteria seems to be achieved only when employing more than one type of selective media.

4.3.2 Fermented Dairy Products

Fermented dairy products have long been an important component of nutritional diet and their virtues are well documented and were well known even during the ancient days of civilization. Historically, fermentation processes involved unpredictable and slow souring of milk caused by the activities of natural flora inherently present in milk or added from the surroundings. Over the period, scientists have tried to isolate and study the characters of such desirable organisms. Among the bacteria, the most important dominant group bringing fermentation is LAB.

Though raw animal milk and fermented animal milks are possible sources of *Bifidobacterium* strains, these samples have been studied to look for new probiotic candidates. Currently, some strains of *B. animalis* subsp. *lactis* are commonly used in many probiotics and their occurrence was reported in various animal hosts (Bunešová et al., 2012; Masco et al., 2005; Mayer et al., 2007). The species *B. crudilactis* was first isolated and described by Delcenserie et al. (2007) in raw milk and raw milk cheese in France (Table 4.1). *B. psychraerophilum* was isolated from kefir grains collected in Taipei and fermented either in cow's or goat's milk (Hsieh et al., 2012), while *B. mongoliense*, from airag, a traditional Mongolian fermented product from mare milk have been described by Watanabe et al. (2008, 2009). They used modified MRS (m-MRS; glucose replaced with lactose) supplemented with 0.01% of both cycloheximide and sodium azide, and 0.2 g L-cysteine hydrochloride. Later, the species *B. crudilactis* and *B. mongoliense* were detected in raw milk cheeses from the Vercors region (Delcenserie et al., 2013). The origin of these bacteria is not known yet: animal feces are likely the source because raw milk was collected from farms (Delcenserie et al., 2007) and other authors also emphasized that raw milk samples could be contaminated by cow dung on the farm (Beerens et al., 2000). However, their presence probably contributes to the specific organoleptic and technologic characteristics of those cheeses (Delcenserie et al., 2013).

Milk products also serve as important delivery vehicles for probiotic bacteria and during the past three decades various bifidobacterial species have been incorporated into a wide range of functional dairy foods or have been used for the production of fermented milks, alone or in combination with other LAB (Roy, 2001; Sule et al., 2014). This is because these bacteria can play a dual role in transforming milk into a diverse array of fermented dairy products (yogurt, kefir, etc.), and also colonizing different sites on the human body, including the mouth and the GIT. Since bifidobacteria populations in functional dairy foods should be over 10⁶ CFU/g at the time of consumption, simple, reliable, and inexpensive methods are needed to routinely determine the initial inoculum size and to estimate the storage time and period during which bifidobacteria remain viable (Roy, 2001). However, in raw milk as well in dairy products, bifidobacteria are in mixed populations, those of starter organisms and mesophilic lactic/aromatic cultures as well. Thus, the presence of multiple and closely related species in these products makes the differential or selective enumeration of bifidobacteria difficult because of the similarity in growth requirements and overlapping biochemical profiles of the species (Sule et al., 2014).

For selective and differential enumeration of bifidobacteria from such food samples a wide range of culture media have been described and extensively reviewed in past decades (Arroyo et al., 1995; Ashraf and Shah, 2011; Beerens, 1990; Cole and Fuller, 1989; Karimi et al., 2012; Kneifel et al., 1993; Lapierre et al., 1992; Lim et al., 1995; Muñoa and Pares, 1988; Resnick and Levin, 1981; Teraguchi et al., 1978; Wijsman et al., 1989).

Few media are truly selective for bifidobacteria and some of them are time consuming to prepare (Lim et al., 1995; Pacher and Kneifel, 1996; Roy, 2001).

4.3 ISOLATION

The International Organization for Standardization (ISO) and the International Dairy Federation (IDF) have jointly published international standards denoted by ISO 29981:2010, wherein TOS propionate agar (TOS, Yakult Pharmaceutical Industry, Co., Ltd., Tokyo, Japan) supplemented with lithium mupirocin (TOS-MUP) agar is recommended for the selective enumeration of presumptive bifidobacteria, in milk products, including fermented and nonfermented milks, milk powders, and infant formulae (ISO and IDF, 2010). The ISO standard uses a colony count technique performed at 37°C under anaerobic conditions.

Isolation and enumeration of bifidobacteria from dairy products are generally the same as that from other sources. After collection and proper refrigerated storage (4°C), milk samples (1 or 10 mL), fermented milk samples (10 g) or cheese samples (25 g) are commonly transferred to stomacher bags (Stomacher 400 Classic Standard Bag; Seward Limited, Worthing, UK) for homogenization (10 min), and then subjected to serial dilutions. Various diluents have been described: Tryptone (0.1%) water, phosphate buffered solution (Lahtinen et al., 2006) with or without L-cysteine hydrochloride (0.5%), Ringer solution (quarter strength), and so forth. However, the most commonly used diluents are Peptone (0.1%) water and Peptone water with saline (0.85%) (Roy, 2001).

According to Roy (2001), the plating technique can also make a significant difference to the results of enumeration of bifidobacteria, and differences in percentage recovery observed using the spread- and pour-plate techniques are not easily explained but to some extent reflect the different requirements of these organisms for an anaerobic environment. It is thus important to compare spread- and pour-plate techniques and to select the combination of medium and plating technique that gives the most accurate representation of the bifidobacteria viable count (Roy, 2001).

Furthermore, reliable enumeration of bifidobacteria seems to be achieved only if a microbiologist knows which particular *Bifidobacterium* strain/species was used in the product (Pacher and Kneifel, 1996; Roy, 2001).

In 1978, Teraguchi et al. modified BL medium by adding the supplement NPNL (100 mg/L neomycin sulfate, 200 mg/L paromomycin sulfate, 15 mg/L nalidixic acid and 3 g/L lithium chloride) to isolate and to enumerate bifidobacteria in dairy products containing mixtures of bifidobacteria, lactobacilli and streptococci. They observed that bifidobacteria grow well in NPNL agar and therefore this medium has been considered the reference medium for the isolation of bifidobacteria from fermented dairy products (Ashraf and Shah, 2011). Usually, L-cysteine (0.05%–0.1%) is also added to the media used for enumeration of bifidobacteria to improve their recovery. However, this medium is time consuming to prepare because it contains 24 ingredients, some of which must be filter-sterilized (Roy, 2001). Moreover, for consistent and reliable results, a careful measurement of ingredients and treatment of the NPNL medium is extremely important and necessary (Roy, 2001). Furthermore, several authors (Lim et al., 1995; Pacher and Kneifel, 1996) emphasized an undesirable inhibitory effect toward some bifidobacterial species (Ashraf and Shah, 2011; Roy, 2001). Indeed Pacher and Kneifel (1996) found that *B. bifidum, B. breve*, and *B. longum* subsp. *infantis* were sensitive to neomycin sulfate in a range of concentrations of 2–8 mg/L.

Sonoike et al. (1986) developed the TOS medium on which viable cells of bifidobacteria in commercial fermented milk could be selectively counted. Subsequently, Yakult Pharmaceutical (Tokyo) has developed TOS propionate agar containing galactooligosaccharides as an elective carbon source with bifidogenic (prebiotics) properties, sodium propionate, and cysteine. TOS propionate agar has been reported to support the growth of almost all species of bifidobacteria used in fermented milks, with the exception of some *B. bifidum* strains (Barrett et al., 2011). In 1989, Wijsman et al. proposed the addition of NPNL at a relative concentration of 30% to TOS medium (TOS-NPNL). The resulting agar had no effect on the colony count of bifidobacteria but inhibited the growth of pinpoint colonies of the streptococci (Ashraf and Shah, 2011).

RCA is a known basal medium suitable for routine enumeration of *Bifidobacterium* from pure cultures. It is commercially available and it is proven to be time and cost effective, in addition to giving excellent recovery of bifidobacteria.

In 1988, Muñoa and Pares supplemented RCA with nalidixic acid (20 mg/L), polymixin B sulfate (8.5 mg/L) and kanamycin sulfate (50 mg/L) constituting RCA17 medium. BIM25 medium was then prepared by adding iodoacetic acid (25 mg/L) and 2,3,5-triphenyltetrazolium chloride (TTC, 25 mg/L) into RCA17 as a basal medium. Iodoacetate inhibits glyceraldehyde-3-phosphate dehydrogenase and reduces the growth of nonbifidobacterial contaminant colonies. BIM25 was found to be somewhat toxic to some of the bifidobacteria and Muñoa and Pares (1988) concluded that, when stressed by adverse environmental conditions, the bifidobacteria may become unable to grow on BIM25 medium and the problem was overcome by incorporating resuscitative incubation on RCA into the enumeration procedure (see Section 4.3.6).

Sozzi et al. (1990) used the antibiotic dicloxacillin for isolating and counting the bifidobacteria present in fermented milks. The addition of 2 μ g/mL of dicloxacillin to TPY medium (TPYD) was found inhibitory to the growth of lactobacilli and streptococci whereas the medium supported the growth of most bifidobacteria very well.

TPYD medium was found to be more suitable than MRS agar supplemented with dicloxacillin (MRSD) to select *Bifidobacterium* spp. The authors concluded that the addition of dicloxacillin at a concentration of 2 µg/mL to MRS or, better still, to TPY media can most certainly be recommended for *Bifidobacterium* isolation and count in fermented milks or dairy products in general (Roy, 2001; Sozzi et al., 1990).

Other supplements were developed as selective agents and tested in different basal media. Samona and Robinson (1991) indicated the addition of PPNL (15 g/L sodium propionate, 200 mg/L paromomycin sulfate, 100 mg/L neomycin sulfate, and 3 g/L lithium chloride) as selective agents in RMS. They observed the highest counts of bifidobacteria, but later Ghoddusi and Robinson (1996) reported that RMS with PPNL resulted in inhibition for *B. bifidum* and *B. animalis* subsp. *lactis*.

Another medium used as a base for developing selective substrates is Columbia agar base (CAB). Beerens (1991) found that CAB when supplemented with propionic acid at pH 5.0 (mCAP) was selective and elective for bifidobacteria in presence of starter cultures. In contrast to previous reports, Payne et al. (1999) stated that mCAP medium gave comparatively poor recovery of *B. longum* and no growth of *B. bifidum* and furthermore it did not inhibit the growth of *Lactobacillus delbrueckii* subsp. *bulgaricus, Streptococcus thermophilus,* and *Lactobacillus acidophilus,* as well as the other selective media tested.

Lapierre et al. (1992) formulated lithium chloride–sodium propionate (LP) agar for selective enumeration of *Bifi-dobacterium* from commercial dairy products by combining lithium chloride (2 g/L) and sodium propionate (3 g/L) to liver–cystine–lactose (LCL) agar to suppress the growth of LAB. Sodium propionate was used as a selective agent in the medium. LCL–LP was found to give similar enumeration results as found on NPNL agar used as reference medium and hence it was given more importance than NPNL in terms of ease of preparation. However, Payne et al. (1999) found LCL-LP as suitable only for the selective enumeration of *B. bifidum* at 37°C by the spread-plate method (Roy, 2001).

Lim et al. (1995), proposed the use of BL agar, available in commercial trade, and tested it either supplemented with NPNL or with OG [Oxgall (0.2 mg/mL) and Gentamicin ($30 \mu g/mL$)]. They stated that BL-OG is simpler to prepare than the original NPNL and gives a higher recovery of bifidobacteria.

The supplement NPNL was also added to commercial MRS (with glucose as the only carbon source) with/or without 0,05% L-cysteine. MRS-NPNL medium was found suitable for selective enumeration of bifidobacteria only with the addition of L-cysteine (Ashraf and Shah, 2011; Roy, 2001). When L-cysteine was not present in the media, bifidobacteria either did not grow or formed pinpoint colonies.

Arroyo, Martin, and Cotton (AMC) agar was developed by Arroyo et al. (1995) by adding the selective components of LP agar into modified BIM25 (mBIM-1) agar. The BIM-25 was modified by either reducing the iodoacetate concentration by one half (mBIM-1), or by completely excluding iodoacetate (mBIM-2). Resuming, AMC medium comprised RCM (3.8%) with added nalidixic acid (2%), sodium propionate (0.3%), lithium chloride (0.2%), and agar (1.3%) with a filter-sterilized antibiotic solution containing polymyxin B sulfate (8.5 mg), kanamycin sulfate (50 mg), iodoacetic acid (12.5 mg), and 2,3,4-triphenyltetrazolin-C (125 mg). With reference to the controversial and contrary reports against AMC, the medium leaves as to its suitability for use as a selective medium and therefore it needs to be evaluated in this regard (Ashraf and Shah, 2011).

Hartemink et al. (1996) proposed the use of RB medium (Section 4.3.1). No nonbifidobacterial strains used in dairy products grow on this medium with the characteristics that are distinctive for bifidobacteria.

RCPB was indicated by Ghoddusi and Robinson (1996) for the differential enumeration of *L. delbrueckii* subsp. *bulgaricus*, *S salivarius* subsp. *thermophilus* and bifidobacteria. *B. bifidum* and *B. adolescentis* appeared as white colonies while *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* formed pale blue colonies surrounded by wide royal blue or thin light blue zones, respectively.

TPPY + PB is another differential agar that might allow *L. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*, bifidobacteria, and *L. acidophilus* to be enumerated on one medium. According to Ghoddusi and Robinson (1996), bifidobacteria gave white colonies, *S. thermophilus* formed pale blue colonies surrounded by a thin pale blue zone, *L. delbrueckii* subsp. *bulgaricus* produced small white colonies surrounded by wide royal blue zones and *L. acidophilus* produced large pale blue colonies surrounded by a wide royal blue zone (Roy, 2001).

Pacher and Kneifel (1996) developed a culture medium for the detection and enumeration of bifidobacteria in fermented milk products by using MRS supplemented with cysteine-HCl (0.05%), lactulose (2%) and whey from human milk (2%) plus a selective supplement of aztreonam (8 mg/L), nalidixic acid (6 mg/L), netilmicin (8 mg/L) and paromomycin sulfate (6 mg/L). This medium reliably enumerated bifidobacteria. Sometimes, streptococci produced pinpoint colonies, which could be easily distinguished from large bifidobacterial colonies.

In 1997, Roy et al. described RAF 5.1, RAF plus Gentamicin and RAF plus NPNL for the enumeration of bifidobacteria from cheese. These media were based on Columbia agar with raffinose (5 g/L), pH 5.1. RAF 5.1 and

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RAF plus NPNL contained the inhibitory agents lithium chloride (2 g/L) plus sodium propionate (3 g/L) (RAF 5.1) or the NPNL solution mixture (RAF plus NPLN), while RAF plus Gentamicin contained only gentamicin (30 mg/L). The RAF 5.1 was used successfully as a selective medium for the enumeration of *Bifidobacterium* from fresh cheese (Roy et al., 1997). On the other hand, Roy et al. (1997) recommended the use of mCAB plus lactose and gentamycin (30 mg/L), for the selective enumeration of *B. bifidum* because some strains of this species are raffinose negative.

In 1997, Meile et al. enumerated bifidobacteria from commercial milk products on a modified agar of Lapierre et al. (1992). They used LCL agar, containing double amounts of sodium propionate (6 g/L; Fluka) and additionally cysteine hydrochloride (Fluka) at 0.5 g/L, supplemented with nalidixic acid (Sigma) at 30 mg/L and 0.001 g/L resazurine, respectively. The authors isolated the species *B. animalis subsp. lactis*.

In 1999, Nebra and Blanch defined another selective, antibiotic-free medium for *Bifidobacterium*. Lactulose is the main carbon source used by *Bifidobacterium* medium, which includes methylene blue, propionic acid, and lithium chloride as inhibitors of some related bacterial species.

Rada and Koc (2000) tested two media for selective enumeration of bifidobacteria in dairy products: WC agar with addition of NPNL (WC-NPNL) and WC agar with mupirocin (100 mg/L). Mupirocin agar was both more elective and more selective in comparison with WC-NPNL. The total counts of bifidobacterial pure cultures on mupirocin agar were practically identical with those found on the control WC agar, while the WC-NPNL medium gave significantly lower numbers of bifidobacteria.

Modified TPY agar (with added mupirocin, 100 mg/L) was claimed to be highly selective and suitable for isolation and enumeration of bifidobacteria in dairy products (Vlková et al., 2004).

The supplement containing neomycin sulfate (100 mg/L), nalidixic acid (15 g/L) and lithium chloride (3 g/L) (NNL) was added to TPY agar (TPY-NNL) and gave highest recovery of bifidobacteria without any growth of lactobacilli and streptococci (Roy, 2001).

MRS is the medium of choice for the enumeration of LAB and probiotic organisms in cultured dairy products, and is effective when *Bifidobacterium* is the only organism present (Roy, 2001). Selectivity of MRS can be enhanced by addition of specific agents, such as cycloheximide at 0.01% (antibiotic inhibiting growth of yeasts) or sorbic acid at 0.2% (inhibiting growth of yeast by reduction of pH of medium from 6.2 to 5.7) (Hartemink and Rombouts, 1999; Reuter, 1985; Watanabe et al., 2008).

More frequently, however, inhibitors or supplements are used in combination with other compounds to enhance selectivity for the bifidobacteria and inhibit the growth of the LAB. MRS supplemented with cysteine hydrochloride (0.5 g/L) and mupirocin (50 mg/L) to obtain *Bifidobacterium* selective medium (BSM) was found to be elective for *Bifidobacterium* species and selective in the presence of bacilli, lactococci, lactobacilli, and streptococci commonly found in animal feed (Simpson et al., 2004a,b).

Lima et al. (2009) proposed MRS agar with added dicloxacillin (2 mg/L), lithium chloride (1.1 g/L), and cysteine (0.5 g/L). Dicloxacillin when present in MRS agar inhibited the growth of yogurt bacteria without affecting that of bifidobacteria.

Tabasco et al. (2007) supplemented with raffinose (1%), LiCl (0.05%), cysteine HCl (0.05%) the basal medium MRS. They found the medium as selective for the enumeration of *B. animalis* subsp. *lactis* in presence of lactobacilli when incubated at 45°C.

Miranda et al. (2014), proposed a selective culture medium to enumerate bifidobacteria in fermented milk (raffinose-propionate lithium mupirocin, RP-MUP) comprised of TOS-propionate broth which had its TOS component substituted by raffinose to a final concentration of 5 g/L. This basal culture medium was supplemented with a MUP solution containing lithium mupirocin in lyophilized form (Merck, 50 mg/L). This medium was compared to TOS MUP, MRS NPNL, and MRS-ABC dicloxacillin (0.5 mg/L), lithium chloride (1.1 g/L), L-cysteine (0.5 g/L). RP-MUP, whether used or not with Petrifilm AC, presented similar performance to TOS-MUP (ISO and IDF, 2010), with no significant differences between the mean bifidobacteria counts.

In summary, the medium called NPNL agar is considered by many authors to be the reference medium for the isolation of bifidobacteria from fermented dairy products. However, CAB with lithium chloride and sodium propionate plus raffinose or CAB with propionic acid and dicloxacillin as additives and MRS medium supplemented with neomycin, paromomycin, nalidixic acid, and lithium chloride can be recommended for selective enumeration of bifidobacteria in dairy products (Roy, 2001).

4.3.3 Breast Milk

Human milk is considered the best food for newborn infants because it contains optimal ingredients for healthy growth and development (Jeurink et al., 2013). Breast milk has been hypothesized to be a continuous source of

commensal and/or probiotic bacteria for the infant gut, thus providing a constant supply, which may contribute to the dominance of bifidobacteria in breast-fed infants (Heikkilä and Saris, 2003; Martín et al., 2003; Perez et al., 2007).

Now we know that breast milk (human and nonhuman) contains bifidobacteria (Gueimonde et al., 2007; Jost et al., 2013; Killer et al., 2013; Kozak et al., 2015; Milani et al., 2015b; Solís et al., 2010; Turroni et al., 2011).

Recently, a sharing of bifidobacterial strains between breast milk and infant species (Martin et al., 2012) has been demonstrated, suggesting vertical mother-to-child transmission from the maternal gut to that of breast-fed infants (Solís et al., 2010; Turroni et al., 2011). Indeed, bifidobacterial strains isolated from breast milk were found in the corresponding infant feces, suggesting that predominant bifidobacterial populations are individual-specific.

Several studies have demonstrated that human milk from healthy women contains approximately 10³–10⁴ CFU/ mL bacteria (Beasley and Saris, 2004; Collado et al., 2009; Gueimonde et al., 2007; Heikkilä and Saris, 2003; Hunt et al., 2011; Martin et al., 2009; Martín et al., 2003; Perez et al., 2007). The first descriptions of the bacterial diversity of human milk from healthy women in 2003 were based on in vitro culturing methods (Heikkilä and Saris, 2003; Martín et al., 2003).

However, breast milk is difficult to sample and microbiological contamination can never be discounted (Solís et al., 2010). Although it is questioned whether it is possible to aseptically collect human milk, culture-dependent methods have confirmed the presence of bacteria in assumedly aseptically collected milk. Cultured genera include *Staphylococcus, Streptococcus, Lactococcus, Leuconostoc, Weissella, Enterococcus, Propionibacterium, Lactobacillus,* and *Bifidobacterium*. The most commonly isolated bacterial species from human milk include *Staphylococcus epidermidis, Staphylococcus aureus, Streptococcus mitis, Streptococcus salivarius, Lactobacillus salivarius, Lactobacillus fermentum, Lactobacillus rhamnosus, Bifidobacterium breve,Bifidobacterium adolescentis, Bifidobacterium bifidum, Bifidobacterium longum* subsp. *longum,* and *Bifidobacterium pseudocatenulatum. Bifidobacterium adolescentis* has been also described in the breast milk of the nonhuman primate *Macaca mulatta* (Killer et al., 2013).

To isolate bifidobacteria from breast milk, the samples may be collected in a sterile tube by a manual breast-pump or by a manual expression using sterile gloves (Martin et al., 2009; Mastromarino et al., 2014; Solís et al., 2010). Previously, nipples and mammary areola have been cleaned with soap and sterile water and soaked in chlorhexidine (Cristalmina; Salvat, Barcelona, Spain). The first drops (~500 μ l) are usually discarded. The milk samples are immediately transported to the laboratory in ice-cooled containers and in anaerobic conditions achieved using anaerobic gas generation kits within 2 h from collection (Awasti et al., 2016). The analysis for bacteria isolation starts as soon as samples arrive to the laboratory. The transported samples are serially diluted in a proper buffer. Dilutions are frequently made in Peptone water (Martin et al., 2009), or in TPY broth (Killer et al., 2013), or in a reducing medium containing BHI broth (Merck) supplemented with 0.5% glucose, 0.5% yeast extract (Merck), 0.25% L-cysteine (Sigma Chemical Co, St. Louis, MO, USA), 10 μ g/L vitamin K1 (Merck) and 0.02 g/L Hemin (Sigma) (Solís et al., 2010).

Proper dilutions of the milk are plated in a *Bifidobacterium* selective or a nonselective medium. Media frequently used are MRS supplemented with L-cysteine (0.5 g/L) (Martin et al., 2009; Solís et al., 2010) and MRS supplemented with 50 mg/L of mupirocin (Delchimica, Italy) (Turroni et al., 2011). Martin et al. (2009) found cultivable bifidobacteria from only 8 out of the 23 samples studied and their isolates belong to three bifidobacterial species: *B. breve* (four milk samples), *B. adolescentis* (two samples), and *B. bifidum* (two samples). In the work of Solís et al. (2010) the bifidobacterial strains isolated from breast milk samples were *B. longum* subsp. *longum* (3 isolates), *B. breve* (3 isolates). Turroni et al. (2009) isolated 25 bifidobacterial strains from three breast milk samples identified as *B. breve*, *Bifidobacterium longum* subsp. *longum/infantis*, and *Bifidobacterium adolescentis*. mTPY agar prepared according to Rada and Petr (2000) was also used to isolate *Bifidobacterium adolescentis* (10 strains) from the milk of a macacae subject (*Macaca mulatta*) (Killer et al., 2013).

4.3.4 Oral Cavity

The microorganisms found in the human oral cavity have been referred to as the oral microbiota, or more recently as the oral microbiome (Dewhirst et al., 2010; Ledeberg, 2001). The oral cavity, or mouth, includes a wide range of niches, such as teeth, gingival sulcus, attached gingiva, tongue, cheek, lip, hard palate, and soft palate (Mantzourani et al., 2010). We define the human oral microbiota as all the microorganisms that are found on or in the human oral cavity and its contiguous extensions (stopping at the distal esophagus), though most of our studies and samples have been obtained from within the oral cavity.

The acidogenic and acidiuric bifidobacteria have been reported to be commensal microorganisms in the oral cavity even if their role in oral health is controversial (Jäsberg et al., 2016). They have been proposed as associated with the caries process in children, adults, and the elderly (Beighton et al., 2010; Mantzourani et al., 2009a,b). In addition,

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bifidobacteria were found only at the sites of active caries when the microbial ecology of the occlusal caries lesions was studied (Dige et al., 2014).

Bifidobacterium dentium is the predominant species of *Bifidobacteriaceae* in the mouths of dentate subjects together with *Scardovia inopinata* and *Scardovia denticolens*; moreover in the edentulous subjects wearing dentures *B. breve*, *B. scardovii*, and *B. longum* subsp. *longum* have been isolated (Mantzourani et al., 2010). *B. dentium*, *B. longum* subsp. *longum*, *B. breve*, and *B. subtile* have been also detected in the oral cavities of children with occlusal caries (Mantzourani et al., 2009a) and in those of caries-free children (Mantzourani et al., 2009b). A new species *B. tsurumiense* was recently described in dental plaque samples from hamsters, suggesting the presence of this genus also in the oral cavity of nonhuman subjects (Okamoto et al., 2008).

Studies on isolation of bifidobacteria from oral cavity relied on analysis of saliva samples (Hojo et al., 2007; Kaur et al., 2013), plaque samples (Mantzourani et al., 2010; Modesto et al., 2006; Tanner et al., 2011), and caries lesions samples (Mantzourani et al., 2009a).

For studying bifidobacterial distribution in mouth, two kinds of saliva samples are commonly collected—stimulated and nonstimulated samples—knowing that many factors, such as age and sex differences of the participants, (oral) health status, medication, physical activity, and level of oral self-care can all influence the composition of saliva and therefore can influence the results of studies focusing on saliva analysis. Even the circadian cycle—and therefore the time of day when saliva is collected—has a significant effect (Prodan et al., 2015). Hojo et al. (2007) collected a nonstimulated saliva sample in a dish in the morning, before breakfast and toothbrushing, whereas Kaur et al. (2013) used the tongue loop method. Briefly, a 10 μ L bacteriological loop was drawn down the center of the anterior two-thirds of the tongue until the loop was full of saliva, equivalent to 10 μ L of saliva. Stimulated saliva samples might be usually obtained using paraffin chewing gum (Mantzourani et al., 2009b).

Plaque samples may be taken with sterile wooden toothpicks (Modesto et al., 2006; Tanner et al., 2011) from the buccal, the posterior, and interproximal surfaces of teeth.

Samples of infected dentine (Mantzourani et al., 2009b) were obtained from active occlusal lesions using a sterile excavator.

The collected samples are immediately dislodged into a prereduced fastidious anaerobic broth (Oxoid, Basingstoke, UK) (Beighton et al., 2008; Mantzourani et al., 2009b) or into a prereduced anaerobically sterilized Ringer's solution (Tanner et al., 2011) as well as into prereduced anaerobically sterilized dilution blanks (Modesto et al., 2006), or into anaerobic broths, such as GAM broth (Hojo et al., 2007) or WC broth (Beighton et al., 2008).

The samples are processed within 1–6 h from sampling. Aliquots of dilutions are spread on the surface of appropriate (selective) media. The main media are acidified TPY (Modesto et al., 2006), Beerens medium (Beerens, 1991) and mTPY (Mantzourani et al., 2009b) medium (mupirocin-containing selective medium) which contained, per litre of deionized water: 7 g proteose peptone (Oxoid), 5 g soya peptone (Oxoid), 5 g yeast extract (Oxoid), 15 g glucose, 5 g raffinose, 0.5 g cysteine HCl, 2 g K₂HPO, 0.5 g MgCl₂ 6H₂O, 0.25 g ZnSO4 7H₂O, 0.15 g CaCl₂, 0.001 g FeCl₃, 15 g agar, and 1 mL Tween-80. Glacial acetic acid (1 mL/L) and 1 mL of a 5 mg/mL mupirocin 50% ethanolic solution were finally added to the sterilized and cooled medium.

Bifidobacteria were also isolated from an acid agar, comprising Trypticase soy agar (20 g/L), brain heart infusion agar (26 g/L), yeast extract (10 g/L), and hemin (5 mg/L), with the pH being adjusted to pH 5.0 with HCl before the agar was autoclaved (Tanner et al., 2011).

Okamoto et al. (2008), inoculated diluted aliquots of the hamster dental plaque samples onto Cadmium Sulfate Fluoride Acridine Trypticase (CFAT) agar, which is a selective medium for human *Actinomycetes*, its composition (per one liter) being as follows: trypticase soy broth (BBL Microbiology Systems), 30 g; glucose, 5 g; agar, 15 g; cadmium sulfate, 13 mg; sodium fluoride, 85 mg; neutral acriflavin, 1.2 mg; potassium tellurite, 2.5 mg; basic fuchsin, 1.3 mg; defibrinated sheep blood, 50 mL.

4.3.5 Vaginal Content

First Blaurock (1940), Roufogalis (1940a,b, 1941), Montagna and Cataldi (1944) reported the isolation of bifidobacteria from the vagina (Poupard et al., 1973). Harrison et al. (1953) studied the occurrence of bifidobacteria in the vaginas of normal nonpregnant women and in women in the second and third trimesters of pregnancy. They noted a significant increase in incidence of bifidobacteria at term.

Few culture dependent studies dealing with the isolation of bifidobacteria from human vagina are present in the literature. From the available data, bifidobacteria are present in the 22%–26% of healthy women (Crociani et al., 1973; Seelinger and Werner, 1963). *B. breve* and *B. adolescentis* have been the most frequently isolated species whereas *B. longum* subsp. *longum* and *B. bifidum* were present to a smaller extent (Korshunov et al., 1999; Mattarelli and

Biavati, 2014). In the work of Tannock et al. (1990) bifidobacteria have been isolated from deep vaginal swabs collected at the first examination soon after admission to a maternity hospital and before the standard procedures of vaginal douching, after delivery. Swabs were added to preweighed bottles containing a transport medium, maintained refrigerated until delivered to the laboratory. The time elapsed between collection of specimens and bacteriological examination seldom exceeded 6 h. Swabs were used to directly inoculate the selective medium for the isolation of bifidobacteria: BIM agar prepared as described by Muñoa and Pares (1988) but with the concentration of iodoacetic acid reduced to 0.0124 g/L. In the study of Crociani et al. (1973) 100 vaginal secretions were obtained and suspended in saline and then inoculated in TPY agar. This medium contained per 100 mL: trypticase (BBL) 1.0 g, Phytone 0.5 g, glucose 1.5 g, yeast extract 0.25 g, cysteine-HCl 0.05 g, dipotassium phosphate 0.15 g, magnesium chloride 0.05 g, zinc sulfate 0.025 g, traces of ferric chloride. Zinc sulfate and ferric chloride were dissolved separately. After 3–4 days of incubation, colonies formed by cells with irregular shape were isolated (Crociani et al., 1973).

4.3.6 Water

In 1958, Mossel proposed the use of the bifidobacteria as potential microbial indicators of human fecal pollution (Resnick and Levin, 1981) due to the following alleged characteristics: (1) bifidobacterial presence in the feces of humans in densities greater than that of *Escherichia coli* by a factor of 10; (2) their inability to multiply outside the intestinal track due to stringent nutrient requirements; and (3) survival characteristics which were considered to be similar to those of *E. coli* (Lamendella et al., 2008; Resnick and Levin, 1981). Moreover, some *Bifidobacterium* species are associated with human feces, whereas others have an exclusively animal origin (Biavati and Mattarelli, 2012; Biavati et al., 2000).

Since then a number of workers (Beerens, 1998; Bonjoch et al., 2009; Lynch et al., 2002; Mara and Oragui, 1985, 1983; Nebra et al., 2003; Resnick and Levin, 1981) have examined and extended the concept of using these organisms as indicators of fecal pollution with the goal to optimize a bacteriologically based methodology that might be easily and economically implemented in existing laboratories, smaller laboratories, and developing countries to enumerate bifidobacteria in environmental waters.

The method for the enumeration of bifidobacteria in a natural aquatic environment with the membrane filter technique had been developed by Evison and James (1973), Evison and Morgan (1978), and Gyllenberg et al. (1960), and it was then described in publications. Bifidobacteria were enumerated on the medium of Gyllenberg et al. (1960), a completely chemically defined medium containing nalidixic acid (5 mg/L) and 2-3-5-triphenyl tetrazolium chloride (0.1 g/L, TTC) as selective agents, ascorbic acid (10 g/L) as reducing agent, and arabinose (10 g/L) as the carbon source (Evison and Morgan, 1978). Later, another two selective media were described: YN-6 (Resnick and Levin, 1981) and YN-17 (Mara and Oragui, 1983). However, as it has been reported by Mara and Oragui (1983), YN-6 medium is inhibitory to some species of *Bifidobacterium* and lacks the desired selectivity to allow a real estimation of the number of viable cells of *Bifidobacterium* spp. (Carrillo et al., 1985). On the other hand, YN-17 medium, which is more selective than YN-6, is inhibitory to a part of the viable bifidobacterial population (Muñoa and Pares, 1988).

In 1983, Mara and Oragui developed a selective medium for the isolation and enumeration of those bifidobacteria associated with human feces, assessing the practicability and applicability of using such bifidobacteria to distinguish between human and animal fecal pollution. Human bifidobacteria sorbitol agar (HBSA) was proposed to give a more reliable indication of the source of fecal pollution, as sorbitol fermenting bifidobacteria (SFB) were previously reported to specifically indicate human fecal bifidobacterial pollution (Mara and Oragui, 1985). This selective medium consistently isolated SFB (mainly *B. adolescentis* and *B. breve*) as yellow growing colonies.

In 1988, Muñoa and Pares described a new method for the quantification of the presence of *Bifidobacterium* spp. in aquatic environments based on the utilization of a new selective and differential medium, *Bifidobacterium* iodoacetate medium 25 (BIM-25), and on resuscitation techniques to allow for the isolation of injured bifidobacteria. By this procedure, enumerations were made as follows. River and sewage samples were diluted in phosphate-buffered saline, and portions (0.1 mL) were surface plated onto 5 mL of RCA and incubated at 37°C for 5 h under anaerobic conditions (GasPak; BBL). Thereafter, 20 mL of BIM-25 was carefully overlaid onto the plates, which were incubated for 5 days under the conditions described above. Appropriate volumes of seawater samples were filtered through 0.45-µm-pore-size membrane filters (Millipore Corp., Bedford, Massachusetts). Filters were first incubated on RCA for 5 h and then transferred to BIM-25. In all cases, enumerations of *Bifidobacterium* spp. were made in parallel on RCA. This rich, nonselective medium served as a reference.

Currently, the bacteriologically assay is based on the enumeration of SFB and total bifidobacteria (Blanch et al., 2006; Luyt et al., 2015).

4.3 ISOLATION

Animal and human sources of fecal contamination are distinguished based on the ratio of the two concentrations in a water sample (Blanch et al., 2006; Luyt et al., 2015). The tracking ratio is the ratio of SFB to the total bifidobacteria concentrations. There is a cut-off point, which for Bonjoch et al. (2005) was 0.2, while for Blanch et al. (2006) it was 3.2. Thus a tracking ratio below the cut-off point indicates animal sources of the fecal contamination, while higher values indicate human sources (Blanch et al., 2006; Bonjoch et al., 2005).

The intestinal anaerobic bifidobacterial populations have been reported to have a rapid die-off in environmental water or wastewater, which are hostile environments because they contain oxygen (Carrillo et al., 1985; Resnick and Levin, 1981). This may be partially addressed by using reducing agents to help bifidobacteria present in such environments to recover from the damage they suffer from exposure to oxidizing conditions (Nebra et al., 2002). The metabolic stress induced by oxidizing conditions is particularly significant in selective media because these media are necessarily stressful even to the bacteria that they seek to select for. Nebra et al. (2002) tested three reducing agents in an attempt to improve the recovery of oxygen-stressed bifidobacteria: L-cysteine, sodium pyruvate, and sodium thioglycolate. The authors used five different pure cultures of bifidobacteria: *B. pseudocatenulatum* DSM 20438, *B. adolescentis* DSM 20083, *B. dentium* DSM 20084, *B. longum* subsp. *suis* DSM 20211, and *B. longum* subsp. *infantis* DSM 20088. These agents were evaluated both individually at various concentrations, and in combination, by measuring recovery on rich and selective media. Selective media used in the recovery of injured bifidobacterial cells were modified Columbia blood agar, CBA, supplemented with 5 g/L of glucose and 0.5 g/L of L-cysteine before sterilization (sCBA). They found that the addition of several mixtures of reducing agents to the water samples and preincubation for 4 h at 37°C increased the recovery of *Bifidobacterium* spp. on culture media.

To isolate bifidobacteria, after sampling, wastewater samples are stored at 4°C or at environmental temperature and analyzed within 8 h of collection. Prior to filtration, samples may be serially diluted in quarter-strength Ringer's solution or PBS if necessary. Polluted water and tap water samples were either diluted or whole volumes (1 mL, 4 mL, 10 mL, 100 mL) were filtered through sterile cellulose nitrate membrane filters (Millipore type HAWG 047 SI, 0.45 µm pore size, 47 mm diameter), which thereafter were placed on HBSA (Mara and Oragui, 1983) or Beerens agar (Balleste and Blanch, 2011; Beerens, 1998). The plates were inverted and incubated at 37°C for 48 h in an anaerobic jar containing anaerobic atmosphere generating system. Enumeration of SFB was undertaken according to the protocols described in previous studies using HBSA medium (Long et al., 2005; Mara and Oragui, 1983). Deep yellow, domed, and mucoid colonies resulting from the fermentation of sorbitol were scored as presumptive SFB.

However, as previously mentioned, due to its faster die-off, as compared to many waterborne pathogens, SFB cannot replace microbiological standard parameters for routine water quality monitoring, but it is highly recommendable as a specific and complementary tool when human fecal pollution has to be localized or verified. Because of its exclusive fecal origin and human specificity it seems also worthwhile to include SFB in future risk evaluation studies.

4.3.7 Probiotics

Bifidobacteria are probiotic microorganisms that are widely used in the food industry (Miranda et al., 2011). In addition to the food probiotics, there are various health products and pharmaceutical preparations containing probiotics on the market (Saad et al., 2013). The most commonly used species of probiotic bacteria in lyophilized form are *B. animalis* ssp. *lactis*, *B. bifidum*, *B. breve*, *B. longum* ssp. *longum*, and *B. longum* ssp. *infantis*. The amount of probiotic bacteria required for therapeutic effect is considered to be in the range of 10° cells of live microorganisms per day (Miranda et al., 2014). To exert a beneficial effect, the bacteria must remain viable in the product until the time of consumption. Commercially available probiotics are usually in the form of freeze-dried, powdered bacteria or in the capsule-packed forms, which can affect their persistence and viability. The manufacturer should correctly inform customers about bacteria amounts and species composition in the product. A widely used method for the microbiological control of food quality, including probiotics, is cultivation. Different culture media have been proposed (Ashraf and Shah, 2011; Karimi et al., 2012; Roy, 2001) and there also exists an ISO standard for the enumeration of bifidobacteria in foods, such as milk products. This method is applicable for milk products, such as fermented and nonfermented milk, milk powders, infant formulas, and starter cultures.

However, no standard method has been described for the selective enumeration of bifidobacteria in probiotic supplements. The use of bifidobacteria in pharmaceutical supplements is becoming increasingly popular, resulting in a wide variety of products being marketed with specific or generic claims of health benefits. Bunesova et al. (2015) evaluated three different mupirocin (Mup; 100 mg/L) selective media for the enumeration of bifidobacteria by colony count technique in 13 probiotic supplements: TOS, BSM (Fluka) and modified WC anaerobe agar with soya peptone (WSP)(5 g/L), L-cysteine (0.5 g/L), and Tween 80 (1 mL/L). All tested mupirocin media were found to be not fully

selective for bifidobacteria. Therefore, the TOS Mup medium recommended by the ISO standard could not be regarded as a suitable medium for the genus *Bifidobacterium*, indeed not all tested strains of *B. bifidum* showed good growth. In contrast, the BSM Mup and WSP Mup media supported the growth of all tested pure bifidobacterial cultures (*B. adolescentis* DSM 20083, *B. animalis* subsp. *lactis* DSM 10140, *B. bifidum* strains DSM 20456, DSM 20082, DSM 20215, and DSM 20239, *B. breve* DSM 20213, *B. longum* subsp. *infantis* DSM 20088, and *B. longum* subsp. *longum* DSM 20219).

4.4 STORAGE

A growing interest in bifidobacterial health promoting effects has required microbiologists to adopt appropriate storage methods. Cultivation and characterization of bifidobacteria alone is not adequate without preservation techniques that do not alter the viability, morphology, physiology, or genetics of pure strains.

The industrial exploitation of bifidobacteria as probiotic cultures depends strongly on the preservation technologies employed, which are required to guarantee long-term delivery of stable cultures, in terms of viability and activity. Therefore, the double aim of storage methods for bifidobacteria is to preserve pure cultures for long periods but also to prepare pure cultures for any applications as probiotics.

Several methods have been described for the short- and long-term preservation of bifidobacterial cultures: repeated subculturing of strains in agar stabs or in liquid medium, cryopreservation, and lyophilization.

4.4.1 Short-Term Methods

Biavati and Mattarelli (2012) indicated a chopped meat medium as the best one for preservation of bifidobacterial strains in liquid media. To avoid dehydration and diffusion of O_2 , sterilized chopped meat medium (prereduced anaerobically) in tubes sealed with butyl stoppers is inoculated, and after growth, the cultures can be stored at room temperature or at 5°C for 4–6 months.

Cultures may be also maintained on stabs (TPY or other basal media with 0.7% agar added) under anaerobic conditions, but they should be subcultured every two or three weeks.

4.4.2 Long-Term Methods

4.4.2.1 Cryopreservation

Cryopreservation can be seen as one of the standard methods to preserve bacterial cultures, and it is the widely utilized technique for storage of bifidobacterial cultures, over a long period of time. The term "cryopreservation" refers to the preservation of biological materials at cryogenic temperatures, generally –80°C, (dry ice) or –196°C, (liquid nitrogen). Proper cryopreservation allows for the maintenance of bacterial stocks over extended time, so decreasing the need for repeated subcultures, which in turn can lead to contamination, genetic drift, or mutation as each next time smaller portions of population are selected. Low-temperature storage significantly reduces phenotypic and genotypic drifts. Low temperature protects proteins and DNA from denaturation and damage and slows the movement of cellular water. Consequently, biochemical and physiological activities of the cells are essentially halted and cells are protected for long periods of time (Prakash et al., 2013a).

Preservation of cells at –20°C is not recommended for long-term preservation. Preservation at –80°C is adequate, but –196°C is considered ideal because the chances of DNA mutations are almost zero at that temperature (Prakash et al., 2013a).

Moreover, during freezing, ice crystals are formed that can damage cells. The growth of the ice crystals is dependent on the freezing rate and temperature. A high freezing rate is preferred over a slow freezing rate, since it will lead to the formation of smaller ice crystals, avoiding extensive cellular damage. A current strategy used to protect the cells from cryoinjuries during cryopreservation is the application of cryoprotectants, viz. water soluble chemicals that lower freezing point of water, promote hydrogen bond formation and vitrification of solvents, and prevent ice crystal formation (Fuller, 2004; Prakash et al., 2013b). Cryoprotectants can be broadly classified as penetrating or nonpenetrating, and cell-penetrating cryoprotectants are generally considered ideal.

Glycerol (10%–20%) and dimethyl sulfoxide (10%, DMSO) are frequently used in cryopreservation of bifidobacteria, and both have cell-penetrating capacity. At physiological temperatures, glycerol works best, but at lower temperatures it does not penetrate well inside the cell and consequently provides less protection. DMSO has a better penetrating ability than glycerol but its use is limited due to toxic effects at higher concentrations (Prakash et al., 2013a).

Watanabe et al. (2009) reported the suspension of *B. mongoliense* strains in nutrient broth (BD Difco) containing 10% (v/v) DMSO and storage at -80°C. Choi et al. (2014) described maintenance of bifidobacterial isolates belonging to *B. faecale* as glycerol suspensions (30%, w/v) at -80°C. Endo et al. (2010) kept their pure cultures at -80°C, suspended in sterile nutrient broth supplemented with 20% (v/v) glycerol. Other authors stored their cultures as frozen stocks in broth media containing 15%-20% glycerol (Celik and O'Sullivan, 2013). Zacarías et al. (2011) reported that their isolates were stored frozen in MRS broth supplemented with 20% (v/v) glycerol at -70° C.

There are other types of compounds employed for cryopreservation apart from glycerol and DMSO. Cryoprotectants, such as skim milk and nonpenetrating compounds (e.g., polysaccharide, polyalcohols, proteins) are also commonly used. Novik et al. (2009) determined the effect of rapid freezing kinetics (direct immersion in liquid nitrogen), type of cryoprotective additive, and composition of cryoprotective media on viability of bifidobacteria. Their study indicates that media traditionally used for bifidobacteria cultivation have good cryoprotective properties and could be effective freezing media for preservation of these organisms. They also stated that rapid cooling kinetics appeared to lead to minimal losses in viability and acidification activity of bifidobacteria.

Modesto et al. (2004) tested the resistance to freezing of several potential probiotic bifidobacteria and compared two different cryoprotective solutions, (1) a skim milk, 20% lactose, 3% yeast extract (0.3%) solution and (2) a sucrose, 12% yeast extract, 0.3% peptone (0.5%) solution. They stated that the performance of each protectant appears to be strain specific and has to be investigated on a case-by-case basis.

Though freezing and thawing can cause cellular damage to the bacterial cells, cryopreservation is a simple method, relatively cheap and not time consuming (Novik et al., 2009). However, from a commercial point of view, it has several disadvantages, such as the need for subzero transportation and storage temperatures, and thus high energy costs (Broeckx et al., 2016; Dubourg et al., 2013).

4.4.2.2 Freeze Drying

Preservation by freeze drying has been the preferred long-term preservation method for bacterial cultures for decades, and also for bifidobacteria due to the low cost of maintenance and ease of transportation of lyophilized cultures. There are extensive Microbial Resource Centers that depend on freeze-drying to preserve a huge diversity of cells for future propagation (Prakash et al., 2013a).

Lyophilization can be divided into three steps: freezing, primary drying, and secondary drying. In the primary drying step, the frozen water is removed by sublimation under vacuum, while in the secondary drying step, the unfrozen water is removed by desorption (Broeckx et al., 2016). Therefore, lyophilization exerts stress on the cells during vacuum desiccation, and a decreased viability of the microorganisms during the drying process is still the most important undesirable side effect that should be controlled (Prakash et al., 2013a).

Many protection strategies have been developed to enhance bacterial viability during freeze-drying and one of the most applied is the addition of cryo- and lyoprotectants to the suspension. As previously mentioned, cryoprotectants are useful in the freezing step, while lyoprotectants protect cells during the drying steps when water is removed. Some sugars can work both as cryo- and lyoprotectant and render positive effects on the viability of bacteria after freeze drying (Broeckx et al., 2016). Protectants commonly used are cellobiose, lactose, sucrose, and trehalose, at 10% concentration (Basholli-Salihu et al., 2014; Saarela et al., 2005). Modesto et al. (2015, 2004) indicated the use of a skim milk 20% lactose, 3% yeast extract (0.3%) solution as cryoprotectant for both freezing and freeze drying.

Moreover, the viability and longevity of microorganisms under preservation depends on some critical factors: (1) composition of the suspension and rehydration medium, (2) type of cryoprotectant used, (3) rate of cooling and thawing, (4) growth stage of the culture, (5) water content and initial density of cells (Prakash et al., 2013a).

The use of stationary phase cultures, a suspension medium with 10⁸–10⁹ cell/mL, borosilicate ampoules, a 1%–2% final moisture content of the lyophilized specimen, and storage at 4°C in the dark are recommended for higher cell viability and longer stability after lyophilization (Prakash et al., 2013a). Even different strains of the same species may respond differently to the same preservation method (Modesto et al., 2004; Prakash et al., 2013a).

In our experience, for freeze-drying bifidobacteria, cells are cultivated in TPY broth or MRS broth supplemented with 0.5% of cysteine. Fresh cells are harvested by centrifugation (10,000 g, 4°C, 15 min) and washed twice with 50 mM phosphate buffer, pH 6.5. The pellets derived from 10 mL cultures are then resuspended in 1 mL of a proper lyoprotectant: a skim milk solution with lactose (Modesto et al., 2004). The suspension is divided into 250 μ L aliquots, poured into sterile glass vials, and then frozen at -135° C prior to freeze-drying.

Bifidobacterial cells are freeze-dried over 22 h in a single chamber freeze-drier at a condenser temperature of -54° C using a Lio5P (Pascal, Italy) lyophilizer. At the end of the process, vials are hermetically sealed under vacuum by melting the thin top with an open flame. Freeze-dried cultures are then stored in the dark at room temperature. Lyophilized cultures of bifidobacteria remain viable and stable after 30 years.

4.4.3 Methods for Drying Probiotics

Drying technologies can be seen as the preferred method to preserve and store bacteria for industrial applications. Several methods can be distinguished, including spray-drying, freeze-drying, vacuum-drying, air-drying, and fluidized-bed drying. Dried cultures have advantages during shipping and storage, but critical parameters, such as temperature, exposure to oxygen, and water activity may affect their viability (Broeckx et al., 2016).

Drying processes induce stress on probiotics, thus decreasing their viability and to some extent their functionality. Several protection strategies can be used to enhance bacterial viability, including addition of protective agents, controlling the process parameters, and prestressing the microorganisms prior to drying (Champagne et al., 1996; Shokri et al., 2015; Vinderola et al., 2012). Researchers have been working for several years on this approach, and although it did not achieve a robust technique suitable for all bacteria, nevertheless they revealed and emphasized the important role of lyoprotectant solutions in affecting not only strain viability but also their functionality (Celik and O'Sullivan, 2013; Iaconelli et al., 2015; Saarela et al., 2005; Vinderola et al., 2012). Studies of the impact of drying processes on the viability of microorganisms often fail to consider cell functionality. Indeed, the survival of probiotic bacteria is not necessarily correlated with the preservation of their beneficial effects. Addition of solutes (trehalose, glycerol, sucrose, skim milk) in the suspension medium is a suitable strategy for protecting bacterial viability and functionality during the drying (Vinderola et al., 2012), though the solute nature is dependent on the chosen drying technique and the bacterial strain itself (Meng et al., 2008).

Moreover, probiotic viability needs to be maintained not only during the drying process but also during storage. This is a critical challenge for commercial production of bifidobacteria for probiotic applications (Saarela et al., 2005) since the abilities to survive during freeze-drying and subsequent storage are not linked. Therefore, factors that affect survival during storage need to be defined. The stability of freeze-dryied bifidobacteria varies depending on the freeze-drying method and subsequent storage conditions. Once again, the important role of lyoprotectants on survival of dried cultures of bifidobacteria has been widely emphasized (Champagne et al., 1996; Tanimomo et al., 2016).

Overall, lower storage temperature and low moisture content result in good survival rates. Careful attention should also be paid to the rehydration conditions of the dried probiotics, as this can exert an important effect on their revival (Celik and O'Sullivan, 2013).

In terms of bacterial viability and functionality, freeze-drying is the preferred method for preservation of probiotics cultures, including bifidobacteria. However, its cost has hindered its use in large-scale processes. Spray-drying is more economic and efficient because of its continuous high production rate behavior, but viability of bacteria is usually affected due to use of extreme heat (Shokri et al., 2015). During spray-drying, bacteria are exposed to multiple stresses, that is, heat (both wet and dry), oxidation, dehydration-related stresses (osmotic, acidic, and thermal shock, accumulation of toxic compounds, etc.) that potentially could lead to cell death. Loss of viability appears to be principally caused by cell membrane damage; moreover, the cell wall, ribosome, and DNA are also affected by higher temperatures.

Though bifidobacteria are very sensitive to heat, nevertheless several studies have confirmed that spray-drying may be applied for some cultures, but the conditions require optimization (Chávez and Ledeboer, 2007). The use of suitable carriers in the culture media (sucrose, galactooligosaccharides, or FOS) before spray-drying and the application of thermal shock to cultures before drying have been described as key factors in this field (Lian et al., 2002; Shokri et al., 2015).

The main characteristics of the different drying technologies are discussed in detail in Chapter 16.

4.5 CONCLUDING REMARKS

TPY together with other commercially available media, such as RCA, BHI, and mMRS containing cysteine-HCl (0.05%) can provide optimal overall growth conditions for cultivation of bifidobacteria. Many selective media have been described for differential enumeration and isolation of *Bifidobacterium* spp. However, no single selective medium has proven satisfactory for recovery of bifidobacteria from various ecological niches. Therefore, it could be noted that the bifidobacteria selective media should be chosen in respect of the origin of the sample tested. Finally, for the general enumeration of bifidobacteria a basal medium supplemented with mupirocin may be considered as the reference medium.

Among the different methods used to preserve bifidobacterial cultures, long-term storage techniques are likely adequate to maintain cell viability and stable characteristics over different periods of time. Cryopreservation and freeze-drying are widely used methods, but require optimization of protection strategies for enhancing bacterial viability and functionality.

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Further Reading

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5

Chemotaxonomic Features in the *Bifidobacteriaceae* Family

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5.1 INTRODUCTION

Chemotaxonomy studies the chemical variation in microbial cells and the use of chemical characteristics in the classification and identification of bacteria; it can be very helpful in the modern approach of bacterial polyphasic taxonomy. For some groups it is one of the most important taxonomic criteria for identification (e.g., *Sphingomonas* sp.; Busse et al., 1999) while for others, such as *Bifidobacteriaceae*, it is important but not sufficient for strain identification. It has been recommended in the Bifidobacteriaceae Minimal Standard guidelines for the description of new species (Mattarelli et al., 2014).

Chemotaxonomic markers applied in polyphasic approach of Bifidobacteriaceae are here described singularly.

5.2 CELL WALL STRUCTURE

5.2.1 Peptidoglycan Structure

Peptidoglycan (referred to also as murein) is the common cell wall component of most Gram-positive bacteria (about 30%–70% of the cell walls) and Gram-negative (only a minor component of the cell wall <10%) bacteria. Peptidoglycan is essentially composed of glycan strands consisting of repeats of β -1,4-linked *N*-acetylglucosamine and *N*-acetylmuramic acid disaccharide units, cross-linked by short peptides. It is very important for physiological studies involving the mechanism of resistance to the antibiotics, phage susceptibility, and serological behavior and immune response (Schumann, 2011). The composition and structure of the peptidoglycan is rather constant among Gram-negative bacteria, but widely differ among Gram-positive bacteria. Therefore, the nature of the interpeptide bridge of the cell-wall peptidoglycan is markedly involved in bacteral taxonomy and it has been widely studied for interspecies and intraspecies variations. The determination of these features does not necessarily require the purification of the complete cell wall for all groups, focusing on the interpeptide bridge, which is considered an important taxonomic criterion.

The analysis of peptidoglycan has been performed on the total hydrolysate of the peptidoglycan by gas chromatography/mass spectrometry. Two-dimensional thin-layer chromatography of the peptidoglycan partial hydrolysate has been finally utilized to determine the presence of different peptides [for details, see Schumann (2011)].

The peptidoglycan structure has been examined in most *Bifidobacteriaceae* species (54 taxa out of 60 taxa in the *Bifidobacterium* genus and in all the so-called scardovial genera). *Gardnerella vaginalis* cell wall contains major amounts of alanine, glycine, glutamic acid, and lysine different from all *Bifidobacteriaceae* species (O'Donnell et al., 1984). In the genus *Bifidobacterium* there are species where peptidoglycan structure is unique and is not shared with other species (e.g., peptidoglycan type A11.23, A11.25, A11.37); on the other hand, there are species that share the same peptidoglycan structure of the type subspecies (e.g., *B. longum* subsp. *longum*, subsp. *infantis*, subsp. *suis*, and subsp. *suillum* share the same A21.3 structure) (Table 5.1). Moreover, in the genus *Bifidobacterium*, the species usually cluster according to

TABLE 5.1	Peptidoglycan	Type in the	Genus Bifidobacterii	im and Scar	dovial Genera
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Species	Types of cross-linkages	Peptidoglycan types	No. of different peptidoglycan type in the DSMZ catalogue entries
B. asteroides, B. breve, B. reuteri, B. saguini	Α1α	L-Lys-Gly	A11.1
B. catenulatum, B. magnum, B. pseudocatenulatum, B. hapali	Α3α	L-Lys(L-Orn)-L-Ala ₂ -L-Ser	A11.11
B. gallicum	Α3α	L-Lys-L-Ala-L-Ser	A11.13 detected in <i>Weissella</i> but not in bifidobacteria so far
B. minimum, B. biavatii, TRI 7, TRI 1, MRM 8.19	Α3α	L-Lys-L-Ser	A11.14
B. scardovii	Α3α	L-Lys-L-Ser-L-Ala	A11.18 detected in <i>Oenococcus</i> and <i>Lactococcus</i> but not in bifidobacteria so far
B. animalis subsp. animalis and subsp. lactis, B. choerinum, B. cuniculi, B. ruminantium, TRE H	Α3α	L-Lys(L-Orn)-L-Ser-(L-Ala)-L-Ala ₂	A11.21
B. eulemuris	Α3α	L-Lys-L-Ser-L-Thr-L-Ala	A11.23 is typical of several <i>Arthrobacter</i> but not found in bifidobacteria so far
B. aerophilum	Α3α	L-Lys-L-Thr-L-Ala	A11.25 detected in lactococci and streptococci but not in bifidobacteria so far
B. thermophilum	Α3α	L-Orn(L-Lys)-D-Glu	A11.26 detected in <i>Arthobacter</i> but not in bifidobacteria so far
B. angulatum, B. coryneforme, B. gallinarum, B. indicum, B. mongoliense, B. pullorum, B. subtile	Α4α	L-Lys-D-Asp	A11.31
B. adolescentis, B. dentium, B. merycicum, B. saeculare, B. stellenboschense, B. callitrichos	Α4α	L-Lys(L-Orn)-D-Asp	A11.32
B. aesculapii	Α4α	L-Lys-D-Ser-D-Asp	A11.37 is typical of several <i>Cellulosimicrobium</i> but not found in bifidobacteria so far
B. boum	Α4α	L-Lys-D-Ser-D-Glu	A11.38
B. avesanii	Α4β	L-Orn(Lys)-D-Ser-D-Glu	Derived from A11.38
B. pseudolongum subsp. pseudolongum and subsp. globosum	Α3β	L-Orn(L-Lys)-L-Ala ₂₋₃	A21.2
B. longum subsp. longum, subsp. infantis, subsp. suis,subsp. suillum, B. lemurum	Α3β	L-Orn-L-Ser-L-Ala-L-Thr-L-Ala	A21.3
TRE D, TRI 5	Α3β	L-Orn(Lys)-L-Ser-L-Ala-L-Thr- L-Ala	Derived from A 21.3
Bifidobacterium ramosum	Α3β	L-Orn-L-Ser-L-Ala	Not detected in any organism so far
B. bifidum, B. myosotis	Α4β	L-Orn-D-Ser-D-Asp	A21.7
B. tsurumiense		Glu-Lys-Asp-(Ala) ₂	Not in the list of DSMZ
B. kashiwanohense, B. myosotis, B. tissieri		Glu-Ala-Lys	Not in the list of DSMZ
Scardovial genera			
Alloscardovia macacae, A. omnicolens, Bombiscardovia coagulans	Α4α	L-Lys-L-Asp	A11.31
Alloscardovia criceti, Scardovia inopinata	Α4α	L-Lys-D-Ser-D-Glu	A11.38
Neoscardovia arbecensis	Α1γ	meso-Dpm-direct	A31
Pseuscardovia radai	Α4β	L-Orn(L-Lys)-D-Ser-D-Glu	A21.12
Pseudoscardovia suis	Α3β	L-Orn(L-Lys)-L-Ser(L-Ala)-L-Ala ₂	_

5.2 CELL WALL STRUCTURE

TABLE 5.1 Peptidoglycan Type in the Genus Bifidobacterium and Scardovial Genera	(cont.)	i
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Species	Types of cross-linkages	Peptidoglycan types	No. of different peptidoglycan type in the DSMZ catalogue entries
Scardovia wiggsiae		L-(Lys-Orn)-Thr-Glu (Downes et al., 2011)	
	Α4α	L-Lys-L-Thr-D-Glu (in the DSMZ web site)	A11.57
Other genus			
Gardnerella vaginalis		Ala-Gly-Glu-Lys (O'Donnell et al., 1984)	Not in the list of DSMZ

*B. aquikefiri, B. actynocoloniforme, B. bohemicum, B. bombi, B. commune, B. crudilactis, B. faecale, B. psycraerophilum, B. thermacidophilum, Aeriscardovia aeriphila, and Parascardovia denticolens have not been tested for peptidoglycan structure. In bold new bifidobacterial species under description isolated from tamarins (TRE H, TRE D, TRI 1, TRI 5, TRI 7) and from common marmoset (MRM 8.19).

their source, that is, the species derived from human and nonhuman primates cluster together (peptidoglycan type A11.11, A11.32, A21.3), whereas those from other animals have peptidoglycan type A11.1 and A11.31 (Table 5.1). Peptydoglycan structures are rather conservative, as many enzymes are involved in their complex syntheses, but its significance as an evolutionary biomarker is not known. New insights can be obtained by deeper knowledge of peptidoglycan structural diversity in *Bifidobacteriaceae* species.

Knowledge about peptidoglycan structures dates back to the 1970s–80s, but systematic analytical work on peptidoglycans has not been continued after sequence analyses became a more exciting topic (Schumann 2011). However, its importance in polyphasic taxonomy remains relevant (Mattarelli et al., 2014).

5.2.2 Polar Lipids

Polar lipids are widely distributed in membranes of prokaryotes, and these have an enormous variety of structures, most of which have not been examined in any detail. For comparative taxonomic purposes, it is not always necessary to identify the structure of these lipids by nuclear magnetic resonance (NMR) and mass spectrometry (MS), since it is sufficient to show the native lipids on two-dimensional thin-layer chromatography (2D-TLC) and identify some properties of the lipids on the basis of staining (Da Costa et al., 2011).

Members of the genus *Bifidobacterium* are characterized by phosphatidylinositol (PI) type of phospholipids in the cell walls. The PI type is characterized by the absence of nitrogen-containing phospholipids, unbranched saturated fatty acids, and monounsaturated fatty acids with an even number of carbon atoms in the fatty acids chain (Exterkate et al. 1971; Ikawa 1967). For example, glycolipids of B. bifidum are mainly represented by galactolipids—mono-, di-, and trigalactosylglycerides and their acyl derivatives. Monogalactosyl and digalactosylmonoglycerides are represented in small amounts (Exterkate and Veerkamp, 1969). Phosphatidylglycerol, diphosphatidylglycerol, and alanylphosphatidylglycerol are specific to the cell wall of bifidobacteria. Novik et al. (2005) showed that polar lipids from B. longum subsp. longum B 379M, B. bifidum 791, and B. adolescentis 94 BIM exhibited the closest similarity to their counterparts from the propionic acid bacteria; however polar lipid analysis permits us to differentiate members of these bifidobacterial species from other morphologically similar organisms, such as propionic acid bacteria. Polar lipids could be as helpful as sugars and amino acids of the cell walls in the taxonomic characterization of *Bifidobacteriaceae* strains. Unfortunately very few data about polar lipids are available for different species of *Bifidobacteriaceae* family (Table 5.2). Exterkate et al. (1971) examined B. bifidum, B thermophilum, B. asteroides, B. coryneforme, and B. indicum, and recently most of the scardovial genera have been tested for polar lipids, while no data have been presented for other species. As shown in Table 5.2, diphosphatidylglycerol is shared by all the species tested while polyglycerolphospholipids are present only in the tested species belonging to Bifidobacterium. Scardovial genera generally had similar cellular polar lipids profiles.

5.2.3 Teichoic Acids

Teichoic acids are found instead of, or in addition to, polysaccharides in the cell walls of many Gram-positive bacteria. Teichoic acids, composed of anionic glycopolymers, are major cell surface components that can account for over 60% of the mass of the cell wall in a wide range of Gram-positive organisms. They fall into two

Species	DPG	GL	L	PG	PGL	PL	PGPL
B. bifidum	+				+		+
B. thermophilum	+				+		+
B. asteroides	+				+		+
B. coryneforme	+				+		+
B. indicum	+				+		+
Alloscardovia macacae	+	5 ^a			2	1	
A. criceti		6	5			3	
Bombiscardovia	+	6			2	1	
Neoscardovia	+	6		1	1	2	
Pseudoscardovia suis	+				3	1	
P. radai	+	5			3	1	
Scardovia inopinata	+				3	1	
Gardnerella vaginalis ^b	+	5		+		1	

 TABLE 5.2
 Polar Lipid Distribution in Bifidobacteriaceeae

DPG, diphosphatidylglycerol; GL, glycolipid; L, lipid; PG, phosphatidylglycerol; PGL, phosphoglycolipid; PL, phospholipid; PGPL, polyglycerolphospholipid. Data obtained from the original species description except data from bifidobacteria that are from Exterkate et al. (1971).

^a Number of positive spots on TLC plate; +, positive.

^b Contains also phosphatidylinositol (O'Donnell et al., 1984).

categories: the wall teichoic acids that are covalently linked to the peptidoglycan (WTAs) and the lipoteichoic acids (LTAs) that are macroamphiphilic components with their glycolipids anchored in the membrane and its poly(glycerophosphate) (Gro-P) chain extending into the wall (Neuhaus and Baddiley, 2003). As wall teichoic acids differ more in structure than do lipoteichoic acids, the former are more valuable as chemical markers. Teichoic acids are water-soluble polymers containing the polyols glycerol, mannitol, or ribitol linked through phosphodiester bridges and substituted by amino sugars, sugars, or D-alanine. Teichoic acids are immunologically active, and teichoic acids of different structure and serology have been used in the classification of staphylococci and lactobacilli (Brondz and Olsen, 1986). Moreover, together with peptidoglycan, WTA and LTA make up a polyanionic network with ion-exchange properties required for not only maintaining metal cation homeostasis and control but also assisting in the "trafficking" of ions, nutrients, proteins, and antibiotics. In the high-GC (>55 mol.%) bacteria, LTA is generally replaced by lipoglycans. For example, B. bifidum contains a macroamphiphile with single Gro-P units attached to the glycan backbone by phosphodiester linkages and substituted with L-alanyl esters rather than the D-alaninyl esters typical of LTA (Sutcliffe and Shaw, 1991). Lipoglycans with essentially the same structure as that proposed by Fischer (1987) have been confirmed to be present in *B. breve* and *B. longum* strains (Iwasaki et al., 1990). However, so far, there is a paucity of information regarding the presence of WTAs and LTAs in the cell envelope of bifidobacteria. In silico analyses targeting the chromosomes of all 48 (sub)species that currently represent the genus Bifidobacterium revealed the presence of genes responsible for TA biosynthesis, suggesting that bifidobacteria contain both wall WTAs and LTAs (Colagiorgi et al., 2015). When new knowledge on WTAs and LTAs composition of *Bifidobacteriaceae* species becomes available, it will be possible to consider their value as chemotaxonomic markers.

5.2.4 Cell-Wall Polysaccharides

Cell-wall polysaccharides (CWP) are attached to different surface structures on the PG, such as the peptides or NAG. They are believed to play a role in the structural stability of the cell. CWP from different species vary greatly. In contrast to CWP, exopolysaccharides can also be produced in some bacteria as a slime, and are believed to have a protective function against the environment, such as preventing desiccation and phage attack, and to be involved in cell adhesion. The following CWP structures have been found in different bifidobacterial species: a polymer consisting of a galactofuranose repeating unit, branched, with galactofuranose residues in *B. catenulatum* YIT4016 (Nagaoka et al., 1995); a structure containing L-rhamnose and galactose in *B. longum* subsp. *longum*YIT3028 (Nagaoka et al., 1996);

galactofuranose and galactopyranose with glucopyranose as part of the branching units in *B. longum* subsp. *infantis* (Tone-Shimokawa et al., 1996); a repeating unit containing not only galactofuranose and galactopyranose, but also glucopyranose in *B. bifidum* BIM B-465 (Tone-Shimokawa et al., 1996); galactofuranose and galactopyranose in *B. adolescentis* M101-4 and in *B. pseudocatenulatum* MBL8320 CPS (Hosono et al. 1997, 1998); a galactan polymer of unknown structure in *B. gallicum* (Lauer, 1990); a repeating tetra saccharide unit composed of $[\rightarrow3)\beta$ -Glc($1\rightarrow3)\beta$ -Gal($1\rightarrow4$) α -Gal($1\rightarrow2$) α -Glc($1\rightarrow1$) was branched on O-6 of the α -Gal residue in *B. breve* YIT 4010 (Habu et al., 1987); a mixed acetal moiety and a galactofuranose moiety as a head group (1-(β -D-Galf)-3-glyceroplasmalomonoglyceride), representing an entirely new class of glycolipids, in *B. longum* subsp. *infantis* ATCC 15702 (Timmer et al., 2014).

Glycolipid can be analysed using thin-layer chromatography (TLC), a variety of nuclear magnetic resonance spectroscopy techniques and more traditional lipid and carbohydrate analyses and recently a matrix-assisted laser desorption ionization (MALDI) in combination with collision induced dissociation (CID) (Timmer et al., 2014).

CWP can also be a chemotaxonomic marker but due to the scarce data in *Bifidobacteriaceae* species distribution it is not possible to establish its value in species identification at present.

5.3 WHOLE CELL CHEMICAL COMPOUNDS

5.3.1 Fatty Acid Analysis

The variability in chain length, double-bond position and substituent groups and relative abundances of fatty acids produced within a cell are largely determined by an organism's genotype and can be used for identification of different species (Vandamme et al., 1996). For fatty acid analysis the more frequently used methodology comprises extracting the lipids and converting their fatty acids to methyl esters, which are examined by gas-chromatographic analysis (Killer et al., 2014). This method is successfully applied in the taxonomic characterization of some Grampositive microorganisms. Particular attention must be paid, however, to the influence of culture conditions and growth temperature in particular, which greatly influence bacterial fatty acid composition. Therefore, standardization of media and growth conditions is necessary to obtain highly reproducible fatty acid profiles that may be used as chemotaxonomic markers.

Concerning the *Bifidobacteriaceae* family, 20 out of 60 taxa of the *Bifidobacterium* genus, all the scardovial genera and *G. vaginalis* have been tested for fatty acid composition. Five to twelve different fatty acids have been found in *Bifidobacterium* and in *Gardnerella* genera while 10–27 ones in scardovial genera (Table 5.3).

Palmitic acid (C16:0) is the only fatty acid present in all species of *Bifidobacteriaceae* that have been tested and it is generally the most abundant fatty acid. Myristic acid (C14:0) is present in the *Bifidobacterium* genus but not in all tested species and in scardovial genera it is present only in *Aeriscardovia, Parascardovia,* and *Pseudoscardovia.* C14:0 DMA (dimethyl acetal) is present in bifidobacterial species isolated from nonhuman primates (*B. callithricos, B. hapali, B. myosotis, B. stellenboshense,* and *B. tissieri*) and in *B. angulatum, B. catenulatum, B. dentium, B. longum* subsp. *suis,* and *B. longum* subsp. *suillum.* C18:0, even if in low amount, is present in all *Bifidobacteriaceae* species tested except *B. callithricos* and *B. myosotis.* C18:1ω9C and C18:1ω9C DMA are present only in the genus *Bifidobacterium* and interestingly the bifidobacterial species from insects (*B. actinocolooniforme, B. bohemicum,* and *B. bombi*) do not possess them.

Due to the limited coverage of fatty acid composition in *Bifidobacteriaceae* family (only about 50% of the species are represented), it is not possible to utilize fatty acid composition as a solid taxonomic tool. Furthermore, the interspecific variability of fatty acid composition is not known because this analysis has not been performed on enough strains for it to be possible to make definitive statements about distributions in different species. Therefore, the fatty acid analysis should be coupled with other genotypic and phenotypic characteristics for correct characterization of a novel genus or species.

5.3.2 Acetic and Lactic Acid Production

Bifidobacteriaceae metabolize glucose to produce generally about three molecules of acetic acid and two molecules of lactic acid per two moles of glucose. Separation of volatile compounds is usually performed by gas chromatography with a coupled mass selective detector or by HPLC method (Van Der Meulen et al., 2006). The acetic and lactic acid production is a taxonomic characteristic of *Bifidobacteriaceae*that is not sufficient for their identification but constitutes an important feature that should be present for their description. A list of ratio of acetic to lactic acid described in the original description of the species is shown in Table 5.4; a high variability in the ratio of acetic to/lactic acid production under the same conditions is present. Data are not available for all the species.

									Ger	nus Bifid	obacterii	ım		
Species		B. actinocoloniiforme	B. adolescentis	B. angulatum	B. bohemicum	B. bombi	B. callitrichos	B. catenulatum	B. dentium	B. faecale	B. hapali	B. kashiwanohense	B. longum subsp. longum	B. longum subsp. infantis
Fatty acids		DSM 22766 ^T	ATCC 15705 ^T	ATCC 27535 ^T	DSM 22767 ^T	DSM 19703 ^T	DSM 23973 ^T	ATCC 27539 ^T	ATCC 27534 ^T	JCM 19861 ^T	$DSM 100202^{T}$	DSM 21845 ^T	ATCC 15707 ^T	ATCC 157697 ^T
	Saturated													
Caprylic acid	C _{8:0}													
Capric acid	C _{10:0}			1.5				0.7	0.7			2.1		
Undecylic acid	C _{11:0}													
	C _{11:0} DMA						1.94	0.7				0.8		
Lauric acid	C _{12:0}		1.1	2.7			2.8	1.3	1.2	TR	1.68	8.1		1.06
	C _{12:0} - aldehyde													
Myristic acid	C _{14:0}	1.97	5.1	8.4	2.86	<0,01	16.74	5.3	5.1	5.7	9.25	15.9	2.84	9.83
	C _{14:0} DMA		TR	2.1			13.47	3.5	2.9	1.2	7.21	3.3	3.08	4.02
Pentadecylic acid	C _{15:0} Iso-C _{15:0}													
	Anteiso-C _{15:0}													
Palmitic acid	C _{16:0}	20.17	27	18.1	15.97	7.14	23.63	25.7	25.8	27.7	23.13	26.8	24.68	14.26
	C _{16:0} DMA Iso-C _{16:0}							0.5			1.53	0.7	1.78	0.26
Margaric acid	C _{17:0}	2.11			2.56	4.21								
	Anteiso-C _{17:0}						4.74							
Stearic acid	C _{18:0}	7.05	5.5	3.7	6.56	5.91		3.3	6.9	5.7	1.61	2.5	4.2	1.38
	C _{18:0} DMA								0.6				1	
	iso-C _{19:0}		TR							TR				
Arachidic acid	C _{20:0}	3.25	1.1		4.62	7.18				4.0				
Heneicosylic acid	C _{21:0}													
Behenic acid	C _{22:0}	<0,01			<0,01	5.87								
Tricosanoic acid	C _{23:0}	<0,01			<0,01	5.38								
Lignoceric Acid	C _{24:0}	<0,01			<0,01	4.68								

TABLE 5.3 Fatty Acids Composition in Bifidobacteriaceae Species

										Scardov	vial gene	ra			Other genus
B. longum subsp. suillum	B. longum subsp. suis	B. myosotis	B. pseudocatenulatum	B. ruminantium	B. stellenboschense	B. tissieri	Aeriscardovia aeriphila	Alloscardovia omnicolens	Bombiscardovia coagulans	Neoscardovia arbecensis	Parascardovia denticolens	Pseudoscardovia suis	Scardovia inopinata	Scardovia wiggsiae	Gardnerella vaginalis
DSM 28597 ^T	ATCC 27533 ^T	DSM 100196 ^T	ATCC 27919 ^T	$ATCC 49390^{T}$	DSM 23968 ^T	$DSM 100212^{T}$	DSM 22365 ^T	$DSM 21503^{T}$	$DSM 22924^{T}$	$DSM 25737^{T}$	$DSM 10105^{T}$	$DSM 24744^{T}$	$DSM 10107^{T}$	DSM 22547 ^T	
		1.26	1.2				0.21 0.14	3.15 0.33 2			3.91 2.6	1.04 0.55 1.34	3.26 0.31 2.6 0.14	0.12	
		4.26	2.9	TR	1.78	1.62	1.86	0.97		1.7 0.3	0.74	2.2	2.3	0.43	
7.7 5.34	9.14 8.34	27.22 10.33	10.5 2.7	3.1 TR	12.63 7.45	15.26 6.91	10.11	4.53		5.5	8.38	12.01	14.73 0.87	3.85	4.9
							0.76	0.38		0.4 0.4			0.28		
24.46 0.82	28.57 0.66	17.48	13.7	26.3	23.6 1.4	30.49	15.86	9.29	6.31	42.3	18.15	15.64	28.92 0.24	49.76	35.3
			0.7				3.46	1.96	5.71	0.3 0.5 0.5	2.1	3.12	1.88 0.88		
1.92 0.44	2.42 0.2		2.3	6.3	1.69	1.8	5.54	3.69	6.99	3.1	3.13		1.82	5.71	17
				1			4.16 4.37 3.7	2.66 2.9 2.53	7.63	4.7	3.03 3.18 3.06	3.99 4.49 3.26	0.26 2.61 2.67 2.51	0.28	
							3.93 <0,01	2.65 <0,01	5.52 4.47		3.11 <0,01	0.20	2.22 <0,01		
							5.54	3.69		3.1	3.13	4.82	3.1	5.7	

(Continued)

									Ge	nus Bifid	lobacteri	ım		
Species		B. actinocoloniiforme	B. adolescentis	B. angulatum	B. bohemicum	B. bombi	B. callitrichos	B. catenulatum	B. dentium	B. faecale	B. hapali	B. kashiwanohense	B. longum subsp. longum	B. longum subsp. infantis
Fatty acids		DSM 22766 ^T	ATCC 15705 ^T	ATCC 27535 ^T	$DSM 22767^{T}$	$DSM 19703^{T}$	DSM 23973 ^T	ATCC 27539 ^T	ATCC 27534 ^T	JCM 19861 ^T	DSM 100202 ^T	DSM 21845 ^T	ATCC 15707 ^T	ATCC 157697 ^T
	Unsaturated C _{13:1} C _{14:1} C _{14:1} $\omega 5C$													
Pentadecenoic acid	C _{15:1}	<0,01			<0,01	2.47								
Palmitoleate acid	C _{16:1} C _{16:1} ω5C C _{16:1} ω6C			2.6				2.2	1.8			6.3		
Palmitoleic acid	C _{16:1} ω7C C _{16:1} ω7C DMA		2.1	2.6				1.8 1.1	1.1 0.6	2.1				
Olois acid	C _{16:1} ω9C Iso I-C _{17:1}	0.00			4.60	7 40	3.64				1.41			
	C _{18:1} C _{18:1} ω6C C _{18:1} ω6C DMA	9.99			4.07	7.47					2.49		2.09	
Vaccenic acid	C _{18:1} ω7C C _{18:1} ω7C DMA							0.6						
Elaidic acid	C _{18:1} ω9C C _{18:1} ω9C DMA		32.6 14.8	33.5 14.3			8.2 13.34	18.3 23.3	22.8 23.2	27.4 15.5	9.38 23.07	19.4 9.1	11.82 26.5	24.62 27.48
	C _{18:1} ω11C DMA C _{19:1} Iso-C _{19:1} Iso I-C _{19:1}		1							TR				

TABLE 5.3 Fatty Acids Composition in Bifidobacteriaceae Species (cont.)

5.3 WHOLE CELL CHEMICAL COMPOUNDS

										Scardov	vial gene	ra			Other genus
B. longum subsp. suillum	B. longum subsp. suis	B. myosotis	B. pseudocatenulatum	B. ruminantium	B. stellenboschense	B. tissieri	Aeriscardovia aeriphila	Alloscardovia omnicolens	Bombiscardovia coagulans	Neoscardovia arbecensis	Parascardovia denticolens	Pseudoscardovia suis	Scardovia inopinata	Scardovia wiggsiae	Garánerella vaginalis
DSM 28597 ^T	ATCC 27533 ^T	$DSM 100196^{T}$	ATCC 27919 ^T	ATCC 49390 ^T	$DSM 23968^{T}$	$DSM 100212^{T}$	DSM 22365 ^T	$DSM 21503^{T}$	$DSM 22924^{T}$	$DSM 25737^{T}$	DSM 10105 ^T	DSM 24744 ^T	$DSM 10107^{T}$	DSM 22547 ^T	
							1.44 <0,01	0.98	3.3	0.3 0.3 0.3	<0,01		0.77 0.1 <0,01		
							3.23	3.89		9 0.3	0.51	3.01	2.65 0.14	1.9	2.3
			2.2 3.9 0.5	TR						7.7			1.52	1	
		4.68			1.8	2.74	16.66	22.02	12.62	1.2 0.4	12 /1		3.77	0.87	25.2
6.13		1.8					10.00	32.03	12.05		12.41		14.01		33.3
													0.48	1.91	
5.25 25.08	11.27 18.83	7.52 11.17	33.6 12.2	41.7 12.6	9.98 19.77	16.85 11.6	16.66	32.03		26.6	12.41	13.08	37.3 1.79	35.78	
				TR						2.1 1.6 0.5				0.3 0.31	

(Continued)

									Ge	enus Bifi	dobacter	ium			
Species		B. actinocoloniiforme	B. adolescentis	B. angulatum	B. bohemicum	B. bombi	B. callitrichos	B. catenulatum	B. dentium	B. faecale	B. hapali	B. kashiwanohense	B. longum subsp. longum	B. longum subsp. infantis	
Fatty acids		DSM 22766 ^T	ATCC 15705^{T}	ATCC 27535 ^T	$DSM 22767^{T}$	DSM 19703 ^T	DSM 23973 ^T	ATCC 27539 ^T	ATCC 27534 ^T	JCM 19861 ^T	$DSM 100202^{T}$	DSM 21845 ^T	ATCC 15707 ^T	ATCC 157697 ^T	
	Polyinsaturat	ted													
Linoleic acid	С _{18:2} С _{18:2} w6,9С С _{20:5} w3С	<0,01			<0,01	7.34						1.1			
	Cyclopropane C _{19:0} cyclo 9,10	2									1.14		2.09	0.42	
	C _{19:0} cyclo 9,10 DMA						1.34				2.39		5.51	1.02	
	Hydroxy fatty acids														
	C _{13:0} 3OH														
	C _{14:0} 2OH														
	C _{16:0} 3OH								0.4					4.45	
	$C_{18:0}$ 120H							1.5	0.6				1.67	1.17	
	Summed features	-													
	1						2.73				1.65		0.48	0.46	
	2												6.49	5.05	
	3												1.82	3.59	
	4												0.92	0.88	
	5												0.65	1	
	7		2.7				4.76			3.1	10				
	10		5.3							4.6					

TABLE 5.3 Fatty Acids Composition in Bifidobacteriaceae Species (cont.)

Summed features represent groups of two or more fatty acids that could not be separated by GLC with the MIDI system.

Summed feature 1: C13:1 at 12–13, C14:0 aldehyde, C11:1 2OH.

Summed feature 2: C17:2 at 16.760, C17:1 $\omega 9c.$

Summed feature 3: C18:1 ω 7c, unknown 17.834.

Summed feature 4: C17:0 iso 3OH, C18:2 DMA.

Summed feature 5: unknown 18.622, C19:0 iso.

Summed feature 7: 17:2 at 16.760/17:1 ω 9c.

Summed feature 10: one or more of an unknown fatty acid of ECL 17.834 and/or C18: 1v11c/v9t/v6t.

Data are expressed as a percentage of total fatty acid content for each particular strain. Data from the original paper of species description. DAM, Dimethyl acetal.

5.3 WHOLE CELL CHEMICAL COMPOUNDS

										Scardo	vial gene	ra			Other genus
B. longum subsp. suillum	B. longum subsp. suis	B. myosotis	B. pseudocatenul atum	B. ruminantium	B. stellenboschense	B. tissieri	Aeriscardovia aeriphila	Alloscardovia omnicolens	Bombiscardovia coagulans	Neoscardovia arbecensis	Parascardovia denticolens	Pseudoscardovia suis	Scardovia inopinata	Scardovia wiggsiae	Gardnerella vaginalis
DSM 28597 ^T	ATCC 27533 ^T	$DSM 100196^{T}$	ATCC 27919 ^T	$ATCC 49390^{T}$	DSM 23968 ^T	DSM 100212 ^T	DSM 22365 ^T	$DSM 21503^{T}$	$DSM 22924^{T}$	DSM 25737 ^T	$DSM 10105^{T}$	$DSM 24744^{T}$	$DSM 10107^{T}$	$\mathbf{DSM}\ 22547^{\mathrm{T}}$	
							< 0.01	< 0.01	4.20		< 0.01		< 0.01		
								2.29			2.96	1.31	2 62		
								2.2)			2.90	1.01	2.02		
1.76	2.57	2.38			1.56	2.58									
8.36	5	2.75			1.93	2.12									
										0.8					
										0.3					
										0.3			0.29		
1.5	0.59				1										
							3.93	2.65			3.11	2.98	2.22		
0.92	1.14	1.55			1.43	1.01									
6.78	3.31														
0.07	4.18														
0.87	0.86														
0.62	0.67	2 22		2.2	62	1.65									
		2.33		2.2 2.3	0.2 2.27	1.03									
				2.0	4.41										

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								Genus Bifido	bacterium
Species	1. B. actinocoloniiforme DSM 22766 ^T	3. B. adolescentis ATCC 15705 ^T	4. B. aerophilum CCUG 67145 ^T	5. B.aesculapii DSM 26737 ^T	6. B. angulatum ATCC 27535T	7a. B. animalis subsp. animalis ATCC 25567 ^T	7b. B. animalis subsp. lactis DSM 10140 ^T	8. B. asteroides ATCC 25910 ^T	11. B. bohemicum DSM 22767 ^T
Lactic/acetic ratio	1:1.4	1:1.76	1:4.83 ± 0.85	1:2 to 1:5	1:2.2±0.06	1:3.6	1:10	1:2	1:1.5
							G	enus Bifidob	acterium
Species	28. B. hapali DSM 100202 ^T	29. B. indicum ATCC 25912 ^T	30. B. kashiwanohense DSM 21845 ^T	22c. B. longum subsp. suis ATCC 27533 ^T	39. B. pseudocatenulatum ATCC 27919 ^T	40. B. pseudolongum subsp. globosum ATCC 25865 ^T	41. B. psychraerophilum LMG 21775 ^T	42. B. pullorum ATCC 27685T	45. B. ruminantium ATCC 49390 ^T
Lactic/acetic ratio	1:3.8 to 1:4.2	1:3	1:3	1:17 to 1:2	1:2	1:3	1:2.77±0.16	$1:1.35\pm0.2$	1:2 to 1:3



^a Bifidobacterium species are numbered according to Table 2.1. All the data are obtained from the original paper of species description.

12. B. bifidum ATCC 29521 ^T	13. B. bombi DSM 19703 ^T	14. B. boum ATCC 27917 ^r	16. B. catenulatum ATCC 27539 ^T	18. B.choerinum ATCC 27686 ^T	19. B. commune LMG 28292 ^T	20. B. coryneforme ATCC 25911	21. B. crudilactis LMG23609 ^T	22. B. cuniculi ATCC 27916 ^T	23. B. dentium ATCC 27534 ^T	25. B. faecale JCM 19861 ^T	
1:3	1:3.3	1:3	1:2.15±0.15	1:4 to 1:8	2:3	1:3.5	1:1.49±0.1	1:5 to 1:20	1:2.5±0.15	1:1.57	
						Scardovial genera					
27. B. saeculare DSM 23967T	B. thermacidophilum subsp. thermacidophilum DSM 15837 ^T	B. thermacidophilum subsp. porcinum DSM 17755 ^T	31. B. thermophilum ATCC 25525 ^T	54. B. tsurumiense JCM 13495 ^r		Neoscardovia arbecensis DSM 25737 ^T	Parascardovia denticolens DSM 10105 ^T	Pseudoscardovia suis DSM 24744 ^T	Scardovia inopinata DSM 10107 ^T	Scardovia wiggsiae DSM 22547 ^T	
1:3.3 to 2.0	1:2.46 to 1:2.72	1:4.9	1:3	2:3		1:2 to 1:10	1:2	1:1.2	1:2.9	1:1.9	

5.3.3 MALDI-TOF MS

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is an emerging analytical tool for detecting and identifying microorganisms. It offers high sensitivity, simple sample preparation processes, low sample consumption volumes, and the possibility of automated and high-throughput analyses. MALDI-TOF facilitates the generation of molecular fingerprints for entire microorganisms producing complex spectra of complex biological macromolecules, through their specific degradation products, which are characteristic for the molecular content of a bacterial cell. Comparison of these spectra offers a discriminatory power at species level. In some cases, subspecies discrimination might be possible (Zeller-Péronnet et al., 2013). This type of analysis is routinely used in some public microbial collections (e.g., DSMZ) for taxonomic identification control. MALDI-TOF mass spectrometry is cost effective and allows for highly accurate identification of *Bifidobacteriaceae* in a faster way than traditional methods. The drawback is the necessity to have a database comprising all the *Bifidobacteriaceae* species.

5.3.4 Polyacrylamide Gel Electrophoresis

Whole cell protein analysis by polyacrylamide gel electrophoresis (PAGE) has been utilized mostly in the 1980s in the taxonomy of bifidobacterial species. The protein profiles obtained, even if they are rather complex, were considered to be useful for quickly grouping a large number of strains. Protein profiles from whole cell extract correlates with the results of DNA–DNA hybridization suggesting its use in rapid bacterial identification at both species and subspecies levels (Berber, 2004; Biavati et al., 1982; Kim et al., 2010). For example, in *B. animalis* it is possible to discriminate between subspecies *animalis* and *lactis* by PAGE pattern (Mattarelli et al., 1992). Computer-assisted numerical comparisons of protein patterns and a created database can be helpful for identification purposes provided that highly standardized conditions are used for cultivation and electrophoresis; this allows large numbers of strains to be compared and grouped into clusters of closely related homology (Pot et al., 1994). However, even if this technique has a high discriminatory power for identification of *Bifidobacteriaceae* species, it is not per se sufficient in the identification of strains and it should be coupled with others genotypic techniques.

Currently, PAGE has been replaced by DNA fingerprinting molecular analysis. RAPD, rep-PCR and BOX-PCR proved to be the most effective and reliable method, allowing rapid differentiation of *Bifidobacterium* strains at all taxonomic levels (Jarocki et al., 2016)

5.3.5 Isoenzyme

The most direct and reliable property for assigning an organism to the family *Bifidobactericeae* is the presence of fructose-6-phosphate phosphoketolase (F6PPK) activity in cellular extracts. F6PPK is the characteristic key enzyme of "the bifid shunt." This enzyme activity is apparently absent in anaerobic Gram-positive bacteria with a "pseudobifid" morphology, that is, *Actinomyces, Arthrobacter, Corynebacterium*, and *Propionibacterium* (Scardovi and Trovatelli, 1965). Isozymes of transaldolase and 6-phosphogluconate dehydrogenase (6PGD) were studied by starch gel horizontal electrophoresis (Smithies, 1955) in 1206 strains belonging to 24 *Bifidobacterium* species. Fourteen isozymes of transaldolase and 19 of 6PGD were identified and numbered and patterns or zymograms thus obtained for each species (Scardovi et al., 1979); 60% of the strains were identified on this basis. An additional 20% of the strains were assigned to species based on the electrophoretic behavior of their 3-phosphoglyceraldehyde dehydrogenase (Scardovi et al., 1979). The same reliability of taxonomic bifidobacterial species groupings using electrophoretic mobility of transaldolase in 75 strains of different species was obtained by Lauer and Kandler (1983), using disc electrophoresis in polyacrilamide. The observation that the amino acid sequences of the isoenzymes of transaldolase gene is variable between species has been utilized to set up a PCR technique targeting the transaldolase gene followed by denaturing gradient gel electrophoresis (Requena et al., 2002).

Antisera against eight purified transaldolases further established natural relationships among *Bifidobacterium* species (Sgorbati, 1979; Sgorbati and London, 1982; Sgorbati and Scardovi, 1979). The segregation of the *Bifidobacterium* species into four distinct clusters which correlated with the groups made on the basis of their ecological distribution suggests that a "subdivision of the genus into four groups would more accurately reflect the group's natural history" (Sgorbati and London, 1982). It would be interesting to apply this analysis to the currently recognized *Bifidobacteriaceae* species to confirm the above statement.

Also the electrophoretic mobility of isozymes of L-LDH has been tested by Lauer and Kandler (1983); however, these isozymes are only species-specific if the species are defined in a very narrow sense, as is the case at present.

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5.4 CONCLUDING REMARKS

Chemotaxonomy analysis is a helpful support in the polyphasic taxonomy. It contributed remarkably to the knowledge of the diversity of bifidobacterial species in the 1960s–80s while today increasing trust in sequence data has determined its apparent decline. A very important point for its applicability in systematics is to create library entries prior to utilizing the chemotaxonomic analysis as a rapid screening method or for complementary identification. The use of MALDI-TOF in checking the identification of strains in public collections achieved after the construction of a database of species profiles is an example. The knowledge of peptidoglycan composition, which has a rather conservative structure, is also a very important feature for *Bifidobacteriaceae* taxonomy. Whenever possible, it remains important to test the different chemotaxonomic markers on multiple strains of the same species, allowing the comparison of intraspecies with interspecies variability and the detection of possible overlaps. The chemotaxonomic analyses could probably also offer interesting suggestions about the evolutionary history of *Bifidobacteriaceae*, as well as producing information about chemical composition of molecules that can be very promising as health promoting agents.

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6

Nutritional Requirements of Bifidobacteria

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6.1 CHARACTERISTICS OF BIFIDOBACTERIA AND THEIR METABOLISM

Bifidobacteria are Gram-positive, strictly anaerobic, nonsporulating rods, frequently found in association with humans (Khoroshkin et al., 2016). First discovered in 1900 by Henry Tissier, bifidobacteria were initially found in infant feces and named for their distinctive bifurcations (Russell et al., 2011). Bifidobacteria in humans predominate in infants; in breast-fed infants, bifidobacteria comprise approximately 95% of their microbiota (Yoshioka et al., 1991).

Bifidobacteria are saccharolytic and derive the majority of their energy from "indigestible" carbohydrates, such as oligosaccharides of human and plant origin. Bifidobacterial metabolism involves a unique pathway for hexose catabolism and ATP production, known as the "bifid shunt" (Fig. 6.1). Hexoses from the hydrolysis of complex carbohydrates are all subject to this type of metabolism (Pokusaeva et al., 2011). The key enzyme in the bifid shunt is fructose-6-phosphate phosphoketolase (de Vries and Stouthamer, 1968); its presence is considered a taxonomic marker of family Bifidobacteriaceae (Russell et al., 2011).

The characteristic fructose-6-phosphoketolase enables bifidobacteria to produce lactate, acetate, and ATP via the bifid shunt (Pokusaeva et al., 2011; Sela et al., 2008). Fructose-6-phosphoketolase converts the intermediate fructose-6-phosphate product from glucose and galactose catabolism into D-erythrose-4-phosphate and acetyl phosphate (Sela et al., 2008). Further degradation results in a 3:2:5 ratio of acetate to lactate to ATP as metabolic end products (Sela et al., 2008). Lactate and acetate are classified as short chain fatty acids (SCFA), which confer a multitude of benefits to the host organism, including utilization by enterocytes for energy (Musilova et al., 2015). Catabolism of carbohydrate substrates via the bifid shunt results in a higher energy yield and different end products than typical fermentative pathways (Pokusaeva et al., 2011).

SCFA, such as lactate and acetate, are important metabolites for human health. SCFA are a key source of energy for colonic enterocytes (Steinmeyer et al., 2015; Thomas et al., 2011), act as ligands for G-protein coupled receptors involved in antiinflammatory signaling (Kasubuchi et al., 2015; Steinmeyer et al., 2015), and modulate the maturation and function of microglia in the central nervous system (Erny et al., 2015). The addition of lactose to the infant diet was associated with increased counts of *Bifidobacterium* and concentrations of SCFA (Francavilla et al., 2012), likely due to bifid shunt metabolism of lactose.

Knowledge of the bifid shunt has allowed scientists to coopt its properties. Certain prebiotics have been discovered to promote the growth of bifidobacteria specifically. The so-called bifidogenic effects of certain substrates, both carbohydrate and protein, select for bifidobacteria and are an important consideration for their growth in addition to regular nutritional needs, particularly during multispecies and in vitro experiments. Both the nutritional requirements of bifidobacteria and the bifidogenic effects of particular substrates are presently discussed.

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FIGURE 6.1 Simplified schematic highlighting the key metabolic steps of the bifid shunt. *Xfp*: fructose-6-phosphate phosphoketolase.

6.2 NUTRITIONAL REQUIREMENTS

6.2.1 Carbohydrate Sources

The genomic variability of bifidobacterial carbohydrate transport and degradation allows these bacteria to consume a variety of carbon substrates. Some carbohydrate sources only promote the growth of certain species or strains of bifidobacteria, but the array of potential substrates available creates the opportunity for multiple species to survive and proliferate within the same microbial community. The characteristics of bifidobacterial growth on different carbohydrates and purported bifidogenic effects are discussed here.

6.2.1.1 Lactose

Lactose is a disaccharide composed of one glucose and one galactose, and is the main carbohydrate human infants encounter in breast milk (González-Rodríguez et al., 2013). Lactose is the most abundant solid constituent of breast milk (Katayama, 2016), and comprises about 80% of all carbohydrate found in breast milk (Urashima et al., 2012). Not only a critical aspect of infant nutrition, lactose is also a significant prebiotic for commensal microbes in the infant gut. The infant digestive system remains immature during the first few months of life. For this reason, lactose is not fully enzymatically hydrolyzed in the small intestine, and reaches the community of bacteria in the colon intact at higher rates in infants than in adults (Francavilla et al., 2012).

Once lactose reaches the colon, β -galactosidases can hydrolyze the glucose–galactose bond. β -galactosidases, wellcharacterized among species of bifidobacteria, are involved in the first step of enzymatic lactose breakdown and serve as the final point before entry into the bifid shunt (González-Rodríguez et al., 2013). A novel β -galactosidase, not previously identified in any sequenced bacterial genome, was identified in the genome of *B. longum* subsp. *infantis* (COG3250, Blon_2334). This unique β -galactosidase, located in a novel gene cluster dedicated to carbohydrate metabolism and transport, suggests *B. longum* subsp. *infantis* evolved this enzyme in addition to the other sugar transporters within the cluster in a coregulatory fashion (Sela et al., 2008). Another *B. longum* subsp. *infantis* β -galactosidase, INF1, was also found to have minimal homology with the enzymes of *B. bifidum*, but phylogenetic analysis based on enzyme active site DNA sequences showed it was most closely related to that of *B. breve* (Møller et al., 2001). Significant amounts of bifidobacterial genomes are dedicated to expression of carbohydrate importers and enzymes for lactose hydrolysis and utilization (Sela et al., 2008). *B. bifidum* has three characterized β -galactosidases: BIF1, BIF2, and BIF3. Although these three enzymes also showed little homology with other hydrolases of the same family, assays performed demonstrated high levels of substrate specificity for the galactosidic bond present in lactose (Møller et al., 2001). Intracellular concentrations of β -galactosidases significantly increased in *B. adolescentis* grown on lactose, but enzymatic activity evaluated at the cell surface was negligible (Amaretti et al., 2007). The lack of surface enzymes, coupled with observations that extracellular β -galactosidases in this species were in very low concentrations, indicates lactose is imported for intracellular hydrolysis. One subset of genes consistently expressed across the transcriptomes of all species and strains of bifidobacteria includes a β -galactosidase with lactase activity (Garrido et al., 2015). Despite the range of habitats for these bacteria and the discrepancies in the genes encoding these hydrolases, the presence of these enzymes in a number of bifidobacterial genomes implies a common necessity for lactose metabolism.

Growth experiments with different bifidobacterial strains on lactose in growth medium have demonstrated species-specific patterns of carbohydrate utilization preference and metabolite profiles. Bacterial growth of *B. adolescentis* MB 239, with different sugars as sole carbon sources, was quantified based on the parameters of specific growth rate and the amount of biomass produced. Metabolic and energetic outputs were determined on the basis of the bifid shunt. When lactose is the sole carbon source, its utilization by *B. adolescentis* is less robust than those bacteria grown on galactose or galactooligosaccharides (GOS), but greater than on glucose alone (Amaretti et al., 2007). *B. animalis* subsp. *lactis* also preferentially uses lactose over glucose. Bacteria grown with lactose and glucose had similar growth curves at 25 h, but ¹³C-NMR analysis in vivo found greater end product incorporation of carbons from catabolism of lactose and lactose moieties (González-Rodríguez et al., 2013). Additionally, 21 strains of *B. longum* subsp. *infantis* grew exceedingly well on lactose, based on optical density (OD₆₀₀) greater than or equal to 0.8 (Garrido et al., 2015), while only some *B. bifidum* (6 strains) had OD₆₀₀ values greater than 0.8, with an equal amount falling within the range of 0.5–0.8. Comparative transcriptomics demonstrated the gene clusters for lactose utilization in *B. longum* subsp. *infantis* while those in *B. bifidum* were more similar. In general, smaller oligosaccharides stimulate similar mechanisms to lactose metabolism (Garrido et al., 2015).

In very early experiments of bifidobacterial growth on different sugar substrates by de Vries and Stouthamer (1968), three strains of *B. bifidum* grown on lactose had carbon recovery rates of 81%–93%. Most significantly, *B. bifidum* S234 had almost double the molar growth rate on lactose compared to other experimental carbohydrate sources. Additionally, fermentation of one mole of lactose produced significantly higher quantities of lactate as an end product, compared to breakdown of glucose, galactose, or mannitol (de Vries and Stouthamer, 1968).

6.2.1.2 Galactose and Galactooligosaccharides

Galactose is a six carbon monosaccharide, found in lactose, and one of the building blocks of free human milk oligosaccharides (HMO) found in breast milk (Garrido et al., 2015). Different strains of bifidobacteria have displayed varied success in growth on galactose as a substrate: *B. adolescentis* showing the highest specific growth (Amaretti et al., 2007), and *B. animalis* subsp. *lactis* demonstrating very poor growth on galactose (González-Rodríguez et al., 2013). *B. bifidum* had about half the molar growth yield on galactose as that of growth on lactose (de Vries and Stouthamer, 1968). This growth differential is potentially explained by the lack of transporters, and presence of only catabolic enzymes, in the galactose regulons of bifidobacterial genomes, including those of *B. adolescentis, B. animalis* subsp. *lactis, B. bifidum, B. breve*, and others (Khoroshkin et al., 2016). In general, many characterized strains do have the ability to ferment galactose, including *B. breve, B. longum* subsp. *longum*, and *B. mongoliense* (Pokusaeva et al., 2011). It is worth noting that monosaccharide preference in bifidobacteria between glucose and galactose is likely species and strain dependent.

The metabolic fate of galactose and its role in the bifid shunt is unique in bifidobacteria. Canonically, galactose is broken down via the Leloir pathway. The first steps of this catabolism hold true in bifidobacteria; β -D-galactose is epimerized, phosphorylated, and finally acted on by galactose-1-phosphate uridylyltransferase to form glucose-1-phosphate (Holden et al., 2003). In the bifid shunt, however, the glucose-1-phosphate product is redirected to eventually form fructose-6-phosphate, which is acted on by phosphoketolase, a signature enzyme of bifidobacteria (González-Rodríguez et al., 2013; Pokusaeva et al., 2011). Following this key metabolic step, the acetate and lactate characteristic of bifidobacterial metabolism can form (Pokusaeva et al., 2011; Sela et al., 2008).

Galactose is also found in the human diet as a substituent of more complex bifidogenic carbohydrates. GOS are one of the most commercially available prebiotics today (Musilova et al., 2015). Naturally occurring in a variety of foods of plant origin, GOS can also be synthesized using the activity of β-galactosidases, which are a prevalent family

of enzymes in bifidobacteria species (Garrido et al., 2013a,b). Structurally, GOS consist of galactose, glucose, and fructose residues, joined via β 1–3, β 1–4, and β 1–6 linkages. They can range in degrees of polymerization 2–10, and possess a terminal glucose residue (Coulier et al., 2009). Early experiments showed that most GOS are preferentially consumed by bifidobacteria, classifying them as bifidogenic (Bouhnik et al., 1997). Many bifidobacteria species are able to utilize GOS as a primary carbon source.

Twenty-one strains of *B. longum* subsp. *infantis* were experimentally determined to be able to grow on GOS as the sole carbon source. Consumption of GOS with greater degrees of polymerization was correlated with higher growth, as measured by OD₆₀₀ (Garrido et al., 2013a,b). As a whole, species of bifidobacteria measured in dry weight per gram of fecal sample significantly increased in an experimental group of elderly (>65 years) people receiving a GOS prebiotic at weeks 5 and 10 postcontinuous administration of the prebiotic. The increase in number of bifidobacteria was accompanied by an increase in lactate concentrations in those receiving the prebiotic compared to those on the placebo (Vulevic et al., 2015). Bifidobacteria also increase proportionally when grown on a prebiotic composed of mainly GOS, as demonstrated by a change from 7.94% to 25.77% of the total bacterial count after growth on the prebiotic compared to the control medium (Musilova et al., 2015). Metabolically, there were also significant increases in the proportions of acetate, butyrate, and propionate, SCFA produced by bacterial metabolism (Musilova et al., 2015).

A number of in vivo studies have been performed in order to ascertain the efficacy of GOS as a prebiotic. Bouhnik et al. (1997) found that in healthy human subjects, the consumption of 10 g GOS/day resulted in a 10-fold increase in bifidobacteria concentrations after 1 week. Volunteers for an in vivo study of the bifidogenic effect of GOS and maltodextrin had a significant increase of bifidobacteria in log CFU/g in their feces as well (Musilova et al., 2015). Another in vivo study had premenarchal girls consume smoothies supplemented with either 0, 5, or 10 g of a predominantly GOS prebiotic mixture (Whisner et al., 2013). The number of bifidobacteria was significantly increased only under the 5 g GOS per day condition, measured both by bacterial DNA quantitation and weight in the subjects' fecal samples (Whisner et al., 2013).

Nine species of bifidobacteria have been assessed for their ability to grow on galactan, a polysaccharide of galactose. Few of the 34 strains tested were able to reach an OD signifying a high level of growth on galactan or arabinogalactan as a sole carbon source, but of the 11 strains that were able to utilize these substrates, more than half belonged to the species *B. breve*. Genome studies employing DNA microarray analysis revealed five genes found in *B. breve* UCC2003, constituting a novel galactan utilization cluster, were upregulated when the strain was grown on galactan. Strains of *B. longum* subsp. *longum* were noted to have cluster homology >90% for each gene locus. Homology was found to a lesser degree in the other strains that consumed galactan, including strains belonging to *B. adolescentis*, *B. longum* subsp. *infantis*, and *B. dentium*. All of the species that utilized galactan as a sole carbon source preferentially degraded substrates with β -1,4-linkages, and were unable to consume arabinogalactans that had β -1,3-linked galactose units (O'Connell Motherway et al., 2011).

6.2.1.3 Fructose, Fructooligosaccharides, and Inulin

Fructose, a hexose monosaccharide, is viable for consumption by most species of bifidobacteria and can be bound by a glycosidic linkage to glucose to form sucrose. Bifidobacteria, including *B. adolescentis* DSM20083, *B. animalis* subsp. *lactis*, and *B. longum* subsp. *longum* encode a sucrose phosphorylase for the purpose of liberating α-D-glucose-1-phosphate and fructose from sucrose, bypassing the bifid shunt. Additionally, fructofuranosidases are employed to hydrolyze glycosidic bonds formed between fructose and glucose monomers intracellularly (Pokusaeva et al., 2011). *B. breve* UCC2003 in particular has two genetic operons for the utilization of fructose and its oligomers: the *fru* operon for fructose and the *fos* operon for fructooligosaccharides (FOS) and sucrose (Mazé et al., 2007; Ryan et al., 2005). *B. breve* in general has high activity of its β-fructofuranosidase to hydrolyze β-2,1 glucose–fructose linkages, while *B. animalis* subsp. *lactis* has the greatest enzymatic activity on fructose–fructose bonds, such as those found in FOS (Louis et al., 2007; Ryan et al., 2005).

FOS are one of the most effective bifidogenic prebiotics available to date, and the most commonly used (Gómez et al., 2014; Manderson et al., 2005; Musilova et al., 2015; Rockova et al., 2011; Russell et al., 2011). FOS are composed of fructose monomers joined by β -2,1-glycosidic bonds, with a terminal glucose, and degrees of polymerization ranging 2–10 (Rossi et al., 2005). Inulin, another polysaccharide, is also composed of fructose but has a much higher range of polymerization—up to 60—and a flexible backbone usually consisting of 10–60 units of fructose (Mensink et al., 2015). Both FOS and inulin are found in foods common to the human diet, including onion, garlic, banana, and wheat (Mensink et al., 2015; Rossi et al., 2005). In vivo human studies and in vitro studies of human feces have demonstrated both FOS and inulin are bifidogenic (Bouhnik et al., 1999; Rossi et al., 2005).

Rossi et al. (2005) tested the ability of 11 species of bifidobacteria to grow on FOS and inulin. All species, including *B. adolescentis*, *B. bifidum*, and *B. breve* were able to grow on FOS. In contrast, only eight were able to utilize inulin, and

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the only organism of mammalian origin to metabolize inulin was *B. thermophilum* ATCC 25866 (Rossi et al., 2005). Interestingly, *B. longum* subsp. *infantis* was unable to grow on FOS well, despite their almost universal function as a prebiotic for bifidobacteria, and accordingly were not able to use inulin either (Rossi et al., 2005).

Selak et al. (2016) grouped 190 strains of bifidobacteria from different regions of the colon based on characteristics of fructose, oligofructose, and inulin type fructan degradation. The strains were representative of five species of bifidobacteria: *B. adolescentis, B.animalis* subsp. *animalis, B. bidifum, B. longum,* and *B. pseudolongum*. The majority of strains (n = 92) fell into a cluster characterized by positive growth on fructose and oligofructose, but only 10 strains from the entire cohort were able to utilize inulin (Selak et al., 2016). The ability of the bifidobacteria to consume the fructose-based sugars tested seemed to depend on strain specific phenotypes, not the region of the colon in which they reside. This phenotypic specificity may imply there is a certain level of cooperativity between and among the different species and strains of bifidobacteria that are suited to degrade certain substrates better than others. Additionally, evidence suggests bifidobacteria associated with the gut mucosa exhibit different patterns of metabolism compared to those found primarily in the lumen (Selak et al., 2016).

6.2.1.4 Xylose and Xylooligosaccharides

Xylose, a pentose sugar, is frequently found in xylo-oligosaccharides (XOS), polymers of xylose (Kumar et al., 2012; Mäkeläinen et al., 2010). These sugars are naturally occurring in fruits, vegetables, and milk, but are also synthesized from xylan, a structural component of plant lignins and hemicelluloses (Kumar et al., 2012). Naturally occurring XOS have varying degrees of polymerization, anywhere from 2 to 10 monosaccharides, and subunits mostly connected through β -1,4-linkages (Kumar et al., 2012). As these polysaccharides are both acid-resistant and indigestible through human enzymatic activity, there is a high likelihood of xylose reaching the colon intact and numerous studies have shown XOS are prebiotics that stimulate growth of bifidobacteria (Christensen et al., 2014; Mäkeläinen et al., 2010).

Genome sequencing of bifidobacteria has demonstrated that the ability to catabolize pentose sugars in some species and strains is a dispensable function, often in those species that are initially introduced to the infant gut microbiome (Sela et al., 2008). Despite this, some species of bifidobacteria, such as *B. longum* subsp. *longum*, *B. adolescentis*, *B. bifidum*, *B. catenulatum*, and *B. lactis* are still capable of metabolizing pentose sugars, including xylose and arabinose (de Vries and Stouthamer, 1968; Mäkeläinen et al., 2010; Pastell et al., 2009; Pokusaeva et al., 2011; Rivière et al., 2014; Sela et al., 2008). *B. adolescentis* grown on XOS consumed almost all linear sugars (i.e., xylobiose, xylotriose, xylotetraose) but did not completely consume the monosaccharide (Pastell et al., 2009), suggesting intracellular uptake of the oligosaccharide prior to hydrolysis. XOS are responsible for high levels of bifidobacterial proliferation, due to the microbes' preferential use of XOS compared to other carbohydrate oligosaccharides (Kumar et al., 2012).

Growth on xylose by two strains of *B. bifidum* was shown to have carbon recoveries of 90% and 97%, and further metabolites were assessed (de Vries and Stouthamer, 1968). Cultures of *B. bifidum* in log phase produce less formate and ethanol than acetate and lactate, but once they have reached stationary phase there is no production of formate or ethanol to be detected (de Vries and Stouthamer, 1968). In a study done by Mäkeläinen et al. (2010), the growth of bifidobacteria on both XOS and xylose alone in an in vitro colon environment was analyzed. *Bifidobacterium* spp. grew significantly more than the baseline experiments. *B. animalis* subsp. *lactis* and *B. longum* were further analyzed for species specific growth on these substrates. No significant difference in proliferation was observed for *B. longum* on any of the XOS substrates or on xylan alone, compared to baseline simulations (Mäkeläinen et al., 2010). *B. animalis* subsp. *lactis* did grow at a higher rate on two XOS treatments—commercially available XOS Longlive, a powder with degrees of polymerization of 2–5, and XOS with degrees of polymerization ranging 2–10—but other variations were insignificant (Mäkeläinen et al., 2010). High-throughput sequencing of bifidobacteria isolated from murine cecal matter confirmed a higher-fold-increase of bacteria grown on XOS substrates versus the sterile-water control, with additional validation by qPCR with bifidobacteria-specific primers (Christensen et al., 2014).

XOS obtained from agricultural biomass is a renewable resource of current interest due to the high availability of starting material and the relative ease of processing and purifying extracted oligosaccharides (Moniz et al., 2016). Fractions of XOS from corn straw were obtained via autohydrolysis, a method of agricultural processing of grasses, and gel filtration chromatography by Moniz et al. (2016) and were evaluated for probiotic ability, particularly those with medium to low degrees of polymerization. Impurities found in the purified fractions were mostly the monosaccharides xylose, arabinose, and glucose, respectively, in order of abundance. These substrates were then inoculated with microbial communites from three human fecal samples. Bacterial presence and abundance were measured through oligonucleotide probes specific to bacteria at the genus level and fluorescence in situ hybridization (Moniz et al., 2016). At the population level, bifidobacteria grown on the sample fraction and a commercial nonsubstituted XOS showed significant growth at 10 h and 24 h (Moniz et al., 2016).

Key metabolic end products of XOS fermentation are acetate, butyrate, and propionate, all SCFA. Acetate and butyrate were found at higher concentrations in the colonic simulator vessels than propionate, with acetate being most abundant (Mäkeläinen et al., 2010). The xylan variation had significantly less SCFA production quantified, suggesting that xylan alone is not a suitable substrate for bifidobacterial metabolism. It is important to note that bifidobacteria are incapable of producing butyrate; rather, it can be synthesized from acetate or lactate by other microbes that inhabit the human gut. As such, interorganismal interactions must have occurred in the experimental vessels for this metabolite to be detected (Mäkeläinen et al., 2010).

Populations of bifidobacteria that successfully utilized XOS derivatives from corn straw all produced increased SCFA concentrations, on both experimental fractions and the commercially available *n*XOS. The most abundant metabolite at 36 h was acetate, followed by propionate and butyrate (Moniz et al., 2016), the latter two produced by other groups of bacteria from the same fecal inocula. At 24 h, concentrations of products across the three substrates were relatively similar, but at 36 h, the final time point, SCFA concentrations generated from the extracted fractions surpassed that of the commercial oligosaccharides (Moniz et al., 2016). The fermentation of linear oligosaccharides has been well characterized in previous studies, and it is thought these substrates are fermented more rapidly once in the colon (Pastell et al., 2009). This knowledge, when considered with the high final concentration of acetate, implies more highly polymerized XOS are a better substrate for fermentation by bifidobacteria. Lactate was also produced on all substrates, but after a maximum at 10 h there was a sharp decline in concentration until its disappearance at 24 h. This is a result of the lactate's further utilization by bacteria that produce acetate, butyrate, and propionate (Moniz et al., 2016).

6.2.1.5 Arabinose, Arabinoxylans, and Arabinooligosaccharides

Arabinose is another pentose sugar found in plant polymers, such as hemicelluloses and pectins. Xylose and arabinose are the major monosaccharide components of hemicelluloses, comprising 80% and 12%, respectively (Li et al., 2015). Often, the two are conjugated, and arabinoxylans are more common prebiotic compounds encountered by bifidobacteria. On L-arabinose alone, species found to show positive growth are *B. longum* subsp. *longum*, *B. tsu-rumiense*, *B. dentium*, *B. psychraerophilum*, and *B. mongoliense*, among others, all isolated from various mammalian oral and intestinal microbiomes (Pokusaeva et al., 2011; Pastell et al., 2009).

Arabinoxylans (AX), and their arabinoxylo-oligosaccharide derivatives (AXOS) are promising prebiotics, due to their complex and varied structures (Pastell et al., 2009). AX have a backbone of xylan sugars connected via β -1,4-D linkages (Pastell et al., 2009). This backbone can be anywhere from 1500 to 15,000 D-xylopyranosyl units (Rivière et al., 2014). Additionally, each of these units can either be unsubstituted, or have an α -L-arabinofuranosyl residue (Rivière et al., 2014). As such, various kinds of enzymes are required for the breakdown of AX and AXOS, namely, both debranching and depolymerizing enzymes. The sequencing of the genomes of *B. longum* and *B. adolescentis* unveiled large numbers of genes that encode enzymes, which degrade these complex sugars, such as β -D-xylosidases and α -L-arabinofuranosidases, both glycosyl hydrolases (Pastell et al., 2009). Genes for α -L-arabinofuranosidases, the key enzyme for catabolism of AXOS, have been discovered in many strains of *B. longum*, and in *B. adolescentis*, in which the enzymes act extracellularly (Pastell et al., 2009). In general, genes that encode enzymes necessary for the degradation of AXOS are pervasive in the genomes of *B. longum* strains (Rivière et al., 2014). In concordance, genes for α -L-arabinofuranosidase type I were observed in 60% of strains tested, and type A in 80% of strains (Rivière et al., 2014).

Bifidobacterium adolescentis ATCC 15703 demonstrated weak growth on AX from both wheat and rye precursors, but did not grow on L-arabinose alone, despite the presence of α -L-arabinofuranosidase genes in its genome (Pastell et al., 2009). Most likely, this enzyme is produced for the purpose of liberating arabinose from more complex AX or AXOS substrates. Similar growth patterns were observed in *B. longum* ATCC 15707 on arabinoxylan, but *B. adolescentis* had moderately better growth on wheat AX at 140 h than *B. longum*. *Bifidobacterium breve* did not grow on many substrates besides the FOS positive control, but mild growth was seen at 140 h on rye AX. When D-xylose was the sole carbon source *B. adolescentis* was capable of fermenting the sugar, albeit slowly, but in the presence of AX substrates xylose was not utilized (Pastell et al., 2009).

Sugar beet pulp is a significant agricultural by-product of sugar production. Limited uses have been employed thus far, aside from use as an additive to cattle feed, but studies have also demonstrated single culture utilization by *B. adolescentis* and *B. longum* (Holck et al., 2011). Pectin derived from sugar beet pulp can be further processed to arabinooligosaccharides with degrees of polymerization less than 8, which have a prebiotic effect specific to bifidobacteria (Holck et al., 2011). A complex polysaccharide, pectin is a critical component of cell walls, with abundant galacturonic acid (Christiaens et al., 2016). Galacturonans in the backbone can be substituted with arabinan via α -(1,5)-linkages (Holck et al., 2011).

AXOS metabolism varies among *Bifidobacterium* species. Rivière et al. (2014) found that *B. bifidum* LMG 11583 did not grow on AXOS. Two strains of *B. longum* subsp. *longum*, NCC2705 and LMG 11047, both exhibited broad

degradation of AXOS. On the contrary, *B. infantis* LMG 11588 was only able to utilize AXOS to a limited degree. Broad degradation of AXOS was observed in *B. catenulatum* LMG 11043^T as well (Rivière et al., 2014). Furthermore, the metabolism observed in *B. catenulatum* implied this species employs extracellular degradation followed by import of AXOS backbones, because consumption of arabinose groups was not followed by an increased concentration of backbone derived xylose end products (Rivière et al., 2014).

Metabolites quantified in the medium postfermentation, namely xylobiose and xylotriose, imply *B. longum* is able to consume the arabinose portion of the arabinoxylan, but not the xylose sugars that remain—also supported by the experimental observations of no growth on D-xylose or XOS by this strain of *B. longum* (Pastell et al., 2009). No short chain AXOS were detected in the medium postfermentation by the individual strains of bifidobacteria. As such, it is likely the cleavage of arabinose from the XOS is performed extracellularly, due to the high concentrations of xylobiose and xylotriose at the end of fermentation (Pastell et al., 2009). Some strains of *B. longum* subsp. *longum* are characterized by their ability to metabolize arabinose but not the xylan backbone, as evidenced by release of XOS ranging in size from xylobiose to xylohexose (Rivière et al., 2014). Yet other strains of the same subspecies can catabolize the entire AXOS molecule, highlighting that metabolic capability in *Bifidobacterium* can be highly strain dependent.

Inoculation with whole community samples from human feces showed utilization of all carbohydrates—L-arabinose, D-xylose, XOS, rye AX, and wheat AX—suggesting other bacteria found in vivo compensate for the metabolic capabilities that bifidobacteria lack. Furthermore, acetate concentrations were significantly higher post fermentation by the fecal inoculum, also consistent with in vivo observations (Pastell et al., 2009). Less robust increases in propionate and butyrate concentrations were noted as well. Acetate, ethanol, lactate, and formate are produced by *B. longum* NCC2705, with AXOS as a substrate. At 6 h, when growth reaches the stationary phase, acetate, formate, and ethanol were found at stable concentrations, and lactate had been consumed in favor of producing the other metabolites (Rivière et al., 2015).

6.2.1.6 Milk Oligosaccharides

The most abundant components that comprise breast milk are lactose, lipids, and HMO, respectively (Katayama, 2016). Mature milk composition is about 7% carbohydrate; of that, 80% is lactose, and the remaining 20% is largely HMO (Urashima et al., 2012). On average, the amount of HMO in breast milk is about 7 g/L, and 23 g/L in colostrum, the fluid produced perinatally, prior to milk production (Smilowitz et al., 2014). Though there are more than 200 varieties of HMO, there are four key units that comprise these oligosaccharides—2'-fucosylactose, lacto-*N*-tetraose, lacto-*N*-fucopentaose I, and lacto-*N*-difucopentose (Katayama, 2016; Urashima et al., 2012). Each of these building blocks are derived from the same set of monosaccharides, namely glucose, galactose, fucose, sialic acid, and *N*-acetylglucosamine (Garrido et al., 2013a,b; Ruiz-Moyano et al., 2013; Sela et al., 2008). Additionally, many HMO are further modified by these sugars, with 35%–50% of HMO in breast milk being fucosylated, and 12%–14% sialylated (Smilowitz et al., 2014). HMO are characterized by a terminal lactose that is also modified by fucose, sialic acid or one of the larger substituents (Garrido et al., 2013a,b; Ruiz-Moyano et al., 2013).

Two key types of HMO have also aided in their characterization. Type I HMO contain lacto-*N*-biose, and type II have *N*-acetyllactosamine. Both through experimental determination and comparison to other primate species, it has been discovered that a prevalence of type I HMO is a characteristic specific to human milk, likely due to bifidobacterial utilization (Urashima et al., 2012).

Different species of bifidobacteria possess different mechanisms of carbohydrate uptake particular to HMO. Accordingly, many classes of glycosyl hydrolases are enriched in the microbiomes of breast-fed infants (Garrido et al., 2013a,b). *B. longum* subsp. *infantis*, a highly prevalent microbe in breast-fed infants, imports HMO intact, prior to internal degradation (Katayama, 2016). ABC transporters are responsible for intake of both type I and type II HMO in *B. infantis* (Sela et al., 2008; Urashima et al., 2012). Once internalized, an assortment of intracellular glycosyl hydrolases act on the sugar (Smilowitz et al., 2014). For both type I and type II HMO, hydrolysis begins at the non-reducing end by monoreleasing exoglycosidases (Katayama, 2016; Urashima et al., 2012). These enzymes include sialidase, fucosidase, and lacto-*N*-biosidase (Urashima et al., 2012). Similar to *B. longum* subsp. *infantis*, *B. breve* also imports carbohydrates prior to degradation (Katayama, 2016). In contrast, *B. bifidum* exports sialidases, fucosidases, and lacto-*N*-biosidase outside of the cell to hydrolyze type I HMO (Simeoni et al., 2015; Urashima et al., 2012). The bacteria then import the hydrolyzed products via ABC transporters (Urashima et al., 2012).

Since so many species of bifidobacteria are isolated from predominantly breast-fed infants, it is of scientific interest to determine whether bifidobacteria exhibit comparable growth on milk oligosaccharides from mammalian species other than humans, and to compare growth phenotypes on milk substrates, purified HMO, and individual HMO substituents. Comparative growth studies have shown that pure milk oligosaccharides are the best substrates for bifidobacteria, regardless of animal origin (Thum et al., 2015). Strains of *B. adolescentis, B. bifidum, B. longum* subsp.

longum, and *B. breve* isolated from infant feces all had better growth on purified HMO, but *B. bifidum* and *B. breve* grew almost as well on human milk (Rockova et al., 2012). Human milk samples were also found to promote the growth of *B. bifidum* from infant feces and *B. longum* isolated from a commercial probiotic (Rockova et al., 2011). *B. longum* subsp. *infantis* is known to prefer shorter HMO, but is able to utilize both larger molecules and distinct types with high efficiency, resulting in growth to high cell densities (Garrido et al., 2013a,b). Growth of six strains of *B. breve* on HMO ranged in utilization 23%–42% (Ruiz-Moyano et al., 2013). Compared to this moderate level, growth on HMO components lacto-*N*-tetraose and lacto-*N*-neotetraose, was more robust and comparable to that of *B. infantis* ATCC 15697 (Ruiz-Moyano et al., 2013). In general, *B. infantis* imports lacto-*N*-tetraose whole and is able to utilize the sugar through the action of intracellular enzymes encoded by the HMO gene cluster (Katayama, 2016; Sela et al., 2008). *B. longum* subsp. *infantis* is also capable of metabolizing lacto-*N*-difucohexaose I, as is *B. bifidum*, though at different rates (Katayama, 2016). The species *B. longum* and *B. breve* consume lacto-*N*-tetraose completely. Furthermore, *B. bifidum* is able to derive lacto-*N*-biose from lacto-*N*-tetraose (Katayama, 2016). Oligosaccharides containing *N*-acetylglucosamine residues are also reported to be bifidogenic to *B. bifidum* (Thum et al., 2015).

Simeoni et al. (2015) evaluated the efficacy of bovine milk oligosaccharides (BMO) compared to infants consuming formula with no oligosaccharide supplementation and exclusively breastfed infants. For all infants, a significant increase in total bifidobacteria was observed at 6 weeks, and this elevation remained stable until week 12 (Simeoni et al., 2015). Although the shift to a bifidobacteria dominated microbiome was universal across all of the infant fecal matter tested, in the breast-fed infants *B. bifidum* and *B. breve* were most abundant, while the BMO supplemented group had a majority of *B. longum* and *B. bifidum* (Simeoni et al., 2015). While the BMO are bifidogenic, cow's milk itself has low free oligosaccharide content, so extraction of oligosaccharides remains the best option for supplementation (Garrido et al., 2013a,b). Caprine milk shares its dominant forms of sialylated milk oligosaccharides with human milk, making caprine milk oligosaccharides (CMO) another source of potential supplementation or prebiotics (Thum et al., 2015). All tested strains of *B. breve*, *B. longum* subsp. *longum*, and *B. bifidum* grew best on CMO versus individual sialylated substituents (Thum et al., 2015).

6.2.2 Regulation of Carbohydrate Metabolism

At any given time, a microorganism will only encounter the nutrients present in its immediate environment. It is energetically costly, and ultimately unfavorable, for a bacterium to constitutively express all of the genes in its repertoire related to carbohydrate uptake and metabolism. These genes are many and varied in bifidobacteria for, as saccharolytic organisms, their fitness and survival depends on the proper utilization of the carbon substrates available to the microbe. Accordingly, bifidobacteria employ processes of regulation to determine which genes to express in order to best obtain energy and nutrients.

Bacteria often use transcription factor (TF) regulons to regulate catabolic carbohydrate pathways (Khoroshkin et al., 2016). However, not all of the regulons in bifidobacteria are well understood, and pathways can be intricately interconnected. Complex metabolic networks and carbohydrate utilization pathways have been analyzed via comparative genomics and transcriptomics. Ten species of bifidobacteria with fully sequenced genomes were selected for the analysis of the presence of eight families of common carbohydrate utilization TFs (Khoroshkin et al., 2016). Between the 10 genomes, 308 putative TFs specific to sugar utilization were found, with a moderate degree of conservation, and high variability in the number of TFs specific to each genome (Khoroshkin et al., 2016). Of the TFs that were common among the 10 genomes, the most conserved were regulators of the DeoR family for deoxyribose catabolism (Škerlová et al., 2014), the LacI family for regulation of lactose utilization genes (Ravcheev et al., 2014), and ROK family for transcriptional repression in response to hexoses including glucose (Kazanov et al., 2013). Many reconstructed regulons contained these TFs in addition to glycosyl hydrolases and ABC transporters. Some showed redundancy in the type of sugar catabolism that was regulated, implying the existence of nonorthologous TFs in different genomes, or alternate pathways within a genome that require different TFs (Khoroshkin et al., 2016).

The *araQ* gene has been identified as a possible universal regulator of carbohydrate metabolism in bifidobacteria (Khoroshkin et al., 2016). This regulatory element in bifidobacterial metabolism is located in multiple places throughout the catabolic network, playing a role in glycolysis, the bifid shunt, and glucose metabolism, all key facets of central metabolism (Khoroshkin et al., 2016). It is postulated the incidence of this gene in most species of bifidobacteria originated as a part of the arabinose utilization pathway, but while some bifidobacteria lost the ability to metabolize arabinose, the *araQ* gene remained as a regulator (Khoroshkin et al., 2016; Sun et al., 2015). Currently, the effectors of this gene remain unknown.

Comparative transcriptomics between strains of *B. longum* subsp. *infantis* and *B. bifidum* demonstrated different mechanisms of HMO utilization, although growth on HMO and its substituent sugars was universal (Garrido

et al., 2015). In both *B. longum* subsp. *infantis* and *B. bifidum*, two key genes of the bifid shunt were consistently among the most highly expressed, fructose-6-phosphate phosphoketolase and glyceraldehyde-3-phosphate dehydrogenase (Garrido et al., 2015). In *B. longum* subsp. *infantis*, growth on HMO caused consistent upregulation of HMO utilization gene clusters, suggesting that HMO themselves may induce expression of the genes needed for their catabolism. This was largely homogenous among strains of *B. longum* subsp. *infantis*. When *B. longum* subsp. *infantis* encounters individual HMO components 2-fucosyllactose and 3-fucosyllactose, alternative pathways for consumption are induced (Garrido et al., 2015). *B. bifidum* does not appear to be responsive to these substrates, instead likely only utilizing lactose present on the constituents of HMO (Garrido et al., 2015). The key catabolic enzymes of *B. bifidum*, including lacto-*N*-biosidase, lacto-*N*-biose phosphorylase, and endo- α -*N*-acetylgalactosaminidase, were mostly induced when grown in HMO, though induction also occurred to a lesser degree on lactose (Garrido et al., 2015). Many important genes in *B. bifidum* are induced by lacto-*N*-biose and galacto-*N*-biose. The overall utilization strategies by *B. bifidum* in HMO catabolism were more varied than those of *B. longum* subsp. *infantis*, and both had markedly different transcriptional responses even to the same substrates (Garrido et al., 2015). Significantly, key HMO utilization genes were present in equivalent quantities in bacteria that grew well and poorly on the same substrate, indicating that differential regulation is a determining factor in successful utilization.

B. breve UCC2003 has two gene clusters, *rafABCD* and *melABCDE*, dedicated to raffinose and melezitose metabolism, respectively, that are upregulated by the presence of their target sugar, another instance of substrate specific transcriptional regulation (O'Connell et al., 2014). LacI regulatory TFs also have an effect on the *mel* gene cluster; specifically, MelR1 and MelR2 were identified as regulators of this metabolic gene cluster (O'Connell et al., 2014). On the other hand, the TF regulator of raffinose metabolism, RafR, is postulated to be in the ROK family (O'Connell et al., 2014). Transcriptome analysis of *B. animalis* subsp. *lactis* demonstrated the overall gene expression in the organism was dependent on the type of oligosaccharide on which it was grown (Andersen et al., 2013). Gene expression of different transporters upregulated under various conditions demonstrates the genetic variability *B. animalis* subsp. *lactis* possesses, allowing for consumption of many carbohydrate substrates.

Transcriptional gene regulation is also affected by the presence of other species of bifidobacteria. For example, the expression of glycosyl hydrolases in *B. adolescentis* 22L was upregulated more than twofold when associated with *B. bifidum* PRL2010 (Turroni et al., 2010). Upregulation of catabolic genes in *B. longum* subsp. *infantis* ATCC15697 and *B. longum* subsp. *longum* NCC503 has also been observed as a result of their respective coassociations (Turroni et al., 2010). A potential cause of the upregulation is competition within the gut environment; when a competitor microorganism is present, the change in regulation enhances the *Bifidobacterium*'s own abilities to become a successful member of that environment. When the two species are both of genus bifidobacteria, these effects are mutualistic, as ultimately both species obtain their necessary nutrition and the host benefits from the activity of both.

6.2.3 Other Macronutrients

Metabolism of noncarbohydrate macronutrients by bifidobacteria is poorly understood. There are no reports of bifidobacterial utilization of fat as a substrate, and little description of protein metabolism. However, some peptidases found in bifidobacteria have been characterized.

The endopeptidase PepO has been isolated from *B. animalis* subsp. *lactis* and characterized (Janer et al., 2005). The presence of peptidases in bifidobacteria has previously been hypothesized based on sequenced genomes (Schell et al., 2002), but PepO was one of the first to be identified and overexpressed for experimentation. This peptidase is specific to α_{s1} -casein(f1-23), a milk protein (Janer et al., 2005). The presence of this enzyme in *B. animalis* subsp. *lactis* supports the observation that this species grows particularly well on milk-based media (Janer et al., 2005).

6.2.4 Micronutrients

Current knowledge of micronutrient utilization in bifidobacteria is underdeveloped. It is known that bifidobacteria, among other gut commensals, are important producers of vitamins in the human colon, including vitamin K, folate, and biotin (Morowitz et al., 2011), but their own consumption of vitamins and minerals is poorly understood. To date, iron is the only micronutrient that has been studied in the context of bifidobacterial nutrition.

6.2.4.1 Iron

Almost universally, iron is a required nutrient due to its roles in electron transport, energy production, as enzyme cofactors, and nutritional immunity (Ganz and Nemeth, 2015). Many pathogens of the human gut have coopted their iron sequestration capabilities for the promotion of their pathogenesis (Vazquez-Gutierrez et al., 2015). However, as

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the human large intestine is an iron-limited environment, pathogens and commensals must out-compete each other for the utilization of the iron present. When iron in the environment is low, many species of bacteria may upregulate the expression of iron-chelating proteins, called siderophores, for the sequestration of the mineral (Vazquez-Gutierrez et al., 2015). It has been shown that both Gram-positive and negative bacteria use iron accumulation as a mechanism against pathogens in vivo. Studies have also suggested that the ability to withhold iron from pathogens could be a characteristic for effective probiotics (Bailey et al., 2011).

The current opinion on bifidobacterial utilization of iron is somewhat polarized. Early studies of iron metabolism in bifidobacteria observed iron uptake in *B. breve, B. bifidum,* and *B. thermophilum* (Bezkorovainy et al., 1996). Based on experiments with other divalent cations and iron chelators, it was determined that bifidobacteria likely internalized iron via permeases that could be common to other cations as well, and that siderophores were not involved (Bezkorovainy et al., 1996). On the contrary, assays of siderophore activity in bifidobacteria isolated from iron-limited environments found the production of chelation agents was widespread in the genus, albeit strain specific (Vazquez-Gutierrez et al., 2015). Additionally, siderophore activity and iron internalization were not correlated (Vazquez-Gutierrez et al., 2015). Notably, the highest siderophore activity was reported in *B. breve* (Vazquez-Gutierrez et al., 2015). *B. longum* DJO10A was found to have a strong capability to inhibit other bacteria via siderophores (Lee et al., 2008). Another strain, *B. longum* JCM7052, was found to grow freely in the presence of a strong iron chelator, suggesting that this strain also possesses siderophores that allow iron accumulation in otherwise limiting conditions (Cronin et al., 2012).

A clinical nutrition study demonstrated that bifidobacteria are able to grow to significant abundance in iron-limited conditions, holding the implication that they are able to acquire enough iron to survive (Dostal et al., 2013). Along the same lines, another clinical study found iron fortification decreased numbers of bifidobacteria in the microbiota of young children, resulting in an unfavorable ratio of beneficial microbes to pathogens (Zimmermann et al., 2010). Regardless of the mechanism of internalization, it can be concluded that the ability of bifidobacteria to outcompete other microorganisms for iron is an advantage present in most of the genus (O'Sullivan, 2001).

Lactoferrin, a key iron transporter, is among the most abundant proteins in human breast milk and its presence may contribute to the bifidogenic properties of milk (Ballard and Morrow, 2013). Compared to bovine milk, lacto-ferrin is found in much higher concentrations in human milk, suggesting specificity to the human gut environment (Oda et al., 2014). Still, the efficacy of lactoferrin's bifidogenic effect depends on the species and strain of bifidobacteria, as well as the richness of the microbe's environment.

The structure of lactoferrin varies depending on its level of saturation, and can hold up to two Fe³⁺ ions (Adlerova et al., 2008). In an iron-poor environment, the addition of human or bovine lactoferrin unbound to iron, or only partially saturated, inhibits the growth of *B. breve*, *B. bifidum*, and *B. longum* subsp. *infantis* (Oda et al., 2014). In contrast, when hololactoferrin, the iron-saturated protein, is added to iron-limited media, growth of bifidobacteria is not inhibited (Oda et al., 2014). Likewise, when iron is present, the degree of saturation of lactoferrin is irrelevant, and bifidobacterial growth is promoted across many species, including *B. longum* subsp. *longum*, *B. longum* subsp. *infantis B. breve*, *B. thermophilum*, and *B. adolescentis* (Oda et al., 2014). Taken together, these findings indicate that if binding of iron by lactoferrin would be detrimental to bifidobacteria, such as in an iron-limited environment, then there is no bifidogenic effect. However, if iron is plentiful in the environment, bifidobacterial growth is promoted, potentially through iron sequestration as well. Overall, the bifidogenic effect engendered by saturated hololactoferrin is stronger than unbound apolactoferrin (Morshedur Rahman et al., 2010).

Cultures of *B. breve* grown for 24 h to which iron-poor or saturated lactoferrin was added exhibited different growth phenotypes (Miller-Catchpole et al., 1997). The bacteria were inhibited by lactoferrin that was unbound to iron, and growth was promoted by the iron-saturated lactoferrin, corroborating the importance of iron availability. Interestingly, it was also discovered that *B. breve* is able to accumulate iron from the lactoferrin, rather than binding the protein itself (Miller-Catchpole et al., 1997). Other experiments found that bovine lactoferrin, both iron-free and saturated, moderately promoted the growth of *B. breve* ATCC 15700, *B. longum* subsp. *infantis* ATCC 15697, and *B. bifidum* ATCC 15696 (Kim et al., 2004). At a concentration of 0.1 mg/mL, human lactoferrin also had an effect on *B. infantis* and *B. bifidum* (Kim et al., 2004). Overall, it appears that the growth of bifidobacteria found primarily in the infant gut have more positive growth outcomes from lactoferrin than those of the adult gut environment.

Lactoferrin enrichment of infant formula has led to conflicting results. Some studies reported formula supplementation with lactoferrin caused an increase in bifidobacteria in the infant gut, while others found no significant difference between the gut microbiomes of infants who received supplemented formula and those who had not (Mastromarino et al., 2014). There has also been some disparity in whether human or bovine lactoferrin has greater growth promoting effects; one study found that *B. bifidum* was more stimulated by human lactoferrin, but the bovine protein had a greater effect on *B. breve* and *B. longum* subsp. *infantis* (Petschow et al., 1999). A consensus remains to be reached both regarding which peptides are most bifidogenic and the exact mechanism of the effects.

6.3.1 Media

A successful culture environment for bifidobacteria must satisfy two criteria: adequate nutrient content and anaerobic conditions (Roy, 2001). For the most part, the latter is satisfied through proper culture and plating technique and the use of anaerobic chambers and incubators for growth. Selectivity can be achieved both by enhancing bifidobacterial growth and inhibiting that of other microorganisms. Other general considerations for media include the final pH of the medium, its buffering capacity, low redox potential, and the inclusion of antibiotics (Roy, 2001).

The two most recommended basal culture media for bifidobacterial culture are De Man Rogosa Sharpe (MRS) (De Man et al., 1960) and tryptone phytone yeast (Scardovi, 1986). Other popular media are bifidobacteria specific media (BSM) and Beerens media (Ferraris et al., 2010). A common modification to MRS is the addition of 0.05% L-cysteine HCl, recommended for enumeration from pure culture (Roy, 2001). Popular diluents for media are peptone water, with or without added saline in different quantities; often 85% (Roy, 2001). Modified MRS (mMRS) introduced by Pacher and Kneifel (1996) began to incorporate more bifidobacteria-specific nutrients, namely yeast extract, peptone, lactulose, human whey, cysteine, starch, and the vitamins thiamin, riboflavin, and pantothenic acid (Pacher and Kneifel, 1996). Carbohydrate substrates can be used to selectively grow bifidobacteria based on their metabolic capabilities.

The use of a single carbon source specific to bifidobacteria, such as HMO, GOS, or FOS can be an effective method for achieving selectivity. Likewise, the use of antibiotics introduces selectivity for bifidobacteria at the genus level, and provides the ability to differentiate bifidobacteria from other lactic acid bacteria. The addition of the antibiotic mupirocin (100 mg/L) has proven to be very effective for the enumeration of bifidobacteria (Ferraris et al., 2010).

On Wilkins-Chalgren for bifidobacterium mupirocin (WCBM) media, many species and strains of bifidobacteria, such as *B. bifidum*, *B. breve*, and *B. angulatum*, grew to significantly greater log CFU/mL compared to BSM (Ferraris et al., 2010). In another study, B. *animalis* subsp. *lactis* BB-12 and *B. breve* M-16V grew on transgalactosylated oligo-saccharides-mupirocin lithium salt (TOS-MUP) agar significantly better than on MRS (Süle et al., 2014). The medium raffinose-propionate lithium mupirocin (RP-MUP) was developed to contain multiple factors for bifidobacteria selection—a tripartite collaboration of substrate, SCFA, and antibiotic (Miranda et al., 2014). Growth on RP-MUP by *B. animalis*, *B. lactis*, and *B. longum* demonstrated similar efficacy to TOS-MUP (Miranda et al., 2014).

TOS-MUP is typically utilized for the enumeration of bifidobacteria from milk products, so a study of its efficacy for probiotic uses was performed (Bunesova et al., 2015). Growth studies comparing TOS-MUP to WBCM and BSM + mupirocin tested both pure cultures of six species and a range of commercial probiotics to assess proliferation of bifidobacteria. While most results, in log CFU/mL, were comparable, *B. bifidum* DSM 20456 and *B. bifidum* DSM 20239 grew to significantly lower quantities on TOS-MUP (Bunesova et al., 2015). Overall, the enumeration of bifidobacteria from probiotic supplements on all three media was successful, but particularly on WCBM and BSM (Bunesova et al., 2015).

One potential concern regarding the use of mupirocin as a selective agent is the resistance of other microorganisms to this antibiotic (Patel et al., 2009; Vlková et al., 2015). To address this issue, one group compared susceptibility to antibiotics between species of bifidobacteria and species of clostridia, a genus often resistant to mupirocin (Vlková et al., 2015). They found, of the antibiotics tested, all clostridia were susceptible to norfloxacin, while most bifidobacteria, encompassing 13 species, were resistant (Vlková et al., 2015). For this reason, it is recommended that norfloxacin be added as a second selective antibiotic to WCBM at 200 mg/L (Vlková et al., 2015).

6.3.2 Auxotrophy

In addition to the general nutritional needs of bifidobacteria, there are certain critical organic compounds without which their growth would not be supported. Bifidobacteria are largely incapable of synthesizing these compounds on their own, and as such are classified as auxotrophic for these compounds. It is important to know what they are in order to supplement growth media adequately and assess key factors in growth promotion.

While it is known that certain proteins and their peptides are bifidogenic, the breakdown and utilization of peptides and amino acids in bifidobacteria is poorly understood (Ferrario et al., 2015). It is thought that host–microbial interactions assist in acquisition and synthesis of amino acids by the microbiota, and that amino acids may also act as precursors for common metabolic end products, such as SCFA (Davila et al., 2013). A growth study on a chemically defined medium for *B. bifidum* PRL2010 removed individual amino acids one by one to determine any effects on the growth of the microbe, and found that only when cysteine was removed was there a significant decrease in

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growth, suggesting auxotrophy (Ferrario et al., 2015). Subsequent experimentation with 48 additional species and subspecies of bifidobacteria confirmed auxotrophy for cysteine is ubiquitous across the genus. Of these, only six species—*B. boum* LMG10736, *B. minimum* LMG11592, *B. pullorum* LMG21816, *B. ruminantium* LMG21811, *B. saguini* DSM23967, and *B. scardovii* LMG21589—were able to grow in the absence of cysteine, and only to very low OD (Ferrario et al., 2015).

Additionally, folate has been found to be another determining growth factor for certain species of bifidobacteria. Many species are known to be able to synthesize folate, which plays a role in nucleic acid biosynthesis among others (Sugahara et al., 2015). Bifidobacterial species endogenous to humans are typically capable of folate production, while bifidobacteria of nonhuman origin are not (D'Aimmo et al., 2012; Sugahara et al., 2015). When species do not possess the genetic equipment for folate biosynthesis, such as *B. animalis* subsp. *lactis*, it can be presumed they are auxotrophic for the vitamin (D'Aimmo et al., 2012). Other species that are folate auxotrophs are *B. magnum* and *B. pseudolongum* (Sugahara et al., 2015).

6.4 CONCLUDING REMARKS

In vivo, nutritional requirements are more complex due to the dynamic nature of digestion and the presence of other microbial species. In addition to the metabolic substrates and nutritive additives found in culture medium, the gut exhibits motility, such as mass movement and contractions that may affect the distribution of nutrients and the microbes themselves. Furthermore, different species of bifidobacteria can be associated with the mucosa or found in the intestinal lumen, which also determines which nutrients are available in that particular microenvironment. Finally, the presence of other microorganisms influences competition for resources, as well as introducing new metabolites into the environment through secondary degraders.

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Further Reading

Garrido, D., Ruiz-Moyano, S., Mills, D.A., 2012. Release and utilization of *N*-acetyl-D-glucosamine from human milk oligosaccharides by *Bifidobacterium longum* subsp. *infantis*. Anaerobe 18 (4), 430–435, Available from: http://www.sciencedirect.com/science/article/pii/S1075996412000649.

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Stress Responses of Bifidobacteria: Oxygen and Bile Acid as the Stressors

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7.1 INTRODUCTION

Although oxygen (O_2) and bile acids are well-known abiotic stressors that inhibit the growth of gut microbes, molecular mechanisms for sensitivity/resistance to these stressors in gut microbes, especially their effects on the overall gut microbiota composition await clarification. In this chapter, progress in the related areas was summarized focusing mainly on bifidobacteria.

7.2 O₂ AND GUT MICROBES

 O_2 comprises 21% of atmospheric gases and can permeate into everywhere by dissolving in water. An ecosystem where absolute anoxic environment is sustained is a rarity in nature because the gas–liquid phase on Earth is constantly circulating by convection. Hence, all living organisms that currently inhabit this planet have adapted to highly reactive O_2 .

Gut microbes consist of hundreds of species in genera (Arumugam et al., 2011; Qin et al., 2010; Yatsunenko et al., 2012) that include *Bacillus* (obligate aerobe), *Escherichia, Staphylococcus*, and *Salmonella* (facultative anaerobes that possess respiratory chain), and the lactic acid bacteria *Lactobacillus*, *Streptococcus*, and *Enterococcus* (O₂-tolerant anaerobes that do not possess respiratory chains), *Bifidobacterium* and *Propionibacterium* (anaerobes), and *Bacteroides* and *Clostridium* (obligate anaerobes). Aerobes and facultative anaerobes with respiratory chains actively consume O₂ by respiration, which is hypothesized to reduce O₂ concentrations in gut environments (Espey, 2013; Fanaro et al., 2003; Orrhage and Nord, 1999). Microaerophiles and aerotolerant anaerobes grow in the reduced O₂ conditions, and then obligate anaerobes ferment residual nutrients by anoxic fermentation. Therefore, the gut microbial ecosystem may change with microbial density and composition, or with O₂ concentration, which varies with intake of fermented foods, vegetables, beverages, and nutrients.

O₂ tension in the gastrointestinal tract is known to decrease progressively from the proximal to the distal gastrointestinal tract (Espey, 2013; Lind Due et al., 2003). This O₂ gradient is believed to be generated by the O₂ consumption of gut microbes. In healthy adults, the proximal part of the gastrointestinal tract is dominated by *Streptococci* (Cheng et al., 2013), whereas the distal part of the gastrointestinal tract is occupied by bacteria in the class Clostridia and Bacteroidia, most of which are obligate anaerobes in the genera *Clostridium* and *Bacteroides* (Qin et al., 2010). Recently, an increase in O₂ tension in the large bowel was hypothesized to be the cause of dysbiosis in patients with inflammatory bowel disease, in which a decrease in obligate anaerobic Firmicutes and increase in facultative anaerobic Enterobacteriacea are observed (Rigottier-Gois, 2013). A similar imbalance is observed after antibiotic treatment, when an increase in O₂ availability drives uncontrolled luminal expansion of Enterobacteriacea (Rivera-Chávez et al., 2016). These findings indicate that changes in O₂ concentrations or a partial increase in O₂ tension around colonocytes,


FIGURE 7.1 O₂ inducible O₂- and ROS-reducing detoxification enzyme complex in the obligate anaerobe *Clostridium acetobutylicum*. The apparent K_m values for O₂ and H₂O₂ (2.9 ± 0.4 μ M and less than 1 μ M, respectively) are enough to reduce trace amounts of O₂ and H₂O₂ in the growth environments (dissolved-O₂ concentration in air-saturated medium is approximately 210 μ M at 37°C) (Kawasaki et al., 2009a). Similar enzyme complexes have been found in obligate anaerobes, such as *Bacteroides* species and sulfate-reducing bacteria. *Dsr*, Desulfoferrodoxin; *FprA*, A-type flavoprotein; *NROR*, a master electron donor protein NADH :rubredoxin oxidoreductase; *Rbr*, rubrerythrin homolog; *Rd*, rubredoxin.

which is caused by a disturbance in the gut microbiome ecosystem, can promote gut dysbiosis. O_2 plays a critical role in the growth and metabolic activity of gut microbes, and especially that of anaerobes. It is important to understand the physiology of anaerobes under a variety of O_2 conditions to maintain a healthy gut shaped by gut-anaerobes.

7.2.1 Response of Obligate Anaerobes to O_2 and Reactive Oxygen Species

Aerobes possess a respiratory chain that utilizes O_2 as an efficient electron acceptor and produces nontoxic H_2O by a four-electron reduction. O₂ is also reduced by several biological and chemical reactions in vivo by a one-electron reduction that produces superoxide anion O_2^- , a two-electron reduction that produces hydrogen peroxide (H₂O₂), and a three-electron reduction that produces hydroxyl radical (·OH). These O2-reduced products are called reactive oxygen species (ROS), and they are highly toxic to cells. Aerobes harbor efficient systems to detoxify ROS, with enzymes, such as catalase and superoxide dismutase (SOD). The growth of anaerobes is inhibited in the presence of O_2 . Specifically, obligate anaerobes are defined as microbes that cannot form colonies at levels above $0.5\% pO_2$ (Loesche, 1969). Deleterious effects of ROS scavenging have long been speculated to contribute to growth inhibition of these obligate anaerobes in aerated conditions, because anaerobes lack catalase and SOD (McCord et al., 1971). However, recent studies have identified O₂-inducible oxygen and ROS-reducing enzyme complex composed of flavoproteins, rubrerythrin, superoxide reductase, and rubredoxin. This is a valuable system to scavenge O_2 and ROS with high affinity for substrates (Kawasaki et al., 2009a) (Fig. 7.1). This enzyme complex is distributed only in obligate anaerobes, such as sulfate-reducing bacteria (Lumppio et al., 2001), Clostridium (Kawasaki et al., 2005, 2009a), and Bacteroides (Mishra and Imlay, 2013). By using these systems, Clostridium and Bacteroides species can consume O₂, which may be important in generating an anoxic environment for anaerobiosis (Baughn and Malamy, 2004; Kawasaki et al., 2004, 2009a; Mishra and Imlay, 2013). These findings indicate that obligate anaerobes possess useful systems to maintain their obligate anaerobiosis in aerated environments and avoid oxidative damage.

7.2.2 Effect of O₂ on the Growth of Bifidobacterium

The O_2 and ROS-reducing enzyme complex described earlier is not conserved in the genomes of O_2 -tolerant anaerobes and anaerobes, such as lactic acid bacteria, *Bifidobacterium*, and *Propionibacterium*. Bifidobacteria and lactic acid bacteria are considered beneficial in the low O_2 environment of human intestines. Bifidobacteria and O_2 -sensitive lactic acid bacteria share similar niches in the human gut, but bifidobacteria are more sensitive to O_2 than lactic acid bacteria and produce acetic acid, in addition to lactic acid, under anoxic conditions.

Bifidobacterial species are classified as anaerobes and their O_2 sensitivities are reported to differ among species. Since Tissier (1900) reported the isolation of *Bifidobacterium bifidum*, bifidobacterial species have been isolated using anaerobic cultivation techniques. The O_2 sensitivities of isolated strains have been estimated using several culture methods, such as stab and plate cultures (Jones and Collins, 1986), static cultures with stirring (Ahn et al., 2001), or liquid culture with adjusted shaking speeds in the presence of O_2 (Kawasaki et al., 2006; Simpson et al., 2005). The influence of O_2 on the growth of *Bifidobacterium* was investigated by de Vries and Stouthamer (1969). They



FIGURE 7.2 Growth of *Bifidobacterium* strains in liquid shaking cultures under various O_2 concentrations. *Circles*, 100% N_2 ; squares, 5% O_2 –95% N_2 ; triangles, 10% O_2 –90% N_2 ; diamonds, 20% O_2 –80% N_2 . Strains tested were the type strains of species obtained from the culture collection centers DSM or JCM.

cultured several bifidobacterial strains in liquid shaking flasks under aerated conditions and reported a correlation between O_2 sensitivity and H_2O_2 production in the tested strains. Shimamura et al. (1992) reported that *B. adolescentis* showed an O_2 -sensitive growth profile, with a low level of NADH peroxidase activity detected. Meile et al. (1997) isolated highly O_2 -tolerant *B. animalis* subsp. *lactis* UR1 (DSM 10104^T) from fermented milk. Simpson et al. (2004) isolated aerotolerant *B. psychraerophilum* from a pig caecum. This species is reported to develop colonies on MRS agar plates under aerobic conditions; therefore, it is one of the most O_2 -tolerant species in the genus *Bifidobacterium*.

To evaluate and standardize O_2 sensitivities among bifidobacteria, several type strains of *Bifidobacterium* species were cultured in liquid at a constant shaking speed under different O_2 concentrations (Hayashi et al., 2013; Kawasaki et al., 2006). The growth of representative species is shown in Fig. 7.2. The resulting growth of bifidobacterial



FIGURE 7.3 Classification of strains based on O_2 tolerance. Strains tested were the type strains of species obtained from the culture collection centers DSM or JCM. The growth of *B. boum*, *B. thermophilum*, and *B. longum* was enhanced in the presence of O_2 in comparison with anoxic conditions (listed in the column of "With growth stimulation").

species was classified into four groups: O_2 -hypersensitive, O_2 -sensitive, O_2 -tolerant, and O_2 -hypertolerant (Fig. 7.3). *B. longum* subsp. *infantis* and *B. adolescentis* have O_2 -hypersensitive profiles, with growth inhibited in 5% O_2 conditions. The O_2 -sensitive group, which grows in a 5% O_2 atmosphere in liquid shaking culture, contains *B. bifidum* (the type species of the genus *Bifidobacterium*), *B. breve*, *B. longum*, and *B. animalis* subsp. *animalis*. The O_2 -tolerant group contains *B. asteroides*, *B. indicum*, *B. minimum*, and *B. animalis* subsp. *lactis*. *B. asteroides* and *B. indicum* are isolates from a honeybee hindgut (Scardovi and Trovatelli, 1969), and *B. minimum* is an isolate from sewage (Scardovi and Trovatelli, 1974). The O_2 -hypertolerant group, which shows enhanced growth in the presence of O_2 (Fig. 7.3), contains *B. boum* and *B. thermophilum*—both isolated from bovine intestinal lumens (Scardovi et al., 1979). The bee hindgut, bovine rumen, and pig cecum are considered rich in O_2 , suggesting that isolates from nonhuman sources have adapted to the O_2 conditions present in their host organisms. For example, bifidobacteria isolated from bee hindgut, such as *B. asteroides* and *B. indicum*, are shown to possess catalase activity (Hayashi et al., 2013; Jones and Collins, 1986) and *B. actinocoloniiforme* (Killer et al., 2011) and *B. bombi* (Killer et al., 2009) conserve genes of catalase homolog, which is a rare characteristic among bifidobacteria and suggests the evolution of these species in response to their growth environments. The classifications based on O_2 -sensitivities of bifidobacterial strains described in this chapter generally coincide with those from previous reports by Simpson et al. (2005) and Talwalkar and Kailasapathy (2003).

7.2.3 Enzymes for ROS Detoxification in Bifidobacterium

Bifidobacterium species are known to produce H_2O_2 in the presence of O_2 (de Vries and Stouthamer, 1969; Kawasaki et al., 2006; Shimamura et al., 1992). The production of H_2O_2 is detected only in O_2 -sensitive and O_2 -hypersensitive species, but not in O_2 -hypertolerant and O_2 -tolerant species, suggesting that H_2O_2 production or decomposition may inhibit bifidobacterial growth. CO_2 can have a stimulatory effect on bifidobacterial growth and influences on its O_2 tolerance (Kawasaki et al., 2007). Previous reports have revealed that the addition of catalase to medium (Kawasaki et al., 2006) or introduction of ROS-detoxifying enzymes into O_2 -sensitive *Bifidobacterium* strains (He et al., 2012; Zuo et al., 2014) improved the growth shown by these bacteria in the presence of O_2 , indicating that H_2O_2 production strongly correlates with aerobic growth inhibition. H_2O_2 is produced directly from the enzymatic reduction of O_2 or by the spontaneous reaction of O_2^- . SOD is an enzyme that detoxifies O_2^- and that is widely conserved in bacteria, including both aerobes and anaerobes. Although almost every bifidobacterial species lacks SOD, genes encoding SOD homologs are conserved in the genomes of a few species, such as *B. psychraerophilum*, *B. mongoliense*, *B. subtile*, and *B. actinocoloniiforme*. As for the enzymes involved in H₂O₂ decomposition, previous studies show the existence of H₂O₂ splitting activities in some *Bifidobacterium* species (Shimamura et al., 1992; Uesugi and Yajima, 1978); however, none of the enzymes that decompose H_2O_2 in bifidobacteria have been purified or characterized, except for hemecatalase from B. asteroides (Hayashi et al., 2013). Genes that encode homologs of ROS-detoxifying enzymes found in lactic acid bacteria are conserved among *Bifidobacterium* species. A homolog of the gene encoding NADH peroxidase, which is well characterized in Streptococcus species, including Streptococcus faecalis (Enterococcus faecalis, P37062)



FIGURE 7.4 Reaction of *b*-Type dihydroorotate dehydrogenase (DHODb) under anaerobic and aerobic conditions. *B. bifidum* DHODb is a heterotetramer composed of two PyrDb subunits and PyrK subunits.

(Ross and Claiborne, 1991), and *Streptococcus pneumoniae*, is conserved (with low amino acid sequence identities) in the genomes of bifidobacteria (i.e., YP_003971452 in *B. bifidum*, which has been annotated as NADH oxidase), but the function of this protein has not been determined through enzyme purification. Alkylhydroperoxide reductase, which decomposes H_2O_2 through the AhpF-AhpC system in lactic acid bacteria, is also conserved in bifidobacterial genomes (i.e., YP_003970217 in *B. bifidum*), and thioredoxin reductase-like protein (i.e., YP_003970218 in *B. bifidum*) is believed to function in place of AhpF. This enzyme complex in the genus *Bifidobacterium* has not been characterized, but Zuo et al. (2014) reported that overexpression of the gene encoding AhpC in *B. longum* subsp. *longum* NCC2705 increases the ability of this bacterium to scavenge endogenously generated H_2O_2 .

7.2.4 Enzymes for H_2O_2 Production in Bifidobacterium

As mentioned earlier, enzymes for H₂O₂ detoxification appear to be encoded in the genomes of *Bifidobacterium* species; however, H₂O₂ production is detected in many O₂-sensitive species. Therefore, high levels of H₂O₂ production or weak H_2O_2 decomposition activity in O_2 -sensitive bifidobacteria may explain this phenomenon. Pyruvate oxidase (POX), lactate oxidase, and NAD(P)H oxidase are enzymes expected to play roles in the production of H_2O_2 in these bacteria. POX in lactic acid bacteria, including Lactobacillus plantarum POX (accession no. P37063) (Muller and Schulz, 1993) and S. pneumoniae SpxB (accession no. Q54970) (Spellerberg et al., 1996), are well characterized. These POX enzymes show high similarity and both play a key role in O₂ metabolism. A homology search using L. plantarum POX returned homologs in the genomes of restricted bifidobacterial species, such as *B. psychraerophilum* (accession no. WP_051921305, 60% identity), B. crudilactis (accession no. WP_034254413, 60% identity), B. subtile (accession no. KFJ05302, 59% identity) and B. asteroides (accession no. WP_045924872). Other species, including B. bifidum and B. longum subsp. longum, do not harbor gene encoding POX homologs. In contrast, homologs of lactate oxidase and glucose oxidase have not been found in the genomes of bifidobacteria. The activities of these H₂O₂-forming oxidases, except for NAD(P)H oxidase, have not been detected in the tested Bifidobacterium strains (Kawasaki et al., 2006). In the type species of the genus *Bifidobacterium*, *B. bifidum*, activity of an H₂O₂-forming type of NADH oxidase has been detected in crude extracts from microaerobically grown cells (Kawasaki et al., 2009b). A purified enzyme with heterodimer subunits was identified as a *b*-Type dihydroorotate dehydrogenase (DHODb), a flavoenzyme that contains FAD and FMN. DHODb is an enzyme that catalyzes the oxidation of dihydroorotate to orotate in pyrimidine biosynthesis. The purified protein catalyzes the DHODb reaction under anaerobic conditions and H_2O_2 -forming NADH oxidase reaction under aerobic conditions (Fig. 7.4). According to kinetic studies, B. bifidum DHODb is believed to be involved in H₂O₂ production in *B. bifidum* under highly aerated environments, but its function has not been confirmed in vivo owing to the lack of gene-disruption techniques for this bacterium.

 O_2 -sensitivities differ among bifidobacterial species, and these sensitivities are used to classify *Bifidobacterium* species and strains. Probiotic activities also differ among species and strains, indicating that these valuable activities are affected by O_2 conditions of food products or the intestinal status of host organisms. To date, only a few enzymes have been implicated in H_2O_2 production and H_2O_2 decomposition. Additional approaches to investigate the mechanisms underlying O_2 sensitivities and O_2 tolerances of bifidobacteria improve these probiotic activities, as well as giving scope to develop commercially valuable O_2 -sensitive strains for manufacturing processes in aerobic environments.

7.3 BILE ACIDS AS ANTIMICROBIALS FOR GUT MICROBES

In the intestines, gut microbes experience various kinds of environmental stresses, including antimicrobials and nutrient availability, according to their location. Interactions between gut microbes and such environmental stresses seem to define, in part, the gut microbiota composition at different locations in the intestine. It is generally believed that various types of host factors, such as digestive enzymes, bile acids, α -defensin, immunoglobulin A antibodies, and short chain fatty acids (SCFAs) produced by fermentation in the intestine, constitute the major growth inhibitors of gut microbes. Nutrient availability for gut microbes differs in the intestines; mono- and disaccharides, such as glucose, fructose, sucrose, and lactose are abundant in the small intestine while indigestible oligosaccharides and fibers are the major carbon sources in the large intestine. Thus, the variation in available carbon sources throughout the intestine contributes to the selective pressures affecting gut microbes according to their assimilation capacities. Our studies have demonstrated that bile acids are deeply involved in the determination of gut microbiota composition, based on their strong antimicrobial activities (Islam et al., 2011; Kurdi et al., 2006). In this section, bile acids will be described in terms of their typical role in lipid digestion and their antimicrobial activity to regulate gut microbiota composition in vivo.

7.3.1 Functions of Bile Acids in Humans: Lipid Digestion and Antimicrobial Activity

Bile acids are steroid compounds synthesized in the liver as primary bile acids and are subsequently secreted into the duodenum as the main component of bile. In humans, cholic acid (CA), which has α -hydroxy groups at C3, C7, and C12, and chenodeoxycholic acid (CDCA), which has α -hydroxy groups at C3 and C7 of the steroid nucleus, are the typical primary bile acids (Fig. 7.5). In the liver, CA and CDCA are synthesized in their conjugated forms; their carboxyl groups are amide bonded either with taurine or glycine, rendering their p K_a values ~4–5 for glycine conjugates and ~1 for taurine conjugates (Cantafora et al., 1987). These conjugated bile acids exhibit emulsifying activity as detergents to help lipid digestion and absorption in the small intestine. This ability results from their unique molecular structures. Bile acid molecules form hydrophilic α -surfaces and hydrophobic β -surfaces (Fig. 7.5), thus rendering them biplanar amphiphiles. A larger amount of bile is excreted into the duodenum on a high-fat diet than on a normal diet, as it is necessary for lipid digestion (Reddy, 1981). Thereafter, bile acids are absorbed in the distal ileum and are returned to the liver through the portal vein for recycling. This process is known as enterohepatic circulation. However, small amounts of bile acids (\sim 5% of the total flow; excretion into feces amounts to 300–600 mg/ day) escape this recycling process and flow into the large intestine where they undergo biotransformation by gut microbes (Ridlon et al., 2006). The first step of biotransformation is deconjugation to yield free bile acids, such as CA and CDCA. Deconjugation increases the pK_a of the bile acid molecules to ~5 (Cantafora et al., 1987), which increases their hydrophobicity and in turn decreases their emulsifying activity as detergents. In the subsequent step, modifications of their steroid nuclei occur, yielding various types of secondary bile acids. These free bile acids, which are either primary or secondary, exhibit increased affinity for bacterial cell membranes, and bactericidal activity through interaction with the phospholipid bilayer resulting in membrane damage. As described in the next section, deoxycholic acid (DCA), a representative secondary bile acid in the intestines of humans and rodents, exhibits quite strong bactericidal activity (Kurdi et al., 2006). Therefore, bile acids may act as environmental stressors to gut microbes, suggesting a dual role of bile acids in both helping lipid digestion in the small intestine and controlling gut microbiota composition in the large intestine.

7.3.2 Bile Acid Metabolism by Gut Microbes and the Resulting Bile Acid Composition in the Large Intestine

As mentioned in the previous section, gut microbes metabolize the primary bile acids CA and CDCA into various types of secondary bile acids in the large intestine. Briefly, the bioconversion reactions that occur include dehydrogenation and epimerization of the α -hydroxy groups at C3, C7, and C12, which are catalyzed by hydroxysteroid dehydrogenases (HSDHs) in a single (dehydrogenation) step or in two successive (epimerization) steps (Ridlon et al., 2006). Furthermore, the α -hydroxy group at C7 is removed by multistep reactions catalyzed by a limited number of species of *Clostridium*, yielding DCA and lithocholic acid (LCA) from CA and CDCA, respectively. DCA may be further metabolized by HSDH (Ridlon et al., 2006). In addition to CA and CDCA, rodents use muricholic acids (MCAs), which contain hydroxy groups at C3, C6, and C7. Epimers at C6 and C7 are synthesized by host metabolism, resulting in the formation of four types of MCAs, namely α , β , λ , and ω MCAs (Fig. 7.5). Elimination of the α -hydroxy group at C7 occurs to yield hyodeoxycholic acid by biotransformation by a gut microbe (Eyssen et al., 1999). Thus, the molecular bile acid species of humans and rodents are quite complex (Fig. 7.5).

In spite of such complexity, bile acid composition in vivo comprises only several major bile acid species. In the human large intestine, DCA and LCA account for ~65% of the total bile acids, and other molecular species include CA, CDCA, 12-oxo-LCA (the C12 dehydrogenated derivative of DCA), and ursodeoxycholic acid (the 7 β epimer of CDCA), which each account for ~2%–3%, amounting in total for 75% of all bile acids present. The remaining bile acids constitute only minor components (Ridlon et al., 2006). In rodents, the fecal bile acid composition of rats

Bile acids

Orientation of substituents



	No. of carbon atom at bile acid skeleton			
	3	6	7	12
Cholic acid (CA)	αΟΗ	Н	αOH	αΟΗ
Chenodeoxycholic acid (CDCA)	αΟΗ	Н	αOH	н
Deoxycholic acid (DCA)	αΟΗ	Н	н	αOH
Ursocholic acid (UCA)	αΟΗ	Н	βОН	αOH
Ursodeoxycholic acid (UDCA)	αOH	н	βОН	Н
7-oxo-deoxycholic acid (7-oxo-DCA)	αΟΗ	Н	0	αOH
7-oxo-lithocholic acid (7-oxo-LCA)	αΟΗ	Н	0	Н
12-oxo-lithocholic acid (12-oxo-LCA)	αΟΗ	Н	Н	0
3-oxo-12 α -hydroxy-5 β -cholan-24-oic acid	0	н	н	αOH
α -Muricholic acid (α MCA)	αOH	βОН	αOH	Н
β-Muricholic acid (βMCA)	αΟΗ	βОН	βОН	Н
λ -Muricholic acid (λ MCA)	αΟΗ	αΟΗ	αΟΗ	Н
ω-Muricholic acid (ωMCA)	αΟΗ	αΟΗ	βОН	н
Hyodeoxycholic acid (HDCA)	αOH	αΟΗ	н	н

FIGURE 7.5 Structure of bile acids detected in human and rodent intestines. "R" in the bile acid skeleton represents either a carboxyl group for free bile acids or a conjugation with taurine or glycine for conjugated bile acids. *Source: Adapted from Islam, K.B.M.S., Fukiya, S., Hagio, M., Fujii, N., Ishizuka, S., Ooka, T., et al., 2011. Bile acid is a host factor that regulates the composition of the cecal microbiota in rats, Gastroenterology 141, 1773–1781.*

fed a normal diet comprises mainly ω -MCA, β -MCA, HDCA, and DCA (up to 92% of the total bile acids) (Hagio et al., 2011). Thus, particular bile acid compositions are formed according to a combination of diet and gut microbiota in different animal species.

7.3.3 Antimicrobial Activity of Bile Acids: Mechanism of Action and Structure-Activity Relationship

Bile acids have been studied mainly in terms of their molecular diversity and their physicochemical properties in relation to micelle formation with lipids (Armstrong and Carey, 1982; Roda et al., 1990). On the other hand, the

Strains	CA (mM)	DCA (mM)
Bifidobacterium breve JCM 1192^{T}	5.0	0.5
B. breve JCM 7017	3.0	0.55
B. pseudocatenulatum JCM 1200^{T}	3.0	0.45
B. gallicum JCM 8224 ^T	7.0	0.45
B. longum JCM 1217^{T}	9.0	0.55
B. bifidum JCM 1255 [™]	5.0	0.3
<i>B. adolescentis</i> JCM 1275^{T}	5.0	0.4

 TABLE 7.1
 Concentrations of CA and DCA Yielding Complete Growth Inhibition in Bifidobacteria

All data are the means of two separate experiments. For detailed experimental conditions, refer to

Kurdi et al. (2006). CA, Cholic acid; DCA, deoxycholic acid.

Adapted from Kurdi, P., Kawanishi, K., Mizutani, K., Yokota, A., 2006. Mechanism of growth inhibition by free bile acids in lactobacilli and bifidobacteria. J. Bacteriol. 188, 1979–1986.

antimicrobial activity of bile acids, although recognized for more than a century (Stacey and Webb, 1947), has not been a popular topic of research. While the membrane-damaging effects of bile acids was deduced to be an underlying factor contributing to their bactericidal activity, little information is known concerning the contribution of the effects of micelle formation. Recently, bile tolerance was recognized as a requirement for probiotics, and an increasing number of studies characterizing bile tolerant mutants of bifidobacteria and lactic acid bacteria have been published (Margolles et al., 2003). However, the antimicrobial activity of only a limited number of bile acids, including CA, CDCA, DCA, 7-oxo-DCA, and 3 β -DCA, has been studied using *Staphylococcus aureus* or other bacterial strains as indicators (Binder et al., 1975; Devlin and Fischbach, 2015; Floch et al., 1970, 1971, 1972; Stacey and Webb, 1947). In terms of MCAs, the antimicrobial activities of α -, β -, and ω -MCAs against *Clostridium difficile* have been reported only recently (Francis et al., 2013).

Previously, we investigated the antimicrobial activity of CA and DCA against various lactobacilli (nine strains in six species) and bifidobacteria (seven strains in six species) and found that CA (~6 mM) and DCA (~0.3–0.8 mM) completely inhibited the growth of these bacteria, with DCA exhibiting 10-fold higher antimicrobial activity than CA (Kurdi et al., 2006) (Table 7.1). Similarly, CDCA also showed strong antimicrobial activity when tested using *B. breve* (Kurdi et al., 2006). Based on these results, we investigated the mechanism underlying the bactericidal activity of bile acids using *B. breve* as a model strain (Kurdi et al., 2006). We found that the antimicrobial activity of bile acids stemmed from a disruption of cell physiology caused by damage to the cell membrane. In the presence of CA, DCA, and CDCA at minimum concentrations required for complete growth inhibition, decreases in both internal pH (Fig. 7.6A for DCA) and membrane potential leading to the dissipation of the proton-motive force (i.e., loss of



FIGURE 7.6 Effects of (A) DCA and (B) SCFAs on the internal pH of *B. breve* JCM1192^T. *Glc*, glucose; *Nig*, nigericin; *SCFAs*, short-chain fatty acids. *Source: Adapted from Kurdi*, *P., Tanaka*, *H., van Veen*, *H. W., Asano*, *K., Tomita*, *F., Yokota*, *A., 2003. Cholic acid accumulation and its diminution by short-chain fatty acids in bifdobacteria*. *Microbiology* 149, 2031–2037; *Kurdi*, *P., Kawanishi*, *K., Mizutani*, *K., Yokota*, *A., 2006. Mechanism of growth inhibition by free bile acids in lactobacilli and bifdobacteria*. J. Bacteriol. 188, 1979–1986. For detailed experimental conditions, refer to *Kurdi et al.* (2003, 2006).

7.3 BILE ACIDS AS ANTIMICROBIALS FOR GUT MICROBES

			· · · · ·	-	
		Membrane integrity (%)		Viabi	lity (%)
Treatments		1 h	3 h	1 h	3 h
CA	0.1 mM	87.9 ± 5.29	93.12 ± 13.51	112.5 ± 12.02	106.4 ± 16.33
	1 mM	92.68 ± 5.73	90.71 ± 1.22	49.85 ± 8.84	14.5 ± 1.41
	2 mM	88.53 ± 12.41	89.04 ± 1.36	14.67 ± 2.83	3.96 ± 2.06
	4 mM	47.56 ± 6.07	62.00 ± 13.66	0.86 ± 0.06	0.14 ± 0.04
	6 mM	14.19 ± 3.54	18.73 ± 0.19	0.16 ± 0.11	0.03 ± 0.01
	8 mM	13.63 ± 0.52	18.31 ± 3.42	0.03 ± 0.02	0.006 ± 0.003
DCA	0.01 mM	75.1 ± 5.87	68.51 ± 3.80	111.0 ± 17.0	101.61 ± 5.23
	0.1 mM	67.15 ± 18.35	58.97 ± 2.30	82.39 ± 14.83	67.65 ± 3.61
	0.2 mM	67.6 ± 18.34	60.24 ± 8.89	42.42 ± 4.41	27.46 ± 14.35
	0.4 mM	27.08 ± 4.06	32.09 ± 5.52	4.06 ± 2.50	0.19 ± 0.09
	0.6 mM	6.11 ± 2.67	6.44 ± 1.81	0.10 ± 0.04	0.05 ± 0.02
	0.8 mM	4.47 ± 0.19	7.77 ± 0.17	0.007 ± 0.003	0.002 ± 0.001
SCFA	117 mM	91.73 ± 2.34	84.17 ± 3.50	33.18 ± 16.33	39.39 ± 3.89

TABLE 7.2 Membrane Integrity and Viability of Bifidobacterium breve JCM 1192^T Upon Exposure to CA, DCA, and SCFA Mixture

Results are shown as means \pm SD, where $n \ge 2$. For detailed experimental conditions, refer to Kurdi et al. (2006). CA, Cholic acid; DCA, deoxycholic acid; SCFA, short-chain fatty acids.

Adapted from Kurdi, P., Kawanishi, K., Mizutani, K., Yokota, A., 2006. Mechanism of growth inhibition by free bile acids in lactobacilli and bifidobacteria. J. Bacteriol. 188, 1979–1986.

biological energy) were observed. These results indicate dissipation of the transmembrane proton gradient caused by membrane damage. Indeed, potassium ions also leaked from the cells in the presence of CA above the minimum concentration required to inhibit cell growth completely. The degree of membrane damage was correlated with a decrease in viable cells after exposure to CA and DCA (Table 7.2). From these observations, it can be concluded that the mechanism of growth inhibition by bile acids at a minimum concentration required for growth inhibition was not by membrane solubilization (micelle formation) but by membrane damage, since the bile acid concentrations were far lower than the critical micellar concentrations (CMCs) (CA, \sim 11–13 mM; DCA, \sim 3–10 mM; CDCA \sim 4–9 mM) (Roda et al., 1990). On the other hand, macromolecules, such as proteins leaked from the cells only under conditions where CA was applied above its CMC (Kurdi et al., 2006), indicating that solubilization of the membrane did not occur at the minimum CA concentration required for growth inhibition.

As mentioned in the previous section, the steroid skeleton of a bile acid molecule forms a hydrophilic α -surface and a hydrophobic β -surface, rendering the molecule a biplanar amphiphile (Fig. 7.5). Two models can explain the interaction of bile acid molecules with biological membranes (Fig. 7.7) (Hofmann, 1994). In the first, the hydrophobic β -surface rests on the membrane surface and hydrophobic–hydrophobic interactions force the phospholipid membrane surface apart, allowing the β -surface to contact the hydrocarbon chain of the phospholipid molecules. In the other model, bile acid molecules enter into the cell membrane as a face-to-face dimer, with the hydrophilic α -surface on the inside and the hydrophobic β -surface on the outside. Bile acids can damage bacterial cell membranes in either of these arrangements at concentrations lower than the CMC, thus exhibiting bactericidal activity. According to these models, the higher the hydrophobicity of the bile acid molecule, the stronger the interaction between the bile acid molecule and the membrane. This explains the fact that the more hydrophobic DCA molecule with two functional hydroxy groups has higher bactericidal activity than the less hydrophobic CA molecule, which has three functional hydroxy groups. On the other hand, conjugated bile acids do not have antimicrobial activity (Binder et al., 1975; Floch et al., 1971). Although conjugated bile acids can form micelles as amphiphiles, the negatively charged hydrophilic (polar) moiety of these molecules prevents them from accessing the negatively charged phospholipid bilayer due to electrostatic expulsion. In fact, taurocholic acid (TCA) has been reported not to cross the phospholipid bilayer (Kamp and Hamilton, 1993). This is in agreement with our observation that *L. lactis*, a cheese starter lactic acid bacterium, was able to grow in the presence of 100 mM TCA, while 6 mM CA completely inhibited its growth under the same culture conditions (Yokota et al., 2000).



FIGURE 7.7 Schematic representation of the interaction between DCA molecules and phospholipid molecules in bacterial cell membrane. Closed ovals and open circle represent α-hydroxy groups and carboxyl group in a DCA molecule, respectively. Carbon atom numbers are indicated. See text for description.

7.3.4 Cytotoxicity of SCFAs: Comparison With the Antimicrobial Activity of Bile Acids

In the large intestine of humans and rodents, SCFAs, such as acetic acid, propionic acid, and butyric acid accumulate due to the fermentation of indigestible carbohydrates by gut microbes. However, little is known concerning the cytotoxicity effects of these SCFAs on gut microbes. Thus, we investigated the effect of SCFAs on the intracellular pH of *B. breve* cells (Kurdi et al., 2003). When a mixture of SCFAs at a total concentration of 117 mM, mimicking the in vivo concentration of SCFAs in the large intestine, was added to glucose-energized *B. breve* cells, the internal pH instantly lowered from 7.3 to 6.8. However, the pH returned to the neutral range (~7.0) within a few minutes (Fig. 7.6B). This response was different from that observed when cells were challenged with free bile acids, such as DCA, during which the internal pH showed a stepwise decrease upon each addition of DCA (Fig. 7.6A) (Kurdi et al., 2006).

These different responses can be interpreted as follows. *B. breve* cells obtain biological energy, ATP, by glycolysis to produce fermentation end-products, such as acetic acid and lactic acid, thus lowering the internal pH. To prevent this acidification, the cells pump out protons using the F_1F_0 -ATP synthase to maintain an internal pH higher than that of the external environment, at around 7.3 (formation of the concentration gradient of protons across the cell membrane, ΔpH). Because SCFAs are weak acids with pK_a values of ~4.7–4.8, protonated molecules can permeate the cell membrane. Under these conditions, some external SCFAs can be instantly internalized into the cells and dissociate to release protons, thus acidifying the intracellular environment. The dissociated SCFA molecules accumulate in the cells due to their polarity. These processes are driven by ΔpH and continue according to the Henderson-Hasselbalch equation until the concentration of the protonated SCFA molecules on each side of the membrane becomes equal. Under these conditions, the F_1F_0 -ATP synthase works to return the internal cellular pH to neutral. However, the internal pH is unable to return to the original value due to the accumulated SCFAs. These processes are possible as long as the integrity of the cell membrane is conserved.

On the other hand, when CA, CDCA, and DCA are applied, they result in acidification of the intracellular space, similar to SCFAs, but also damage the cell membrane, and thus a decrease in Δ pH also occurs in response to the added levels of bile acids. There is an effort to return the internal pH to neutral by the F₁F₀-ATP synthase and a steady state proton concentration is reached in response to bile acid addition, resulting in the stepwise decrease in internal pH.

From these observations, it was considered that the cytotoxicity of SCFAs was due to metabolic disorders resulting from acidification of the intracellular pH. However, the extent of growth inhibition caused by SCFAs does not seem prominent. It has previously been reported that only a slight decrease in growth rate was observed after addition of 100 mM acetic acid to *S. bovis* cultures when the external pH was less than 6.0 (Russell, 1991). We also demonstrated that exposure of *B. breve* cells to a mixture of SCFAs (117 mM for 1–3 h) did not cause membrane damage or viability loss (Kurdi et al., 2006). Therefore, SCFAs do not seem to cause strong environmental stress in the large intestine, unlike bile acids.

7.3.5 Bile Acids as Host Factors to Control Gut Microbiota Composition

Our efforts to clarify the bactericidal activity and associated underlying mechanism of bile acids using lactic acid bacteria and bifidobacteria led us to investigate how bile acids affect the structure of gut microbiota in vivo. In this



FIGURE 7.8 Alterations of cecal microbiota populations at the phylum level in rats upon feeding a CA-supplemented diet for 10 days. Source: Adapted from Yokota, A., Fukiya, S., Islam, K.B.M.S, Ooka, T., Ogura, Y., Hayashi, T., et al., 2012. Is bile acid a determinant of the gut microbiota on a high-fat diet? Gut Microbes 3, 455–459. See text for description.

context, several studies performed in the early 1970s reported growth inhibition of representative intestinal bacteria by free bile acids, such as CA, CDCA, and DCA, and predicted their role in the control of intestinal bacterial populations in vivo (Binder et al., 1975; Floch et al., 1971, 1972). Thus, we investigated this possibility (Islam et al., 2011).

Rats were fed either a control diet (control group) or a CA-supplemented diet (CA group), in which 0.05% (w/w) sodium cholate was added to the control diet, for 10 day. Analysis of fecal bile acids revealed increased total bile acids, especially of DCA in the CA group. Interestingly, a dramatic alteration of cecal microbiota composition at the phylum level was observed in the CA group, in which Firmicutes predominated at the expense of Bacteroidetes (Fig. 7.8). At the class level, increases in Clostridia and its closely related class, Erysipelotrichi, accounted for the predominance of Firmicutes. These alterations accompanied with the reduction of total bacterial counts to 62%. Furthermore, bacteria were isolated from cecal samples and their DCA sensitivities were investigated to understand the basis of gut microbiota alterations. Several Firmicutes isolates showed higher DCA resistance than Bacteroidetes isolates. These results coincided with the observed shift of gut microbiota populations, and thus, we concluded that bile acids are a host factor that controls the gut microbiota population in vivo.

To our surprise, these changes were very similar to gut microbiota alterations seen in humans eating a Western diet, which is equivalent to a high-fat diet (Yokota et al., 2012). As bile excretion increases upon exposure to a high-fat diet, the elevated antimicrobial activity in the large intestine may provoke similar changes to those observed in the CA group. Thus, we have proposed the "bile acid hypothesis" in which we speculate that bile acids may be a factor responsible for gut microbiota alterations upon high-fat feeding (Yokota et al., 2012). Recently, it has been shown that the gut microbiome is deeply involved in host health and disease development (Clemente et al., 2012). It is well known that diet-induced obesity leading to metabolic syndrome is accompanied by dysbiosis. However, the driving forces that alter gut microbiota composition upon administration of high-fat diet and the causality between altered gut microbiota and disease development are not yet clearly understood. Therefore, proof of the "bile acid hypothesis" is warranted to clarify the possible role of bile acids in disease development through gut microbiota alterations upon high-fat feeding.

7.4 CONCLUDING REMARKS

In this chapter, we describe toxicity of O_2 and bile acids on the growth of gut microbes, including bifidobacteria and the recent progress in the study of underlying mechanisms for O_2 tolerance in anaerobic gut microbes. Clarifying the mechanisms of sensitivity/resistance to these factors may contribute to improve prospective development of efficient probiotics, as well as the maintenance of host health, as these stress factors may induce imbalanced gut microbiota composition.

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Carbohydrate Metabolism in Bifidobacteria

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8.1 INTRODUCTION

Bifidobacteria are Gram-positive, anaerobic, nonmotile, generally Y-shaped bacteria that typically inhabit the gastrointestinal tract (GIT) of mammals and certain insects (Milani et al., 2014). As saccharolytic organisms, defined as those "capable of hydrolyzing or otherwise metabolizing a sugar molecule resulting in the production of energy," bifidobacterial colonization of the GIT is dependent on their ability to utilize the complex carbohydrates present in this environment. The term *carbohydrate* covers a range of biological molecules consisting of carbon, hydrogen, and oxygen and can be categorized depending on their degree of polymerization (DP) into monosaccharides, disaccharides, oligosaccharides (DP between 3 and 10) and polysaccharides (DP greater than 10) (Fig. 8.1). For many years, bifidobacterial carbohydrate metabolism has been the subject of a large number of investigations. Understanding carbohydrate utilization by various bifidobacterial species may explain the prevalence of such species in different niches; for example, it is believed that *B. longum* subsp. *infantis* is able to colonize the infant gut because of its ability to utilize particular oligosaccharides present in breast milk (Sela et al., 2008; Turroni et al., 2012a). The ability to utilize certain dietary carbohydrates may also be exploited to increase the abundance or metabolic activity of a particular species or strain. Such carbohydrates are known as prebiotics, defined as "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health" (Gibson et al., 2004).

8.2 CARBOHYDRATE AVAILABILITY IN THE GASTROINTESINTAL TRACT

The carbohydrates available to the microbiota in the large intestine can be diet or host derived. The carbohydratecontaining fraction present in the human diet can be separated at a basic level into two groups, based on their digestibility by host-encoded enzymes in the GIT. The first group consists of simple sugars, such as the monosaccharides glucose and fructose, and the disaccharides, lactose and sucrose, which contain just one glycosidic linkage (Koropatkin et al., 2012) and are absorbed in the small intestine either directly or following hydrolysis by host-encoded enzymes. The second group consists of complex carbohydrates, often plant cell wall polysaccharides, such as cellulose, pectin, (arabino) xylan, arabinan, and resistant starch, that are resistant to digestion by host-encoded glycosyl hydrolases (GHs) and absorption in the small intestine (Bond et al., 1980; Englyst et al., 1992; Lattimer and Haub, 2010). These complex carbohydrates may incorporate many glycosidic linkages, while certain plant-based polysaccharides, such as pectin can vary in structure between sources (Caffall and Mohnen, 2009; Koropatkin et al., 2012; Mohnen, 2008). Processes, such as milling and cooking can also affect the abundance of nondigestible (to the host) carbohydrates (Koropatkin et al., 2012). It has been estimated that between 10 and 60 g of dietary carbohydrate reaches the colon on a daily basis (Cummings and Macfarlane, 1991), thus providing a substantial nutrient supply to the gut microbiota.

While dietary glycans can fluctuate in both abundance and composition, host-derived glycans represent a more consistent nutrient source. The mucus layer, which can extend up to 150 µm from the epithelial surface (Johansson et al., 2011), provides a source of carbohydrate in the form of the mucin glycoprotein (Salyers et al., 1977). It is



FIGURE 8.1 Haworth projections of carbohydrates discussed in this chapter. Haworth projections of some of the mono-, di-, oligo-, and polysaccharides discussed in this chapter. In the case of monosaccharides, the projections represent the D- or L- form, which is predominant in the gut.

estimated that 3–4 g of mucin are fermented by the intestinal microbiota per day (Stephen et al., 1983). Other hostderived carbohydrates in the large intestine include heparin sulfate, which is present in low quantities in the mucus layer of the large intestine (Oshiro et al., 2001) and chondroitin sulfate, which is also present in the colonic mucosa (Eliakim et al., 1986). In the breast-fed infant gut, in which the *Bifidobacterium* genus is dominant (Avershina et al., 2013; Turroni et al., 2012a), human milk oligosaccharides (HMO) represent another host-derived, yet dietary, nutrient source. There are at least 200 known HMO structures, although it is thought that in an individual mother's milk, at most 50 of these structures are present at relevant levels (Ninonuevo et al., 2006; Wu et al., 2010). These structures are resistant to digestion by host enzymes (Engfer et al., 2000; Gnoth et al., 2000) and thus reach the large intestine where they can be utilized by, among other microbes, certain bifidobacteria, namely *B. bifidum*, *B. breve*, and *B. longum* subsp. *infantis* (James et al., 2016; Ruiz-Moyano et al., 2013; Sela et al., 2008; Turroni et al., 2010).

8.3 THE BIFIDOBACTERIAL GLYCOBIOME

When the first bifidobacterial genome, that of *B. longum* subsp. *longum* NCC2705, was sequenced in 2002, it was noted that more than 8.5% of the predicted proteins were assigned to the cluster of orthologous groups in the carbohydrate transport-metabolism category (Schell et al., 2002), a characteristic that was later found to be shared among the genomes of many other bifidobacterial species (Lugli et al., 2014; Milani et al., 2014; Ventura et al., 2007). The glycobiome of the *Bifidobacterium* pangenome is (relative to the corresponding genome size) among the largest of the commensal gut microbiota, with genes predicted to be involved in carbohydrate metabolism representing 13.7% of the bifidobacterial pangenome (Milani et al., 2015b). Members of the genus Bacteroides, which are dominant members of the adult microbiota, have a similarly high proportion of potential carbohydrate utilization-related genes on their genomes, usually between 10% and 20% of the identified genes of a given Bacteroides genome (McNulty et al., 2013; Turnbaugh et al., 2008; Xu et al., 2003, 2007). B. scardovii and B. biavatii encode the largest number of predicted carbohydrate utilization genes among the currently available bifidobacterial species, including carbohydrate modifying proteins, such as glycosyl hydrolases, carbohydrate esterases, glycosyl transferases, as well as carbohydrate-binding molecules (Milani et al., 2015a). Recently published pangenome analyses of specific bifidobacterial species revealed that predicted carbohydrate-active genes represent 13.23% and 12.5% of the *B. breve* and *B. longum* pangenomes, respectively (Bottacini et al., 2014; O'Callaghan et al., 2015). The genes required for the metabolism of a particular carbohydrate are frequently organized in gene clusters, typically encompassing genes predicted to encode a LacI-type transcriptional regulator, an ATP-binding cassette (ABC)-type transporter system, and one or more GHs (Schell et al., 2002).

8.4 THE FRUCTOSE-6-PHOSPHATE PHOSPHOKETOLASE PATHWAY

Bifidobacteria possess a saccharolytic metabolism and their ability to colonize and survive in the GIT is largely dependent on the ability to utilize certain diet- and/or host-derived carbohydrates. Bifidobacteria, with the possible exception of those isolated from the insect hindgut (Bottacini et al., 2012), do not possess a respiratory pathway, therefore energy production is by fermentation only. The majority of the gut microbiota is assumed to employ the glycolytic pathway or the pentose phosphate pathway when metabolizing carbohydrates (Macfarlane and Macfarlane, 2003). In contrast, the characteristic central metabolic pathway of bifidobacteria is the fructose-6-phosphate phosphoketolase (F6PPK) pathway, also known as the bifid shunt (De Vries and Stouthamer, 1967; De Vuyst et al., 2014; Scardovi and Trovatelli, 1965). The key enzyme of this pathway is F6PPK, an enzyme that is also produced by all other members of the Bifidobacteriaceae family (Gavini et al., 1996; Killer et al., 2010; Palframan et al., 2003). From 1 mole of fermented glucose, the F6PPK pathway theoretically yields 2.5 ATP molecules (Palframan et al., 2003). In comparison, fermentative glycolysis, employed by lactic acid bacteria, theoretically yields two ATP molecules from one mole of glucose. However, it should be noted that this does not take into account the energy cost of internalizing the carbohydrate substrate. In general, bifidobacteria internalize sugars through ABC-type transport systems, which require ATP (Davidson and Chen, 2004), over cost-neutral phosphoenolpyruvate-phosphotransferase systems (PEP–PTS; discussed further in this chapter) (Postma et al., 1993). Another advantage of the F6PPK pathway is that it allows for the assimilation of both pentose and hexose sugars (Fig. 8.1). The F6PPK pathway produces short-chain fatty acids (SCFAs), some of which are (directly or indirectly) perceived to be beneficial to the host (Palframan et al., 2003). The bifid shunt-mediated fermentation of hexose sugars theoretically yields 1.5 moles of acetate and 1 mole of lactate (which is not considered a SCFA), while pentose sugars result in a 1:1 ratio of acetate to lactate, although these ratios can vary depending on the bifidobacterial strain, carbon source, growth phase of the cells, and the environmental

pH (Macfarlane and Macfarlane, 2003; Palframan et al., 2003). For example, in a recent study of the carbohydrate utilization profiles of 22 different strains of bifidobacteria (across multiple species), it was shown that the ratio of lactate and acetate produced varied greatly depending on strain and/or carbohydrate source. In most strains acetate was the principal organic acid produced, with the exception of *B. pseudocatenulatum* NCIMB 8811, *B. longum* subsp. *longum* NCIMB 8809, and *B. bifidum* NCIMB 8810, which predominantly produced lactate (McLaughlin et al., 2015). In a separate study, growth rate was also shown to affect the production ratio of such metabolites. In *B. longum* BB536 and *B. animalis* subsp. *lactis* Bb12, faster growth was associated with higher lactate production and smaller amounts of acetate, formate, and ethanol. Slower growth was associated with higher amounts of acetate, formate, and ethanol. Slower growth was associated with higher amounts of acetate, formate, and ethanol. Slower growth was associated with higher amounts of acetate. (Once again, growth to fructose, oligofructose, and inulin by 18 different bifidobacterial strains was investigated. Once again, growth on less readily fermentable sugars was associated with the production of acetate, formate, and ethanol, at the expense of lactate (Falony et al., 2009). An overview of the bifid shunt and the various metabolic routes is provided in Fig. 8.2.

8.5 CARBOHYDRATE UPTAKE BY BIFIDOBACTERIA

Carbohydrate uptake by bifidobacteria is achieved through a combination of ABC-type transporters, proton symporters, and proton motive force-driven permeases, typically known as major facilitator superfamily (MFS) transporters (Pokusaeva et al., 2011a; Schell et al., 2002). An in-depth study of the carbohydrate transport systems of B. *longum* subsp. *longum* NCC2705 identified 13 ABC-type transport systems predicted to be involved in the transport of di-, tri-, and higher-order oligosaccharides, three MFS transporters predicted to internalize glucose, lactose, and sucrose, and just a single PEP–PTS for the transport of glucose (Parche et al., 2007). In contrast, on the notably smaller genome of *B. animalis* subsp. *lactis* Bl-04, just two genes specifying carbohydrate-specific ATP-binding proteins, which form part of ABC transporters, were identified (Barrangou et al., 2009). PEP-PTS systems are less frequently identified in bifidobacterial genomes compared to other uptake systems, with *B. bifidum* PRL2010 being a notable exception. This strain is predicted to encode just two ABC-type transport systems, four PEP-PTS systems, and four secondary transporters (Turroni et al., 2012b). In contrast, B. longum subsp. infantis ATCC15697 encodes 14 predicted ABC-type transport systems (Sela et al., 2008). This disparity may reflect the difference in strategies for carbohydrate utilization between these two species (see later). Four putative PEP–PTS systems were identified on the B. breve UCC2003 genome, one of which was shown to internalize fructose (Maze et al., 2007); however, carbohydrate uptake in this strain typically involves ABC-type transport systems (Egan et al., 2014b; James et al., 2016; O'Connell et al., 2013; O'Connell Motherway et al., 2013; Pokusaeva et al., 2011a). The predominance of ABC-type transporters on bifidobacterial genomes is perhaps a reflection of the availability of more complex carbohydrates rather than monosaccharides in the large intestine. ABC transporters, in particular the carbohydrate uptake transporter-1 subfamily, transport a variety of oligosaccharides (Schneider, 2001), whereas the PEP–PTS system is typically limited to monosaccharides or certain disaccharides, such as sucrose (Deutscher et al., 2006; Lengeler et al., 1982).

8.6 GLYCOSYL HYDROLASES (GHS) IN BIFIDOBACTERIA

As mentioned earlier, the carbohydrates present in the large intestine are believed to be predominantly di-, tri-, or higher order oligosaccharides, thus their metabolic processing requires them to be degraded to their constituent monosaccharides. In most cases this necessitates the involvement of a GH. GHs are responsible for the hydrolysis of the glycosidic linkage between connected monosaccharides and are found in all three kingdoms (*Archaea, Bacteria*, and *Eukarya*) (Cantarel et al., 2009; Henrissat, 1991). A glycosidic linkage is the chemical bond, in the α - or β -conformation, between two monosaccharides through a shared oxygen atom (Koropatkin et al., 2012). Hydrolysis of the glycosidic bond requires two critical residues on the enzyme, namely a proton donor and a nucleophile base. Depending on the distance between these two residues, GHs can be classified as retaining or inverting enzymes. Hydrolysis of a substrate by a retaining GH results in a product with the same anomeric configuration, while a reaction catalyzed by an inverting GH results in an inversion of the configuration (Davies and Henrissat, 1995). This characteristic is crucial in determining if an enzyme may have transglycosylation ability (discussed later).

In 1991, Bernard Henrissat devised a method to classify GHs based on their amino acid sequence similarity and, as of December 2016, there are 135 recognized GH families, which can be found at www.cazy.org (Henrissat, 1991; Lombard et al., 2014). According to the current information on www.cazy.org (December 2016), bifidobacterial genomes are predicted to encode between 25 and 126 GHs, which in turn represent between 12 and 38 GH families per genome (Table 8.1). Of these, the genome of *B. scardovii* JCM12489 encodes the highest number of GHs (126),



FIGURE 8.2 The fructose-6-phosphate phosphoketolase pathway in bifidobacteria. A schematic representation of carbohydrate degradation through the fructose-6-phosphate phosphoketolase pathway in bifidobacteria. *AckA*, Acetate kinase; *Adh2*, aldehyde-alcohol dehydrogenase 2; *Aga*, α-galactosidase; *Agl*, α-glucosidase; *AraA*, L-arabinose isomerase; *AraB*, Ribulokinase; *AtsA*, sulfatase; *Bgl*, β-glucosidase; *Eno*, enolase; *GalE1*, UDP-glucose 4-epimerase; *GalA*, β-endogalactanase; *GalG*, β-galactosidase; *GalK*, galactokinase; *GalM*, glactosemutarotase; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *GlkA*, glucokinase; *Gpi*, glucose 6-phosphate isomerase; *Gpm*, phosphoglycerate mutase; *FrK*, frucktokinase; *FoPPK*, fructose-6-phosphotetolase; *FucI*, L-fucuose isomerase; *FucA*, L-fuculose kinase; *FucA*, L-fuculose-1-phosphate aldose; *FucO*, lactaldehyde reductase; *Ldh2*, lactate dehydrogenase; *LNBP*, lacto-*N*-biose phosphorylase; *NagA*, *N*-acetylglucosamine-6-phosphate deacetylase; *NagB*, glucosamine-6-phosphate deaminase; *Pgm*, phosphoglucomutase; *Pfl*, formate acetyltransferase; *Pi*, phosphate; *Pyk*, pyruvate kinase; *RsPi*, ribokinase; *R5PE*, ribulose-5-phosphate epimerase; *Tal*, transaldase; *Tkt*, transketolase; *UgpA*, UTP-glucose-1-phosphate uridylyltransferase; *XylA*, xylose isomerase; *XylB*, xylulose kinase [based on a figure from previous review articles Pokusaeva et al. (2011a), De Vuyst et al. (2014), O'Callaghan and Van Sinderen (2016)].

TABLE 8.1 GH-Encoding Genes on Bifidobacterial Genomes

Strain names	GH families	Number of GHs
Bifidobacterium actinocoloniiforme DSM 22766	16	30
Bifidobacterium adolescentis 22L	22	61
Bifidobacterium adolescentisATCC 15703	22	58
Bifidobacterium adolescentis BBMN23	20	60
Bifidobacterium angulatum JCM 7096	15	40
Bifidobacterium angulatum GT 102	15	39
Bifidobacterium animalis A6	16	40
Bifidobacterium animalis RH	16	39
Bifidobacterium animalis subsp. animalis ATCC25527	13	34
Bifidobacterium animalis subsp. animalis	13	35
Bifidobacterium animalis subsp. lactis AD011	16	38
Bifidobacterium animalis subsp. lactis ATCC 27673	17	35
Bifidobacterium animalis subsp. lactis B420	16	38
Bifidobacterium animalis subsp. lactis BB12	16	42
Bifidobacterium animalis subsp. lactis BF052	16	40
Bifidobacterium animalis subsp. lactis Bi-07	16	40
Bifidobacterium animalis subsp. lactis Bl-04	16	38
Bifidobacterium animalis subsp. lactis Bl-12	15	36
Bifidobacterium animalis subsp. lactis BLC1	16	38
Bifidobacterium animalis subsp. lactis CNCM I-2494	16	40
Bifidobacterium animalis subsp. lactis DSM 10140	16	35
Bifidobacterium animalis subsp. lactis KLDS2.0603	16	40
Bifidobacterium animalis subsp. lactis V9	16	38
Bifidobacterium asteroides PRL2011	22	48
Bifidobacterium bifidum BGN4	22	41
Bifidobacterium bifidum ATCC29521	24	48
Bifidobacterium bifidum BF3	24	44
Bifidobacterium bifidum PRL2010	22	43
Bifidobacterium bifidum S17	22	39
Bifidobacterium breve 12L	22	47
Bifidobacterium breve 689b	27	55
Bifidobacterium breve ACS-071-V-Sch8b	22	49
Bifidobacterium breve BR3	24	53
Bifidobacterium breve DSM 20213	21	45
Bifidobacterium breve JCM 7017	24	51
Bifidobacterium breve JCM 7019	26	54
Bifidobacterium breve NCFB 2258	25	51
Bifidobacterium breve S27	24	50
Bifidobacterium breve UCC2003	26	55

TABLE 8.1 (GH-Encoding	Genes on	Bifidobacterial	Genomes	(cont.)
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Strain names	GH families	Number of GHs
Bifidobacterium catenulatum DSM 16992	20	57
Bifidobacterium coryneforme LMG18911	14	26
Bifidobacterium dentium Bd1	25	87
Bifidobacterium dentium JCM 1195	25	88
Bifidobacterium indicum LMG 11587	13	25
Bifidobacterium kashiwanohense JCM 15439	26	63
Bifidobacterium kashiwanohense PV20-2	19	44
Bifidobacterium longum 105-A	23	70
Bifidobacterium longum 35624	21	52
Bifidobacterium longum BG7	27	70
Bifidobacterium longum BXY01	28	65
Bifidobacterium longum DJO10A	25	67
Bifidobacterium longum NCC2705	24	56
Bifidobacterium longum Su859	24	50
Bifidobacterium longum subsp. infantis 157F	28	61
Bifidobacterium longum subsp. infantis ATCC15697	22	46
Bifidobacterium longum subsp. infantis BT1	21	46
Bifidobacterium longum subsp. infantis JCM1222	23	49
Bifidobacterium longum subsp. longum AH1206	23	67
Bifidobacterium longum subsp. longum BBMN68	24	60
Bifidobacterium longum subsp. longum CCUG30698	26	70
Bifidobacterium longum subsp. longum F8	23	54
Bifidobacterium longum subsp. longum GT15	25	59
Bifidobacterium longum subsp. longum JCM 1217	23	59
Bifidobacterium longum subsp. longum JDM301	28	64
Bifidobacterium longum subsp. longum KACC91563	22	55
Bifidobacterium longum subsp. longum NCIMB8809	26	65
Bifidobacterium pseudocatenulatum D2CA	20	60
Bifidobacterium pseudocatenulatum DSM 20438	23	74
Bifidobacterium pseudolongum PV8-2	15	42
Bifidobacterium scardovii JCM 12489	38	126
Bifidobacterium thermophilum RBL67	12	31

GH, Glycosyl hydrolase.

followed by *B. dentium* Bd1 with 87 predicted GHs. When the genome of *B. dentium* Bd1 was sequenced, it was suggested that the relatively high number of predicted GHs is a reflection of the typical niche of this bacterium, that is, the oral cavity, where bacteria are exposed to the full contents of the ingested foods (Ventura et al., 2009). The genome *B. indicum* LMG 11587 is predicted to encode the smallest number of GHs with 25. The number of putative GHs on the genomes of *B. animalis* subsp. *lactis* DSM 10140 and Bl-04 are among the lowest (35 and 38, respectively) and this correlates with the relatively small size of the typical *B. animalis* subsp. *lactis* genome as compared to other bifidobacteria (Barrangou et al., 2009). Genes encoding predicted GHs belonging to the GH13 family are

most commonly found on currently available bifidobacterial genomes (Milani et al., 2015a). Predicted substrates for this family of GHs include complex carbohydrates, such as starch and amylopectin, as well as the simpler di- and trisaccharides, namely sucrose, maltose, maltotriose, palatinose, panose, melibiose, stachyose, and raffinose (Kelly et al., 2016; O'Connell et al., 2013; Pokusaeva et al., 2009). Interestingly, the genome of B. adolescentis 22L appears to encode the largest number of predicted GH13 enzymes (Duranti et al., 2014). Members of this bifidobacterial species are typically isolated from adults and thus the presence of such enzymes may reflect an adaptation to the adult diet, as opposed to HMO (Duranti et al., 2014). Members of the B. scardovii, B. longum, and B. bifidum species encode an extensive number of GHs belonging to the GH29, GH95, GH20, GH112, GH38, GH125, GH101, and GH129 families, which are typically involved in the degradation of host-derived carbohydrates, such as mucin and HMO (Milani et al., 2015b). Considering the size and complexity of some of the prevalent carbohydrates in the gut, such as plantderived starch and arabinoxylan and host-derived mucin, it is interesting to note that 10.9% of the putative GHs encoded by bifidobacteria are predicted to be extracellular (Milani et al., 2015a). Such GHs are critical for the degradation of carbohydrates whose direct uptake is not possible due to the size and complexity of the glycan. B. biavatii, B. scardovii, and B. bifidum each encode at least 11 predicted extracellular GHs, while the remaining bifidobacterial genomes examined encode 7 or less (Milani et al., 2015a). Despite being the second-highest producer of different GHs, the genome of *B. dentium* Bd1 encodes just two predicted extracellular GHs, a putative cellulase and xylosidase (Ventura et al., 2009). This could be considered as an adaptation to the oral cavity, unlike other bifidobacteria, this strain may have access to sugars with a low DP, which do not normally reach the large intestine (as discussed previously).

A small number of bifidobacterial sugar phosphorylases have been characterized, representing enzymes that degrade a glycosidic bond, but in the same reaction add a phosphate to one of the released monosaccharides. For example, the LnbP enzyme produced by *B. bifidum* JCM 1254 is a lacto-*N*-biose (LNB) phosphorylase, which cleaves both LNB and galacto-*N*-biose (GNB) to produce galactose-1-phosphate and *N*-acetylglucosamine (GlcNAc) or *N*-acetylgalactosamine (GalNAc), respectively (Kitaoka et al., 2005; Nishimoto and Kitaoka, 2007). Likewise, a sucrose phosphorylase encoded by *B. animalis* subsp. *lactis*, cleaves sucrose-producing glucose-1-phosphate and fructose (Trindade et al., 2003). An advantage of sugar phosphorylases over GHs is the phosphorylation of a released monosaccharide without the consumption of ATP, thus saving energy (Fushinobu, 2010).

8.7 METABOLISM OF PLANT-DERIVED CARBOHYDRATES BY BIFIDOBACTERIA

A considerable amount of research has been dedicated to investigate the metabolism of particular complex carbohydrates by bifidobacteria. This is not surprising given that many of these carbohydrates may function as prebiotics. As mentioned earlier, a considerable proportion of dietary/plant-derived carbohydrates ingested by the host reach the colon undigested, thus making them prebiotic candidates.

A significant fraction of dietary starch is resistant to digestion by host enzymes, thus providing a potential carbon and energy source for colonic bacteria. Starch consists of 2 high molecular-weight components, namely amylose, a linear polymer consisting of α -1,4-linked glucopyranose, and amylopectin, a branched polymer containing α -1,4-glycosidic linkages and α -1,6-linked branch points occurring every 17–26 glucose units (Wronkowska et al., 2008). Similarly, pullulan is a linear polymer of α -1,6-linked maltotriose units, with internal α -1,4 linkages. Members of the species B. adolescentis, B. angulatum, B. boum, B. breve, B. cuniculi, B. dentium, B. longum subsp. longum, B. longum subsp. infantis, B. merycicum, B. minimum, B. reuteri, B. pseudocatenulatum, B. ruminantium, and B. ther*mophilum* have been shown to be capable of utilizing starch as a sole carbon source, although within certain species, such as B. adolescentis, this was shown to be a strain-specific trait (Duranti et al., 2014). Utilization of starch by *B. breve* UCC2003 was shown to require the *apuB* gene, which specifies a presumed extracellular, cell wall–anchored amylopullunase enzyme, which liberates maltooligosaccharides from starch and amylopectin, and maltotriose from pullulan (O'Connell Motherway et al., 2008). The resulting maltooligosaccharides are further degraded to glucose by various α-glucosidases encoded on the *B. breve* UCC2003 genome (Kelly et al., 2016; O'Connell et al., 2013; Pokusaeva et al., 2009). Two putative amylopullulanases (one of which is predicted to be extracellular) were also identified on the genome of *B. adolescentis* 22L, as well as multiple enzymes with predicted α -amylase and α -glucosidase activities (Duranti et al., 2014).

Arabinoxylan is a large polysaccharide found in cereal grains, such as wheat, rye, rice, barley, and oats. It consists of a linear backbone of 1,500–15,000 β -1,4 linked xylose monomers, which can be mono- or disubstituted at the C2 or C3 positions with arabinose monomers (Izydorczyk and Biliaderis, 1995). Arabinoxylan, as well as unsubstituted xylo-oligosaccharides (XOS), are not digested by host enzymes in the upper GIT and have been shown to support the

growth of certain bifidobacterial species, such as *B. longum* subsp. *longum*, *B. animalis* subsp. *lactis*, and *B. adolescentis* (Lagaert et al., 2010; Mäkeläinen et al., 2010; McLaughlin et al., 2015; O'Callaghan et al., 2015; Pastell et al., 2009; Rivière et al., 2014). Due to its size and complexity, the degradation of arabinoxylan requires a multitude of enzymes. These include α -arabinofuranosidases, which cleave arabinose from the xylose backbone, β -xylosidases, which cleave xylose from the nonreducing end of the xylose backbone, and β -endoxylanases, which cleave arabinoxylan into shorter-chain arabinoxylan oligosaccharides. The combined action of these enzymes gives rise to several arabinoxylan oligosaccharides of varying chain length and substitutions (Grootaert et al., 2007). Three arabinofuranosidases have been characterized from *B. adolescentis*, which remove arabinose monomers from wheat arabinoxylan, although their substrate specificity is greatly influenced by the degree of substitution of the substrate (Lagaert et al., 2010). In *B. longum* subsp. *longum* NCIMB 8809, the *arfB* gene, encoding a putative arabinofuranosidase, was found to be essential for growth of this strain on both rye and wheat arabinoxylan (O'Callaghan et al., 2015). Finally, a β -1,4 xylanase capable of the complete hydrolysis of XOS was characterized from *B. adolescentis* (Amaretti et al., 2013).

Arabinogalactans are polysaccharides derived from pectin, an important cell wall polysaccharide in plants. They can consist of β -1,4 (Type I) or β -1,3 (Type II) galactose linkages. The β -1,3 linked chains can be substituted with β -1,6 galactose residues, while both chains can be further substituted with β -1,3-linked arabinofuranose chains (De Vries and Visser, 2001). In a study of bifidobacterial utilization of galactan, 11 of 34 strains tested grew on potato galactan. Six of these strains were from the *B. breve* species, with the remaining strains being members of the *B. pseudocatenulatum* and *B. longum* subsp. *longum* species (O'Connell Motherway et al., 2011). In *B. breve* UCC2003, an extracellular β -1,4 endogalactanase (GalA) hydrolyzes galactan to produce galacto-oligosaccharides, which are then internalized and further metabolized to galactose by a β -galactosidase (GalG) (O'Connell Motherway et al., 2011). An endogalactanase was also characterized from *B. longum* subsp. *longum* s

Galacto-oligosaccharides (GOS) are synthetic carbohydrates produced by microbial β -galactosidases, composed of galactose moieties in β -1,3/4/6 linkages with a terminal glucose residue (Gosling et al., 2010). GOS have previously been shown to exhibit bifidogenic effects and are generally considered to be prebiotics (Davis et al., 2010; Fanaro et al., 2009). In a recent study, 36 of 39 bifidobacterial strains tested, which included members of the *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, *B. dentium*. *B. longum* subsp. *longum*, and *B. pseudocatenulatum* species, were capable of substantial growth on GOS as the sole carbon source (Watson et al., 2013). Four β -galactosidases have been characterized from *B. longum* subsp. *infantis* ATCC15697, which release galactose from GOS (Garrido et al., 2013), while an exo- β -1,3-galactanase from *B. longum* subsp. *longum* JCM 1217 was active on β -1,3 linked GOS, as well as arabinogalactan (Fujita et al., 2014). In *B. breve* UCC2003, the aforementioned *galA* gene was shown to be required for the degradation of GOS components with a DP greater than three. The previously mentioned GalG β -galactosidase was also shown to be involved in GOS utilization in this strain (O'Connell Motherway et al., 2013).

Other prebiotic candidates that support growth of (some) bifidobacteria include fructooligosaccharides (FOS) and inulin. Inulin is a large polysaccharide consisting of fructose moieties in a β -1,2 linkage, while FOS is produced from the partial degradation of inulin (Roberfroid, 2005). Inulin is a poor growth substrate for many bifidobacterial strains, with just 2 of 39 tested capable of growth above OD_{600 nm} of 0.8, namely *B. breve* JCM7019 and *B. longum* CIP64.63 (Watson et al., 2013). In contrast, 25 of the 39 strains tested were capable of good growth on FOS (Watson et al., 2013). In contrast, 25 of the 39 strains were examined for their ability to utilize fructose, oligo-fructose (DP 2–8), and inulin (DP 12–65). Members of the species *B. adolescentis, B. angulatum, B. bifidum, B. breve, B. catenulatum, B. dentium, B. gallicum, B. longum* subsp. *infantis, B. longum* subsp. *longum, B. pseudocatenulatum,* and *B. thermophilum* were capable of using oligofructose or inulin (Falony et al., 2009). A β -fructofuranosidase protein was characterized from *B. breve* UCC2003, which hydrolyzed the β -1,2 bond between glucose and fructose in sucrose and FOS with a low DP, but exhibited relatively low activity against inulin (Ryan et al., 2005). Low activity toward long-chain inulin was also observed using a β -fructofuranosidase from *B. animalis* subsp. *lactis* DSM 10140T (Janer et al., 2004).

Shorter-chain plant-derived α -galacto-oligosaccharides, such as raffinose, stachyose, and melibiose have also been found to support bifidobacterial growth. The ability to utilize these three sugars was found to be well-conserved among *B. breve* strains, as well as *B. longum* subsp. *infantis* ATCC15697 (O'Connell et al., 2013). The ability to utilize such sugars is dependent on the presence of α -galactosidase enzyme activity. The RafA enzyme from *B. breve* UCC2003 was shown to hydrolyze the α -galactose linkage in raffinose and stachyose to produce sucrose and galactose, as well as producing glucose and galactose from melibiose. The MelE α -galactosidase from the same strain elicited hydrolytic activity against the synthetic disaccharides, α 1,4- and α 1,3-linked galactobiose (O'Connell et al., 2013). An α -galactosidase encoded by *B. adolescentis* DSM 20083 was also shown to hydrolyze the α -1,6 linkage between glucose and galactose in raffinose and stachyose (Leder et al., 1999).

8.8 MUCIN METABOLISM BY BIFIDOBACTERIA

The digestive tract is coated with a continuous layer of mucus, the main component of which is mucin glycoprotein. Given its ubiquity, and the fact that carbohydrate constitutes approximately 80% of the total mucin mass (Larsson et al., 2009), it is surprising that only a small proportion of the culturable intestinal microbiota is believed to encode enzymes required for (partial) mucin degradation into free sugars, including members of the *Bifidobacterium*, *Bacteroides*, and *Ruminococcus* genera, and the more recently characterized gut commensal *Akkermansia muciniphila* (Boulding and Hoskins, 1981; Crost et al., 2013; Derrien et al., 2004; Sonnenburg et al., 2005; Turroni et al., 2010). Mucins are both highly complex and diverse. The original investigations on the (bio) chemical composition of human colonic mucin described 21 discrete oligosaccharide structures (Podolsky, 1985). It is now believed that MUC2, the prominent secretory mucin in the colon, contains more than 100 structurally different *O*-linked glycans. The *O*-linked glycans of human colonic mucin have four distinct core structures bound to a serine or threonine residue, namely Galβ-1,3GalNAc [Core 1, galacto-*N*-biose (GNB)], GlcNAcβ-1,6(Galβ-1,3)GalNAc (Core 2), GlcNAcβ-1,3GalNAc (Core 3), and GlcNAcβ-1,6(GlcNAcβ-1,3)GalNAc (Core 4). These core structures can be elongated by the addition of galactose, GalNAc, and GlcNAc, and substituted with sialic acid, fucose, or sulfate residues in terminal or branched positions (Capon et al., 2001; Larsson et al., 2009; Podolsky, 1985; Robbe et al., 2004; Varki et al., 2009) (illustrated in Fig. 8.3).

The first observation of mucin-degrading bifidobacteria was described by Hoskins et al. (1985), who reported on the isolation of two bifidobacterial strains that constitutively expressed extracellular enzymes capable of degrading oligosaccharide side chains of gut mucins. Cell surface-anchored GHs were later characterized, including two α-L-fucosidases, AfcA and AfcB, from B. bifidum JCM1254 (Ashida et al., 2009; Katayama et al., 2004) and an endo-α-N-acetylgalactosaminidase, EngBF, from B. longum subsp. longum JCM1217, which hydrolyzes the linkage between GalNAc of the Core 1 disaccharide and the serine or threonine residue of the proteinaceous backbone (Fujita et al., 2005). Additionally, an extracellular, membrane bound, exo- α -sialidase that releases sialic acid from porcine gastric mucin was characterized from *B. bifidum* JCM1254 (Kiyohara et al., 2011). Recently, a cell-membrane anchored α -*N*-acetylglucosaminidase, designated AgnB, which is specific to the GlcNAc- α -1,4-Gal structure found in gastroduodenal and colonic mucin, was characterized from B. bifidum JCM1254 (Shimada et al., 2014). Interestingly, the only intracellular mucin-degrading enzyme characterized from B. bifidum is an α -N-acetylgalactosaminidase (designated NagBb), which hydrolyzes the α -1-linkage between GalNAc and the serine or threonine residue of mucin Core 3 structures. It was hypothesized by the authors that extended mucin Core 3 structures are extracellularly hydrolyzed by sialidase, lacto-N-biosidase, β -galactosidase and/or β -N-acetylhexosaminidase activity, resulting in GalNac α -1-Ser/Thr (also known as the Tn antigen), which is assimilated through unknown transporters and intracellularly hydrolyzed by NagBb (Kiyohara et al., 2012).

When the genome of *B. bifidum* PRL2010 was sequenced in 2010, it was observed that 60% of the identified GHs can be linked to mucin degradation (Turroni et al., 2010). This included two putative exo- α -sialidases, two putative α -L-fucosidases and a predicted endo- α -N-acetylgalactosaminidase, all of which are presumed to be extracellular due to the presence of a signal peptide (Turroni et al., 2010). Other possible mucin-degrading enzymes encoded by the genome of *B. bifidum* PRL2010 include four *N*-acetyl- β -hexosaminidases (two of which are predicted to be extracellular) and four β -galactosidases (one of which is presumed to be extracellular) (Turroni et al., 2010). Comparative genome hybridization analysis revealed that most of the genes encoding the aforementioned enzymes are conserved within the examined members of the *B. bifidum* species and, of a number of bifidobacterial strains tested, only members of this species were capable of growth in media containing porcine gastric mucin as the sole carbon source (Turroni et al., 2010). That such a large number of the predicted GHs are extracellular seems significant, especially when it is considered that despite the presence of genes encoding predicted sialidases and fucosidases on its genome, sialic acid and fucose support no growth and poor growth, respectively, of B. bifidum PRL2010 (Turroni et al., 2012b). In a recent in vitro study it was shown that fucose and galactose accumulate in the medium during B. bifidum PRL2010 growth on porcine gastric mucin (Egan et al., 2014a). Indeed, it has been suggested that the function of these enzymes is to provide the strain access to specific mucin components, for example, GNB and galacto-N-tetraose (Galβ1-3-GalNAc-β1-3Galβ1-4GlcNAc; GNT) (Turroni et al., 2010, 2014). The metabolic cluster required for the utilization of GNB, as previously characterized in *B. longum* subsp. *longum* JCM1217, is conserved among B. bifidum strains (Kitaoka et al., 2005; Nishimoto and Kitaoka, 2007; Turroni et al., 2010).

Mucin-derived oligosaccharides and HMO share a number of primary monosaccharide components, including galactose, GlcNAc, glucose, sialic acid, and fucose (Bode and Jantscher-Krenn, 2012; Urashima et al., 2012). Given the similar oligosaccharide structures and monosaccharide components of mucin and HMO, it is not surprising that *B. bifidum* PRL2010 was shown to achieve good growth on HMO (Turroni et al., 2010). However, compared to mucin,

HMO have also been found to be more accessible to other bifidobacterial species, such as *B. longum* subsp. *infantis* ATCC15697 and *B. breve*. Due to the importance of breast milk to the development of the infant gut microbiome, it is not surprising that the utilization of HMO by bifidobacterial species has come under intense scrutiny. This topic will be discussed in greater detail in Chapter 9.

8.9 METABOLISM OF N-LINKED GLYCOPROTEINS

In mucin, glycans can also be N-linked to an asparagine residue via a GlcNAc monomer (Froehlich et al., 2010; Picariello et al., 2008). All N-linked glycans contain the core pentasaccharide Man₃GlcNAc₂, which can be further modified to produce one of the three structures. In high mannose structures, the core pentasaccharide is extended by another two to six mannose residues. Complex structures contain no additional mannose residues but may have up to five antennae consisting of GlcNAc, galactose, and fucose, and are often decorated with sialic acid residues. Hybrid glycans comprise of a combination of high mannose and complex branches (Morelle and Michalski, 2007). Bacterial metabolism of N-glycans has primarily been associated with pathogens, such as *Enterococcus faecalis*, which utilizes the high mannose-type N-glycan, RNaseB (Roberts et al., 2000), and *Capnocytophaga canimorsus*, which utilizes the complex-type N-glycans, fetuin, and IgG (Renzi et al., 2011). The ability to utilize N-glycans is associated with the presence of an extracellular enzyme, endo-β-*N*-acetylglucosaminidase, which cleaves the pentasaccharide core between the GlcNAc residues, releasing the oligosaccharide chain (Roberts et al., 2001).



FIGURE 8.3 Examples of complex O-GalNAc glycans from human mucin. Representative examples of complex O-GalNAc glycans from human mucin. The core structures, 1–4, are boxed in broken lines. Each of the core structures can be further elongated by the addition of galactose, GalNAc, and GlcNAc. The oligosaccharide chains can also be substituted with sialic acid, fucose or sulfate residues in terminal or branched positions. *Ser*, Serine; *Thr*, threonine. The monosaccharide key is shown on the right. *Source: Modified from Varki, A., Cummings, R.D., Esko, J.D., Freeze, H., Stanley, P., Bertozzi, C.R., Hart, G.W., Etzler, M.E., 2009. Essentials of Glycobiology, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (pp. 115–127).*

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Recentresearchhasshownthatsomebifidobacterialspeciesalsodisplayextracellularendo- β -*N*-acetylglucosaminidase activity (Garrido et al., 2012). *B. longum* subsp. *infantis* ATCC15697 was shown to degrade the high mannose-containing glycoprotein RNaseB, yet interestingly, other *B. longum* subsp. *infantis* strains exhibited very little hydrolytic activity toward this substrate, suggesting that this is a strain-specific characteristic (Garrido et al., 2012). Of the nine *B. longum* subsp. *longum* strains tested, only four demonstrated endo- β -*N*-acetylglucosaminidase activity, an observation that is consistent with this subspecies' preference for plant-derived polysaccharides (Schell et al., 2002), while members of the *B. bifidum* species display no activity toward this substrate (Garrido et al., 2012). In contrast, *B. breve* demonstrated high activity toward this substrate, completely deglycosylating RNaseB and consistent with this it was shown that heavily N-glycosylated cell wall proteins from *Saccharomyces cerevisiae* can support growth of *B. breve* strains SC139 and KA179 (Garrido et al., 2012). Characterization of two endo- β -*N*-acetylglucosaminidases, EndoBl-1 and EndoBl-2, from *B. longum* subsp. *infantis* ATCC15697 and *B. longum* subsp. *infantis* SC142, respectively, revealed activity against both high mannose and complex glycans. Endo-BL1 was also active on the complex glycans found in lactoferrin from human milk, another example of this strain's adaptation to the infant gut (Garrido et al., 2012). In a more recent study, N-glycans released from bovine colostrum by EndoB1-1 activity were shown to support growth of *B. longum* subsp. *infantis* ATCC15697, but not *B. animalis* subsp. *lactis* UCD316 (Karav et al., 2016).

8.10 GLYCOSULFATASE ACTIVITY IN BIFIDOBACTERIA

Host-derived glycoproteins, such as mucin, as well as proteoglycans, such as chondroitin sulfate and heparin sulfate, which are found in the colonic mucosa and human milk, are often sulfated (Eliakim et al., 1986; Newburg et al., 1995; Oshiro et al., 2001). Human colonic mucin is also heavily sulfated, a potential purpose of which is to protect the mucin against bacterial glycosidases (Brockhausen, 2003). However, glycosulfatase activity has been identified in enteric bacteria, such as *Bacteroides thetaiotaomicron, Bacteroides ovatus*, and *Prevotella* strain RS2 (Roberton et al., 1993; Salyers et al., 1977).

Sulfatase enzymes are unique in that they require a 3-oxoalanine (typically called Cα-formylglycine or FGly) residue at their active site (Bond et al., 1997; Lukatela et al., 1998). Prokaryotic sulfatases carry either a Cys or a Ser residue, which requires posttranslational conversion to FGly in the cytosol in order to activate the enzyme (Marquordt et al., 2003; Miech et al., 1998; Szameit et al., 1999). In bacteria, two distinct systems have been described for the post-translational modification of sulfatase enzymes. In *Mycobacterium tuberculosis*, modification of Cys-type sulfatases was shown to require a formylglycine-generating enzyme, an enzyme, which is strictly dependent on oxygen (Carlson et al., 2008). In *Klebsiella pneumoniae, Clostridium perfringens*, and *Ba. thetaiotaomicron*, the conversion of the Ser or Cys residue to FGly is catalyzed by an iron–sulfur protein, designated AtsB, which a member of the *S*-adenosyl-L-methionine (AdoMet)-dependent enzyme family (Benjdia et al., 2008; Berteau et al., 2006; Szameit et al., 1999). Crucially these enzymes are active under anaerobic conditions and were thus designated anaerobic sulfatase-maturing enzymes (anSME) (Berteau et al., 2006).

Recently, sulfatase activity was described for the first time in bifidobacteria (Egan et al., 2016). Two gene clusters were identified in *B. breve* UCC2003, each encoding a (predicted) sulfatase and accompanying anSME, as well as an associated transport system and transcriptional regulator. These putative sulfatase clusters are well conserved among the *B. breve* species (Bottacini et al., 2014). *B. breve* NCFB2258, *B. breve* 689B, *B. breve* 12L, and *B. breve* S27 encode clear homologues of both identified putative sulfatase gene clusters. The genomes of *B. breve* JCM7017, *B. breve* JCM7019, and *B. breve* ACS-071-V-Sch8b contain just a single, but variable putative sulfatase cluster, while one of the two clusters was also identified on the genome of *B. longum* subsp. *infantis* BT1. The sulfated monosaccharide, *N*-acetylglucosamine-6-sulfate was shown to support the growth of *B. breve* UCC2003 and insertion mutagenesis identified the predicted sulfatase required for growth. However, the strain appeared unable to grow on the three sulfated monosaccharides, *N*-acetylglucosamine-3-sulfate, *N*-acetylgalactosamine-6-sulfate, or *N*-acetylgalactosamine-3-sulfate, thus indicating that either the transport system or sulfatase is highly specific to the 6-O-sulfated form of GlcNAc (Egan et al., 2016).

8.11 CARBOHYDRATE CROSS-FEEDING BY BIFIDOBACTERIA

Different bifidobacterial species have adopted divergent strategies in the utilization of carbohydrates. For example, *B. longum* subsp. *infantis* ATCC15697, an infant-derived bifidobacterial isolate that is particularly well-adapted to HMO utilization (see Chapter 9) is predicted to internalize various intact HMO structures through ABC transport

systems (of which there are 14 predicted on the genome, as discussed previously) (Sela et al., 2008). It then degrades the HMO structures to their monosaccharide components using a variety of intracellular GHs (Sela and Mills, 2010). The advantage of this strategy is that HMO are degraded intracellularly, therefore the strain does not face competition for the resulting mono- and disaccharides. However, there is an energy cost to this method, as internalizing sugars through ABC-type transport systems requires ATP (Davidson and Chen, 2004). In contrast, as described previously, members of the *B. bifidum* species typically degrade large, complex carbohydrates, such as mucin, employing extracellular GHs, and are predicted to internalize the released monosaccharides through PEP–PTS systems (Turroni et al., 2012b). This strategy has the advantage of requiring less energy for uptake of the sugars as PEP–PTS systems are cost neutral (Postma et al., 1993), but the cells may face competition for the resulting degradation products of mucin. In fact, it can be said that cross-feeding on the breakdown products released from the extracellular degradation of large, complex carbohydrates, such as mucin represents a third strategy of carbohydrate utilization.

It has long been hypothesized that extracellular degradation of large polysaccharides, such as mucin may provide nutritional support to other enteric bacteria (Hoskins et al., 1985), while it was later suggested that cross-feeding on HMO may occur between bifidobacterial species, with particular reference to B. bifidum and B. breve (Ward et al., 2007). A number of studies have investigated cross-feeding behavior in bifidobacteria. It was recently shown that *B. breve* UCC2003 can cross-feed on sialic acid released by the extracellular sialyllactose (a prominent HMO)– degrading activities of *B. bifidum* PRL2010 (Egan et al., 2014b). *B. breve* UCC2003 was also shown to cross-feed on various mucin constituents, such as galactose, sialic acid, GNB, and fucose released by *B. bifidum* PRL2010 activity (Egan et al., 2014a). A similar cross-feeding effect was observed when *B. magnum* and *B. cuniculi* were cocultivated on starch (Milani et al., 2015a). Two extracellular pullulanases encoded by B. cuniculi, as well as extracellular α-glucoside phosphorylase-specifying genes from both strains were upregulated during cocultivation. The authors speculated that the combined action of these enzymes may result in a larger amount of starch-derivatives compared to when the strains are grown individually, thus resulting in an increase in cell numbers for both strains (Milani et al., 2015a). A recent study investigated the transcriptomic effect of cocultivation of four strains, namely B. bifidum PRL2010, B. breve 12L, B. adolescentis 22L, and B. longum subsp. infantis ATCC15697, either in pairs (biassociation) or a combination of all four strains (multiassociation), under in vivo or in vitro conditions. During simulated in vivo conditions, it was found that a significant number of predicted carbohydrate metabolism-related genes on each genome were upregulated when the strains were cocultivated (bi- and multiassociation) as compared to when the strains were cultivated individually (Turroni et al., 2016). The authors found that the upregulation of certain genes was dependent on the combination of strains being cultivated together. For example, a predicted α -amylase from *B. adolescentis* 22L was upregulated when the strain was biassociated with *B. bifidum* PRL2010 or *B. longum* subsp. *infantis* ATCC15697, but not in biassociation with *B. breve* 12L or multiassociation. Likewise the entire predicted sialic acid cluster of *B. breve* 12L was upregulated in multiassociation, but in biassociation with the other strains only certain genes of this cluster were upregulated. Genes predicted to be involved in the metabolism of xylose-containing carbohydrates were upregulated in all strains when they are cocultivated in the cecum (bi- and multiassociation). The authors speculated that in bi- or multiassociation, strains switch their metabolic activities toward a less-coveted substrate, as preferred substrates are more rapidly depleted as compared to monoassociated conditions. Genes predicted to be involved in starch metabolism were also upregulated during cocultivation of B. adolescentis 22L and B. breve 12L. In terms of host-derived carbohydrates, genes putatively involved in hexosamine metabolism were upregulated in *B. bifidum* PRL2010 and *B. adolescentis* 22L, particularly when these two strains were cultivated together (Turroni et al., 2016). Interestingly, considering the strategy of HMO utilization by *B. longum* subsp. *infantis* ATCC15697 described previously, it was notable that transcription of the predicted carbohydrate transporters of this strain was not altered by the presence of the other bifidobacterial strains, consistent with the somewhat solitary strategy of carbohydrate utilization by this strain.

Interestingly, another type of cross-feeding has been described, whereby *Eubacterium hallii* utilizes lactate produced by *B. adolescentis* L2-32 during growth on starch, resulting in butyrate production by *Eu. hallii* (Belenguer et al., 2006). A similar study was performed using *B. longum* subsp. *longum* NCC2705 cocultivated with *Eubacterium rectale* ATCC 33656 on arabinoxylan (Rivière et al., 2015). The metabolism of arabinose by *B. longum* subsp. *longum* NCC2705 resulted in the production of acetate, which was then converted to butyrate by *Eu. rectale* ATCC 33656. Butyrate is a major source of energy for colonocytes and has also been implicated in protection against diseases, such as colonic carcinogenesis, diabetes and obesity (reviewed in Hamer et al., 2008). The arabinoxylan-degrading activities of *Eu. rectale* ATCC 33656, resulting in the extracellular release of arabinose and xylose, also had a bifidogenic effect, as *B. longum* subsp. *longum* NCC2705 utilized these free monosaccharides resulting in an increase in its cell concentration (Rivière et al., 2015).

8.12 TRANSGLYCOSYLATION ACTIVITY IN BIFIDOBACTERIA

As described above, GHs can be classified as retaining or inverting, depending on the spatial configuration of its active residues (Davies and Henrissat, 1995). An advantage of retaining GHs is their ability, in high concentrations of substrate, to form new oligosaccharides with a higher DP, a process known as transglycosylation (Van den Broek and Voragen, 2008). Transglycosylation by bifidobacterial GHs is of particular interest, as it is thought that the resulting oligo- and polysaccharides may function as bifidogenic prebiotics (Van den Broek and Voragen, 2008). An α-galactosidase from *B. adolescentis* DSM 20083, designated AGA, was shown to exert transglycosylation activity toward raffinose and stachyose (Van den Broek et al., 1999). This enzyme was further characterized in 2005 when it was shown that in high concentrations of melibiose (a disaccharide), a trisaccharide and a tetrasaccharide were produced. Their structures, identified by NMR, consisted of α -Gal(1,6)- α -Gal(1 Gal(1,6)- α -Glc (Hinz et al., 2006). Three β -galactosidases from *B. bifidum* NCIMB 41171, namely BbgII, BbgIII, and BbgIV, were shown to have transglycosylation activity toward lactose, producing GOS with a DP greater than 3 (Goulas et al., 2009). Likewise, β-galactosidases from B. longum subsp. longum BCRC 15708 and B. longum subsp. infantis HL96 were also shown to have transglycosylation activity toward lactose, resulting in the production of GOS (Hsu et al., 2007; Hung and Lee, 2002). In B. breve UCC2003, two α -glucosidase enzymes were shown to possess transglycosylation activity toward palatinose, trehalulose, trehalose, panose, and isomaltotriose, although the chemical structure of the newly synthesized oligosaccharides was not determined (Pokusaeva et al., 2009). In B. bifidum JCM1254, a lacto-*N*-biosidase, LnbB, which cleaves LNB from lacto-*N*-tetraose (LNT), a prominent HMO, can synthesize LNT using lactose and $pNP-\beta$ -LNB (Wada et al., 2008). This is particularly interesting given that LNT supports growth of typically infant-derived bifidobacterial species, such as *B. bifidum*, *B. breve*, and *B. longum* subsp. *infantis* (Asakuma et al., 2011; Locascio et al., 2007; Ruiz-Moyano et al., 2013).

8.13 REGULATION OF CARBOHYDRATE METABOLISM IN BIFIDOBACTERIA

Carbon catabolite repression (CCR) is a regulatory process whereby, when presented with a number of carbon sources, bacteria will preferentially utilize the substrate that most effectively yields (the highest amount of) energy, while inhibiting the expression or activity of proteins involved in the uptake and catabolism of other substrates (Stülke and Hillen, 1999). The methods by which CCR operates in the Gram-negative bacterium Escherichia coli and the low-GC, Gram-positive bacterium Bacillus subtilis have been studied extensively (Postma et al., 1993; Saier and Ramseier, 1996). CCR systems comparable to those described in *E. coli* and *B. subtilis* have so far not been identified in bifidobacteria, although the preferential use of one sugar over another has been described. In B. breve UCC2003, expression of an operon involved in the metabolism of FOS was shown to be induced in the presence of sucrose and Actilight (a commercial source of short-chain FOS), yet repressed when the strain was grown on a mixture of glucose and sucrose, or fructose and sucrose, indicating the preferred utilization of glucose or fructose (Ryan et al., 2005). Similarly, when this strain was grown in ribose or a combination of ribose and glucose, genes encoding the predicted fructose-specific PEP-PTS, as well as other carbohydrate-related ABC-type transport systems, were downregulated (Pokusaeva et al., 2010). The *B. longum* subsp. *longum* strain NCC2705 was shown to preferentially utilize lactose over glucose, a phenomenon previously only observed in Streptococcus thermophilus and another B. longum strain, SH2 (Kim et al., 2003; Parche et al., 2006; Van Den Bogaard et al., 2000). The preferential utilization of lactose over glucose was achieved by transcriptional downregulation of the *glcP* gene, a glucose-specific MFS transporter, in the presence of lactose (Parche et al., 2006). In both cases for *B. longum*, the authors speculated that this preference for lactose was an adaptation to the infant gut, where lactose is the predominant carbon source (Kim et al., 2003; Parche et al., 2006). In B. animalis subsp. lactis, sucrose-metabolizing activity was induced in the presence of sucrose, raffinose, or oligofructose, but repressed in the presence of glucose (Trindade et al., 2003). Another CCR-related metabolic control was observed when *B. longum* subsp. infantis ATCC15697 was grown on a complex mixture of HMO. It was observed that of the five fucosidase-encoding genes identified on the B. longum subsp. infantis ATCC15697 genome, one was upregulated, two were downregulated and two showed no change in transcription, compared to when the strain was grown in lactose (Sela et al., 2012).

Transcriptional repressors are DNA binding proteins that bind to the operator sequence of a promoter, thus preventing the binding of RNA polymerase and the initiation of transcription. Perhaps the best-characterized transcriptional repressor in bacteria is the LacI protein of *E. coli*, which represses transcription of the *lac* operon, required for the uptake and utilization of lactose (Gilbert and Müller-Hill, 1966; Jacob and Monod, 1961). LacI monomers are divided into three distinct domains. The *N*-terminus contains a helix-turn-helix DNA-binding domain, which binds to an operator sequence upstream of the *lac* operon, thus preventing transcription, as well as a short hinge region required for recognition of the operator sequence. The core domain, or effector binding domain, binds sugar ligands, while at the C-terminal, the multimerization domain is essential for the formation of the LacI dimer, the native form of LacI, or the LacI tetramer (Lewis et al., 1996).

In a recent study on the comparative genomics of LacI-family transcription factors (LacI-TFs), the largest average number of such transcription factors per genome was found in lineages of the *Actinobacteria* phylum, including the *Bifidobacteriaceae* family (Ravcheev et al., 2014). Bifidobacterial genomes are predicted to encode a particularly high number of repressor proteins, a characteristic believed to allow the bacteria to quickly adapt to fluctuations in carbohydrate sources (Schell et al., 2002). For example, the genome of *B. longum* subsp. *longum* NCC2705 encodes 22 predicted LacI-TFs, all of which contain a sugar-binding motif, suggesting their involvement in the regulation of carbohydrate metabolism (Schell et al., 2002). Four NagC/XylR-type transcriptional repressors predicted to be involved in sugar metabolism were also identified (Schell et al., 2002). In a recent in silico analysis of potential carbohydraterelated transcription factors encoded on ten bifidobacterial genomes, 308 transcription factors were identified in total, 63% of which were predicted to represent LacI-TFs (Khoroshkin et al., 2016).

Six predicted LacI-type regulators encoded by *B. breve* UCC2003 have so far been characterized, including LacI_{fos}, which regulates transcription of the *fos* operon (Ryan et al., 2005), GalR, which controls transcription of a galactan utilization cluster (O'Connell Motherway et al., 2011), CldR, which controls cellodextrin utilization (Pokusaeva et al., 2011b) and RbsR, which controls transcription of the *rbsACBDK* cluster for ribose utilization (Pokusaeva et al., 2010). Two LacI-type regulators, designated MelR1 and MelR2, were shown to control transcription of the melezitose utilization cluster in *B. breve* UCC2003 (O'Connell et al., 2014). Furthermore, a GntR-type repressor, designated NanR, has been characterized from this strain, which regulates transcription of the sialic acid utilization cluster (Egan et al., 2015) and a NagC/XylR-type repressor, designated AtsR2, regulates a sulfatase-encoding gene cluster in *B. breve* UCC2003 (Egan et al., 2016). Just one transcriptional activator involved in carbohydrate metabolism has so far been characterized in a bifidobacterial species: the ROK (repressor open reading frame kinase)–type regulator RafR, which was shown to be required for the transcriptional activation of the raffinose utilization cluster in *B. breve* UCC2003 (O'Connell et al., 2014).

8.14 CONCLUSIONS

As the number of complete bifidobacterial genome sequences continues to grow, so does our understanding of the species and strain-specific attributes responsible for the metabolic diversity described in this review. An understanding of the characteristic metabolic traits of the various species of bifidobacteria, and their effects on other members of the gut microbiota is of crucial importance in the development of the next generation of probiotics and prebiotics and to provide the greatest health advantage from such functional foods. Knowledge concerning the factors that contribute to bifidobacterial utilization of mucin is also of increasing importance as questions are raised on the potential harm caused by degradation of the protective mucus layer and the effect of the Western-style diet and its associated microbiota on mucin degradation (Mahowald et al., 2009; Marcobal et al., 2013). Furthermore, it has become apparent through various cross-feeding studies that many species of bifidobacteria exist in a cooperative relationship with each other in vivo, and how this can be manipulated, perhaps in the development of improved probiotics, will be of interest in the future.

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9

Interactions Between Bifidobacteria, Milk Oligosaccharides, and Neonate Hosts

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9.1 INTRODUCTION

The genus *Bifidobacterium* has been associated with the infant gut from the moment of its discovery. In 1900, Tissier observed a split-rod shaped bacterium that had been isolated from breast-fed infant feces, which he named *Bacillus bifidus*. In the early years, the organism was placed under the family *Lactobacteriaceae*, owing to the fact that lactic acid was one of the by-products of its fermentation (Orla-Jensen, 1924). The organism subsequently underwent several name changes, being identified as *Lactobacillus bifidus* in the 1950s (Breed et al., 1957) and eventually given its own genus, *Bifidobacterium*, with more than 20 species by the 1980s (Scardovi, 1986), culminating in 54 named species and 10 subspecies today (Chapter 2). As early as 1957 (Dehnert, 1957), the taxonomists of bifidobacteria and other lactic acid bacteria suggested a focus on the ability to consume specific sugars as a mechanism for differentiating organisms. As more bifidobacteria were isolated from increasingly diverse sources, classification began to divide the species by association to different hosts, be they the gastrointestinal (GI) tracts of humans, nonhuman mammals, insects, or sewage. Predictably, these very diverse ecological niches selected for different phenotypic features, leading todiversity within the *Bifidobacterium* genus. As technologies and microbiological tools have improved much has been learned about the progression of microbes in the infant gut since the time of Tissier.

9.2 PROGRESSION OF MICROBIOTA IN INFANTS

The microbiota of the human intestinal tract is a subject of intense study. Following in the in the footsteps of the human genome project, the human microbiome project (HMP, http://hmpdacc.org/) is an effort to explore the symbiotic microorganisms inhabiting our bodies, which is increasingly referred to as a "human organ" (Peterson et al., 2009; Turnbaugh et al., 2007). Both within and outside of the HMP, many researchers are describing the human gut microbiome as a function of health and disease, revealing enormous personal (alpha) and interpersonal (beta) diversity, even between closely related individuals (Turnbaugh et al., 2010; Yatsunenko et al., 2012). Within adults, the conventional wisdom suggests increased microbial diversity in the gut as a sign of health, with a decrease in diversity being linked to obesity (Turnbaugh et al., 2009) and inflammatory GI disease (Manichanh et al., 2006), among other pathologies.

Interestingly, breast-fed newborn microbiota tends to display significantly lower alpha diversity (Yatsunenko et al., 2012). While it was historically believed that fetuses were sterile during gestation (as suggested by Tissier as far back as 1900; Mackie et al., 1999), some studies have shown that the placenta may harbor its own microbiota (Aagaard et al., 2014), although more recent analyses contradict these conclusions (Lauder et al., 2016). Regardless, the first stool produced by an infant is not sterile; analysis of the infant fecal microbiota over the first few days of

The Bifidobacteria and Related Organisms. http://dx.doi.org/10.1016/B978-0-12-805060-6.00009-0 Copyright © 2018 Elsevier Inc. All rights reserved. life reveals an aerotolerant (aerobic and facultatively anaerobic) microbial consortium dominated by *Firmicutes* and some *Proteobacteria* (Adlerberth et al., 2007; Koenig et al., 2011), a pattern reminiscent of that observed in the placenta.

In the very early stages of life, the predominant organisms in the infant gut vary by mode of delivery. Vaginally born infants begin with a microbial consortium that overlaps partially with their mother's vaginal microbiome, while infants delivered by caesarian-section share more species with their mothers' skin (Dominguez-Bello et al., 2010). Regardless of their provenance, these organisms eventually turn their environment anaerobic, paving the way for a new consortium comprising principally obligate anaerobic bacteria, including members of the *Bacteroidetes, Firmicutes, Proteobacteria*, and *Actinobacteria* phyla (Dogra et al., 2015; Mackie et al., 1999). Of the *Actinobacteria, Bifidobacterium* is consistently the predominant genus in breast-fed infants, suggesting a consistent selection for these organisms by their environment.

9.3 HUMAN MILK GLYCANS

Another reason breast-fed infants have microbiomes with low alpha diversity is due to the strong selective pressures exerted by the principal carbon source available in the large intestine, human milk glycans. The relationship between certain bifidobacteria and human milk has been established for decades. Gyorgy et al. (1954) showed that human milk contains a factor (termed bifidus factor) that encouraged the growth and acid production of one strain of Bifidobacterium (then termed L. bifidus var. Penn), while this effect was not observed with some other known strains. He noted that unlike other L. bifidus strains, the Penn "biovar" utilized lactose as a sole carbohydrate source, but could not use maltose. Thus, 60 years ago a connection was made between the carbohydrates found in human milk and the successful growth of bifidobacteria. By fractionating the components of human milk, Gauhe et al. (1954) also determined that this "bifidus factor" was not the fatty acids, proteins, or lactose component in milk, but instead some glycan structure or structures that contained glucose (Glc), galactose (Gal), fucose, and glucosamine (GlcNAc). Today, it is recognized that these human milk oligosaccharides (HMOs) are made up of the above monomers, as well as N-acetylneuraminic acid (NeuAc, also known as sialic acid) (Bode and Jantscher-Krenn, 2012) (Fig. 9.1). With varying degrees of polymerization and multiple linkage isomers, and more than 200 different structures observed (Ninonuevo et al., 2006), the variety of these sugars is remarkable. These carbohydrates are present at concentrations of up to 15 g/L in human milk, a level that represents a heavy energetic burden on the mother. Considering this burden, it may be surprising that infants do not have the metabolic capability to metabolize these sugars (Gnoth et al., 2000), suggesting they do not function as a nutritional source. HMOs, however, serve several functions that justify the production cost to the mother. Their similarity in structure and composition to gut mucosal glycans allows them to bind to bacteria, acting as "decoys" and preventing pathogens from establishing an infection in the intestine (Newburg et al., 2005). Additionally, the infant's inability to break down these sugars means that they arrive at the colon intact, where they can function as a growth substrate for those select bacteria that can metabolize them. In addition to free HMOs, glycoconjugates in human milk possess similar potential for bioactivity.



FIGURE 9.1 Structures of hypothetical human milk oligosaccharides. (A) Hypothetical free, (B) N-linked, and (C) O-linked human milk oligosaccharide showing the monomer constituents and linkage types. *Light gray (yellow* in the web version) *circles*, galactose; *dark gray (blue* in the web version) *circle*, glucose; *light gray (yellow* in the web version) *squares*, *N*-acetylgalactosamine; *dark gray (blue* in the web version) *squares*, *N*-acetylgalactosamine; *triangles (red* in the web version), fucose; *diamonds (purple* in the web version), sialic acid; *gray (green* in the web version) *circles*, mannose.

Glycoproteins in milk come in two varieties: N-linked and mucin-type O-linked. N-linked glycans are oligosaccharaides bound via a β -*N*,*N* chitobiose linkage to the asparagine residue of a protein in the sequence Asn-xxx-Ser/Thr, where xxx is any residue except proline (Dallas et al., 2011; Garrido et al., 2013; Kobata, 2000). There are three classes of N-glycans: high mannose, complex, and hybrid, all attached to the protein with a Man3GlcNAc2 core. O-linked glycans by contrast are bound to a serine (Ser) or a threonine (Thr) residue via an α -glycosidic linkage. There is no single core structure for O-linked glycans. Instead, there are eight recognized core structures each with a GalNAc as the moiety bound to the Ser/ Thr. From these core structures, GalNac, GlcNac, galactose, fucose, and sialic acid can be linked to form extended structures (Brockhausen et al., 2009), but few are polymerized as extensively or have the molecular weight of N-linked glycans. Core 1 and 2 are common on most soluble glycoproteins, while cores 3 and 4 are found almost exclusively bound to secreted GI mucin. In human milk, 50%–70% of all proteins are glycosylated (Froehlich et al., 2010; Peterson et al., 2013).

N-glycosylation is nearly ubiquitous in the decorated milk protein fraction; with nearly all of the population having at least one N-glycan. N-glycosylation patterns can vary depending on individual, protein type, and lactation stage (Froehlich et al., 2010; Garrido et al., 2013; Mechref et al., 1999). N-glycoprofile of representative milk samples yielded 52 unique structures (Dallas et al., 2011). Lactoferrin is the most common N-glycosylated protein in human milk, with observed N-glycans that are complex-type with biantennary structure. There are a multitude of other N-glycosylated proteins in both the skim and milk-fat globule membrane fractions. Other important glycoproteins with N-glycans include α -lactoalbumin, serum albumin, xanthine oxidase, and tenascin (Froehlich et al., 2010).

Four highly abundant milk glycoproteins with characterized O-glycans are κ -casein (κ CN), secretory Immunoglobulin A (sIgA), bile salt stimulated lipase (BSSL), and mucin (Froehlich et al., 2010; Xue et al., 2013). Human κ CN is a heavily glycosylated protein that represents approximately 25% of total casein (Peterson et al., 2013). The κ CN protein is common in all mammalian milk, but human κ CN is unique in the increased number of O-linked glycans (10 glycosylation sites compared to 5 in bovine milk) and the fucosylations present on said glycans (Nwosu et al., 2013; Saito et al., 1988; Strömqvist et al., 1995). The structure of milk mucin resembles a bristle brush, with heavy O-glycosylations on a core protein on the solution-facing region, with N-glycans bound near the base where the protein spans the membrane (Patton et al., 1995).

The actual structures of human milk glycans (both free and conjugated) are not easy to elucidate. Gyorgy et al. (1954) did not attempt to describe the structures themselves, but rather only partly described their monomer compositions. Such a description is a challenge in part due to the sheer number of structural permutations possible given degrees of polymerization ranging from 4 to 10 with various isomeric linkages combined with different "decoration" possibilities (i.e., the presence or absence of fucose and sialic acid residues). However, much progress has been made to this endeavor since Gyorgy's early thin-layer chromatography of individual monosaccharide components, and the majority of structural information on the human milk glycome has come from a combination of high-performance liquid chromatography (HPLC) and mass spectrometry (MS) (Ninonuevo et al., 2006). The strategies to elucidate these structures via separation, ionization, identification, and quantification are under constant development, as are methods of extracting and purifying these glycans from total milk (reviewed in Kailemia et al., 2014).

9.4 BIFIDOBACTERIAL CONSUMPTION OF MILK GLYCANS

The presence of multiple bifidobacterial species in the infant gut might be explained by their differential ability to consume various human milk oligosaccharides. Analyses of the abilities of common infant-type bifidobacteria to consume these complex sugars are summarized in Table 9.1. Several patterns are of note based on these studies. Lacto-neo-tetraose (LNT) and other relatively small HMOs seem to be the sugars that are most readily digested (LoCascio et al., 2007; Sela et al., 2012). This makes sense, as the structure of LNT (a linear oligosaccharide with the structure of Gal β 1-3GlcNAc β 1- $3Gal\beta1-4Glc$) serves as the core for other glycans, be they fucosylated, sialylated, or decorated with other sugar moieties. Consequently, whether a microorganism is able to remove these decorations, is critical to that organism's ability to break down this core sugar. The catabolism of LNT, as described in *B. bifidum*, relies on a lacto-*N*-biosidase to separate the lacto-N-biose from the lactose (Wada et al., 2008). The ability to grow on lacto-N-biose has been observed across many infant-associated Bifidobacterium species, such as B. longum subsp. infantis, B. longum subsp. longum, B. breve, B. pseudocaten*ulatum*, and *B. bifidum*, while the ability was not present in the adult-associated *B. adolescentis* (Xiao et al., 2010). Interestingly, there have been a few studies that have shown that lacto-N-neotetraose (LNnT) is not as readily digestible, despite the fact that it has a very similar structure (Galβ1-4GlcNAcβ1-3Galβ1-4Glc) (Asakuma et al., 2011; Garrido et al., 2016). The difference in the structure is a single linkage that is Gal\beta1-3GlcNAc in LNT and Gal\beta1-4GlcNAc in LNnT. While no clear mechanism has been determined for the difference in consumption ability between the two isomers, there is evidence that the enzyme responsible for cleaving that first galactose may be stereo-specific (Miwa et al., 2010).
Species/subspecies	Total HMOs	LNT	LNnT	2FL	3FL	3SL	6SL	References
Bifidobacterium longum subsp. infantis	+	+	+	+	+	+	+	Ward et al. (2006); Ruiz-Moyano et al. (2013); LoCascio et al. (2009)
B. longum subsp. longum	+	+	v	V	v	-	-	Yu et al. (2013a); Garrido et al. (2016)
B. breve	+	+	+	v	v	-	-	Ruiz-Moyano et al. (2013)
B. bifidum	+	+	v	v	+	+	+	Wada et al. (2008); Ashida et al. (2009); LoCascio et al. (2009); Turroni et al. (2010); Moon et al. (2016); Garrido et al. (2015)
B. animalis subsp. lactis	-	-	-	-	-	-	-	Ruiz-Moyano et al. (2013); Garrido et al. (2016)

 TABLE 9.1
 Pooled and Individual HMO Consumption by Bifidobacterial Species and Subspecies

+, All or most strains are able to grow; –, no strains are able to grow; v, some strains are able to grow; HMOs, human milk oligosaccharides; 2FL, α -1,2-fucosyllactose; 3FL, α -1,3-fucosyllactose; LNT, lacto-N-tetraose; LNT, lacto-N-neotetraose; 3SL, α -2,3-sialyllactose; 6SL, α -2,6-sialyllactose.

Another noteworthy trend is that sialylated HMOs, such as 2,3- and 2,6-sialyllactose, serve as an available carbon source for few bifidobacteria, and, to date been shown to support growth of only *B. longum* subsp. *infantis* and *B. bifidum*. The ability to consume these sugars relies on the presence of a sialidase, which is consistently present in these two bifidobacteria, as well as *B. breve*, though in this latter species the presence of a sialidase does not confer a strong ability to consume sialylated sugars (Ruiz-Moyano et al., 2013). Interestingly, while *B. breve* cannot consume these sugars, it is capable of growing on free sialic acid, suggesting a more complex relationship may exist between *B. breve* and this sugar (Egan et al., 2014).

The consumption of fucosylated sugars is more common in bifidobacteria than sialylated sugars. Fucosidases have been found in *B. longum* subsp. *infantis* (Sela et al., 2012), *B. breve* (Ruiz-Moyano et al., 2013), *B. bifidum* (Ashida et al., 2009), *B. longum* subsp. *longum* (Yu et al., 2013a,b), and *B. pseudocatenulatum* (Matsuki et al., 2016). While many of these organisms possess only one fucosidase, of glycosyl hydrolase (GH) family 95, some also possess a fucosidase from family 29. Of most interest is *B. longum* subsp. *infantis*, whose genome encodes five fucosidases, though only three have been shown to have activity associated with HMO consumption. Interestingly, these different fucosidases have different specificities, with GH family 29 (a family more rare in bifidobacteria; Matsuki et al., 2016) associated more with catabolism of 3'-fucosyllactose (Sela et al., 2012).

In addition to free oligosaccharides, several enzymes have been characterized with regard to their activity on glycoproteins. In *B. longum* subsp. *infantis*, an endo-β-*N*-acetylglucosaminidase has been shown to have strong activity on N-linked glycoproteins, making the sugar available as a growth substrate (Garrido et al., 2012b; Karav et al., 2016), while in *B. longum* subsp. *longum* and *B. bifidum*, enzymes have been shown to target O-linked glycoproteins (Fujita et al., 2005; Kiyohara et al., 2012).

The consumption of HMOs by bifidobacteria is generally done by one of two strategies: glycans can be transported intact into the cell where they are then broken down into monosaccharides for utilization, or they can be broken down extracellularly and transported into the cell as mono- and disaccharides for consumption (Fig. 9.2) (reviewed in Garrido et al., 2012a). The first strategy (termed here as "inside-eaters"), observed in most species of bifidobacteria, including *B. longum* subsp. *infantis*, *B. longum* subsp. *longum*, and *B. breve*, requires a wider array of transporters and solute-binding proteins for a broader set of HMOs; the second strategy ("outside-eaters"), observed primarily in *B. bifidum*, requires fewer transporters but also requires cell wall–associated glycosyl hydrolases. The consequence of this outside-eater strategy is that mono- and disaccharides released by the cell wall–associated glycosidases may be consumed by other organisms that are not able to consume the more complex oligosaccharides. This has been observed in the case of *B. breve*, which cannot consume sialyllactose but can grow on the residual sialic acid produced by *B. bifidum* (Egan et al., 2014).

9.5 NONBIFIDOBACTERIAL HMO CONSUMPTION

The ability to consume HMOs is not entirely exclusive to the *Bifidobacterium* genus. *Bacteroides* species are commonly found in breast-fed infant microbiomes, and have been shown to consume certain HMO structures (Marcobal and Sonnenburg, 2012; Pudlo et al., 2015). *Bacteroides* spp. possess many predicted and observed metabolic pathways



FIGURE 9.2 The two HMO consumption strategies of bifidobacteria. Human milk oligosaccharide structures encountered by bifidobacteria can either (A, outside-eater) be broken down by extracellular glycosidases (C) followed by mono- and disaccharides transported into the cell via permeases (C) for digestion (or left outside the cell undigested), or (B, inside-eater) be transported into the cell intact for subsequent glycosidase activity.

associated with the consumption of mucin glycoproteins, generally O-linked glycans similar to the ones described earlier (Marcobal et al., 2011). To do this, Bacteroides species can express membrane- and periplasm-associated GHs that extracellularly cleave the glycan structure from the mucin protein, followed by further glycosidase activity to cleave oligosaccharides into smaller components for transport across the membrane (Xu et al., 2003). This classifies Bacteroides as an outside-eater, similar to B. bifidum in its consumption behavior. In a similar manner, and using some of the same genes, *Bacteroides thetaiotaomicron* is capable of cleaving and utilizing HMO as a sole carbon source (Marcobal et al., 2011). The same ability, to differing extents, has been observed in other members of the Bacteroides genus. The authors note that this capability is a modification of mucin degradation, as some, but not all, of the enzymes expressed for the latter function are activated during HMO growth (Marcobal et al., 2011). When compared with B. longum subsp. infantis, growth of Bacteroides thetaiotaomicron was slower on HMO (Marcobal et al., 2011), and with certain sugars (such as LNnT), B. longum subsp. infantis was able to outcompete Bacteroides thetaiotaomicron in vivo (Marcobal et al., 2011). This suggests that the use of a subset of mucin utilization genes for HMO consumption is advantageous, but is not optimized for growth on HMOs as is seen in some inside-eater *Bifidobacterium* species. As suggested by these data, homologous genes with slightly different targets allow an organism to expand its niche in the host, explaining at least in part why some species are more likely to predominate the infant gut, while others are less so.

9.6 BIFIDOBACTERIAL HMO CONSUMPTION AND COLONIZATION OF INFANTS

As discussed earlier, not all bifidobacteria are equally adapted to consume milk glycans, which would, in turn, suggest that select strains are better able to colonize a breast-fed infant. A recent study addressed this concept in preterm infants—infants who experience a delayed colonization by bifidobacteria by comparison to term infants (Butel et al., 2007). In a study of preterm infants receiving breast milk, Underwood et al. (2013) gave probiotic *B. longum* subsp. *infantis* (HMO consumer) or *B. animalis* subsp. *lactis* (HMO nonconsumer). The results clearly demonstrated that the breast milk–fed infants were colonized by *B. longum* subsp. *infantis*, while no colonization by *B. animalis* subsp. *lactis* was observed. This suggests that the connection between milk glycans (in this case, as part of whole human milk) and colonization of the gut is a directed relationship, specific to certain bifidobacterial species and strains but not others.

9.7 MATERNAL GENOMIC INFLUENCE ON COLONIZATION

Given both the difference in metabolic capacity of different bifidobacteria and the difference in their demonstrated colonization abilities, one could expect that the specific content and composition of HMOs could affect which bifidobacteria dominate the environment. The specific linkages in any given mother's milk depend on the activity of glycosyltransferases in the mammary gland, for example, the fucosyltransferase 2 gene (FUT2) codes for an enzyme that creates α -1,2-linked fucosylated sugars. When active in the mammary tissue, it acts on a large portion of HMOs, creating a broad range of α -1,2-linked fucosyloligosaccharides (Castanys-Muñoz et al., 2013; Chaturvedi et al., 2001). In a subset of the human population (varying by geographic region), point mutations in the FUT2 gene lead to a nonfunctioning protein. Subjects with at least one functioning FUT2 gene are referred to as secretors, while those without a functioning FUT2 gene are referred to as nonsecretors, reflecting the role of the gene in the expression of ABO blood types in various secreted body fluids. In nonsecretors (approximately 20% of the population of the United States), α -1,3-, and α -1,4-linkages are still present, but α -1,2-linkages are absent, yielding a different pattern of HMOs. A study on infants fed milk from secretor and nonsecretor mothers showed that consuming secretor milk was associated with an increase in bifidobacteria in general and *B. longum* subsp. *infantis* in particular, whereas nonsecretor milk was positively correlated with levels of *B. breve*, though the total amount of bifidobacteria was, on average, significantly lower (Lewis et al., 2015b). As explained earlier, the wider array of (complementary) fucosidases in B. longum subsp. infantis provides it with the ability to consume this wider array of fucosylated sugars.

9.8 GEOGRAPHIC VARIATION IN BIFIDOBACTERIAL COLONIZATION

While the presence of bifidobacteria in breast-fed infants is observed consistently on a global scale, there is geographical variation in the percentage of breast-fed infants in which bifidobacteria is the most-abundant taxon (Tannock et al., 2016). Analysis of the infant gut microbiota shows meaningful variation in bifidobacterial abundances when comparing cohorts from different countries, such as Sweden (Abrahamsson et al., 2014), Canada (Azad et al., 2013), Switzerland (Jost et al., 2012), Ireland (Fouhy et al., 2012), Bangladesh (Huda et al., 2014), Finland and Malawi (Grześkowiak et al., 2012), and the USA (Lewis et al., 2015b). In addition to overall bifidobacterial levels, there are likely differences in the species of bifidobacteria present in infants in each of these regions, however, most studies do not robustly account for the populations at the species and subspecies level. In a study of infants from Dhaka, Bangladesh, Huda et al. (2014) showed that breast-fed infants are predominantly colonized by bifidobacteria (average level of bifidobacteria > 70%), and specifically colonized by the HMO-degrading *B. longum* subsp. *infantis*, regardless of vaginal or C-section delivery method. This contradicts the conventional wisdom from studies of infants in the developed world which suggests infants delivered by C-section tend to have lower levels of bifidobacterial colonization, especially early on (Biasucci et al., 2008; Grönlund et al., 1999). In contrast, the microbiomes of infants from California, USA are more heterogeneous, with average bifidobacterial colonization levels of < 40%, and colonized by multiple bifidobacterial species (Lewis et al., 2015b). Interestingly, this phenomenon in the US infants appears to be driven not by each infant being colonized by lower amounts of bifidobacteria, but by fewer infants having appreciable amounts of bifidobacteria at all (Fig. 9.3). Sixty-seven of the one hundred six (63%) samples from the Californian infants were dominated by a bacterial taxon other than bifidobacteria, while only 14 of 144 (9.7%) Bangladeshi samples were dominated by nonbifidobacteria. Fig. 9.3 shows the average infant microbiome of the two countries' data broken down by time, and a Principal Coordinates Analysis plot to visualize the distribution of communities. Given the predominance of Bacteroides-colonized infants in the USA, it is assumed that the HMO consumption abilities of Bacteroides species allow it to thrive in the absence of bifidobacteria. Further research is needed to elucidate the mechanism that leads breast-fed infants from different regions to exhibit such distinct colonization patterns, but it does seem to be in part due to bifidobacteria being absent from many infants.

9.9 CHALLENGES IN IDENTIFICATION AND ENUMERATION OF BIFIDOBACTERIA

9.9.1 Isolate ID Challenges

Given the many associations made between *Bifidobacterium* species and infant health (reviewed by Arboleya et al., 2016), this phenomenon of missing bifidobacteria may cause concern. One possible solution would be to add bifidobacteria to infants in the form of a probiotic. However, existing quality control of specific products to verify



FIGURE 9.3 The gut microbiomes of infants from Dhaka, Bangladesh and Davis, USA. (A) The average microbiome composition of infants over the time points available (Davis, weeks of life 1, 3, 10, and 17; Bangladesh weeks of life 6, 10, and 15). (B) Principal coordinates analysis of the Davis and Bangladeshi infant microbiomes. Dots represent individual samples, and dot color represents day of life. The shaded ovals depict the dominant taxa of the dots in their area.

specific species or subspecies content has been lacking, resulting in a slew of products with potentially (or explicitly) misidentified species and subspecies. Masco et al. (2005) analyzed dozens of probiotic products and showed that many of them contained misidentified species or subspecies. More recently, Lewis et al. (2015a) showed that differentiation between bifidobacteria, especially between *B. longum* subsp. *longum* and *B. longum* subsp. *infantis*, has not always been accurate in commercial probiotics.

There are consequences to such misidentifications. VSL#3, one of the leading probiotic products commonly prescribed by doctors and used in several clinical trials (Brigidi et al., 2000; Gionchetti et al., 2003) was historically labeled as containing *B. longum* subsp. *longum* and *B. longum* subsp. *infantis*. Recently, the VSL#3 product label indicated these two strains were "reclassified" as *B. lactis* (presumably *B. animalis* subsp. *lactis*, a bifidobacterial species quite distant from the *B. longum* cluster). While this misidentification may not have adverse effects on the subjects of trials employing VSL#3, such studies can be erroneously reported as employing *B. longum* subsp. *infantis* and *B. longum* subsp. *longum* (Ewaschuk et al., 2008), thereby leading to inaccurate scientific literature and confusion among clinicians.

Some techniques to identify species and strains within *Bifidobacterium* employ marker genes, such as the 16S ribosomal RNA gene (Leblond-Bourget et al., 1996; Miyake et al., 1998), the 60 kDa heat-shock protein gene (Jian et al., 2001), and the RecA gene (Kullen et al., 1997), as well as combinations of gene targets applied via multilocus sequence typing (Delétoile et al., 2010; LoCascio et al., 2010; Ruiz-Moyano et al., 2013; Ventura et al., 2006). However, given the advent of inexpensive next generation sequencing, the gold standard of strain identification is now whole-genome sequencing and analysis (Sun et al., 2015).

9.9.1.1 Community Analysis of Bifidobacterial Species

Numerous 16S rRNA marker gene-sequencing studies have been used to reveal bifidobacteria in infant feces, though challenges have been encountered in this field. Indeed, methodological errors may have produced results

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suggesting a lack of bifidobacteria in infant feces. High-throughput sequencing technologies often rely on amplified segments of the bacterial 16S ribosomal RNA gene. Unfortunately, one standard "universal" 16S rRNA gene primer, the 27F primer, contains several mismatches with the 16S rRNA genes in *Bifidobacteriaceae* (Walker et al., 2015). Consequently, studies wishing to enumerate (relatively or absolutely) the bifidobacterial representation in a microbial community using the 27F primer must supplement with *Bifidobacterium*-specific primers that correct the mismatch (Palmer et al., 2007).

In addition to primer choice problems, differential DNA extraction is another challenge, described generally by de Boer et al. (2010). The thick cell wall of *Bifidobacterium* renders it more resistant to chemical lysis, especially in the matrix of feces. Consequently, mechanical disruption of the cells by bead beating was shown to be necessary for bifidobacteria to be robustly profiled as part of a fecal microbial community (Walker et al., 2015). These two challenges (DNA extraction difficulty, as well as primer specificity) may explain the inconsistencies in previous reports on bifidobacteria as members of a microbial community. However, these new tools enable future studies on the diversity of *Bifidobacterium* in the context of the human infant, and to elucidate the mechanism of their relationship with human milk.

Another challenge is that many popular 16S rRNA marker gene primer sets (such as the V4 region primers) do not amplify enough of the 16S rRNA gene to accurately define species membership. Researchers have employed secondary analysis, such as Bif-TRFLP (terminal restriction fragment length polymorphism) for identification of bifidobacterial species in a community (Lewis et al., 2013). Other tools selectively differentiate at the subspecies level, such as by distinguishing between *B. longum* subsp. *longum* and *B. longum* subsp. *infantis* (Lewis et al., 2015a). This latter method uses PCR with three primers, one that is shared by both subspecies and two that are exclusive to one or the other. More recently, primers designed for both genus-wide and specific identification of bifidobacteria in complex communities have been introduced (Ferrario et al., 2015), providing hope for more consistent analyses in the future.

9.10 CONCLUSIONS

Our understanding of the association of bifidobacteria and human milk is one that goes back more than 100 years, long predating the mainstream understanding of host-microbe symbiosis. Tissier and Gyorgy's discoveries led the way to a deeper comprehension of the relationship between human milk, the gut, and the microbiome. This three-way relationship, between the mother, the infant, and the bacteria, has been well studied over the years. Through a better understanding of the diverse structures of human milk oligosaccharides, researchers have been able to better explain direct relationships with individual species or subspecies of bifidobacteria. With a more thorough investigation of the genomes of these organisms, it is now possible to describe the interconnectedness of specific glycan structures and specific HMO consumption strategies, and therefore better explain why certain bifidobacteria have such a close relationship to the breast-fed infant. Using genomic approaches to describe microbial ecology, more information has been generated to describe geographical differences in bifidobacterial presence, with the hopes to explain downstream health consequences. To accomplish this, new methods must be embraced as newer technologies allow for exploration of these relationships. Thus, a better understanding of the relationship between bifidobacteria and human milk may lead to the development of products in response to constantly evolving global health needs.

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Further Reading

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Biological Activities and Applications of Bifidobacterial Exopolysaccharides: From the Bacteria and Host Perspective

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Exopolysaccharides (EPS) are carbohydrate polymers present as an extracellular layer surrounding diverse cellular types, from plants to animals also including different microorganisms (Moscovici, 2015). Microbial EPS have received great attention due to their industrial, medical, pharmaceutical, and food applications, although only a few polymers have successfully been used (Freitas et al., 2011; Rehm, 2010). Lactic acid bacteria (LAB) are a group of Gram-positives widely used as functional cultures in food fermentations (Leroy and De Vuyst, 2004). Particularly in food manufacture, strains able to synthesize EPS are selected because they positively influence the viscosity and texture of certain products (Zannini et al., 2016). LAB polymers have been well characterized from a technological perspective mainly in the dairy industry sector (Mende et al., 2016; Torino et al., 2015). This bacterial group has been empirically used from ancient times in the preservation and manufacture of fermented foods; thus, they are considered as "generally recognized as safe" bacteria by the "Food and Drug Administration" in the USA and some species are in the "qualified presumption of safety" list reported by the "European Food Safety Authority" in Europe (EFSA, 2015). Additionally, it has been proved that the consumption of specific LAB, such as Lactobacillus strains, have positive effects on health and they can be considered as probiotics, some of the beneficial properties being attributed to their EPS (Caggianiello et al., 2016; Ruas-Madiedo et al., 2002). Therefore, although from a taxonomic point of view Bifidobacterium genus (belonging to Actinobacteria phylum) is far from the LAB group (belonging to Firmicutes phylum), both share common characteristics, such as their capability to produce lactic acid from sugar metabolism, their presence in similar ecological niches (e.g., the intestinal mucosa of animals) and the probiotic potential of some strains. Additionally, most bifidobacteria species also carry in their genomes eps clusters having equal functional homology to those of LAB, and both types of polymers also have a similar chemical composition (Hidalgo-Cantabrana et al., 2014). Finally, some of the beneficial health properties of EPS-producing LAB and bifidobacteria are related with their polymers because these are a type of microbial-associated molecular pattern that could interact with host pattern recognition receptors, then being involved in a cross-talk established between both cellular types (Bron et al., 2012; Hidalgo-Cantabrana et al., 2012; Lebeer et al., 2010).

10.1 EXOPOLYSACCHARIDE SYNTHESIS IN BIFIDOBACTERIUM SPP.

EPS synthesized by bifidobacteria, as with other bacterial polymers, are molecules present on the extracellular surface that can be visualized as a refracting layer shrouding the bacterium under phase-contrast optical microscopy. Different electron microscopy techniques reveal that these polymers can be linked to the cell wall (Fig. 10.1A); only a few of them are released to the cellular environment when they are produced in large amounts. Besides, the production of EPS is evidenced by the characteristic phenotypes of the colonies growing on the surface of agar-medium



(B) B. animalis subsp. lactis B. reuteri

FIGURE 10.1 Visualization of the EPS layer surrounding bifidobacteria grown in MRSc (Man-Rogosa-Sharpe medium supplemented with 0.25% L-cysteine) broth using different microscopy techniques: optical microscopy (*OM*), transmission electron microscopy (*TEM*), and cryoscanning electron microscopy (*SEM*) (A). Mucoid and ropy colonies showing the production of EPS by bifidobacteria grown in the surface of agar-MRSc (B).

(Ruas-Madiedo and de los Reyes-Gavilán, 2005); bifidobacterial mucoid colonies have a rounded, glistening, and smooth appearance, and, additionally, those having ropy phenotype are demonstrated by the formation of a long filament unbreakable when an inoculation loop touches the colony (Fig. 10.1B). According to the chemical composition and the way of synthesis, two types of EPS can be distinguished: homopolysaccharides (HoPS) and heteropolysaccharides (HePS). In LAB both polymer types have been reported, but in the case of Bifidobacterium spp. only HePSproducing strains have been described to date. Generalizing, HoPS are built on repeating units of glucose (α - and β -glucans) or fructose (β -fructans) linked in different carbon positions, and a single enzyme (glycosyl hydrolase, GH) is involved in their synthesis: GH70 family or glucan sucrases for α -glucans and GH68 family or fructan sucrases for β -fructans (Meng et al., 2016; van Hijum et al., 2006). β -Glucans are synthesized by means of glucosyltransferases (Dols-Lafargue et al., 2008). HePS-like polymers have a more complex composition, which is reflected by the number of genes involved in their synthesis that are organized in eps clusters. They are built on repeating units composed of three main monosaccharides, D-glucose, D-galactose, and L-rhamnose, although in LAB others could also be present in lower proportions (N-acetyl-glucosamine and N-acetyl-galactosamine), as well as different substituents (e.g., glycerol or phosphate) (Ruas-Madiedo et al., 2009b). The eps clusters of most LAB have an operon-like organization with genes orientated in the same direction and in the same order; a core region of different glycosyltransferases (GTF), involved in the synthesis of the repeating units, is flanked by a 5' region with genes of regulation, polymerization, and chain-length determination, and a 3' region with genes encoding proteins for polymerization and export (Berger et al., 2007; Broadbent et al., 2003; Jolly and Stingele, 2001). In the case of bifidobacteria, similar genes are present, but in this genus there is no common structural organization among species nor even among strains of the same taxon (Ruas-Madiedo, 2014). Hereafter, and in the context of this review, heteropolymers will be called EPS.

Most studies carried out with EPS-producing bifidobacterial strains have been focused on the biological properties of the polymers, either for the producing bacteria or for the host. However, there is scarce information about the physiological parameters that in vitro, under laboratory conditions, are involved in the EPS synthesis by *Bifidobacterium* spp. In the case of LAB, it is well known that the medium composition (mainly carbon/nitrogen ratio), growth temperature, pH, and oxygen, among others, affect the final yield and EPS physical-chemical composition (Degeest et al., 2001). In bifidobacteria, it seems that the carbon source could also modify the amount of EPS synthesized by *Bifidobacterium longum* given that the polymer yield of strain CRC002 was higher in the presence of lactose, in comparison with glucose, galactose, or fructose (Audy et al., 2010). It has also been proven that CO_2 , at a concentration of higher than 20%, improves the synthesis of EPS by *B. longum* JBL05 (Ninomiya et al., 2009). Additionally, it has been reported that stressing factors, such as bile salts or acidic conditions, induce the EPS production in bifidobacteria probably as a mechanism of protection (Alp and Aslim, 2010; Ruas-Madiedo et al., 2009a). However, to date, the synthesis of EPS by bifidobacteria under in vivo conditions has not been proved. Indirect evidence, using animal models fed with EPS-producing bifidobacteria, suggest that polymers could be produced in the gut given that these bacteria, in contrast with their non-EPS-producing mutants, exerted protection against pathogens or chemical agents (Fanning et al., 2012; Hidalgo-Cantabrana et al., 2016). Therefore, the production of EPS by bifidobacteria in the gut is still unknown that needs to be answered. Finally, in order to demonstrate the beneficial effects or to study the mechanisms of interaction between EPS and host-cells, it is necessary to purify the polymers from the producing bacterium, which could be a challenging issue. This will also allow the physical-chemical characterization of the polymers to allow insight into the intrinsic properties of the polymers that could be directly related to their biological effects (Hidalgo-Cantabrana et al., 2012). Basic protocols for the isolation and characterization of bifidobacterial, as well as LAB, EPS have been described, which begins with the election of a suitable culture medium to avoid the copurification of nonbacterial polymers (Alhudhud et al., 2014; Vaningelgem et al., 2004). A basic procedure involves the release of the polymer from the biomass suspension using a weak NaOH treatment, followed by ethanol precipitation of the polymer from the supernatant under cold conditions and a final step of intensive dialysis of the precipitate against ultrapure water before lyophilization. Additional purification protocols, directed to reduce the protein and DNA content or to separate EPS fractions of different sizes, could be applied as well (Leroy and De Vuyst, 2016; Ruas-Madiedo, 2014).

10.1.1 Genetic Background

The common strategy used to search for genes related with EPS production is the location of the priming-GTF (*p-gtf*), which is the gene encoding the enzyme that initiates the intracellular synthesis of the repeating unit by transferring a sugar-1-phophate to the lipid carrier anchored to the cytoplasmic membrane. This approach was used to select strains harboring *p-gtf* in a collection of bifidobacteria isolated from human intestinal microbiota (Ruas-Madiedo et al., 2007); based on the amplification of *p-gtf* using hybrid primers developed for lactobacilli (Provencher et al., 2005), several strains belonging to species *B. adolescentis*, *B. longum*, and *B. pseudocatenulatum* showed positive amplification and their EPS were purified. However, polymers were obtained from other strains of these species whereas the PCR amplification failed, as it did for all *B. animalis* subsp. *lactis* strains tested (Ruas-Madiedo et al., 2007). This indicates that the primers used, designed for lactobacilli, were not totally suitable for bifidobacteria. Currently, specific primers have been developed for this genus (Hidalgo-Cantabrana et al., 2015a), which are able to detect the two types of *p*-GTF genes described in bifidobacteria: *rfbP* (coding for undecaprenyl-phosphate sugar phospho-transferase) and *cpsD* (coding for galactosyltransferase). These genes were annotated in the first genome published of this genus belonging to the strain *B. longum* NCC2705 (Schell et al., 2002) and both, or at least one of them, are often present in all *Bifidobacterium* taxa, as revealed by the analysis of their genomes.

The first in silico analysis of bifidobacterial genomes detecting the presence of *eps*-clusters in this genus was reported by Lee and O'Sullivan (2010) who, based on the nine genomes publicly available at that time, found that all carried clusters of genes predicted to be involved in EPS synthesis, although none of the strains were proved to produce the polymer. One year later the sequence of the *eps* cluster involved in the EPS synthesis of *B. animalis* subsp. lactis, as well as the structure of the repeating unit of the rhamnose-rich, high molecular weight (HMW)-EPS produced by the strain IPLA-R1 was published (Leiver et al., 2011). Recently, the EPS-genetic determinants in all taxa (48 to date) from *Bifidobacterium* spp. have been in silico analyzed and they have received the generic name of "*eps*ome" (Ferrario et al., 2016). These authors confirm several findings previously reported by Hidalgo-Cantabrana et al. (2014) with a lower number of different species and strains: there is no common structural-functional organization in the *eps* clusters of bifidobacteria, *B. bifidum* is the only species that does not harbor any *eps* clusters, and some of them carry more than one. It has also been shown that there is a high interspecies variability related with the length of the *eps* clusters, as well as with the presence of a variable number of genes; this is underlined by the 44 unique gene cassettes that are exclusively present in the bifidobacterial genomes. In general, the G + C content of most bifidobacterial *eps* clusters is lower than the total genome and only in a few cases (*B. cuniculi* and *B. subtile*) present similar content. This suggests a horizontal acquisition of genes, the most probable donors being inhabitants sharing a common environment with bifidobacteria, such as members of *Lactobacillaceae* and *Lachnospiraceae* (Ferrario et al., 2016). High intraspecies variability in *eps* clusters from *B. longum* has also been reported, whereas the opposite was detected

for *B. animalis* subsp. *lactis* because all strains publically available had the same cluster with a few single nucleotide polymorphisms (SNP) (Hidalgo-Cantabrana et al., 2014). This fact was recently confirmed, and a higher variability was also detected for strains of *B. adolescentis* and *B. breve* suggesting that the wide diversity among *eps* clusters is, in general, a strain-dependent trait (Ferrario et al., 2016).

In spite of the lack of a common functional structure in the *eps* clusters of bifidobacteria, most of the genes found in these bacteria are also found in LAB with the only exception being transcriptional regulators. In the described "consensus" LAB eps structure (Hidalgo-Cantabrana et al., 2014), regulators are commonly found upstream, in the 5' region, but to date this function has not been assigned to any of the genes enclosed in the bifidobacterial eps clusters (Ferrario et al., 2016). As previously indicated, all taxa harbor at least one p-gtf, being a strain-associated (but not species) characteristic. Additionally, the analysis of the 28 publically available genomes in 2014 revealed that the RfbP and CpsD priming-GTF are located in two separated phylogenetic branches, and this division is also detected when in the same strain, such as *B. animalis* subsp. *lactis* DSM10140 or *B. longum* NCC2705, both enzymes are present (Hidalgo-Cantabrana et al., 2015a). In fact, the divergence between both priming-GTF could be found in the domains involved in recognizing the specific sugars to be transferred, whereas those more conserved are related to the interaction with the lipid carrier (Hidalgo-Cantabrana et al., 2015a; Provencher et al., 2005). Apart from the *p-gtf*, other genes coding for GTF are present in the bifidobacterial *eps* cluster; these enzymes catalyze the glycosidic linkage between the sugar moieties building the EPS repeating units. Genes predicted to be involved in polymerization and export, as well as in chain-length determination, were also annotated in the bifidobacterial *eps* clusters. Two types of genes related with the transport of the repeating units across the cell envelope have been found: ABC-transporters and the "flippase-like" transport/polymerization system (Ferrario et al., 2016), whereas it seems that the first one is absent in LAB (Hidalgo-Cantabrana et al., 2014). Another remarkable feature is the presence of genes coding for precursors involved in the biosynthesis of rhamnose. At least three genes (coding for dTDP-glucose pyrophosphorylase, dTDP-4-dehydrorhamnose 3,5-epimerase, and dTDP-D-glucose) were present in almost all genomes, showing a high percentage of similarity (Ferrario et al., 2016). Moreover, the C + G content of these rhamnose-precursor genes in B. animalis subsp. lactis strains DSM 10140 and ATCC 25527, B. longum NCC2705, and B. dentium Bd1 was higher than that of the eps cluster (Hidalgo-Cantabrana et al., 2014). Finally, another common feature shared between bifidobacterial and LAB eps clusters is the presence of IS elements/transposase-encoding genes. Most often these mobile elements flanked the eps cluster in LAB, whereas in the case of bifidobacteria transposases and derivatives could be also found within the cluster. The presence of these mobile genetic sequences favor the hypothesis of horizontal genetic transfer of the eps genes between bifidobacteria and other bacteria inhabiting a common ecological niche (Ferrario et al., 2016; Hidalgo-Cantabrana et al., 2014).

It is worth noting that for the genes described earlier as being involved in EPS synthesis in bifidobacteria, have been proposed based on homology analyses. As far as we know, only two genes present in eps cluster of bifidobacteria have been functionally characterized to date. Two derivative strains, showing a different bacterial surface, were obtained from the EPS-producing *B. breve* UCC2003 by insertion or by fortuitous deletion of a region in the single *p-gtf* found in its eps cluster. In addition, the transcriptional analysis of this eps cluster was also achieved showing that, of the two operons found, only the *eps1* is constitutively transcribed whereas the *eps2* operon is silent (Fanning et al., 2012). Recently, our group has proved the function of the gene *balat_1410* that encodes a hypothetical membrane-anchored protein with a predicted soluble domain, which is theoretically involved in determining the chain elongation of EPS (Hidalgo-Cantabrana et al., 2015b). Using a double-crossover marker-less strategy, a mutant strain (DSM10140- Δ Balat_1410) lacking balat_1410 was obtained from the type strain B. animalis subsp. lactis DSM10140; afterward, this deletion was complemented with a plasmid harboring the same *balat_1410* or the gene *balat_1410^{S89L}* in which an SNP was present. This SNP consists of a C to T transition, which causes a codon change (a serine is substituted by a leucine in position 89) and it was detected in the genome of *B. animalis* subsp. *lactis* IPLA-R1 displaying a mucoid-ropy phenotype and producing a HMW-EPS, which is synthesized in lower amounts by other strains lacking this SNP. In our study, we proved that although *balat_1410* is not essential for EPS synthesis, the three mutant strains have different surface traits and the strain Balat_1410^{S89L}, harboring the plasmid with the SNP gene, acquired a ropy phenotype and produced the rhamnose-rich, HMW-EPS fraction in a higher proportion. Besides, the three *balat_1410* mutants displayed different effects on immune modulating capabilities, as well as in the interaction with the intestinal epithelium, showing the involvement of the HMW-EPS on bifidobacteria-host interactions (Hidalgo-Cantabrana et al., 2015b).

10.1.2 Hypothetical Biosynthesis Pathway

The ubiquitous presence of *eps* clusters in *Bifidobacterium* genus points to the implication of these polymers in essential physiological or ecological roles for the producing bacterium. Indeed bifidobacteria, as well as LAB, invest

part of their energy in biosynthesis of the EPS in a process that is in direct competition with the synthesis of other structural molecules and with the central carbohydrate metabolism (Hidalgo-Cantabrana et al., 2014). This could explain the reduced biomass yield obtained from most EPS producing strains, either LAB or bifidobacteria, which, in general, is lower than 600 mg/L (range between 25 and 600 mg/L; Ruas-Madiedo et al., 2009c). The pathway of EPS biosynthesis in bifidobacteria is not known, but according to the predicted function of the proteins encoded by the genes of the *eps* clusters a hypothetical biosynthetic route can be predicted (Fig. 10.2). This theoretical pathway is based on that proposed for bacterial polymers used as additives in multiple applications, which are synthesized at high yields, mainly by Gram-negative bacteria, such as *Sphingomonas* spp., *Xanthomonas campesris*, or *Pseudomonas aeruginosa*, among others (Schmid and Sieber, 2015), or on that investigated for pathogens in which the EPS could be a virulent factor, such as *Eschericia coli* and *Streptococcus pneumoniae* (Cuthbertson et al., 2009; Yother, 2011), as well as on that proposed for LAB (Hidalgo-Cantabrana et al., 2014).

The first step in the synthesis of EPS is the intracellular formation of the repeating units for which nucleotide-activated sugar precursors, acting as donors of monosaccharides in the reactions catalyzed for GTF, are required. These sugar precursors could be obtained from different metabolites, such as glucose-1-phosphate and fructose-6-phosphate that are also intermediates in the central sugar metabolism (Boels et al., 2001; De Vuyst et al., 2001). In the case of bifidobacteria, the enzymes required for the synthesis of UDP-glucose, UDP-galactose, and UPD-rhamnose from glucose-1-phosphate have been found in their genomes according to the information compiled in the Kyoto Encyclopedia of



FIGURE 10.2 Hypothetical pathway of heteropolysaccharide biosynthesis in *Bifidobacterium* spp. and proposed role of EPS for the producing-bacteria in the gut ecosystem. The codes inside the boxes are the E.C. numbers (http://www.brenda-enzymes.org) of the putative enzymes needed for obtaining the sugar-nucleotide precursors, which are involved in the synthesis of EPS repeating units in *Bifidobacterium* spp. according to the information of the KEGG (Kyoto Encyclopedia of Genes and Genomes) database (http://www.genome.jp/kegg/pathway.html).

10. FROM THE BACTERIA AND HOST PERSPECTIVE

Species	Strains	Glc	Gal	Rha	Others	References
B. adolescentis	YIT 4011	3			Deoxytalose (6)	Nagaoka et al. (1988)
	M101-4	2	1			Hosono et al. (1997)
B. animalis subsp. lactis	C64MRa	2.5	1.5	1		Salazar et al. (2009a,b)
	E43	2.5	2	1		Salazar et al. (2009a,b)
	A1	2.5	2	1		Ruas-Madiedo et al. (2010)
	A1dOxR (IPLA-R1)	1	1	1.5		Ruas-Madiedo et al. (2010)
	HMW-IPLA-R1	1	2	3		Leiver et al. (2011)
	RH	1	3	2	Mannose (2)	Shang et al. (2013)
	LKM512	1	2	3		Uemura and Matsumoto (2014)
B. bifidum	BIM B-465	1.3	1			Zdorovenko et al. (2009)
	ALM 35	1	3	2.4		Prasanna et al. (2012)
B. breve	YIT 4010	1.5	1			Habu et al. (1987)
	NCIMB 8807-EPS1	2.5	1	1.4		Prasanna et al. (2012)
	NCIMB 8807-EPS2	1	1.9			Prasanna et al. (2012)
B. catenulatum	YIT 4016		1			Nagaoka et al. (1996)
B. longum subsp. longum	ATCC 15707 ^T	1	1			Abbad-Andaloussi et al. (1995)
B. longum subsp. infantis	ATCC 15697 ^T	a	1			Tone-Shimura et al. (1996)
	35624 ^{тм}	2	2	_	Deoxytalose Galacturonic acid	Altmann et al. (2016)
B. longum	YIT4028		1.5	1		Nagaoka et al. (1995)
	BB-79	1	1.5			Roberts et al. (1995)
	JBL05	2	4	1		Kohno et al. (2009)
	H73	2.5	1	2		Salazar et al. (2009a,b)
	L55	4	1	1		Salazar et al. (2009a,b)
	H67	4	1	4		Salazar et al. (2009a,b)
	E44	1	1			Salazar et al. (2009a,b)
	CCUG52486	1.3	1			Prasanna et al. (2012)
	NCIMB702205	1	2.5			Prasanna et al. (2012)
	NB667	1	2.2	1		Salazar et al. (2012)
	N667dCo	1.2	2.3	1		Salazar et al. (2012)
B. pseudocatenulatum	A102	2	1			Salazar et al. (2009a,b)
	C52	1	1			Salazar et al. (2009a,b)
	E515	2	1			Salazar et al. (2009a,b)
	E63	1	1			Salazar et al. (2009a,b)
	H34	15	1			Salazar et al. (2009a b)

TABLE 10.1 Monosaccharide Composition of EPS Synthesized by Bifidobacterium ss	ssp.
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 a This backbone is partially substituted with $\beta\text{-D-Glcp}$ linked in the carbon C-6 of both monosaccharides.

Modified and updated from: Hidalgo-Cantabrana, C., Lopez, P., Gueimonde, M., de Los Reyes-Gavilán, C.G., Suarez, A., Margolles, A. and Ruas-Madiedo, P., 2012. Immune modulation capability of exopolysaccharides synthesised by lactic acid bacteria and bifidobacteria. Probiotics Antimicrob. Prot. 4, 227–237, with permission of Springer Editorial.

Genes and Genomes (KEGG) pathways database (Fig. 10.2). These are the nucleotide-sugar precursors needed for the incorporation of glucose, galactose, and rhamnose, which are the main monomers found in the repeating units of bifidobacterial EPS up to date (Table 10.1). The enzymes for the synthesis of GDP-mannose and GDP-fucose, which are present in a few LAB EPS, have been found as well in bifidobacteria; however, mannose has only been found in

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a bifidobacterial polymer and fucose has not been reported yet (Table 10.1). Interestingly, N-acetylated amino-sugars have also not been reported, which could be correlated with the absence of known enzymes, according to the KEGG database, involved in the synthesis of these sugar precursors in bifidobacteria (Fig. 10.2).

As previously stated, two putative secretion-polymerization systems have been in silico described in bifidobacteria, ABC transporters and the flippase-polymerase complex (also named Wzx-Wzy-dependent pathway). The experimental studies supporting these models of EPS secretion and polymerization have mainly been performed with *Escherichia coli*, but also with Gram-positive pathogens forming capsular polysaccharides, such as some *Streptococcus* pneumoniae serotypes. Additionally, the knowledge gained about the biosynthesis of other surface molecules, for example the O-antigen lipolpolysaccharide, also contributed to shed some light on the secretion and polymerization of bacterial EPS (Cuthbertson et al., 2009; Freitas et al., 2011; Rehm, 2010; Yother, 2011). The polymerization of ABC transporter-dependent EPS is apparently carried out without the participation of the lipidic carrier (or undecaprenylphosphate); in this case the formation of the polymer occurs in the cytoplasmic side of the cellular membrane by the sequential addition of sugar residues to the nonreducing terminus of the chain, although the acceptor molecule in the membrane for the chain in formation is still not known. Afterward, the polymer is transported across the (inner) membrane to the periplasmic side by means of the ABC-transporter in Gram-negatives (Cuthbertson et al., 2009). In the case of bifidobacteria, although several ABC transporters have been found in the eps clusters (Ferrario et al., 2016), it has not been established whether they are involved in the export of the EPS chains. However, it can be hypothesized that this ABC-transporter/polymerization system could be involved in the formation of EPS in those bifidobacterial taxa for which the priming-GTF has not been detected since this enzyme seems to be present in the Wzx-Wzy-dependent pathway. In this flippase-polymerase system the intracellular assembly of the EPS repeating units begins with the activity of the priming-GTF, which links the first sugar to the lipid carrier. This is a limiting step not only due to the availability of the nucleotide-sugar precursors, but also to that of the undecaprenyl-phosphate, which could be involved in the synthesis of other surface molecules, such as peptidoglycan and teichoic acids in Gram-positives (Yother, 2011). Once the undecaprenyl-phosphate-linked repeating units are built, through sequential enzymatic glycosylations, they are exported across the membrane by the putative flippase (Wzx) and they are extracellularly polymerized by the putative polymerase (Wzy). Finally, a tyrosine kinase phospho-regulatory system, named Wzz (E. coli) or Wzc (S. pneumoniae), seems to be involved in the chain length determination of the polymer, given that mutations in this system enable the formation of polysaccharides of different chain lengths (Woodward et al., 2010; Yother, 2011). In the case of bifidobacteria, the protein encoded by *balat_1410* in *B. animalis* subsp. *lactis* shows a high homology with a hypothetical membrane-anchored protein theoretically involved in the chain length determination of the polymer. This protein is homologous to Etk-like tyrosine kinases, suggesting that phosphorylation might regulate EPS synthesis in this bifidobacterial species. As indicated in the previous section, a single mutation in *balat_1410* induced a change in the phenotype of *B. animalis* subsp. *lactis*, which correlates with an increase in the amount of the large-size EPS fraction synthesized (Hidalgo-Cantabrana et al., 2015b). Modifications in the phenotype of Lactobacil*lus johnsonii*, toward a smooth colony, were also associated with a mutation in *epsC* belonging to the *eps* cluster that is predicted to encode a tyrosine-protein kinase (Horn et al., 2013). Thus, it seems that in LAB and bifidobacteria a tyrosine kinase phosphoregulatory system could be involved in the elongation of the EPS chain.

10.1.3 Physical–Chemical Composition

Some studies have been conducted in order to elucidate the chemical and structural composition of the bifidobacterial EPS. The main techniques involved in such characterizations are analytical chromatography, either liquid or gas chromatography, and nuclear magnetic resonance (NMR), both requiring a previous purification of the polymer (Ruas-Madiedo and de los Reyes-Gavilán, 2005). In spite of the widespread presence of *eps* clusters in *Bifidobacterium* spp., the physical–chemical composition of the polymers was undertaken only for a few species (Table 10.1). As was indicated earlier, the monosaccharides that build the bifidobacterial polysaccharides are D-glucose, D-galactose, and L-rhamnose (Table 10.1) and they are, in general, the only monomers present in the seven species studied to date. The exceptions are the presence of mannose in the EPS of the strain *B. animalis* subsp. *lactis* RH (Shang et al., 2013) and 6-deoxytealose in the EPS from *B. adolescentis* YIT 4011 (Nagaoka et al., 1988) and *B. longum* subsp. *longum* 35624TM (Altmann et al., 2016). Galactose and glucose are present in almost all bifidobacterial EPS described to date, while rhamnose is present in half of them (16 out of 32 polymers) (Table 10.1). Curiously, two *B. bifidum* strains have been reported as EPS producers although no *eps* clusters have been found in this species (Ferrario et al., 2016; Hidalgo-Cantabrana et al., 2014). This fact could be related with a misclassification of the strains, given that the authors do not clearly report the identification procedure of the isolates, or it could be related with a specific strain-dependent characteristic. In any case, the studies reporting the chemical composition of bifidobacterial EPS demonstrate that

10. FROM THE BACTERIA AND HOST PERSPECTIVE

Repeating unit sizes	Strains and EPS repeating unit structures ^a	References
Disaccharide	B. infantis ATCC15697: 3)-β-D-Galf-(1→3)-α-D-Galp-(1→ (both partially occupied with β-D-glucopyranose at position 6)	Tone-Shimur et al. (1996)
Trisaccharide	B. catenulatum YIT 4016: \rightarrow 6)- β -D-Galf-(1 \rightarrow 5)- β -D-Galf-(1 \rightarrow	Nagaoka et al. (1996)
	6 ↑ 1 α-D-Gal <i>p</i>	
Pentasaccharide	<i>B. breve</i> YIT 4010: →3)-β-D-Glcp-(1→3)-β-D-Galp-(1→4)-α-D-Galp-(1→2)-α-D-Glcp-(1 → 6 ↑ 1 α-D-Glcp	Habu et al. (1987)
Pentasaccharide	B. longum YIT 4028: →2)-α-L-Rhap-(1→3)-α-D-Galp-(1→2)-α-L-Rhap-(1→3)-α-D-Galp-(1→ 6 ↑ 1 α-D-Galp	Nagaoka et al. (1995)
Hexasaccharide	<i>B. animalis</i> subsp. <i>lactis</i> HMW-IPLA-R1: α -D-Galf-(1→2)-α-L-Rhap 1 ↓ 2 →4)-β-D-Glcp-(1→3)-α-L-Rhap-(1→2)-α-L-Rhap-(1→4)-β-D-Galp-(1→	Leiver et al. (2011)
Hexasaccharide	<i>B. animalis</i> subsp. <i>lactis</i> LKM512: α -D-Galf-(1 \rightarrow 2)- α -L-Rhap 1 \downarrow 2 \rightarrow 3)- β -D-Glcp-(1 \rightarrow 3)- α -L-Rhap-(1 \rightarrow 3)- α -L-Rhap-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow	Uemura and Matsumoto (2014)
Hexasaccharide	B. longum subsp. longum 35624 TM : →4)-β-D-Glcp-(1→4)-α-L-6dTalp-(1→4)-α-D-GalpA-(1→4)-α-D-Galp-(1→4)-α-D-Galp-(1→ 1 ↓ 2 α-D-Glcp	Altmann et al. (2016)
Heptasaccharide	B. bifidum BIM B-465: →6)-α-D-Glcp-(1→3)-β-D-Galf-(1→3)-α-D-Glcp-(1→2)-β-D-Galf-(1→3)-α-D-Glcp-(1→3)-α-D-Glcp-(1→ 6 ↑ 1 α-D-Glcp	Zdorovenko et al. (2009)
Heptasaccharide	B. longum JBL05: β-D-Glcp	Kohno et al. (2009)
	↓ 6 →4)-α-D-Galp-(1→4)-α-D-Galp-(1→4)-β-D-Glcp-(1→3)-α-D-Galp-(1→3)-β-L-Rhap-(1→ 4	
	↑ 1 β-D-Gal <i>p</i>	

TABLE 10.2	Structure of the Repeating Units of Bifidobacterial EPS Determined by NMI	R

TABLE 10.2 Structure of the Repeating Units	f Bifidobacteria	al EPS Determined	l by NMR (cont.)
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Repeating unit sizes	Strains and EPS repeating unit struc	tures ^a			References
Heptasaccharide	<i>B. animalis</i> subsp. <i>lactis</i> RH:				Shang et al.
	α-D-	Galp	α-D-Gal <i>p</i>		(2013)
		1	1		
		Ţ	\downarrow		
		3	3		
	→4)-α-D-Glcp-(1→4)-α-D-l	Man <i>f</i> -(1→4)-α-L-Rha-(1→4)-α-D-Man <i>f</i> -(1→4)-α-D-G	Sal <i>f</i> -(1 →	
Nonasaccharide	B. catenulatum YIT 4011:				Nagaoka
	β-Glc		β-Glc	β-Glc	et al. (1988)
	1		1	1	
	\downarrow		\downarrow	\downarrow	
	3		3	3	
	ightarrow 2)- $lpha$ -6dTal-(1 $ ightarrow$ 3)- eta -6dTal-(1	l→3)-β-6dTal-(1→3)-β-6dTa	al-(1→2)-α-6dTal-(1→2)-	α-6dTal-(1→	

^a dTal, Deoxytalose; f, furanose ring conformation; Gal, galactose; Glc, glucose; Mn, mannose; Rha, rhamnose; p, pyranose ring conformation.

some of the *eps* clusters described for *Bifidobacterium* spp. are functional although, as far as we know, only in *B. animalis* subsp. *lactis* IPLA-R1 and *B. longum* subsp. *longum* 35624TM have both the genetic determinants and the structure of the repeating unit been studied in the same strain (Leiver et al., 2011).

The repeating unit that builds some bifidobacterial EPS has been determined by NMR for only 11 strains and all of them present a unique structure of variable length (Table 10.2). The shortest one is that present in the EPS synthesized by *B. longum* subsp. *infantis* ATCC15697 that has two 1,3-linked β -D-galactofuranosyl and α -D-galactopyranose moleties, although this galactan backbone can be occupied (90% and 30%, respectively) with β -D-glucopyranose at position C6 (Tone-Shimura et al., 1996). The strain B. catenulatum YIT 4016 also synthesizes a galactan composed of a trisaccharide repeating unit (Nagaoka et al., 1996) and this is the only polymer presenting a single monosaccharide type in their EPS, because the remaining polymers present, at least, two different sugars. In general, most bifidobacterial EPS have repeating units composed of five or more monosaccharides and the highest reported is the nonasaccharide synthesized by *B. catenulatum* YIT 4011 (Nagaoka et al., 1988). The size of the repeating units of bifidobacterial polymers is not correlated with a given producing species but is a strain-associated characteristic, as previously indicated for the *eps* clusters. Even in *B. animalis* subsp. *lactis*, which showed a very low variability at EPS genetic level, three different structures are described, although the hexasaccharides reported for strains IPLA-R1 (Leiver et al., 2011) and LKM512 (Uemura and Matsumoto, 2014) are very similar. In the bifidobacterial repeating units both α - and β -anomers are present mainly in pyranose ring conformation and furanose is only adopted by galactose and mannose (Table 10.2). With no exceptions, glucose and galactose have D-absolute configuration, while rhamnose has L-absolute configuration. In general, these characteristics have also been found in LAB EPS (Mozzi et al., 2006; Ruas-Madiedo et al., 2009b) although, due to the limited number of bifidobacterial repeating units described, these generalizations should be taken with precaution and more studies are needed to know if bifidobacterial EPS could have other distinctive traits.

10.2 BIOLOGICAL PROPERTIES

As previously indicated, bifidobacteria invest part of their energy in the biosynthesis of EPS, even when in this process several housekeeping enzymes are shared with the central catabolic pathway and with other anabolic routes. This indicates that EPS may play a beneficial role for the producing bacterium in terms of acting as a physical barrier in response to stressing environmental conditions, intermediating in cell–cell interactions, and/or modifying the colonization and persistence in different ecological niches. Additionally, as a surface microbial-associated molecular pattern these polymers are also involved in the interaction with host cells and, therefore, they are able to exert beneficial effects (Hidalgo-Cantabrana et al., 2014; Ryan et al., 2015). Finally, EPS-producing LAB are selected as functional starters because of their ability to positively modify the sensorial characteristics of fermented dairy products. Bifidobacteria do not have good technological aptitudes to be used as starters for acidification and food fermentations, mainly due to their reduced ability to survive in the presence of oxygen; however, in spite of the limited growth in milk some EPS-producing *B. animalis* subsp. *lactis* and *B. longum* strains are able to improve the viscosity and texture of fermented dairy products (Prasanna et al., 2014; Ruas-Madiedo et al., 2009c). Therefore, it is clear that the diverse



FIGURE 10.3 Biological functions and potential applications proposed for EPS-producing bifidobacteria and their polymers.

biological and biotechnological functions attributable to bifidobacterial EPS, some of them summarized in Fig. 10.3, have focused the attention of researchers working in different fields.

10.2.1 Bacterial Protection and Colonization

The role that EPS play for the producing bifidobacteria has not been determined yet, but it is expected that they could have similar functions to those proposed for other bacteria sharing common (food and gut) ecosystems, such as LAB. It has been reported that EPS could act as a protective shield against bacteriophages in some LAB, as is the case of Lactococcus lactis (Gopal and Crow, 1993; Sijtsma et al., 1988) or Lactobacillus delbrueckii subsp. bulgaricus (Durlu-Özkaya et al., 2005). However, other authors also pointed to EPS as receptors for phage adsorption in L. lactis strains (Ainsworth et al., 2014; Deveau et al., 2002), as well as in *Streptococcus thermophilus* (Rodriguez et al., 2007). Therefore, the role that EPS could play in relation to phage resistance or sensitivity is not clear, it seems to be a characteristic dependent on the surface of the LAB-host strain, as well as on the phage infection system. In the case of *Bifidobacterium* genus, the in silico analysis of their genomes revealed the wide presence of prophages (Lugli et al., 2016), as well as CRISPR-Cas systems (Briner et al., 2015). In fact, Lugli et al. (2016) were able to induce the release of complete phage particles after mitomycin C induction in three species; the phage obtained from *B. boum* have tail decorations that resemble those of the phage 1358 from L. lactis, which were postulated to be involved in the nonspecific adsorption of the phage to the lactococcal EPS (Spinelli et al., 2014). Therefore, it could be possible that EPS surrounding bifidobacteria interplay with specific phages in the gut ecosystem, contributing to the modulation of the bifidobacterial population in this niche. In any case, this hypothesis needs further confirmation and the ability of bifidobacterial EPS to adsorb, or to avoid the adsorption of, phages would be a specific trait dependent on the characteristics of both microorganisms.

EPS from LAB are also involved in protection against antimicrobial agents, such as nisin (Durlu-Özkaya et al., 2005; Looijesteijn et al., 2001), lysozyme (Coulon et al., 2012), or heavy metals (Polak-Berecka et al., 2014), among others. In bifidobacteria the protective function of these polymers was studied under conditions mimicking gastrointestinal stresses, mainly acid and bile salt challenges. By means of adaptive evolution to low pH, a variant strain from *B. longum* BBMN68 was obtained, which presented two nucleotides altered in *cpsD* and a concomitant modification in the amino acid sequence of the encoded protein that modified the EPS production in this variant strain (Jiang et al., 2015). Similarly, an acid-resistant derivative strain was obtained from *B. breve* BB8 and the transcriptional and physiological analyses of both strains showed that EPS production was improved in the acidic-adapted *B. breve* BB8dpH strain (Yang et al., 2015). The presence of 0.3% bovine bile salt (oxgall) in the culture medium induces *eps* gene expression, as well as polymer production in *B. animalis* subsp. *lactis*; the amount of EPS synthesized proportionally increased with the percentage of bile salt added (Ruas-Madiedo et al., 2009a). Therefore, the positive correlation between EPS production and the resistance to bile salts or low pH suggests that bifidobacteria could synthesize these polymers as a mechanism of protection to face the harsh conditions that they encounter in the intestinal tract.

The role of EPS in the colonization and persistence of the producing bacteria in the gut remains largely unclear. Some knowledge advances have been made with one of the most widely used probiotic lactobacilli strains, that is, *Lactobacillus rhamnosus* GG. This strain produces two different EPS; the presence of a long galactose-rich polymer prevents the in vitro adhesion of *L. rhamnosus* GG to intestinal Caco-2 cells, given that it partially masks the exposure

10.2 BIOLOGICAL PROPERTIES

of the pili involved in adhesion. However, the absence of this galactose-rich polymer reduced the in vivo (murine model) survival of a mutated strain and its presence in the parental L. rhamnosus GG protects it from the activity of host-antimicrobial peptides (Segers and Lebeer, 2014). Similar findings have also been reported for EPS producing Lactobacillus johnsonii strains (Denou et al., 2008; Dertli et al., 2015). In the case of bifidobacterial EPS most studies related with in vivo colonization and survival have been performed with two model species: B. breve and B. animalis subsp. lactis. Fanning et al. (2012) found that the EPS located on the surface of B. breve UCC2003 has an impact on the persistence, but not colonization, of the producing strain in mice gut, given that bifidobacterial counts from feces of animals fed with the non-EPS producer mutant were significantly lower that the parental strain 9 days posttreatment. Our group has been working with EPS-producing *B. animalis* subsp. *lactis* for more than a decade. We have observed that the daily oral administration for 24 days of the ropy strain IPLA-R1 (producing a rhamnose-rich HMW-EPS, also named A1dOxR, which has been obtained from the bile-adapted A1dOx strain) to Wistar rats promoted higher levels of *Bifidobacterium* spp. and *B. animalis* subsp. *lactis* quantified by specific PCR in rat feces and cecum content than those of the placebo-fed group; this shows a high survival rate of the IPLA-R1 strain in the mice gut and suggests a certain colonization capability that could be related with the presence of the polymer (Salazar et al., 2011). In fact, in vitro studies carried out with intestinal epithelial cells (Caco-2 and HT29) showed that stain IPLA-R1, which was spontaneously obtained from the bile salt-adapted A1dOx strain, had a higher adhesion capability than the parental one, which produced the HMW-EPS in a lower amount (López et al., 2012). Thus, the intrinsic characteristics of the polymers might be important factors influencing the capability for colonization, survival, and/or persistence of the producing strain in the gut environment. In this regard, the polymer purified from IPLA-R1 was able to compete, in a dose-dependent manner, with the producing *B. animalis* subsp. *lactis* strain for its attachment to human mucus (Ruas-Madiedo et al., 2006). From these results we hypothesized that EPS could directly adhere to mucus, and then competitively inhibit the adhesion of the bifidobacteria, or they could stick to the bacterial surface, thus masking other bacterial molecules involved in mucus attachment (Ruas-Madiedo et al., 2006). This second option is supported with studies of the two Lactobacilli species indicated earlier; in the first option, the EPS could be forming a biofilm layer in the intestinal mucosa, which is a possibility later suggested by other authors (Dertli et al., 2015; Fanning et al., 2012) but that, as far as we know, has not been demonstrated.

10.2.2 Beneficial Effect for the Host

10.2.2.1 Immune Modulation Capability

Traditionally, it was considered that carbohydrates were not able to induce an adaptive immune response, but nowadays it is well known that they are acting on T-cell and antigen-presenting cells; some bacterial EPS, such as the zwitterionic polysaccharides, have been extensively studied and they are known as potent immune activators (Avci et al., 2013). The capability of EPS synthesized by LAB and bifidobacteria to modulate the immune response was reviewed by Hidalgo-Cantabrana et al. (2012) and specifically updated for bifidobacterial EPS in 2015 (Hidalgo-Cantabrana et al., 2015a). Most of the studies carried out with bifidobacterial EPS aimed to establish their involvement in the beneficial properties of the producing bacteria, but no mechanisms of immune action of the polymer itself have been elucidated to date. One of the earliest studies showed that the polymer synthesized by *B. adolescentis* M101-4 was able to in vitro induce the proliferation of cultured spleen cells and Peyer's patch cells isolated from BALB/c mice (Hosono et al., 1997). Later, it was demonstrated that the polymer purified from B. longum BCRC 14634 acted as a mild immune modulator of J77A.1 macrophages due to its capability to induce the secretion of IL-10 (antiinflammatory cytokine) or, after J77A.1 induction with a proinflammatory stimulus, to reduce the levels of TNF α (proinflammatory cytokine) (Wu et al., 2010). Similarly, polymers purified from 18 strains of *B. animalis*, *B. longum*, and *B. pseudocatenulatum* were capable of slightly stimulating the proliferation of human peripheral blood mononuclear cells and modify their cytokine production pattern (López et al., 2012). Differences dependent on the size and chemical composition of the polymers were observed; generalizing, the EPS of small molecular weight induced higher levels of cytokines, while higher polymers diminished the immune response toward a lower release of cytokines or a decrease in the TNF α /IL-10 ratio that is used as an indicator of the antiinflammatory profile (López et al., 2012). Thus, these results show that the physical-chemical characteristics of bifidobacterial-EPS are key parameters determining their ability to act as immune effector molecules. In fact, the studies carried out with the model of three isogenic B. animalis subsp. lactis strains presented in previous sections demonstrate that the strain producing the rhamnose-rich, HMW-EPS increased the IL-10 production of peripheral blood mononuclear cells cultures and reduced the $TNF\alpha$ secretion of ex vivo cultivated human colonic biopsies in comparison with the non-EPS producer strains; that is, the levels of the TNF α /IL-10 ratio were reduced at systemic and intestinal mucosa levels (Hidalgo-Cantabrana et al., 2015b). Besides, the strain B. animalis

subsp. *lactis* IPLA-R1 was proven to increase the suppressor-regulatory TGF-β cytokine and reduce the levels of the proinflammatory IL-6 in an in vivo model of Wistar rats (Salazar et al., 2014). Similarly, the treatment of mice with *B. breve* UCC2003 reduced the levels of proinflammatory cells and cytokines in comparison with the non-EPS producing strain (Fanning et al., 2012). These findings suggest that HMW-EPS-producing bifidobacterial strains could be used for attenuating symptoms in mild inflammatory processes. Indeed, the mutant *B. animalis* subsp. *lactis* producing the HWM-EPS was in vivo effective for the reduction of colitis induced by a chemical agent in C57BL/6J mice (Hidalgo-Cantabrana et al., 2016).

10.2.2.2 Modulators of Intestinal Microbiota

In a recent review, Salazar et al. (2016) compiled studies related to the capability of EPS from LAB and bifidobacteria to act as fermentable substrates by the intestinal microbiota. The earliest demonstration of the use as a carbon source of EPS, purified from different LAB by members of the intestinal microbiota, was performed with homogenized fecal cultures from healthy donors (Ruijssenaars et al., 2000). Later works confirmed that the biodegradability of EPS was directly correlated with the complexity (structural organization and chemical composition) of the polymers, as well as with the pool of degrading enzymes (GH family) available, which are supplied for specific members of the intestinal microbiota (Salazar et al., 2016). Regarding bifidobacterial EPS, in vitro studies with nonpH-controlled batch cultures of human fecal slurries (Salazar et al., 2008), or pH-controlled batch cultures simulating the conditions in the distal part of the gut (Salazar et al., 2009a), as well as in vivo approaches (Salazar et al., 2011) carried out with purified polymers, or with the EPS-producing bifidobacteria, have demonstrated that they are able to modify the profile and diversity of the intestinal microbiota, also inducing shifts in their metabolic activity (production of short chain fatty acids). Similar findings have been reported latterly with other EPS-producing bifidobacteria (Li et al., 2014a). Thus, these bifidobacterial polymers were able to modulate the dynamic of the intestinal microbiota. In fact it was shown that Bacteroides fragilis, one of the most abundant commensal bacteria of the human gut, was able to metabolize bifidobacterial EPS when added to a basal semidefined medium; generalizing, by means of proteomic and transcriptomic approaches, changes driven by EPS consumption were detected toward an improvement in the reducing power and energetic capability of bacteroides (Rios-Covian et al., 2015). The effectiveness of Bac. fragilis to degrade bifidobacterial EPS was recently proven using a minimal medium supplemented with the polymers as the sole carbon source. An increase in propionate and acetate levels, in parallel to a decrease in lactate production, and a concomitant reduction in the size of the polymer added was detected (Rios-Covian et al., 2016). Thus, it could be suggested that in the intestinal ecosystem the EPS synthesized by bifidobacteria could act as carbon-source reservoirs for microbiota, which could use them through different cross-feeding pathways leading to changes in the profile of short chain fatty acids and, ultimately, promoting health benefits (Salazar et al., 2016).

10.2.2.3 Other Functions

Several bifidobacterial strains have been found to be able to inhibit the activity of intestinal pathogens and their EPS could play a relevant role in this antagonism (Sarkar and Mandal, 2016). Indeed, some works report the antimicrobial activity of bacterial EPS and preliminary studies suggest as possible targets of action the cell wall, cytoplasmic membrane and DNA of pathogens (He et al., 2010). It was shown that the EPS-producing B. breve UCC2003 strain was able to reduce the colonization of *Cytrobacter rodentium* in gut mice; the authors proposed that the potential mechanism of action would be through the formation of an EPS biofilm (Fanning et al., 2012). Indeed, previous in vitro studies have hypothesized that the antagonistic mechanism of the EPS purified from *B. animalis* subsp. *lactis* IPLA-R1 counteracting the effect of bacterial toxins upon the intestinal epithelium (Caco-2 cells) was the formation of a physical barrier by the polymers; thus, in a physiological situation either the EPS-producing bacterium or the polymer released to the surroundings could form a protective shield on the intestinal mucosa (Ruas-Madiedo, 2014; Ruas-Madiedo et al., 2010). Another hypothesis that does not exclude the previous one was proposed after the observation that EPS purified from the same IPLA-R1 strain was able to in vitro increase the adhesion of enteric pathogens to human mucus (Ruas-Madiedo et al., 2006); in this case the EPS could have a "lectin-like" activity, being able to act as analogs of eukaryotic receptors for the adhesion of pathogens (or their toxins), or they could act as scavenging agents reducing the levels of harmful agents (Hidalgo-Cantabrana et al., 2014). In relation to this last function, some articles in literature report the capability of bifidobacterial EPS to act as antioxidants by reducing levels of reactive oxygen species (Li et al., 2014b). In support for this hypothesis, an in vivo study shows that EPS purified from B. animalis subsp. lactis RH had a potent antioxidant activity (Xu et al., 2011). Finally, other beneficial effects for human health have been reported for EPS producing LAB, such as a cholesterol lowering capability or in vitro antiproliferative effect on carcinogenic cells, but as far as we know no data are available regarding bifidobacterial EPS (Hidalgo-Cantabrana et al., 2014; Ryan et al., 2015).

10.3 POTENTIAL APPLICATIONS

As reviewed in the previous section, the beneficial effects that have been attributed to bifidobacterial EPS open up a wide range of applications. However, the low yield reported in laboratory conditions for these polymers makes their use as additives a very challenging issue. Indeed, in the case of EPS from LAB several attempts have been made to increase the yield of EPS synthesized by LAB, but no significant overproduction was obtained, and this was not even tried for bifidobacterial EPS. Therefore, the current application of these polymers will be limited to the use of the EPS-producing strains either for foods or food supplements (Ruas-Madiedo et al., 2009c).

10.3.1 As Probiotics

Bifidobacteria have been included in the manufacture of dairy products, mainly yogurt-like fermented milks since the last few decades of the past century, due to the "probiotic" character of this bacterial group (Prasanna et al., 2014). Probiotics have been defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2006; Hill et al., 2014). Several positive effects have been attributed to specific bifidobacterial strains whose efficacy was demonstrated in human intervention studies (Tojo et al., 2014). In some of them, the strain was orally delivered as a food supplement (e.g., in a capsule), but in other cases the study was carried out with a fermented food, thus this additional beneficial effect cannot be separated. In fact, apart from the use of fermented milks as a delivery vehicle for probiotic bifidobacteria, some strains are able to modify milk components generating a product with beneficial properties; this is the case for the strain *B. bifidum* MF 20/5 that is able to release antihypertensive peptides encrypted in milk proteins (Gonzalez-Gonzalez et al., 2013). It has been reported that a few EPS-producing bifidobacteria are able to slightly grow in milk (Salazar et al., 2009b) and even some specific strains have influence in the rheological and textural properties of the fermented product; this is the case of *B. longum* subsp. *infantis* CCUG 52486 that reduces syneresis and improves viscosity of milk fermented with a starter yogurt (Prasanna et al., 2013). Thus, this indicates that at least some specific strains are metabolically active in milk and they are able to synthesize EPS.

10.3.2 As Prebiotics or Synbiotics

The capability of the EPS to act as fermentable substrates for beneficial members of the intestinal microbiota prompts the proposal that these polymers have potential as prebiotics. There are several definitions (reviewed by Roberfroid et al., 2010), which finally propose the prebiotic concept as "the selective stimulation of growth and/or activity(ies) of one or a limited number of microbial genus(era)/species in the gut microbiota that confer(s) health benefits to the host." Nowadays, most prebiotics substrates used are nondigestible carbohydrates; some of them, such as inulin, have a similar chemical composition, but shorter length, than LAB-EPS (i.e., inulin-like β -fructans). Thus, the EPS produced by bifidobacterial strains able to modulate the composition of the intestinal microbiota, as reviewed before, could fit into the concept of prebiotic. However, due to the limited production capability of these polymers by bifidobacteria, or LAB in general, the application of the EPS-producing bifidobacteria strain as a "synbiotic" product will be more plausible, that is, a combination of the probiotic and the prebiotic potential in a single strain (Salazar et al., 2016). In any case, this possible application deserves further research since no human intervention studies have been carried out with EPS-producing bifidobacteria aiming to demonstrate their suitability as effective modulators of the intestinal microbiota for improving the health and wellbeing state.

10.4 CONCLUDING REMARKS

EPS production is a widespread phenotypic trait in bifidobacteria. For its synthesis, a relevant amount of cell molecules must be put into action, including several transport proteins, GTF, polymerases, and other carbohydrate-modifying enzymes; the energetic cost of this process for the producing bacteria is justified by the key functional role of the polymers. EPS constitute the most external shield of the bifidobacterial envelope and, as such, it is the first cell structure exposed to the contact with the surrounding environment. Thus, it is a relevant player involved in the interactions with members of the intestinal microbiota, the gut environment and host cells. In recent times, solid scientific evidence has come to light supporting a role of bifidobacterial EPS in protection against environmental factors, colonization/persistence in the intestine, immune modulation and as substrates for other commensal bacteria. However, the molecular mechanisms underlying these processes are still far from being understood, and some

scientific challenges are waiting to be tackled to have a complete picture of the EPS role in bifidobacteria. Perhaps one of the most exciting ones is to clarify the particular structural features and chemical properties that determine the different functions attributed to these polymers because, until now, there exists only fragmentary information and nonconclusive evidence. Thus, the establishment of the structural–functional relationships of EPS will be critical to understand the interactions between bifidobacteria and their host.

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Folate and Bifidobacteria

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11.1 INTRODUCTION

Folates are involved in some of the most fundamental metabolic reactions taking place in cells of all types, and probably in all forms of life. Cells can, for instance, neither properly synthesize DNA and proteins nor methylate these macromolecules without folates. Some organisms synthesize their own folate and do not need external supply. The rest of the living world, however, depends on external folate as food, presynthesized by folate synthesizing organisms, such as bifidobacteria, many other microorganisms, and plants. Mammals including humans are auxotrophic for folate. This chapter reviews folate biosynthesis, metabolism, analysis, deficiency, and biotechnological potential—all with some emphasis on bifidobacteria. We also discuss whether there may be a link between the human need for folate and the ability of bifidobacteria to synthesize folate and be part of the gut microbiota, and whether there is a potential for folate trophic probiotics.

11.2 NOMENCLATURE AND MOLECULAR STRUCTURE

Folate is a term referring to a family of compounds having similar chemical structure to that of folic acid, and has received much attention due to its importance for health and increasing understanding of consequences from deficiency (Section 11.7). Mitchell et al. (1941) isolated a compound from spinach, and called it folic acid after the Latin name *folium* (leaf). Folate has since been studied in different animals, plants, and microorganisms and many synonyms have arisen along the way, such as "vitamin M" (Langston et al., 1938), "*Lactobacillus casei* factor" (Stokstad, 1943), and the currently accepted name vitamin B₉.

From a chemical point of view folic acid is part of a group of compounds called pteridines. More precisely, its structure is made of three components: a pteridine ring and a *para*-aminobenzoic acid unit (*p*ABA) linked to each other by a methylene bridge to form the pteroic acid, which is joined by a peptide linkage to glutamic acid. The acyl group derived from the pteroic acid is a pteroyl group. Therefore, folic acid is also known under the name ptero-ylglutamate (pteroyl-L-glutamic acid). The atoms are numbered as indicated in Fig. 11.1. Folic acid is the synthetic analogue of folate. It is used for fortification of foods and in dietary supplements.

In nature, folates are mainly present in their reduced, one-carbon-substituted (or unsubstituted) forms of pteroylglutamates. Reduced folates are specified by prefixes and numbers of the added atoms of hydrogen ("dihydro-folate": H_2 folate, "tetrahydro-folate": H_4 folate, THF). The pteridine ring can have three different oxidative states: fully oxidized (folic acid), partially reduced (as in H_2 folate), or fully reduced (as in THF). A tetrahydro-folate is assumed to have substituents in the 5, 6, 7, and 8 positions, and a dihydro-folate in the 7 and 8 positions, unless otherwise stated. The reduction of the pteridine moiety of folate to THF and its various physiologically active derivatives occurs in two stages (Fig. 11.2). Only the reduced forms of folate are biologically active.

Naturally occurring folate forms differ in types of substituent and number of glutamyl residues attached to the pteroyl group. The majority of folates have a side chain of five (in liver) to seven (in yeasts and plants) glutamate residues linked with γ -peptide bonds (Gregory, 1996). More precisely, the molecules of glutamate are conjugated through amide bonds and each is linked to the preceding molecule of glutamate with the γ -carboxyl group (Fig. 11.1). These



FIGURE 11.1 The structure of folic acid (monoglutamate derivate). pABA, para-aminobenzoic acid.



FIGURE 11.2 The structure of folic acid, dihydrofolate, and tetrahydrofolate. In nature folates exist in their dihydro- (as in DHF) and tetrahydro- (as in THF) form, while the pteridine ring is fully oxidized in folic acid.

bonds are different from those present in proteins because they involve α -carboxyl groups. The polyglutamate chain is responsible for linking the coenzyme to the apoenzyme while the pteridine ring side participates to the passage of the one-carbon unit (Talwar et al., 2015) (Section 11.5).

Depending on which one-carbon unit acts as a substitute, different derivatives of folic acid are formed. So far, six of these substitutes are commonly found, and are named as defined in the general Organic Nomenclature Rules (Series, 1979): hydrogen (H), methyl (CH₃), formyl (HCO), formimino (NHCH), methylene (CH₂), and methenyl (CH⁺), together with the numbers related to the substituted positions. The nomenclature of these folate derivatives depends on the oxidative status of the pterioline ring and on the type of substituent (added one-carbon unit) at the position N⁵ and/ or N¹⁰ of the pteroyl group. Pteroic acid can be linked to one or more molecules of L-glutamate to form pteroyl-glutamate, pteroyl-diglutamate, and so on. The number of glutamate units linked to the ring can be up to 10, making the amount of possible folate forms in nature more than 100 (Gregory, 1989; Scott et al., 2000). Some of the most studied derivatives of folates are unsubstituted THF, 5-methyl-tetrahydro-folate (5-CH₃-THF), 5-forminino-tetahydro-folate (5-NHCH-THF), 5-formyl-tetrahydro-folate (5-HCO-THF or folinic acid), 10-formyl-tetrahydro-folate (10-HCO-THF), 5,10-methenyl-tetrahydro-folate (5,10-CH⁺=THF), and 5,10-methylene-tetrahydro-folate (5,10-CH₂-THF) (Talwar et al., 2015) (Fig. 11.3).

Folate belongs to the water-soluble B-vitamin family in which it has been given the name B₉. The IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN) (1979) indicated how the term folate might be used for any members of the family of pteroylglutamates, or a mixture of them, with different levels of oxidation of the pteridine ring, one-carbon substitutions and numbers of glutamate units. The term folic acid should only be used when referring to the fully oxidized and synthetic form of vitamin B₉ (Scaglione and Panzavolta, 2014).

11.2.1 Stability and Interconversion

Folates are chemically unstable reduced compounds (Forssén et al., 2000), especially susceptible to oxidative cleavage that splits them at the C⁹—N¹⁰ bond, often into inactive molecules of pteridine and *p*-aminobenzoylglutamate. Folates with a substituent in the position N⁵ or N¹⁰ are more resistant to the cleavage (Lucock, 2000). This higher resistance could be due to a steric hindrance action against oxidative compounds. The stability of folates depends also on the pH of the environment, temperature, and on the presence of metal ions, such as copper and iron as well (Gregory, 1989, 1996). Different folate forms have different pH-ranges of stability. 5,10-CH⁺—THF is stable at pH below 2, whereas folic acid and 5-HCO-THF are more stable at pH above 5 (Jagerstad and Jastrebova, 2013; Strandler et al., 2015). 10-HCO-THF is highly unstable at all pH values. THF is also unstable, but its stability improves at pH



Folate

γ-Glutamyl tail

Folates	R subst	Bridge (NS N10)	
derivatives	N ⁵	N ¹⁰	Bridge (N°=N°)
THF	-H	-H	
5-CH ₃ -THF	-CH ₃	-H	
5-NHCH-THF	-CH=NH	-H	
5-HCO-THF	-HCO	-H	
10-HCO-THF	-H	-HCC	C
5, 10-CH ₂ -THF			-CH ₂₋
5, 10-CH ⁺ =THF			-CH ⁺ =

FIGURE 11.3 The structure of folate and its derivatives. Folates have commonly a γ -polyglutamyl tail linked to the first glutamate ($n \le 8$ residues). Different one-carbon units (R substitutes) can be linked to the N⁵ and/or N¹⁰ positions.

above 8 (De Brouwer et al., 2008; Strandler et al., 2015). 5,10-CH₂-THF is stable at pH above 9.5 (Strandler et al., 2015), whereas 5-CH3-THF is relatively stable in pH range 2–10 (De Brouwer et al., 2008). All folate forms are sensitive to photodegradation and need therefore to be protected from UV-light. The length of the glutamate tail does not interfere with the stability of the molecule (Gregory, 1996; Matella et al., 2005). As a consequence, folates can be lost during food preparation and storage and therefore food folate content can decrease already before ingestion. Usually this is due to their exposure to oxygen, heat, and light or simply leakage into the cooking water. Different types of cooking for instance can lead to different degree of loss. Dang et al. (2000) studied the amount of folates lost during soaking, boiling, and pressure cooking in chickpeas and peas. Pressure-cooking resulted in the best preservation of folates in peas (P < 0.05). Soaking legumes in the cooking medium represented another cause of folate loss. Losses due to leakage were greater in field peas as compared to chickpeas. McKillop et al. (2002) found that boiling green vegetables in water may cause 51% loss of folate in spinach (P < 0.005), and 56% in broccoli (P < 0.0001). Steaming, however, did not cause any significant decrease in folate content (McKillop et al., 2002). From a public health perspective, provision of practical advice on cooking techniques may be a useful strategy to improve folate intake.

Apart from chemical degradation, folates may also alter in structure due to molecular interconversion from one form to another with potential further effects on their bioavailability (Horne, 2001; Quinlivan et al., 2006). According to a study by O'Broin et al. (1975), folate stability depends to large extent on the one-carbon unit present in the molecular structure, where 5-HCO-THF is more stable than 5-CH₃-THF, followed by 10-HCO-THF and THF. However, folates were quantified by the authors with a microbiological assay (MA) that detects all biologically active forms, without qualitative information. Therefore, it is not possible to exclude that the higher stability of 10-HCO-THF over THF was due to a conversion of 10-HCO-THF to more stable 5-HCO-THF and/or 10-HCO-folic acid.

Folic acid has better stability than reduced folate forms. Although some folate oxidation to folic acid has been observed in cooked or stored food, folic acid is virtually unknown in nature (Forssén et al., 2000). This is due to the fact that THF or H₂folate only rarely would lose their hydrogen and form folic acid while remaining uncleaved (Scott et al., 2000).

11.3 SOME CRUCIAL ASPECTS IN MEASURING BACTERIAL FOLATE PRODUCTION

Measuring bacterial folate production can be performed either by microbiological assays (MA) (Masuda et al., 2012; Sugahara et al., 2015; Sybesma et al., 2003b) or high performance liquid chromatographic (HPLC) techniques (D'Aimmo et al., 2012). Both methodologies are widely used for folate analysis and have their advantages and disadvantages.

11. FOLATE AND BIFIDOBACTERIA

It may be convenient and inexpensive to use the MA when studying folate production by different bacterial strains because of availability of equipment and specialist expertise at microbiological laboratories. The application of HPLC methods requires expensive equipment and expertise in using these techniques and interpreting the data.

MA provides only the total folate content in samples whereas HPLC methods determine concentrations of different folate derivatives and give more detailed picture of the folate production by bacteria. Independently of the method used, it is important to be aware of some crucial aspects in the folate analysis in order to obtain reliable analytical results. The most crucial aspects include stabilization of folates during all steps of analytical procedure, efficient extraction of folates from the matrix, and deconjugation of folate polyglutamates to mono- or diglutamates (Eitenmiller et al., 2008; Jagerstad and Jastrebova, 2013; Strandler et al., 2015). The right choice of test microorganism for MA or detection technique for HPLC method is also of great importance (Eitenmiller et al., 2008).

Natural folate forms are highly susceptible to thermal and oxidative degradation and need to be stabilized during the whole analytical procedure (Strandler et al., 2015). They should be protected from UV-light, oxygen, and metal ions, such as copper and iron that can promote free radical oxidation reactions. All analytical steps should be optimized toward shorter incubation times and lower temperatures everywhere it does not deteriorate the yield of folate (Strandler et al., 2015). The combination of two antioxidants, ascorbic acid and a thiol, has been shown to be the most efficient method to protect folates from degradation (Wilson and Horne, 1984). Many HPLC methods for folate analysis employ a combination of ascorbic acid with 2-mercaptoethanol (Eitenmiller et al., 2008; Wilson and Horne, 1984). However, 2-mercaptoethanol is less effective and more toxic than other thiols, such as 2,3-dimercaptopropanol, 1,4-dithiothreitol, or 2-thiobarbituric acid (Patring et al., 2005). In recently developed HPLC methods, 2-mercaptoethanol has been successfully replaced by more efficient and more environmentally friendly thiols, 2,3-dimercaptopropanol (D'Aimmo et al., 2012) and 1,4-dithiothreitol (De Brouwer et al., 2008).

Less attention is paid to stabilization of folates when measuring bacterial folate production using MAs. In some works, no antioxidant use for folate stabilization is reported (Masuda et al., 2012), whereas other works report the use of ascorbic acid (Sugahara et al., 2015), 2-mercaptoethanol (Hugenschmidt et al., 2010), or a combination of ascorbic acid with 2-mercaptoethanol (Sybesma et al., 2003a). However, the stabilization of folates in MA is as important as in HPLC methods because the test microorganisms used in MA do not respond to all degradation products of folates, which results in underestimation of folate content (Buehring et al., 1974). A combination of ascorbic acid with non-toxic 2-thiobarbituric acid can be used for stabilization of folates in MA without inhibitory effects on the growth of *L. casei* subsp. *rhamnosus*.

Instability of natural folate forms may also be caused by interconversion between different folate forms. These interconversion reactions are strongly pH- and temperature-dependent and should be taken into account when developing sample pretreatment procedure for folate analysis by HPLC methods. Different folate derivatives have different pH-ranges of stability (Jagerstad and Jastrebova, 2013, 2014; Strandler et al., 2015), which makes it impossible to analyze them all simultaneously in the same run. The pH range of buffers commonly used for sample pretreatment (extraction step, deconjugation procedure, and purification by solid phase extraction using strong-anion exchange sorbents) is between 4.5 and 7.85 (Eitenmiller et al., 2008; Jagerstad and Jastrebova, 2013). Folate derivatives that are unstable in this pH range, such as 5,10-CH₂-THF (stable at pH over 9.5) and 5,10-CH⁺ = THF (stable at pH below 2), may easily convert to more stable THF and 5-HCO-THF, respectively. This means that analytical results obtained for individual folate forms do not necessarily reflect the exact relative proportions of folate forms in the original sample. Instead, they reflect the distribution between folate forms in the sample extract, which, in turn, depends on pH and other conditions during the sample pretreatment procedure. Therefore, the analytical conditions for sample pretreatment should be carefully selected considering the stability pH-ranges for different folate forms.

Bacterial intracellular folate presents mostly in the form of polyglutamates. The number of glutamate residues varies commonly between 2 and 11 glutamates and distribution between polyglutamates depends on the strain of bacteria and on the growth conditions (Bassett et al., 1976; Sybesma et al., 2003b). Polyglutamates containing three or more residues are often predominant (Shane et al., 1983; Sybesma et al., 2003b). However, the current methods for folate analysis have limitations regarding which polyglutamate forms may be determined. The MA using *L. casei* subsp. *rhamnosus* ATCC 7469 produces similar response only to mono-, di-, and triglutamates, whereas response to polyglutamates with longer chains is much lower or absent (Eitenmiller et al., 2008; Goli and Vanderslice, 1992). Other test microorganisms, such as *Enterococcus hirae* ATCC 8043 or *Pediococcus acidilactici*, ATCC 8081 provide even lower response rates compared to *L. casei* subsp. *rhamnosus* (Eitenmiller et al., 2008). Most HPLC methods allow determination of only folate monoglutamates (Eitenmiller et al., 2008; Jagerstad and Jastrebova, 2013; Strandler et al., 2015). Therefore, the deconjugation of folate polyglutamates is an essential prerequisite for reliable results in the folate analysis. Omitting this step may easily lead to underestimated and even false negative analytical results in both MA and HPLC methods.

Rat serum/plasma is commonly used as a source of folate conjugase in many current methods. It provides efficient deconjugation of folate polyglutamates to monoglutamates without compromising stability because the pH-optimum of rat plasma conjugase is 6.2–7.5 (Horne et al., 1981). Conjugase from chicken pancreas deconjugates folate polyglutamates to diglutamates and can therefore be used only for MA, its pH-optimum is 7.8 (Arcot and Shrestha, 2005). Folate conjugases from other sources, such as hog kidney or human plasma, provide deconjugation to monoglutamates, but these conjugases have pH-optimum 4.5, which result in losses of folates due to stability problems (Goli and Vanderslice, 1992). All sources of conjugase enzyme may contain small amounts of endogenous folate, therefore, enzyme blanks need to be prepared and analyzed in the same way as real samples and their endogenous folate content should be used for correction of folate content in real samples.

Bacterial folate is a mixture of several folate derivatives. According to the recent studies, polyglutamates of 5-CH₃-THF, THF, and 5-HCO-THF are the most abundant in bacterial cells (D'Aimmo et al., 2012, 2014; Leung et al., 2013; Lin and Young, 2000). This knowledge should be taken into account when developing methods for measuring bacterial folate. In the case of MA, it helps to select the most suitable test organism. An example of such microorganism is L. rhamnosus ATCC 7469; it responds almost equally to all main forms of bacterial folate (Eitenmiller et al., 2008). In contrast to L. rhamnosus ATCC 7469, other microorganisms, such as E. hirae ATCC 8043 and P. acidilactici ATCC 8081, exhibit no response to 5-CH₃-THF (Eitenmiller et al., 2008), which can result in strongly underestimated folate content. These test microorganisms should be avoided in MA for bacterial folate. In the case of HPLC, the knowledge on main folate derivatives helps to choose the most suitable detection technique, as well as to optimize all steps of the analytical procedure. THF and 5-CH₃-THF exhibit high fluorescence and can be determined with good selectivity, sensitivity, and accuracy by using fluorescence detector, whereas UV-detector cannot provide similar analytical quality because of much lower UV-response and more interference from the sample matrix (Patring, 2007). In contrast to THF and 5-CH₃-THF, 5-HCO-THF does not exhibit high fluorescence; therefore, it is not possible to achieve the same selectivity, sensitivity, and accuracy as for earlier mentioned folate forms when determining 5-HCO-THF by using either fluorescence or UV-detector. Small amounts of 5-HCO-THF may therefore remain undetected.

Mass spectrometric (MS) detection provides new opportunities for HPLC determination of folates because of its high selectivity and sensitivity. Different types of mass spectrometric detectors have been used for folate HPLC analysis: single quadrupole (MS), triple quadrupole or tandem mass spectrometer (MS/MS), and matrix-assisted laser desorption/ionization mass spectrometer (MALDI/MS) (Arcot and Shrestha, 2005; Arnold and Reilly, 2000; Eitenmiller et al., 2008; Lu et al., 2007; Patring and Jastrebova, 2007). These detectors have been shown to be superior over UV- and fluorescence detectors regarding limit of determination, selectivity, and sensitivity. Recently, novel methods combining HPLC with mass spectrometric detection, such as LC-MS/MS and LC-MALDI/MS have been successfully applied to profiling of different folate derivatives in bacteria (Arnold and Reilly, 2000; Leung et al., 2013; Lu et al., 2007). However, there are some challenges associated with mass spectrometric detectors, particularly ion suppression and/or ion enhancement of MS signal, which can result in either under- or overestimation of analytical results (Annesley, 2003; Gosetti et al., 2010). Therefore, the development of these methods requires very careful optimization of sample pretreatment, especially purification steps, as well as separation on HPLC column in order to minimize the matrix effects that can result in ion suppression/enhancement of MS signal.

11.4 MICROBIAL BIOSYNTHESIS OF FOLATE

Each gram of the intestinal content of the human colon contains up to 10¹¹ microorganisms mainly represented by the phyla *Bacteroidetes*, *Proteobacteria*, *Cyanobacteria*, *Actinobacteria*, *Firmicutes*, and *Fusobacteria* (Eckburg et al., 2005; Qin et al., 2010). This gut microbiota is composed of a vast diversity of species within these phyla, representing thousands of different bacterial strains , most of which are anaerobic. In addition, fungi are also a significant, although much smaller, component of the human gastrointestinal (GI) microbiota.

The GI biota can play a beneficial role, for instance, by providing enzymes that aid digestion of complex food components, such as polysaccharides. It may also continue breakdown of molecules that escaped the digestive enzymes in the upper part of the intestine. Moreover, the GI biota also has a role in energy recovery by production of metabolites, such as short chain fatty acids (e.g., acetic, lactic, propionic, and butyric) used as an energy source by colonocytes (O'Keefe, 2008).

Another important action of colonic microbiota is generating certain vitamins that animals cannot synthesize autonomously but need to consume with the diet. Folate is certainly a vitamin that attracts attention in this regard. Given the large quantity of bacteria present in the intestine of animals, folates produced by the GI biota could

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contribute to the daily folate intake required by the host. Therefore, probiotic strains of lactobacilli and bifidobacteria have been studied with the aim of producing naturally folate-enriched products and to exploit their potential capacity of producing folate in vivo in the colon of the host (Section 11.6).

All cells require folate in its reduced form for the production of their cellular components. Tetrahydrofolate plays a role as cofactor in essential metabolic pathways, such as DNA synthesis and methylation pathways (Jacob, 2000). Prokaryotic and eukaryotic cells differ in their strategies for obtaining cellular folate. Plants and most microorganisms can produce folate de novo through the same folate biosynthetic pathway with few variations (Bermingham and Derrick, 2002; Hanson and Gregory Iii, 2002; Levin et al., 2004). Mammals instead are auxotrophs for folate, and obtain it through the diet (e.g., leafy green vegetables, yeast extracts, liver, beans) or folate-synthesizing bacteria in the intestine (Aufreiter et al., 2009; Strozzi and Mogna, 2008). Membrane-associated folate transport proteins in the intestinal epithelia facilitate the uptake of folate from food and GI biota (Henderson and Huennekens, 1986).

The biosynthesis of folate is a complex pathway that was first elucidated by Brown and colleagues in the 1960–70s. Their work resulted in identification of the folate pathway intermediates, reactions, and the enzymes involved (Brown, 1971; Brown et al., 1961; Burg and Brown, 1968; Shiota et al., 1969). The chemical structure of folate contains a pterin moiety originating from 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHPPP), linked to a unit of *p*ABA unit (*p*ABA), which is then joined to glutamic acid. The basic building blocks to synthetize this higher structure are: guanosine triphosphate (GTP), chorismate, and glutamate, because DHPPP is produced from GTP and *p*ABA from chorismate. This biosynthesis process requires purines for the production of GTP. GTP can then form DHPPP in four consecutive steps. During the first step GTP is apparently subjected to an Amadori rearrangement from which the pterin ring structure 7,8-dihydroneopterin triphosphate is formed. This step is catalyzed by GTP cyclohydrolase I (GTPCHI, EC 3.5.4.16). This enzyme has been conserved during evolution and is used to produce biopterin. Mammals, however, lack the other enzymes needed to produce folate, which explains their dependence on exogenous sources of the vitamin.

In the second and third step, from the pterine ring and under the action of aldolase and pyrophosphokinase, DHPPP is produced. More precisely, 7,8-dihydroneopterin triphosphate first undergoes a dephosphorylation to 7,8-dihydroneopterin catalyzed possibly by the enzyme dihydroneopterin triphosphate pyrophosphohydrolase (DHPP, EC 3.6.1.67); then, 7,8-dihydroneopterin is converted, by the action of dihydroneopterine aldolase (DHNA, EC 4.1.2.25), to 6-hydroxymethyl-7,8-dihydropterin. In the fourth and last step, DHPPP is produced with an ATP-dependent reaction of phosphorylation catalyzed by the enzyme dihydroneopterine pyrophosphokinase (HPPK, EC 2.7.6.3).

The *p*ABA can be produced by plants and many bacteria. The *p*ABA biosynthesis requires parts of the glycolysis, the pentose phosphate pathway, and the shikimate pathway in order to produce chorismate. Chorismate forms 4-amino-4-deoxychorismate via a reaction catalyzed by the enzyme aminodeoxychorismate synthase (ADCS, EC 2.6.1.85), and then *p*ABA as a result of a lyase catalyzed by aminodeoxychorismate lyase (ADCL, EC 4.1.3.38).

Further, the pterin ring structure and *p*ABA are linked together with a C—N bond by the enzyme dihydropteroate synthase (DHPS, EC 2.5.1.15) to form 7,8-dihydropteroate (DHP). At this point, glutamate is joined to the newly formed dihydropteroate to make DHF by the action of the enzyme dihydrofolate synthase (DHFS, EC 6.3.2.12). DHF is reduced by a dihydrofolate reductase (DHFR, EC 1.5.1.3) to the biologically active cofactor tetrahydrofolate (THF). To add a polyglutamyl tail, other glutamate molecules can be linked to THF by the action of folylpolyglutamate synthase (FPGS, EC 6.3.2.17) (Fig. 11.4). Different folate derivatives are in their turn synthesized in the one-carbonmetabolism and then used in specific metabolic reactions.

The polyglutamilation step may also occur before the reaction of reduction, either catalyzed by DHF synthase or, in many bacteria, by the bifunctional enzyme folate synthetase/polyglutamyl folate synthetase (EC 6.3.2.12/17) (de Crécy-Lagard et al., 2007). However, in most eukaryotes investigated to date these actions are exerted by two different proteins (Ravanel et al., 2001).

11.4.1 Production of Folate by Bifidobacteria

The complete biochemical pathway for folate biosynthesis in bifidobacteria is still under investigation. Genome information can, however, be used to find genes and corresponding proteins presumptively involved in the folate biosynthesis, by comparing identified folate biosynthesis genes present in other organisms with bifidobacterial genomes.

Different databases and web resources have become available for the studies of microbial genomes. The online Integrated Microbial Genome System (IMG) (Nordberg et al., 2014), for instance, is a genome search and annotation platform established by the US Department of Energy Joint Genome Institute (DOE JGI), which supports distribu-



FIGURE 11.4 Folate biosynthesis pathway and relevant enzymes involved. For the correspondence between the EC system, and the enzyme names refer to the text.

tion and genome analysis. With this and similar databases (e.g., MicrobesOnline; Dehal et al., 2010) it is possible to study and compare genomes of microorganisms. This allows checking whether a microbe has all necessary genes to produce a vitamin, such as folate. However, presence of genes does not automatically mean functioning corresponding proteins.

A useful website is the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (Kanehisa et al., 2016). Using KEGG it is possible to see which enzymes are necessary and present in a microbial genome and how this compares to the reference pathway (LeBlanc et al., 2013; Ventura et al., 2007). This allows us to predict whether a particular bacterium would be able to produce folate de novo.

Based on existing bifidobacteria genome sequences all bifidobacteria have thus far shown the required genes to yield shikimate and thus produce chorismate (Barrangou et al., 2009; Turroni et al., 2010; Ventura et al., 2009). However, not all species are able to transform this into *p*ABA. In fact, even though all *Bifidobacterium* species have the aminodeoxychorismate synthase (ADCS) enzyme, only *B. adolescentis* and *B. dentium* species possess the genes encoding for the production of aminodeoxychorismate lyase (ADCL) and can therefore synthetize *p*ABA de novo (Ventura et al., 2009). The other species would need *p*ABA supplementation in order to be able to produce folate. However, *B. animalis* subsp. *lactis* misses the genes needed for 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHPPP) production, likely rendering it auxotrophic for folates or 7,8-dihydropteroate (DHP) even if *p*ABA is present in the environment (Rossi and Amaretti, 2010).

Not all genomes of species of bifidobacteria have yet been sequenced. At present, the IMG database contains genome information about 18 species of bifidobacteria (24 strains), but recently the total number of sequenced genomes increased to 43 out of 54 species so far described (Lugli et al., 2014; Sun et al., 2015). Available genome information for bifidobacteria led Rossi et al. (2011) and LeBlanc et al. (2013) to conclude that all bifidobacteria have the potential to transform *p*ABA to DHPPP, because they possess both the enzyme dihydropteroate synthase (DHPS) and some enzymes for DHPPP production. Moreover, *B. adolescentis, B. dentium*, and *B. longum* likely synthesize DHPPP with a thus far unknown enzyme. Considering all genes and enzymes necessary for the biosynthesis of DHPPP, THF-polyglutamate, chorismate, and *p*ABA it appears that *B. adolescentis* and *B. dentium* can produce folate de novo, *B. longum* requires *p*ABA, and *B. animalis* requires folate supplementation. More studies are needed to study these aspects at the subspecies level.

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Parallel in vivo studies of these pathways are, however, scarce. Studies on folate production and content in bifidobacteria have shown a large biodiversity between species and even between strains within species. Certain strains were found to produce folate to very high levels (D'Aimmo et al., 2012, 2014; Pompei et al., 2007a) (Section 11.6.1), whereas in others, folate biosynthesis was absent (auxotrophs). Some in vivo studies further confirmed the potential of certain bifidobacteria strains, able to produce high folate levels, to act as an in situ source of this vitamin by studying its release inside the gastrointestinal tract of animals (Pompei et al., 2007b; Strozzi and Mogna, 2008) (for reviews, see Asrar and O'Connor, 2005; Di Gioia et al., 2014; LeBlanc et al., 2015).

We conclude that microbial genomics have identified the most likely pathways responsible for bacterial folate biosynthesis, yet more genome sequencing data is needed from more *Bifidobacterium* species and strains showing differences in folate phenotypes are needed. In addition, these studies should be complemented with studies of structure and function of proteins in order to elucidate the full folate biosynthesis process for better understanding of the mechanisms causing variation in folate content among strains and species.

11.5 METABOLISM AND BIOLOGICAL FUNCTION OF FOLATE

The core molecule of bioactive folates is the fully reduced THF normally with a polyglutamate tail conjugated to the *p*ABA part of the molecule. Mutant studies on, for instance, plants have shown that polyglutamylation enhances coenzyme affinity, molecular stability, affects folate homeostasis, subcellular compartmentation in eukaryotes and helps with retaining folate species inside cells (Mehrshahi et al., 2010). Polyglutamated folates are poor substrates for folate transport systems and hence restricted to intracellular movement (Shane and Stokstad, 1975), and passive diffusion over intact membranes is negligible due to the negatively charged carboxyl groups on each glutamate unit (Zhao et al., 2009). Glutamation degree also affects bioavailability of folates originating from food or from the gut biota. The intestinal uptake of natural folates is increased by the removal of the polyglutamate chain by the intestinal mucosal conjugase folylpoly- γ -glutamyl carboxypeptidase (FGCP; EC 3.4.17.21) (Yao et al., 1995) yielding monoglutamate folates (Saini et al., 2016). This enzymatic deconjugation is however rate limiting (Konings et al., 2002), which seems to be a bottleneck for uptake of natural folates, in contrast to synthetic folic acid, which has a relatively high bioavailability (Winkels et al., 2007).

Folylpolyglutamate synthase gene families have been demonstrated also in prokaryotes including Bifidobacteria showing the importance of the polyglutamate tail also in cells lacking organelles. For instance *B. actinocoloniiforme* DSM 22766 has a gene coding for folylpolyglutamate synthase (UniProtKB database). The details on degree of glutamation of folates and its impact on relative transport/leakage and function, such as enzyme activity, are however lacking for bifidobacteria.

The main function of the core vitamin THF is to receive, carry and deliver 1-Carbon (1C) units to other metabolites as an enzyme cofactor, and participate in reactions essential for many fundamental cell functions, including biosynthesis, as well as methylation of nucleic acids and proteins. The different 1C units transiently bound to THF and delivered in a regulated fashion are: methyl (CH₃), formyl (CHO), methenyl (=CH-), methylene (-CH₂--), and formimino (CH = NH). The 1C pathways and general folate metabolism are well characterized and have been reviewed many times (for recent reviews see Stover, 2009; Saini et al., 2016). Less understood, however, are regulation of involved genes, enzymes, and fluxes, and implications on 1C metabolism (e.g., related disease states), for instance, during insufficient folate supply. A condensed overview follows.

The most abundant folate form in most cells and organism groups, as well as in circulation in mammals, such as humans, is 5CH₃-THF. With few exceptions also bifidobacteria and yeasts have higher content of 5CH₃-THF than other folate forms (D'Aimmo et al., 2012; Hjortmo et al., 2005). A pronounced dominance of CH₃-THF seems to reflect high activity, so resting states, such as stationary, surviving nongrowing populations, are likely to have a much lower fraction of CH₃-THF of total folates. In yeasts it has been demonstrated that the level of cellular 5CH₃-THF correlates positively with growth rate (Hjortmo et al., 2008b) as studied in glucose limited chemostat cultures in synthetic medium with varied dilution rate. THF was on the other hand stable at different growth rates and hence the ratio 5CH₃-THF: THF increases with increasing growth rate. It is possible that upregulation of 5CH₃-THF levels at high requirements for methionine and SAM changes the dynamic equilibrium in favor of methionine synthesis. Whether this is the case for bifidobacteria is not known. The methyl on 5CH₃-THF is used to methylate homocysteine to methionine, which can be either used in protein synthesis or further adenolysated to S-adenosylmethionine (SAM). SAM is a key molecule for virtually all cells; the universal methyl donor required for numerous methylation reactions, including methylation of proteins, cytosine bases on DNA, neurotransmitters, phospholipids, and numerous small molecules.



FIGURE 11.5 One carbon metabolism. Folates are shown in *dark gray (blue* in the web version) boxes, *striped (red* in the web version) boxes relate to DNA synthesis, *light gray (green* in the web version) boxes show other 1C metabolites, and vitamin B cofactors are indicated where involved in reactions. *5-Me-THF*, 5-Methyl-tetrahydrofolate; *DHF*, dihydrofolate; *DHFR*, dihydrofolate reductase; *dTMP*, deoxythymidine monophosphate; *dUMP*, deoxyuridine monophosphate; *MS*, methionine synthase; *SAH*, S-adenosylhomocysteine; *SAM*, S-adenosylmethionine; *THF*, tetrahydrofolate; *Thym synt*, thymidylate synthase.

Sufficient supply of 5CH3-THF is therefore necessary in the highly conserved one-carbon (1C) metabolism, which is shared by all living organisms, albeit with a few variations, such as in some halophilic archaea and some bacteria (Levin et al., 2004). The central 1C metabolism is outlined in Fig. 11.5.

The step where 5CH₃-THF delivers its methyl to homocysteine catalyzed by methionine synthase is required by all organisms to ensure regeneration of the methyl group of SAM. This step shows a specific variation between organism groups: the involvement of vitamin B₁₂ (cobalamin) or not. Many organisms, including humans, need B₁₂ as a cofactor in this step catalyzed by different forms of methionine synthase. Land-living plants and fungi, such as yeasts, do, however, not use B₁₂ at this step. In bacteria, the methionine biosynthesis may or may not be B₁₂-dependent. In *Escherichia coli*, for instance, B₁₂ can be synthesized but only when provided with the complex intermediate cobinamide (Lawrence and Roth, 1995). Three cobalamin biosynthetic genes have been cloned and sequenced from E. coli K-12. Both B₁₂-dependent MetH and the B₁₂-independent isoenzyme MetE with lower catalytic rate, can catalyze the methionine forming reaction. With respect to bifidobacteria, biosynthesis of B₁₂ has been reported (Deguchi et al., 1985), however, most statements on B_{12} production in bifidobacteria and B_{12} dependence in methionine synthesis are sweeping and, it seems, poorly studied. With respect to gut and food microorganisms with potential contribution to human B₁₂ status there are instead solid data on, for example, L. reuteri (Santos et al., 2011; Saulnier et al., 2011) and *Propionibacterium freudenreichii* (Piao et al., 2004); the latter used for commercial B_{12} production. In a nutritional perspective, in fermented foods or as trophic probiotics, a Bifidobacterium strain with high levels of both folate and B₁₂ would be very desirable because these two vitamins are, in humans, needed together, and their status is often too low, especially in the elderly, and supplementation is commonly prescribed in combination.

SAM is tremendously important in all kinds of methylations, including epigenetic labelling of genes (methyl groups on cytosine) and histones, which both affect gene expression (and phenotype) with high precision. Insufficient folate status may therefore lead to error in the epigenetic printing with consequences in gene expression we only begin to understand. Many human diseases, including cancers, diabetes, obesity, and celiac disease, show a changed epigenetic pattern of methyl groups on DNA and histones (Hernández-Aguilera et al., 2016; Kaushik and Anderson, 2016; Kocsis et al., 2014; Mayne et al., 2016; Schnekenburger and Diederich, 2015). The serum ratio between SAM and S-adenosyl homocysteine is generally considered a marker for overall methylation capacity (Monteiro et al., 2014). This ratio can be influenced by nutritional intake, for instance by "epi-bioactives," such as folate, betaine, and choline, as well as many other compounds with potential to affect the epigenetic state (Mazzio and Soliman, 2014). Folate metabolism has in fact been shown to modify epigenetic mechanisms, and human intervention trials suggest a role for folates as determinants of colorectal mucosal DNA methylation (Johnson and Belshaw, 2014).
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Epigenetic methyl labelling of DNA occurs also in prokaryotes, as shown in, for example, *E. coli, Salmonella*, and *Vibrio*, however, differently than in eukaryotes, for instance, by methylation of adenine rather than cytosine. This 5CH₃-THF-dependent labeling via SAM has not been studied in bifidobacteria. However, human nutritional epigenetics is an emerging field expected to have large impact on understanding health and disease (Crider et al., 2012; Ji et al., 2016; Mayne et al., 2016; Moreno et al., 2016), and hence *high folate bifidobacteria* may in the future be part of "epi-nutritional" strategies, together with other bioactive nutrients shown able to alter the epigenetic state.

The THF, free of 1C groups, remaining after methionine synthesis, is required for the de novo synthesis of purines and thymidylate (dTMP), necessary for DNA synthesis and repair. THF may also be used by cells to convert the amino acid serine to glycine in a step yielding 5,10-methylene-THF. This reaction may go in both directions meaning that glycine can be changed to serine and THF-formed. Vitamin B_6 is the required cofactor in this reaction. THF can also be converted to 10-formyl-THF, which subsequently may form 5,10-methenyl-THF and thereafter back to 5,10-methylene-THF, which closes the cycle, sometimes referred to as the DNA-cycle. The only folate forms analyzed in bifidobacteria are the two main forms 5CH₃-THF and THF.

11.6 BIOTECHNOLOGY AND BIOFORTIFICATION

The magnitude of folate deficiency in the world is not well known. It appears clear, however, that for many vulnerable groups (the pregnant; the elderly; those not consuming many legumes, leafy vegetables, or fruits; etc.) it is common. This strongly motivates exploring microbes as a vehicle for raising folate in food, and increasing intestinal absorption in humans (Section 11.7). Mandatory folic acid fortification of cereal grain products is implemented in many countries to reduce the incidence of neural tube defects (NTD), which it clearly has, whereas others, for example, in Scandinavia, have chosen not to. The reason given by food and health authorities in some countries for not adopting folic acid fortification is the fear for negative consequences, such as increased growth of existing tumors from overdosing synthetic folic acid (Hirsch et al., 2009; Mason, 2002) (actively debated in the medical literature, without reached consensus) or other unknown consequences. In these countries, and for people without access to high folate food or fortified food, a microbiological approach would be an attractive alternative to fortification by synthetic folic acid, especially since the reduced form of folate (THF) in microbes is identical to the active form in humans.

Bifidobacteria, as well as yeasts, have shown very promising for this, and food fermentation can indeed raise the folate content in food. Model fermentations of togwa—a traditional fermented Tanzanian cereal-based food/beverage—were studied with selected strains, yielding up to 20-fold increase in folate content after fermentation (Hjortmo et al., 2008a). Common wheat bread was bioenriched with folate using a selected strain of yeast and bioprocessing. The bread contained roughly 4 times higher folate level compared with control bread with a commercial bakers' yeast strain (Hjortmo et al., 2008c). Also Kefir-strains of yeast were found to have the potential to increase the folate content (Patring et al., 2006). Moreover, Korhola et al. (2014) showed that oat bran can be fermented and in that process bioenriched with folates. Given that search and development of starter strains with respect to folate production has only just begun, there is a large potential to increase the content of natural folate in fermented foods.

The basis for both biotechnological production of folate and biofortification of fermented foods is twofold: (1) strong *production organisms* and (2) a suitable *technical process*. The organism should have properties suitable for folate production in either a bioreactor, in fermented foods or in the intestinal tract. The last since the strategy of trophic folate probiotics is highly realistic. The technical process must favor biosynthesis and accumulation of folate in a bioreactor or in the food of choice. We discuss both in this section.

11.6.1 The Production Organism

The genus *Bifidobacterium* has been shown to include several species with capacity for de novo folate biosynthesis (D'Aimmo et al., 2012, 2014; Rossi et al., 2011, 2016). It appears, however, that many strains and possibly whole species have lost their ability to synthesize folate. Adaptations to habitats, which frequently contain external folate, mainly intestinal tracts of animals, have presumably led to reduced selection pressure for de novo folate biosynthesis. Because evolution never stops it follows that strains of the same species may differ in either being folate self-supplying or dependent on uptake from the environment. Such strain variations have become evident in several studies where different strains of the same species, screened for folate content, have been shown to be either auxotrophs or autotrophs. What does *not* differ, however, is the necessity for all bifidobacteria to maintain the many folate dependent cell functions—either from uptake of folate from the environment or by biosynthesis. A practical consequence of this

is that some bifidobacteria will not grow in synthetic media without supplementation of folic acid whereas others will. Another implication is that food fermentations may lead to reduced *or* increased folate content of the final product, as has been demonstrated for different lactic acid bacteria (Laiño et al., 2013).

The mere ability to synthesize folate, the content of folate per unit bacteria, and the composition of folate forms, have all been shown to vary substantially between species and strains of bifidobacteria (D'Aimmo et al., 2012, 2014). This is the case also for yeasts. The variations in specific total folate content of different strains of Saccharomyces cerevisiae and close relatives cultured at equal conditions were roughly fourfold (Hjortmo et al., 2005). The explanation for the large biodiversity of yeasts and bifidobacteria is technical, as well as biological. First, folate content is not a static property of a certain bacterium unless conditions are at a steady state, such as in a chemostat, which is never the case in real life. It varies highly with type and degree of activity (discussed in more detail later), which depend on medium composition, state of the population, growth rate, time at harvest, pH, and temperature. Second, the methods for folate analysis vary between studies (see analysis Section 11.3). Third, the biodiversity is real and inherent in the genes as discussed earlier, evident from studies of many strains at equal condition in one lab (D'Aimmo et al., 2012; Pompei et al., 2007a). All of this affects the apparent folate levels. In certain species, however, a functioning folate synthesizing machinery seems to be the dominating phenotype, and some species have frequently shown a high specific folate content, expressed for instance as weight folate per dry weight bacterial biomass. Hitherto, studied species with dominating autotrophy for folate are *B. catenulatum*, *B. adolescentis*, *B. pseudocatenulatum*, and *B. bifidum*. The highest value hitherto found is 93 μ g/g dry matter (DM), which was found in *B. catenulatum* ATCC 27539 (the highest in yeast is near 200 μ g/g DM). For comparison, broccoli, known to be rich in folate, contains approximately 70–80 μ g folate/100 g fresh weight (FW), which (~87% water content) yields approximately 5.5 μ g/g dry weight (17 times less than B. catenulatum ATCC 27539). Obviously, humans rarely eat large amounts of microbial biomass (yeast is sometimes the exception), but the extraordinarily high specific levels in both bifidobacteria and yeasts can make a significant difference in, for example, fermented foods.

Species in which folate biosynthesis have been found absent or a rare exception are *B. animalis*, *B. longum* subsp. *infantis*, and *B. breve* and the lowest level per biomass unit was found in *B. animalis* subsp. *animalis* ATCC 25527 containing 220 µg/100 g DM.

Naturally, the strain must also be robust enough and readily produce suitable levels of biomass with a high specific content of folate at desired conditions. The amount of folate per unit biomass is one of the most important criteria when screening and developing folate production organisms; easy biomass production with a high yield to sufficient level on suitable raw material is another. With respect to the potential application of *folate probiotics* (nutritional or trophic probiotics), a number of other properties must also be fulfilled; for instance, survival at harsh conditions prevailing in the stomach and ability to actively produce folate in the gut ecosystem.

Many bifidobacteria strains isolated from humans have this far been shown to have functioning folate biosynthesis machinery (D'Aimmo et al., 2012). The concept of folate trophic probiotics seems therefore valid and should be explored much more. It seems possible that mutual benefits have selected for folate production in bifidobacteria adapted to the human colon: a constant (perhaps low) folate supply from gut microbes may have led to a selective advantage for the host, whereas low folate status may have led to a number of low fitness conditions, such as birth defects and reduced cognitive function. If humans with folate-producing gut biota have been favored during the evolution, it would at the same time select for microbes able to both colonize the human gut *and* produce folate (symbiotic coevolution). Whether this is true or not cannot be known but the data suggest a large potential to improve human folate status by supplying high-folate live bifidobacteria of human origin.

11.6.2 Bioprocessing

A thorough understanding of the so-called one carbon (1C) metabolism and biology of the production organism is likely to help to optimize biotechnological folate production. The level and composition of folates in any cell depends on its physiological states, including type of metabolism and growth rate. It will also depend on external supply of compounds involved in the 1C metabolism, such as the amino acids methionine, serine, and glycine, also nucleotides and *p*ABA. It is misleading to view folate levels only in terms of production and accumulation (although often practically used terms) because folates are in constant turnover with exchange of 1C groups and in constant recycling. The cellular content of, for instance, 5CH₃-THF—the dominating form—is the net difference between production rate and consumption rate. In actively growing cells, folates are rapidly turned over into different forms depending on the present activities. The rate of folate biosynthesis must be high in a fast growing population, to supply methyl groups for SAM at a rate sufficient for methylation in all new biomass formed. Likewise, THF must be converted to 10-formyltetrahydrofolate (10-CHO-THF) to supply purines at a rate sufficient for rapid DNA synthesis at high

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growth rates. This rapid *turnover* does not by default mean a higher *level* in each cell of a specific folate form, such as 5CH₃-THF. These things have not yet been studied in bifidobacteria, but findings in yeast may be valid also for bifidobacteria, at least as guidance in setting up experiments. A brief overview follows.

Baker's yeast, *S. cerevisiae*, in batch cultures with synthetic medium with glucose as carbon and energy source shows diauxic growth, where fermentation of glucose dominates the first phase and respiration of ethanol the second (and a transition phase in between). Folate content (normalized for cell biomass) was highest during fermentative catabolism on glucose (the respiro-fermentative phase; ~125 μ g/g dry biomass), lower in the transition phase (~90 μ g/g), while throughout the respiratory phase continuously decreasing to 50 μ g/g at the cessation of growth where it continued to decrease to very low levels (approximately 5 μ g/g after 15 h in stationary phase). Hence, one specific strain of a microorganism can contain up to 25-fold different levels of folate depending on physiological state. It matters where cells are harvested for analysis!

In such an experiment, it was clear that the growth rate was important, because *S. cerevisiae* grows much faster by fermentation than respiration. However, studies in rich, complex versus synthetic medium showed that other things matter, too. In rich media (YPD and molasses), in spite of clearly higher growth rate, the folate levels were in yeast dramatically lower than in synthetic poor medium in which the growth rate is lower (Hjortmo et al., 2008b). This was true for yeast, as well as for bifidobacteria. In bifidobacteria, a consistently higher folate level was found in folate free medium as compared to a richer more complex folate-containing medium (D'Aimmo et al., 2012). Hence, medium composition strongly affects folate level—the richer medium the lower folate. The natural interpretation is that providing a microbe with growth factors, otherwise requiring folate to be synthesized, will reduce the folate content because the need is lower.

However, when comparing growth rates in *one* specific medium it was shown that the higher rate the more folate, as demonstrated by chemostat cultures in synthetic medium at different dilution rates (Hjortmo et al., 2008b). Moreover, the strain difference within a species was found to *not* be simply a function of different capacity to grow fast. One high and one low folate strain from the screening were compared in chemostat to force those strains to grow at same rate. The relative difference in folate content earlier observed between the strains in the batch screening remained in chemostat cultures where both strains were compared at exactly the same growth rate (Hjortmo et al., 2008b). This finding tells that there are inherent differences between strains of one species other than differences in growth rate, which determines the folate level and composition per cell or biomass unit.

Because most microorganisms can utilize many growth factors from the environment as a cheaper alternative to corresponding biosynthesis it is logical to test whether metabolites in the 1C-metabolism, would, if added to synthetic medium in concentrations typical for a complex medium, affect the cellular folate levels. A decrease in folate levels (expressed as percent change in folate levels compared to without the compound) was observed in yeast when histidine (-6%), glutamine (-16%), serine (-15%) or mix of amino acids (23%) was present, whereas methionine and glycine increased folate levels by 22 and 32%, respectively (Hjortmo et al., 2008b). The increase was restricted to THF for glycine whereas only 5CH₃-THF increased by adding methionine. A possible explanation is that external surplus of methionine decreases the need for methionine synthesis (from 5CH₃-THF and homocysteine), which would lead to accumulation of 5CH₃-THF, and discontinued requirements for glycine synthesis would favor serine and THF accumulation (Fig. 11.5). Nucleotides, pABA, or 5CH₃-THF added to the medium did *not* alter the folate levels whereas peptone yielded a 90% decrease in 5-CH₃-THF. Clearly, the complex peptone reduces the folate requirements of a microbial cell. These things have not been much studied in bifidobacteria with the exception of Pompei et al. (2007a) who added folate and pABA or folate to cultures of different strains of B. adolescentis and found an increased folate level at 0.3 mM pABA and a decrease in net folate production by supplying exogenous folate. Much remains to be understood, but the data show that it is possible to raise folate content and composition quite dramatically in a production organism by carefully composing the medium, or by adding specific compounds to food fermentation.

Data on total folate content per volume of cultivation medium is also relevant to optimize production and reduce costs. This may be measured with or without cells (the former means intracellular + extracellular folate per volume, the latter folates in cell-free supernatant). It can, however, be meaningfully compared only if it is combined with biomass data. If, for instance, the specific folate content (weight folate per weight biomass) is fixed, double concentration of glucose would for one specific strain (theoretically) result in double folate level per volume (provided sufficient levels of other nutrients). A high volumetric folate concentration can therefore either suggest high biomass concentration, high folate per biomass unit or both. If biomass is included in the equation it can be sorted out, and may show which strains are worthwhile to optimize for higher biomass concentration.

Another factor to consider is leakage by older cultures or from damaged cells (if, e.g., conditions are harsh) versus active secretion to the surrounding medium. Pompei et al. (2007a) reported a generally higher extracellular than

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intracellular folate production by most bifidobacteria strains as analyzed in batch cultures grown for 48 h. The authors also studied one selected strain in a controlled fermenter batch cultivation and found a continuous increase of folate in the medium. This suggests folate export. If bifidobacteria use energy to both produce and actively export folates (passive diffusion is negligible) it would be an altruistic behavior, possibly hard to explain. The fraction of damaged cells increases, however, with the age of a microbial population, which may lead to leakage of folates to the surrounding medium, and a heavily stirred bioreactor may cause some damaged cells. Hence, the relative contribution of leakage versus active export of folate by bifidobacteria remains unclear. In vivo, it seems likely that there is a balance between production of new cells and lysis of old cells. This chemostat-like "steady state" of gut populations will most certainly release folate, which can be absorbed by the colonocytes.

To sum up, a number of strategies can be applied for optimizing microbial folate production. These vary depending on whether a starter *Bifidobacterium* is meant to be produced, and during that step be set into a high-folate state, or if the process to optimize is the actual food fermentation, or even the human gut. All includes careful selection of strains and bioprocessing, and the use of prior art on folate and 1C-metabolism.

11.7 DEFICIENCY

This chapter has shown how crucial folates are. The central biological dogma, for instance, the transfer of information between cells and generations and between genes and proteins, cannot function without folates. It is therefore totally clear that deficiency causes damage and disease, and sufficient folate status protects us. Still, there are many open questions and controversies, such as exactly how folate protects, at which levels disease risk appears, about mechanisms for protection and disease, what good and bad levels actually are, about differences in response between synthetic folic acid and natural folate and much more. This final section only touches on some of these issues and the reader is referred to the vast medical literature on folate for details.

It has long been known that adequate periconceptional folate status (both maternal and paternal), as well as good folate status during pregnancy, are critical for maternal and foetal health. Most established is the link between low folate status and increased frequency of NTDs, which is the most common malformation of the nervous system, with a prevalence of 0.2–2 pregnancies per 1000 in Europe (Jägerstad, 2012). There is no doubt that folic acid supplementation and fortification of staple foods can reduce the severe public health problem of NTDs (Jägerstad, 2012; Zaganjor et al., 2016). More than 60 countries, therefore, practice mandatory fortification of flour, whereas some countries, for example, in Europe, have decided not to. Folic acid can be manufactured synthetically and is often used for fortification in food products or supplements, such as vitamin tablets and where chemical stability is required (Kelly et al., 1997). Although data strongly suggest that the benefit to risk ratio is very high (Jägerstad, 2012), there are still concerns among some researchers as to whether high doses of synthetic folic acid may constitute an increased cancer risk (Lucock and Yates, 2009; Mullin, 2011; Sauer et al., 2009). It has been suggested that natural folates may be of lower risk with respect to overdosing and cancer (Kim et al., 2004). This is still a matter of controversy and we certainly don't have the answer.

If, however, it is proven that overdosing of synthetic folic acid is connected to some kind of risk, obviously, raising the level of natural folates by fermented food or via probiotics would be a safer approach. The reasons are mainly two: first, biofortification by high folate microbes, such as yeast and bifidobacteria, has a good potential to significantly contribute to better folate status, without ever reaching risk levels. Synthetic supplements are, on the other hand, easy to overdose. Second, microbial folates are in the same reduced bioactive form as in human cells, whereas synthetic folic acid must be reduced in a two-step conversion in the liver. The first step, reduction by dihydrofolate reductase (DHFR) to dihydrofolate (DHF), is in humans very slow (Bailey and Ayling, 2009) and easily becomes saturated leading to unmetabolised folic acid entering circulation, which in fact is not natural at all. It is not known what damage this may cause, if any, but concerns are, for example, stimulating existing tumors. We do not believe that a microbiological approach via fermented foods and high-folate probiotics can or should replace fortification and supplementation. We rather believe it can be an important complement, especially considering that many people do not reach recommended levels of folate needed for protection.

Low folate status can also lead to so-called folate-deficiency anemia, or megaloblastic anemia, which is a result of inhibited DNA synthesis during red blood cell production, leading to cellular growth in size without division. The condition can be treated with folic acid, and, of course, avoided by sufficient intake of natural folate via food. A related well-known phenomenon is the risk for masking of vitamin B_{12} deficiency by supplying folic acid and not B_{12} (Asrar and O'Connor, 2005), which may correct red blood cell formation but not neurological damages (which may be much more severe) caused by too low B_{12} . The phenomenon that 5CH₃-THF cannot deliver its methyl group

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to homocysteine to form methionine and SAM due to absent B_{12} is known as the methyl trap. As mentioned before, folate and B_{12} are needed together.

Folate deficiency has been suggested on good grounds to have the potential to increase cancer risk, mainly in two principally different ways. First 5,10-methylenetetrahydrofolate donates a methyl to uracil, converting it to thymine, which is used in DNA synthesis and also repair. If folate is limited, there may be imbalances in the pool of nucleotides required for DNA synthesis, and uracil may be misincorporated into DNA (Hazra et al., 2010). Such misincorporation and inaccurate repair may lead to double strand breaks, chromosomal damage, and, in the worst case, cancer. The second folate-associated cancer risk is insufficient SAM and thereby compromised methylation capacity, which may lead to errors in epigenetic methylation of DNA and histones. Since cancers show changes in DNA methylation, which, for instance, may lead to the silencing of tumor suppressor genes, such hypomethylation may be a cancer risk (Toyota and Yamamoto, 2011).

Adequate folate status thus seems to protect against some forms of cancer; especially for pancreatic, oesophageal, and colorectal cancer data indicate protection (Ding et al., 2016). Consensus has not been reached because the association of folate to cancer is a complex relationship that depends on both dosage and timing of exposure to folate, or lack of folate (Lucock and Yates, 2009). It has been suggested, based on data, that cancer risk and folate status is a U-formed relation where low and excessive folate intake is bad and the range in the middle—high but not too high—is good.

Another subject under discussion is whether sufficient folate protects against heart disease. Elevated levels of homocysteine have for long been associated with cardiovascular problems. Folate deficiency may lead to raise homocysteine, and folate supplementation can lower homocysteine. However, treatment with folic acid has this far not shown to reduce heart disease.

Finally, folate has also been studied in relation to brain function. The role of folate and homocysteine in brain atrophy associated with Alzheimer's disease is not completely understood. However, in a study by Gallucci et al. (2014), a role of folate, as found inversely associated with the severity of brain atrophy, was confirmed. Systemic folate deficiency has also been associated with neuropsychiatric phenotypes. In some of these, despite normal systemic levels, folate transport to the brain is impaired in the so-called cerebral folate deficiency syndromes presenting as developmental and psychiatric disorders (Ramaekers et al., 2016).

11.8 CONCLUDING REMARKS

As a water-soluble vitamin, folate is constantly lost and has to be refilled regularly. The recommendations vary some between countries but are usually 300–400 and 600 μ g/day during pregnancy. The actual dietary intake is, however, very often below that (Jägerstad, 2012). The concluding message of our chapter is: the science of folate and 1C-metabolism is highly active and interesting in so many ways. The fact that it is conserved through all kinds of life shows its tremendous importance, which life cannot be without. There are still many remaining questions, such as when during evolution and why the ability to synthesize folate was lost since microbial eukaryotes can synthesize folate and, for example, mammals cannot, and some bifidobacteria can and others not. Advances in folate-related basic, applied microbiology, and biotechnology can contribute to new understanding, as well as to alleviating the global nutritionally important health problem of folate deficiency.

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Bifidobacteria: Ecology and Coevolution With the Host

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12.1 INTRODUCTION

Bifidobacteria are considered as key commensals in human–microbe interactions, and are believed to play an important role in maintaining a healthy gut. However, despite their generally accepted importance as probiotic microorganisms, the molecular mechanisms by which bifidobacteria act as health-promoting or probiotic bacteria are still largely unknown. Recent genome sequencing has provided intriguing insights into the genetic makeup of some members of the genus *Bifidobacterium*, although the availability of the full genomic sequence represents only the first step in the understanding of the biology and metabolic capabilities of these gut commensals.

12.2 ECOLOGICAL ORIGIN OF BIFIDOBACTERIA AND GENETIC ADAPTATION TO THE HUMAN GUT

The so-far recognized 60 bifidobacterial taxa are basically found within six distinct ecological niches, represented by the human intestine, insect gut, oral cavity, human blood, sewage, and animal gut (Turroni et al., 2011). Altogether, this rather diverse ecological distribution is a reflection of the common origin of bifidobacteria from the gut of those animals who subject their progeny to parental care. This ecological origin is sustained by a particular bifidobacterial colonization route, which involves vertical transmission from mother to offspring (Milani et al., 2015b). In support of this notion, it was recently demonstrated that specific bifidobacterial strains are shared between mother and child (Milani et al., 2015b).

Another intriguing ecological feature of bifidobacteria is represented by their dominance in the gut of mammals at the very early stages of life (Turroni et al., 2012). Such findings are further supported by recent discoveries showing that several taxa of the *Bifidobacterium* genus are genetically adapted to utilize the natural nourishment of infants, that is, breast milk, through the metabolism of particular carbohydrates present in this liquid nutrition (Sela et al., 2008). However, human milk should not only be seen as a crucial reservoir of carbohydrates [e.g., lactose and human milk oligosaccharides (HMOs)] or peptides (Oda et al., 2013) acting as bifidogenic factors to specifically support growth of particular bifidobacterial species, but may also be an important source of bifidobacterial strains for vertical transmission from mother to child (Milani et al., 2015a,b).

Other factors, including the mode of delivery, that is, vaginally delivered versus delivered by caesarian section, and type of feeding, that is, breast-fed versus bottle-fed, are pivotal in shaping the infant gut microbiota and species/ strain composition of bifidobacteria (Fanaro et al., 2003; Penders et al., 2006).

The dominance of bifidobacteria in the infant gut, especially in breast-feeding infants (Turroni et al., 2012) changes following weaning, although absolute numbers of bifidobacteria appear to become only moderately reduced (Arboleya et al., 2015; Koenig et al., 2011). Interestingly, individuals suffering from gastrointestinal disorders/diseases show a marked

The Bifidobacteria and Related Organisms. http://dx.doi.org/10.1016/B978-0-12-805060-6.00012-0 Copyright © 2018 Elsevier Inc. All rights reserved. reduction of the bifidobacterial community compared to healthy controls (Arboleya et al., 2015; Koenig et al., 2011). Such findings support the notion that bifidobacteria play a positive role in the establishment/maintenance of gut homeostasis through host–microbe interactions and/or their direct interplay with other members of the gut microbiota.

12.3 GENOMICS OF THE BIFIDOBACTERIUM GENUS

Since the first bifidobacterial genome was decoded in 2002 (Schell et al., 2002), scientific efforts to explore the genomic contents of various members of this genus have been increasing. With the completion of the Genomic Encyclopedia of *Bifidobacteria* project in 2014, which aimed to decode the genome sequences of all type of strains, each of 47 (sub)species that at that time encompassed the *Bifidobacterium* genus, it has been possible to assess the overall genetic variability within the genus Bifidobacterium (Milani et al., 2014). This evaluation revealed that bifidobacterial genome sizes range from 1.73 (Bifidobacterium indicum) to 3.25 Mb (Bifidobacterium biavatii), corresponding to 1352 and 2557 predicted protein-encoding open reading frames, respectively. Functional classification, based on cluster ortholog (e.g., genes in different species that evolved from a common ancestral gene; normally, orthologs retain the same function in the course of evolution) gene (COG) family analyses, of the genetic arsenal of the *Bifidobacterium* genus, also known as the pan-genome of this taxon, highlighted that about 14% of the reconstructed pangenome encode enzymes are involved in carbohydrate metabolism (Milani et al., 2014, 2015a). Notably, a substantial part of this genetic repertoire involved in carbohydrate metabolism is shared among all 47 bifidobacterial (sub)species, thus belonging to the bifidobacterial core genome, while there are also a large number of genes that are uniquely found within a single clade, thus representing Truly Unique Genes (TUGs) (Milani et al., 2014). Notably, as mentioned earlier, the evolutionary success of bifidobacteria is mostly derived from their specialization to metabolize either a very specific set or sometimes a broad range of dietary and/or host-derived glycans (Duranti et al., 2014; Milani et al., 2016; Pokusaeva et al., 2011). Interestingly, about 15% of the identified TUGs of the bifidobacterial pan-genome, specify glycosyl hydrolases (GHs) and proteins involved in carbohydrate uptake (Milani et al., 2014, 2015a).

The genomic enrichment of genes involved in carbohydrate metabolism is believed to represent the evolutionary reflection of the metabolic commitment of bifidobacteria to a saccharolytic life style, representing a key genetic characteristic of this genus.

12.4 HOW BIFIDOBACTERIAL GENOMES HAVE BEEN SHAPED BY CARBOHYDRATE AVAILABILITY

The availability of the genomes for each bifidobacterial taxon also allowed the reconstruction of the genetic evolution of the taxa of this genus (Lugli et al., 2014; Milani et al., 2014). Bionformatics analyses predicted that the genome of the ancestor of the genus *Bifidobacterium* consisted of about 967 genes (Milani et al., 2014). Thus, the evolutionary development of currently known bifidobacterial taxa appears to have undergone a relatively small number of ancestral gene loss occurrences, but a substantial number of gene acquisition events. Evaluation of bifidobacterial genes that were (predicted to be) acquired during evolution revealed that adaptation to an environment rich in complex carbohydrates, like that of the animal gut, represents the main driving force responsible for speciation among members of the genus *Bifidobacterium*.

A closer look at the predicted functions of genes that have been identified as acquired or lost during bifidobacterial speciation demonstrates a clear involvement of a specific genetic repertoire that encodes GHs. In this context, a small number of GHs are associated with the breakdown of plant polysaccharides, such as the members of the GHs GH3 and GH43 families, and it is believed that these were acquired early in bifidobacterial evolution. Furthermore, other encoded GHs, such as those involving members of the large GH13 family, representing α -amylases/ α -glucosidases, were predicted as acquired during bifidobacterial speciation (Milani et al., 2014). Other (predicted) acquired genes encompass those belonging to the ATP-binding cassette (ABC), phosphoenolpyruvate-phosphotransferase system, and major facilitator superfamily families of transporters (Milani et al., 2014).

12.5 THE PREDICTED GLYCOBIOMES OF BIFIDOBACTERIA

The overall predicted enzymatic arsenal involved in carbohydrate metabolism, that is, GHs, glycosyl-transferases, and carbohydrate esterases, and identified in the pangenome of the genus *Bifidobacterium* was shown to be one of the largest among human gut commensals (Milani et al., 2015a). The bifidobacterial glycobiome is enriched in enzymes

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belonging to the GH13 family, which are involved in the hydrolysis of a wide range of complex carbohydrates, such as starch, glycogen, as well as palatinose, trehalose, stachyose, raffinose, and melibiose (Pokusaeva et al., 2011). Notably, all these sugars are dominant sugars found in the (adult) mammalian diet (El Kaoutari et al., 2013).

Other dominant GH members of the bifidobacterial glycobiome are those that are pivotal in host-derived glycan breakdown, such as GH33 and GH34 members, which represent *exo*-sialidases, GH29 and GH30 members, representing fucosidases, and GH20 members, that include hexosaminidase and lacto-*N*-biosidase activities (Milani et al., 2015a).

As many carbohydrate metabolized by bifidobacteria are polysaccharides, a substantial proportion of the predicted glycobiome of bifidobacteria is extracellular. Notably, about 33% of the predicted extracellular GHs are members of the GH13 family and classified as pullulanases and α -amylases, 24% are members of the GH43 family and annotated as β -xylosidases and α -L-arabinofuranosidases, while 12% are members of the GH51 family and classified as α -L-arabinofuranosidases (Milani et al., 2015a).

An in silico clustering of the predicted GH and carbohydrate-degradation pathway repertoire of each member of the genus *Bifidobacterium* allowed the identification of three groups, that is, designated GHP/A, GHP/B, and GHP/C (Milani et al., 2015a). In this context, group GHP/A encompasses bifidobacterial taxa with a considerable array of GH43 family members, representing enzymes predicted to be involved in the breakdown of complex plant carbohydrates, such as xylan and arabinoxylans. This suggests that the bifidobacterial taxa that belong to group GHP/A have adapted themselves to hosts that enjoy a vegetarian or omnivorous diet. The bifidobacterial species isolated from social insects constitute the GHP/C group, as they encompass a discrete set of GH43 and GH3 family members, and only a very limited number of GH13 members, which is in contrast to all other analyzed bifidobacteria (Bottacini et al., 2012). As described earlier, the GH13 family includes enzymes that are involved in the hydrolysis of poly- or oligosaccharides with α -glucosidic linkages, such as starch, glycogen, and related substrates, thus, this finding can be explained by the paucity of such carbohydrates in the (vegetarian) diet of honeybees and bumblebees. The remaining bifidobacterial taxa, that is, those that do not cluster in groups GHP/A or GHP/C, represent group GHP/B, whose members are characterized by an underrepresentation of GH43 and GH3 enzymes (Milani et al., 2015a, 2016).

12.6 EVALUATION OF THE GENETIC ADAPTATION OF BIFIDOBACTERIA TO THE HUMAN GUT

An important sign of genetic adaptation of bifidobacteria to the human gut is the specific utilization of various complex carbohydrates, such as resistant starch, which are derived from the diet and which escape host-mediated digestion.

Despite the fact that a survey of the bacteria of the adult gut microbiota highlighted that bifidobacteria are not a dominant microbial group of this ecosystem, their biological functional roles in the metabolism of dietary and hostderived glycans have only recently been appreciated. In silico analyses of the glycobiome of the genus *Bifidobacterium* clearly uncovered the widespread occurrence of enzymes that are predicted to metabolize starch or starch-related compounds (Milani et al., 2014), in particular encoded by the chromosomes of *B. adolescentis* (Duranti et al., 2013, 2014), a species that is commonly associated with adults, and *B. breve* (Duranti et al., 2013; Ryan et al., 2006). The predicted glycobiome of the *B. adolescentis* species revealed, compared to other bifidobacterial gut commensals, a much larger set of GH13 enzymes, including amylase, pullulanase, and cyclomaltodextrinase activities, thus suggesting superior growth performance of this taxon on particular plant-derived carbohydrates (Ze et al., 2012). These in silico data were substantiated by the analyses of fermentation profiles of members of the *B. adolescentis* taxon, which highlighted a preference for the utilization of different sugars (e.g., galactose, mannose, and glucose), as well as plant-derived glycans that are typically present in the human diet, such as starch (Duranti et al., 2014).

Interestingly, recent studies reveal that a number of *Bifidobacterium* species have very specific features that allow them to adapt to their particular ecological niche (Schell et al., 2002; Sela et al., 2008; Turroni et al., 2010a; Ventura et al., 2009). An example of such bifidobacterial adaptation to the human gut is represented by the ability of *B. longum* subsp. *longum* to utilize a wide variety of oligo- and polysaccharides found in plant-derived dietary fibers (Schell et al., 2002). Genome analysis of *B. longum* revealed that it contains genes encoding a substantial number of GHs (e.g., xylanase, arabinosidase, galactosidase, neopullanase, isomaltase, inulinase, glucosidase, hexosaminidase, and mannosidase), oligosaccharide transporters and proteins with a cell-surface anchor motif (Schell et al., 2002). Interestingly, the fact that the genome of *B. longum* subsp. *longum* has the coding capacity for these enzymes suggests that this species may partially metabolize "nondigestible" plant polymers or host-derived glycoproteins and glycoconjugates (Schell et al., 2002). In addition, the genome of *B. longum* encodes fimbriae and a teichoic acid-linked surface

polysaccharide that mediates the adhesion to the colon mucosa (Schell et al., 2002). Therefore, the ability of *B. longum* to metabolize different oligosaccharides and to interact with their host reflects the genetic strategies employed by this microorganism to survive and compete in its ecological niche (Schell et al., 2002).

Moreover, genome analyses of *B. longum* subsp. *infantis* ATCC15697 and *B. bifidum* PRL2010 have revealed how these two microorganisms are able to utilize the host-derived glycans HMOs and mucin. In particular, the genome sequences of *B. longum* subsp. *infantis* ATCC15697, a strain that was originally isolated from a breast-fed infant, revealed several genes encoding enzymes predicted to be involved in the degradation (such as a fucosidase, sialidase, β -hexosaminidase, and β -galactosidase) and internalization (such as extracellular solute binding proteins and permeases of ABC transporter systems) of HMO (Sela et al., 2008). Furthermore, the genome of this strain encompasses an operon involved in the metabolism of urea, an important source of nitrogen in human milk (Sela et al., 2008).

In silico analyses of the chromosomal sequence of *B. bifidum* PRL2010, a strain isolated from infant stool, revealed a gene set responsible for mucin metabolism, encoding extracellular enzymes that include sialidases, fucosidases, a putative cell wall-anchored endo- α -*N*-acetylgalactosaminidase, N-acetyl- β -hexosaminidases, and β -galactosidases (Turroni et al., 2010a). Finally, the genome analysis of *Bifidobacterium dentium* Bd1, a strain originally isolated from the human oral cavity, identified genes encoding enzymes involved in the metabolism of simple carbohydrates, such as fucose, mannose, galactose, N-acetylglucosamine, and N-acetylgalactosamine (Ventura et al., 2009). Moreover, the *B. dentium* Bd1 genome encompasses several genes encoding proteins associated with adhesins, acid tolerance, and defense against toxic substances, all useful traits to survive in the oral environment (Ventura et al., 2009). These findings show that the genome of *B. dentium* Bd1 contains various genetic adaptations to successfully colonize, compete, and survive in the oral cavity (Ventura et al., 2009).

12.7 CROSS-FEEDING ACTIVITIES OF BIFIDOBACTERIA

Within the mammalian gut, bifidobacterial populations are believed to establish several trophic interactions with each other and with other members of the gut microbiota, leading to competition for or cooperative sharing of nutrients.

Commonly, microbe–microbe interactions can either positively or negatively influence the fitness of the affected organisms (Pande et al., 2015) through the release of molecules in the environment (Morris et al., 2013; Phelan et al., 2012). Due to cross-feeding strategies gut bacteria promote an expansion of their carbohydrate acquisition abilities, thereby positively influencing the ecological fitness of a specific proportion or even the overall gut microbiota. Cross-feeding activities in the gut are generally carried out by primary microbial degraders like bifidobacteria that, thanks to partial extracellular hydrolysis of specific complex carbohydrates (e.g., host-glycans, as well as plant derived polysaccharides), provide carbohydrates (monosaccharides and oligosaccharides) to other microbial gut inhabitants (De Vuyst and Leroy, 2011). The subsequent fermentative metabolism of these carbohydrates generates end-metabolites, such as acetate and lactate, which, in turn, may act as substrates for secondary microbial degraders like the butyrate-producing enteric bacteria (Barcenilla et al., 2000; Duncan and Flint, 2008; Falony et al., 2009; Morrison et al., 2006; Munoz-Tamayo et al., 2011).

Examples of cross-feeding activities in bifidobacteria have been experimentally demonstrated between two infanttype bifidobacteria, such as *B. bifidum* PRL2010 and *B. breve* UCC2003 cells, when these bacteria were cultivated on sialyllactose as the unique carbon source (Egan et al., 2014a,b). Previous studies have shown metabolic cross-feeding between *B. adolescentis* and lactate-utilizing, butyrate-producing *Firmicutes* bacteria related to *Eubacterium hallii* and *Anaerostipes caccae* (Belenguer et al., 2006). Furthermore, bacterial cross-feeding opportunities, as facilitated by members of the colonic microbiota, have been considered to be pivotal for carbohydrate turnover in this ecological niche (De Vuyst and Leroy, 2011).

Recently, the cross-feeding activities of various bifidobacterial strains, such as *B. bifidum* PRL2010, *B. breve* 12L, *B. adolescentis* 22L, and *B. thermophilum* JCM7017, when cultivated on plant derived carbohydrates, such as starch and xylan, have been evaluated (Turroni et al., 2015). Cocultivation assays coupled with transcriptomic and metabolomic analyses revealed that cooccurrence of the aforementioned bifidobacterial strains results in enhanced metabolic activity of *B. bifidum* PRL2010, thus suggesting that PRL2010 cells benefit from the presence of other bifidobacterial strains.

The existence of cross-feeding activities between various bifidobacterial strains (*B. bifidum* PRL2010, *B. longum* subsp. *infantis* ATCC15697, *B. adolescentis* 22L, and *B. breve* 12L) have been further confirmed by assessment under in vivo conditions in a conventional murine model (Turroni et al., 2016). Remarkably, in this study, transcriptomic experiments coupled with metagenomic analyses of single, dual or multiple-associations of bifidobacterial strains

uncovered cross-feeding behavior that caused an apparent expansion of the murine gut glycobiome toward its enzymatic potential related to the breakdown of plant-derived carbohydrates, such as xylo-oligosaccharides, arabinoxylan, starch, and host-glycan substrates. Furthermore, these analyses revealed distinct strategic responses by the different bifidobacterial strains toward sugars, for example, a "selfish" behavior was exhibited by *B. longum* subsp. *infantis* ATCC15697 as it internalizes HMOs prior to degradation thereby limiting opportunities for resource sharing by other (bifido)bacteria. In contrast, *B. bifidum* strain PRL2010 actively participates in the extracellular breakdown of host-glycans and thus in the release of simple sugars that can then be utilized by to other members of the bifidobacterial community (Turroni et al., 2016).

12.8 INTERACTION OF BIFIDOBACTERIA WITH THE HUMAN GUT

The intestinal microbiota is a complex ecosystem with extensive metabolic activity. Little is known about the mechanism by which bifidobacteria interact with the host, but bifidobacterial colonization seems to play an important role in the host. Previous studies have demonstrated that many bifidobacterial species encode extracellular polysaccharides (EPS), cell surface-encoding proteins, called fimbriae or pili, and a secreted serine protease inhibitor, all of which seem to be involved in host interactions (Foroni et al., 2011; Hidalgo-Cantabrana et al., 2014; O'Connell Motherway et al., 2011; Turroni et al., 2010b). EPS are carbohydrate polymers present as an extracellular layer covering the surface of various microorganisms (Freitas et al., 2011), including Gram-positive and Gram-negative bacteria. The EPS can have two different functions, in fact, it has been shown that bacterial EPS can act as a virulence factor in particular diseases, but also that it may elicit positive effects on human health (Round and Mazmanian, 2009; Round et al., 2011). The EPS produced by certain lactic acid bacteria can be classified in two groups: homopolysaccharides, consisting of just a single repeated monosaccharide type, and heteropolysaccharides, composed of a repeated oligosaccharide (Laws et al., 2001). Specifically, the EPS synthesized by bifidobacteria are built from different monosaccharides, for example, D-glucose, D-galactose, and L-rhamnose, thus are classified as heteropolysaccharides (Hidalgo-Cantabrana et al., 2014). However, the genetic organization of EPS gene clusters is not well conserved in different Bifidobacterium species. Several studies demonstrated that the production of EPS has been correlated with their capability to modulate the host immune response (Fanning et al., 2012; Lopez et al., 2012), their capabilities to act as antioxidants to decrease the damage caused by reactive oxygen species in the tissues (Xu et al., 2011), and/or their ability to modulate the composition and activity of the gut microbiota (Hidalgo-Cantabrana et al., 2014). Another key force that drives interaction with the host is represented by pilus-like structures. Specifically, two different types of pili have so far been described in bifidobacteria: the tight adherence pili (Tad) and the sortase-dependent pili. Tad in bifidobacteria are encoded by a genetic locus, which was originally identified in the genome B. breve UCC2003. The tad locus of UCC2003 is similar to the type IVb pilus-encoding gene cluster of Actinobacillus actinomycetemcomitans that appears to be separated in two different loci (O'Connell Motherway et al., 2011). The first locus encodes proteins involved in the pilus assembly and localization (TadB, TadA, TadC, and TadZ), as well as the Flp prepilin and two pseudopilins (TadE and TadF); the second locus consists of single gene, *tadV*, which is responsible for the processing of pilin precursors to mature pilin proteins (O'Connell Motherway et al., 2011). The tad locus was identified through transcriptome analysis of *B. breve* UCC2003, which showed that upon colonization of mice transcription of this gene cluster is induced, while it is not (or at a very low level) transcribed under laboratory conditions. Thus, such extracellular structures may be produced only when B. breve UCC2003 is found in its natural environment (O'Connell Motherway et al., 2011). Finally, a mutational analysis of the *tadA* gene highlighted the crucial importance of the tad locus in the colonization and persistence of B. breve UCC2003 in the mammalian gut. Notably, the tad locus is highly conserved among all sequenced bifidobacterial strains, which supports the notion that such extracellular structures mediate host colonization by bifidobacteria (Ivanov et al., 2006; O'Connell Motherway et al., 2011; Turroni et al., 2010b; Ventura et al., 2012). Other crucial extracellular appendages that might be crucial for colonization and the interaction of bifidobacteria with the mammalian host are represented by sortase-dependent pili, which were visualized for the first time in bifidobacteria by Foroni et al. (2011). The genomes of bifidobacteria characterized by Foroni et al. (2011) encompass one to three predicted sortase-dependent pilus gene cluster/s, each consisting of three genes predicted to encode a major pilin subunit, a minor pilin subunit, and a sortase that assembles the pilin subunits (Foroni et al., 2011).

Another microbial encoded protein that in the gut bacteria seems to be involved in the interaction with the host is the serine protease inhibitor, which is a member of the serpin superfamily (Ivanov et al., 2006; Roberts et al., 2004). Ivanov et al. (2006) found a gene encoding a serpin-like protease inhibitor in the genome of *B. longum* NCC2705 that was demonstrated to contribute to the interaction with the host gastrointestinal tract. This enzyme seems to inhibit

the human neutrophil and pancreatic elastases, phenomena that are considered to play an important role in the interaction between these commensal bacteria and their host (Ivanov et al., 2006). In addition, Turroni et al. (2010b) characterized a serpin-encoding gene encoded by the genome of other human-bifidobacteria, such as *B. breve* strain 210B. Microarray and quantitative real-time PCR analyses highlighted the polycistronic mRNA encompassing the *ser* operon is strongly induced following treatment of *B. breve* 210B cultures with gut proteases, such as pancreatic elastase, human neutrophil elastase, thrombin, papain, kallikrein, trypsin, α -antitrypsin, chymotrypsin, or plasmin (Turroni et al., 2010b). This study revealed that the activity of the serine protease inhibitor might thus produce an antiinflammatory response in the intestine (Turroni et al., 2010b).

12.9 CONCLUDING REMARKS

For millennia mammals have coevolved with their commensal partners, with the establishment of very complex links between the gut microbiota and their host. Imbalances in the gut commensal composition, such as loss of bifidobacteria, influence the immune system, which compounds the intricate connection between gut microbiota composition and host health. The first decade of genomic exploration of the biology of gut commensals, such as bifidobacteria, has afforded unprecedented insights into the genetic adaptation of these microorganisms to the human gut through the decoding of their genome sequences (probiogenomics). The next decade holds the promise of being even more rewarding as new discoveries on the molecular mechanisms underpinning host–microbe interactions are generated by means of functional genomics efforts.

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Clinical Significance of Bifidobacteria

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13.1 INTRODUCTION

As discussed in other chapters of this book, bifidobacteria are an important group of intestinal commensals that exert a number of beneficial effects on their hosts (Chapters 9, 10, 14, 17, 18; Callaghan and Sinderen, 2016; Foligné et al., 2013; Holmes et al., 2012). Based on these properties, bifidobacteria are extensively marketed as probiotics by the pharmaceutical, food, and dairy industries to positively impact on host health (summarized in Chapter 17). In this chapter we summarize the existing results of clinical trials investigating the use of bifidobacteria for therapeutic purposes. Moreover, the focus of this chapter is to describe clinically relevant aspects of administration of bifidobacteria rather than their (immunological) effects or the underlying molecular mechanisms, which is addressed elsewhere in this book (e.g. Chapter 18).

Using the search term "Bifidobacterium", a total of 276 clinical trials are registered at the time of writing in the publicly available database of the US National Institutes of Health on www.ClinicalTrials.gov, which is a comprehensive registry of phase 1-4 clinical studies worldwide. Of those trials, 162 were listed as "completed." Likewise, searching the National Center for Biotechnology Information (NCBI) literature database PubMed using the terms "Bifidobacterium clinical trial" retrieved 865 results (status end of August 2016), including primary reports on clinical trials, reviews, and metaanalyses. These search results were analyzed to compile a list of randomized, placebocontrolled clinical trials investigating the effect of bifidobacteria on clinically relevant conditions. In recent years, there has been a trend toward the use of multistrain probiotic cocktails containing bifidobacteria, lactobacilli, and streptococci in different combinations. However, in these cases, it is not possible to clearly assign effects to a single Bifidobacterium sp. strain. In consequence, these clinical studies were not considered unless at least one arm of the trial assessed a single *Bifidobacterium* sp. strain. For the same reason, we excluded any study using combinations of a probiotic *Bifidobacterium* sp. with a prebiotic or vitamin as it cannot be excluded that the observed effects are mediated by the prebiotic or vitamin. Moreover, trials assessing the impact of bifidobacteria on the community structure or function of the gastrointestinal microbiota as primary outcome or measuring the effect of bifidobacteria on general health parameters (e.g., endurance performance in extreme athletes or weight loss in postoperative gastric bypass patients) were also excluded.

The remaining trials were grouped according to different pathologies, clinical markers assessed, and target patient groups. This led to identification of medical conditions and other groups of individuals that are most frequently targeted by probiotic intervention. Although, in nondiseased persons it is difficult to find markers that allow assessment of effects on health parameters, a significant portion of the trials that met our inclusion criteria were conducted in healthy individuals. The most frequently assessed outcomes were infant growth, general health, and prevention of illness episodes, adjuvant activity during vaccine regimes, immune activation or modulation, and gastrointestinal function (stool frequency and consistency, constipation). On the other hand, a large number of trials were conducted investigating the effects of different diseased states including prevention of infections and necrotizing enterocolitis (NEC) in preterm infants, supplementation of critically ill patients, prevention of infections or reducing symptoms of infectious and antibiotic associated diarrhea, atopic and allergic diseases, inflammatory bowel disease (IBD), and irritable bowel syndrome (IBS). The findings of clinical trials addressing treatment of these conditions with specific strains of bifidobacteria are summarized in this chapter.

13.2 EFFECTS IN HEALTHY INDIVIDUALS

The focus of this chapter is clinical relevance of bifidobacteria. Although this addresses mainly treatment of clinical conditions, that is, pathologies with defined symptoms or patient groups, there are a number of trials conducted in healthy subjects aiming at improvements in general health parameters, adjuvant activity, and the immune status or reducing the prevalence of common infections.

Bifidobacteria are among the first bacterial colonizers of the gastrointestinal tract and are predominant during the first few months, when the newborn is exclusively breast-fed (Bäckhed et al., 2015; Walker et al., 2015; Yatsunenko et al., 2012). Breast-feeding and high levels of bifidobacteria are thus associated with a normal, "healthy" development of the infant (Matamoros et al., 2013). Conversely, low levels of infant-type bifidobacteria (*Bifidobacterium breve*, *B. bifidum*, *B. longum* subsp. *infantis*) or predominant colonization by adult-type bifidobacteria (*B. longum* subsp. *longum*) are considered abnormal and are hypothesized to contribute to development of different diseased states later in life (Gerritsen et al., 2011; Sekirov et al., 2010). This has fueled the concept of supplementing infant nutrition with different bifidobacteria to support development of a healthy microbiota and prevent disease.

13.2.1 Infant Growth

There are a number of clinical trials investigating the effect of different *B. animalis* subsp. *lactis* strains on growth of healthy infants. Three small-scale trials with a total of 319 subjects investigated the effect of supplementation with *B. lactis* BB-12 [daily doses of approximately $1-10 \times 10^8$ colony forming units (CFU)] on growth parameters (Holscher et al., 2012; Weizman and Alsheikh, 2006; Ziegler et al., 2003). While in all trials the *B. lactis* BB-12 supplement was well tolerated, no effect on anthropometric parameters was observed compared to placebo or other formulas not containing bifidobacteria. Two further small trials investigating supplementation with other *B. lactis* strains (doses not reported) in healthy infants of HIV positive mothers yielded opposing results. While one trial reported significant enhanced head growth and a trend toward increased weight gain of infants fed a formula containing a nonspecified *B. lactis* CNCM I-3446 treated and placebo groups (Velaphi et al., 2008). Overall, the results suggest that supplementation with *B. animalis* subsp. *lactis* strains does not enhance growth of healthy infants.

13.2.2 General Health and Prevention of Illness Episodes

Several trials with larger participant numbers were conducted to assess the potential of different bifidobacteria to maintain health, that is, to prevent illness episodes or, at least, extend periods without illness. In 2 trials, 638 children aged 3-6 years (Merenstein et al., 2010) or 172 children between 2 and 4 years (Merenstein et al., 2011) received a probiotic drink containing *B. lactis* BB-12 (daily dose of 1×10^{10} CFU) or placebo for 90 consecutive days. Both studies report no significant differences in the number of days of missed school between the two groups. Likewise, in a large trial with a total of 727 hospitalized children aged 1–18 years, treatment with *B. lactis* BB-12 at 1×10^{9} CFU per day did not reduce incidence or duration of nosocomial infections (onset > 48 h after hospitalization) compared to placebo (Hojsak et al., 2015). Langkamp-Henken et al. (2015) performed a trial monitoring a cohort of undergraduate students (n = 581) before, during, and after their exam week, that is, during a mentally stressful period. Study subjects consumed one capsule per day containing 3×10^9 CFU of either Lactobacillus helveticus R0052, B. infantis R0033, B. bifidum R0071, or placebo for 7 days and were then followed up for 6 weeks for symptoms of cold or flu. No differences were observed between groups that received B. infantis or L. helveticus or placebo. However, participants that received the *B. bifidum* strain reported significantly fewer days with flu or cold. In another trial, a significantly reduced risk for upper respiratory tract infections and a delay of 0.7 months to first appearance of respiratory symptoms was observed in individuals consuming *B. lactis* Bi-07 (2×10^9 CFU per day) compared to the placebo group (West et al., 2014). Interestingly, administering a combination of B. lactis Bi-07 group and L. acidophilus NCFM yielded no significant effect compared to placebo.

13.2.3 Gastrointestinal Functions

A total of 16 trials investigating the effects of different bifidobacteria on gastrointestinal problems met our criteria for inclusion. It is worth mentioning that readouts in the different trials were somewhat opposing, with some studies aiming at reducing diarrhea while others targeted improvement of constipation or gastrointestinal discomfort.

13.2 EFFECTS IN HEALTHY INDIVIDUALS

Del Piano et al. (2010) conducted a double-blind, randomized, placebo-controlled study with 300 participants to evaluate the efficacy of 2 different probiotic blends in the management of problems in gastrointestinal function. One of the blends contained *B. longum* subsp. *infantis* BS01 as sole bacterial supplement and subjects treated with this strain at 2×10^9 CFU per day reported a significant improvement in the consistency of feces and the number and ease of evacuations. In constipated women consuming a cheese containing *B. lactis* Bi-07 (1 \times 10⁸ CFU per day) or placebo for 30 days (n = 30), a significant improvement in the treatment group was observed (Favretto et al., 2013). However, statistical comparisons were made to baseline, the placebo group also reported significant improvements in some of the criteria assessed, and no statistical analysis of intergroup differences is provided. Another study suffers from the same drawback (Gomi et al., 2015). Here, 27 subjects with gastric symptoms received fermented milk containing 1×10^9 CFU of *B. bifidum* YIT 10347 or placebo daily for 2 weeks in a crossover design and completed a questionnaire covering frequency and severity of 10 gastric symptoms. The authors report a significant decrease in the average gastric symptoms score. However, statistics were performed by comparing both groups to baseline and a slight, yet nonsignificant decrease in the symptoms score was also observed in the placebo group. It is not mentioned if there is a statistically significant effect of treatment over placebo. Another crossover study investigated the effect of a *B. longum* strain in goat yogurt on chronic functional constipation in infants (n = 59; age 5–15 years). During the 5-week study period, stool frequency was significantly improved by treatment with 1×10^{9} CFU daily and this effect was reversed following crossover (Guerra et al., 2011). A group of 100 healthy adults received B. lactis HN019 daily at a high dose (1.7×10^{10} CFU), a low dose (1.8×10^{9} CFU), or placebo (Waller et al., 2011). Both doses but not placebo reduced the time of gastrointestinal transit significantly. The effect of *B. lactis* GCL2505 was investigated in a placebocontrolled double-blind crossover study in 17 young constipated females (Ishizuka et al., 2012). Administration of this strain for 2 weeks at a dose of 1×10^{10} CFU in a milk-like drink significantly improved defecation frequency compared to the placebo phases.

Chouraqui et al. (2004) performed a trial in 90 children younger than 8 months in residential care that received a milk formula supplemented with viable *B. lactis* BB-12 (at least 1×10^8 CFU per day) and recorded acute diarrheal episodes. Incidence of diarrhea, as well as duration of the diarrheal episodes were reduced in the group receiving *B. lactis* BB-12 compared to placebo, although these effects were not significant. However, the number of days with diarrhea was significantly lower. Pitkala et al. (2007) compared *B. lactis* BB-12 and a *B. longum* strain (both at 1×10^9 CFU per day in a fermented oat drink) in elderly persons in a nursing home and both preparations significantly improved bowel movements compared to placebo. In another study, a total of 1248 subjects that received capsules with 1 or 10×10^9 CFU *B. lactis* BB-12 or placebo daily for 4 weeks completed a diary on bowel habits, relief of abdominal discomfort, and symptoms (Eskesen et al., 2015). Overall, supplementation with *B. lactis* BB-12 resulted in significantly higher defecation frequencies but no difference between the two doses were observed.

A yogurt containing *B. animalis* DN-173 010 was analyzed in several trials. In one of the trials with a crossover design, administration of *B. animalis* DN-173 010 3 times a day (total daily dose approximately 1.25×10^{10} CFU) significantly reduced colonic transit time over placebo in 36 healthy women (Marteau et al., 2002). Two further trials were performed in 371 adults (Guyonnet et al., 2009b) or 197 women (Guyonnet et al., 2009a) reporting digestive discomfort using the same yogurt containing the same strain at similar doses. In both studies, self-reported improvements of symptoms were significantly higher in the treatment groups compared to the controls but increasing the dose did not enhance the effect (Guyonnet et al., 2009b). A similar study using the same strain (1.25×10^{10} CFU per day) in 135 constipated Chinese women yielded significant improvements in stool frequency and consistence compared to the placebo group (Yang et al., 2008). Interestingly, another trial in women (n = 324) with minor digestive symptoms (Marteau et al., 2013) repeated the setup of Guyonnet et al. (2009b) but could not confirm the results. Administration of a yogurt containing *B. animalis* DN-173 010 twice daily (each dose 4.25×10^9 CFU) for 3 weeks to chronically constipated children (>3 years, n = 159) increased stool frequency but this effect was not significant and it was also observed in placebo-treated children (Tabbers et al., 2011).

In addition to the drawbacks regarding statistical analyses in several studies, it is intriguing that some of the strains show somewhat opposing effects. For example, treatment with *B. lactis* BB-12 reduces diarrhea (Chouraqui et al., 2004) but also increases bowel movements and stool frequencies (Eskesen et al., 2015; Pitkala et al., 2007) depending on the trial settings and subject groups. Also, the effects of *B. animalis* DN-173 010 on digestive discomfort were significant in some trials (Guyonnet et al., 2009a,b), but not in others (Marteau et al., 2013; Tabbers et al., 2011).

13.2.4 Immune Activation/Modulation

An early study in 25 healthy volunteers assessed the effect of *B. lactis* HN019 on general immune parameters (Arunachalam et al., 2000). Intake of milk supplemented with 1.5×10^{11} CFU of *B. lactis* HN019 twice daily for

6 weeks resulted in increased phagocytosis by isolated peripheral blood mononuclear cells (PBMCs), enhanced bactericidal activity of phagocytes, and higher interferon alpha (IFN- α) production by stimulated PBMC compared to the placebo group. In a second trial with the same strain, administration of capsules containing *B. lactis* HN019 (daily dose of 9 × 10⁹ CFU) to pregnant women before and after delivery increased levels of secretory IgA (sIgA) and transforming growth factor β in milk of the mothers and IFN- γ in cord blood (Prescott et al., 2008).

A number of trials were performed with supplements containing *B. lactis* BB-12. In a group of infants fed a formula containing 6×10^9 CFU *B. lactis* BB-12 daily until 32 weeks of age, fecal sIgA was not affected by the treatment (Bakker-Zierikzee et al., 2006). By contrast, a formula containing the same strain administered at 1×10^8 CFU per day for 6 weeks to another group of vaginally delivered infants significantly improved total sIgA and Poliospecific IgA in fecal samples compared to a control group fed the same formula without B. lactis BB-12 (Holscher et al., 2012). In a study with 62 volunteers, consumption of a daily dose of 3.5×10^{10} B. lactis BB-12 in a milk-based fruit drink yielded no significant effects on serum levels of C-reactive protein (CRP) compared to a placebo group (Kekkonen et al., 2008). Also, production of tumor necrosis factor alpha (TNF- α), IFN- γ , and interleukins 1 β , 6, 8, 10, or 12 by isolated PBMCs following stimulation with S. pyogenes, E. coli, or influenza A virus did not differ between groups fed *B. lactis* BB-12 or placebo. Another study randomized a total of 55 institutionalized elderly subjects into 3 groups: the first group received *B. lactis* BB-12, the second a mix of two *B. longum* strains, and the third group was treated with placebo (Ouwehand et al., 2008). The carrier was a fermented oat-based drink that was consumed daily for 6 months and, in case of the intervention groups contained approximately 1×10^9 CFU per day. However, the results were inconclusive. Three and 6 months after the start of the trial, the *B. lactis* BB-12 group displayed significantly reduced serum IL-10 and TNF- α levels compared to placebo. A similar effect was also observed with the *B. longum* blend, albeit only as a trend. More intriguingly, baseline levels (i.e., before start of the intervention) of IL-10 and TNF- α were already significantly lower in the *B. lactis* BB-12 group compared to placebo.

Maneerat et al. (2013) sampled blood from healthy elderly people (n = 37) that had consumed *B. infantis* Bi-07 (1 × 10⁹ CFU per day) alone or in combination with a prebiotic and found that the *B. infantis* strain significantly enhanced phagocytic activity of monocytes and granulocytes. A significant increase in interleukin 10 (IL-10) secretion from isolated PBMCs from healthy volunteers stimulated with a T-cell activating cocktail of antibodies was observed following intake of *B. infantis* 35624, which was not observed with a placebo (Konieczna et al., 2012). The authors followed up on this observation with in vitro experiments and could show that naive dendritic cells pulsed with *B. infantis* 35624 were able to induce FoxP3 expression in T cells and thus promote regulatory T-cell responses. Collectively, these results suggest that *B. infantis* 35624 has antiinflammatory potential. Another small study compared the immunomodulatory effects of *B. breve* CNCM I-4035 with two *Lactobacillus* sp. strains and a combination of the three strains, and found that administration of the *B. breve* strain significantly increased fecal sIgA (Plaza-Diaz et al., 2013). Moreover, these subjects had increased percentages of regulatory T cells and reduced TNF- α serum levels.

Paineau et al. (2008) compared seven different commercially available probiotic strains including the two *B. lactis* strains Bi-07 and Bl-04 and found no significant effects of the two strains on titers of IgA, IgG, or IgM in serum or saliva after 3 weeks of administration of two capsules per day, each containing 1×10^{10} CFU, compared to the placebo group.

Three trials addressed the question whether immunomodulatory properties of bifidobacteria might be used to enhance vaccine responses. Daily administration of a *B. breve* strain (4 × 10⁹ CFU per day) to children (age 2–5) for 4 weeks with two doses of a cholera vaccine during the trial period did not alter the adverse effects of the vaccine but also had no effect on the immune response to the vaccine (Matsuda et al., 2011). By contrast, supplementation of a small number of elderly subjects (total n = 27) with a daily dose of 1×10^{11} CFU of *B. longum* BB536 for 5 weeks during which they also received two doses of an influenza vaccine significantly reduced the subsequent incidence of influenza infections compared to the control group that received the vaccine and a placebo (Namba et al., 2010). Also, activity of neutrophiles and natural killer cells was enhanced in the *B. longum* BB536 group during the trial period, suggesting that the strain might enhance innate immunity. In a similar setting, daily administration of *B. lactis* BB-12 (1 × 10⁹ CFU) for 6 weeks along with a seasonal influenza vaccine in week 2 of the study protocol increased total antibody titers and vaccine-specific IgG, IgG1, and IgG3 levels in plasma and saliva (Rizzardini et al., 2012), suggesting an effect on humoral immunity.

Among the trials analyzed, there are no consistent readouts that allow a comparison of the effects of a single strain from one study to another or between different strains. The very few trials that compare different strains in the same

experimental setting suggest strain-specific effects. In summary, available clinical trials provide a rather diffuse picture on the immunomodulatory potential of bifidobacteria.

13.3 PRETERM INFANTS AND NECROTIZING ENTEROCOLITIS

Birth of an infant before term (i.e., less than 37 weeks of gestational age) and/or with very low birth weight (VLBW; i.e., <1500 g) is associated with high mortality rates and a number of serious complications later in life (Saigal and Doyle, 2008). These problems are mainly the consequence of the immaturity of most organs including the gastrointestinal barrier and immune system. The consequences range from delayed development to serious infections, and life-threatening conditions, such as sepsis and NEC (Berrington et al., 2012; Saigal and Doyle, 2008). These conditions are characterized by a delayed, reduced, and/or aberrant colonization with bifidobacteria and increased levels of proteobacteria that cause or contribute to pathology (Goldmann et al., 1978; Sakata et al., 1985; Torrazza et al., 2013). As mentioned previously, bifidobacteria are a numerically and functionally important group of the normal microbiota of breast-fed infants and are thought to confer protection against infections by competition with pathogens for nutrients and attachment sites (Buffie and Pamer, 2013). This provides a rationale for their use in the prevention of NEC and neonatal infections.

A prospective randomized clinical study of 91 preterm (gestational age 25–28 weeks) VLBW infants without major anomalies examined the effect of *B. breve* YIT4010 supplementation (5×10^8 CFU per day) for 4 weeks (Kitajima et al., 1997). During the time of administration, the volume of aspirated gas from the stomach was significantly reduced indicating a reduced severity of NEC. Moreover, children that were successfully colonized by *B. breve* YIT4010 received significantly fewer doses of an analgesic, established earlier feeding of greater volumes of formula and showed greater weight gain during the 4 weeks after administration.

In another trial, a formula containing *B. lactis* BB-12 was given to VLBW infants (mean gestational age: 31 weeks) daily at 1.6×10^9 CFU on day 1–3 and 4.8×10^9 CFU from day 4 onward. Feeding this formula significantly increased body weight gain (Mohan et al., 2006). This effect, however, was abolished when children received antibiotics. A similar study was conducted in a total of 75 preterm infants (gestational age 27–36 weeks) with the same strain. The preparation contained 2×10^7 CFU/g of *B. lactis* BB-12 and was administered in increasing doses (starting at 20 mL/kg/day until complete enteral feeding with 150 mL/kg/day). This regime reduced intestinal permeability and increased head growth during the first 30 days of life (Stratiki et al., 2007). By contrast, supplementation of VLBW infants with *B. lactis* BB-12 (total n = 183; six daily doses, each 2×10^9 CFU) did not reduce the incidence of NEC or nosocomial infections as indicated by elevated levels of C-reactive protein in serum (Mihatsch et al., 2010).

A large trial in VLBW infants (mean gestation age 28–29 weeks; n = 400) in five neonatal intensive care units compared administration of different formulas containing a nonspecified *B. lactis* strain, a prebiotic, or a mix of both (synbiotic) for a maximum of 8 weeks after birth. Children in the treatment group received 5×10^{9} CFU per day with a run-in phase with lower doses to test if the formula is tolerated. A highly significant reduction in the incidence of NEC (2% vs. 18% in the placebo group) was achieved by feeding the formula containing the *B. lactis* strain (Dilli et al., 2015). Moreover, *B. lactis* treated infants also had significantly lower rates of nosocomial sepsis and mortality, shorter time to full enteral feeding, and shorter stays in the intensive care unit.

Patole et al. (2016) performed a retrospective cohort study in preterm children (<34 weeks of gestation) in a nursery over the course of 2 years. The authors retrospectively analyzed cases of NEC and observed a significantly reduced incidence of NEC after introduction of a daily supplement containing 3×10^9 CFU of *B. breve* M-16V as standard additional treatment. Mechanistically, this effect might be linked to antiinflammatory properties of the strain since in another small trial a similar group of preterm newborns (total *n* = 19) demonstrated that administration of *B. breve* M-16V (1×10^9 CFU daily in two doses) increased serum levels of TGF- β compared to placebo-fed infants (Fujii et al., 2006). In another large multicenter trial, a total of 1310 infants (gestational age 23–30 weeks) at 24 hospitals were assigned within 48 h of birth to daily receive either a formula containing 2–6 × 10⁸ CFU of *B. breve* BBG-001 or placebo (Costeloe et al., 2016). However, no statistically significant effects on incidence of NEC, sepsis, or mortality rate were observed.

The majority of studies have shown positive effects of bifidobacteria supplementation of preterm infants on severity and incidence of NEC and infections of the infant. Thus, treatment of preterm and very low birth weight children with bifidobacteria (and other probiotics) is discussed as a supplementary therapy to prevent or treat NEC. However, further trials are needed to identify the most effective strain(s) and investigate their mechanism of action.

13.4 CRITICALLY ILL PATIENTS

Besides their already serious to life-threatening condition, cancer patients and other critically ill persons have an increased risk for infections. One factor that contributes to this increased risk is the administration of very potent pharmacological drugs, which often compromise the immune status of the patient. Additionally, intubation for enteral feeding and urinary, intravenous, and other catheters are potential entry sites for infectious agents. Because probiotic bacteria in general are thought to positively impact on the immune system and may inhibit colonization by pathogens, a few clinical studies have investigated the effect of bifidobacteria on infectious complications in critically ill patients.

Wada et al. (2010) tested a potential prophylactic effect of a preparation containing a B. breve strain on infections in cancer patients (n = 42) undergoing chemotherapy. The preparation contained 1×10^9 CFU and was administered 3 times daily starting 2 weeks before chemotherapy until discharge from the hospital. The authors found a lower frequency of fever and a reduced use of antibiotics in the treatment group compared to patients receiving placebo. By contrast, no significant difference in the incidence of postoperative infections was observed in patients undergoing colorectal resection that were administered either a supplement containing 5×10^{10} CFU B. longum BB536 (n = 31) or placebo for 7–14 days before and 14 days after surgery (Mizuta et al., 2016). The same strain was used in two other studies in elderly patients fed by an enteral tube and supplemented with *B. longum* BB536 or a placebo. In one of these trials, patients were treated twice daily (each dose 5×10^{10} CFU) for 12 weeks, received an influenza vaccine after 4 weeks of supplementation, and were followed up for 4 weeks after supplementation was stopped. Readouts were body temperature, bowel movements, and immunological biomarkers in blood. The only statistical significant difference between the two groups was a decrease in the activity of natural killer cells in placebo-treated patients, which was not observed in subjects receiving *B. longum* BB536 (Akatsu et al., 2013). In the second study, two trials were conducted with two doses (2.5×10^{10} or 5×10^{10}) of *B. longum* BB536 administered once or twice daily and compared to placebo. In both cases, patients did not receive a vaccination (Kondo et al., 2013). Irrespective of the dose, administration of B. longum BB536 normalized bowel movements.

In summary, a few studies report a reduction in infectious complications in critically ill patients by administration of bifidobacteria whereas in others no effects are observed. However, the limited number of trials and the heterogeneity in terms of administered strains do not support the conclusion that any of the treatments with bifidobacteria in general have beneficial effects in these patients.

13.5 INFECTIONS

Early colonization of the gastrointestinal tract with healthy microbiota in the correct pattern of succession is considered to be essential for proper development of the immune system (Matamoros et al., 2013). Similarly, microbial dysbiosis early in life is thought to predispose children to repeated infection or allergic and inflammatory diseases later in life (Gareau et al., 2010; Russell and Finlay, 2012). Because bifidobacteria are an essential part of the neonatal gut microbiota with immunomodulatory potential, it is not surprising that a number of studies have been performed to investigate their effects on incidence and duration of infectious diseases.

In a multicenter trial in 14 childcare centers, healthy term infants (4–10 months old) were randomly assigned to a formula supplemented with B. lactis BB-12 or placebo. Children were not breast-fed due to parental decision and no additional probiotics or prebiotics were allowed. During the 12 weeks of feeding and follow-up, children in the control group (n = 60) had significantly more and longer episodes of fever and diarrhea compared with those fed B. lactis (n = 73, daily dose not specified) but no differences in incidence and duration of respiratory infections were recorded (Weizman et al., 2005). By contrast, a daily dose of 1×10^{10} CFU of *B. lactis* BB-12 in a placebo-controlled study reduced episodes of respiratory infections in treated infants (n = 55) compared to the placebo group (n = 54) yet no differences were observed in gastrointestinal symptoms, otitis media, or use of antibiotics (Taipale et al., 2011). In a trial enrolling 50 children aged 1–23 months, which were hospitalized for acute diarrhea, administration of the same strain (*B. lactis* BB-12; 14.5 \times 10⁶ CFU daily for 1 week) significantly reduced mean duration of diarrhea and the number of stools per day (El-Soud et al., 2015). Moreover, discharge from hospital in less than 2 days was more frequent than in the control group. Two other trials also investigated *B. lactis* BB-12. The authors of one trial reported no significant effects on the number of diarrheal episodes in children up to 8 months of age in residential care centers (Chouraqui et al., 2004; daily dose at least 1×10^8 CFU). In the second trial, no changes in serum levels of C-reactive protein as indicator for nosocomial infections upon administration of B. lactis BB-12 (six doses of 2×10^9 CFU daily) to VLBW infants were observed (Mihatsch et al., 2010).

Two small trials were aimed at assessing bifidobacteria as supplementary treatment options in *Helicobacter pylori* infections. A 12-week ingestion of a milk product fermented with *B. bifidum* YIT 4007 ($1-5 \times 10^8$ CFU per day) improved a number of clinical parameters including urea breath test and serum pepsinogen as biomarkers for inflammation or atrophy in *H. pylori* positive patients (Miki et al., 2007). Another trial investigated the effect of *B. infantis* 2036 (3×10^9 CFU twice daily) in addition to the standard *H. pylori* treatment, that is, a triple antibiotic regime. Supplementation with the *B. infantis* strain prior to or concomitant with standard therapy significantly increased eradication rates (Dajani et al., 2013).

Another specific diseased state that has been targeted by oral supplementation with bifidobacteria in three smallscale trials is dental caries. However, in none of the studies treatment reduced occurrence of dental caries or the number of *S. mutans* in caries plaques (Caglar, 2014; Pinto et al., 2014; Taipale et al., 2013).

13.6 ATOPY/ALLERGY

Atopy and allergic diseases include asthma, hay fever, food allergy, and atopic dermatitis (AD). These hypersensitivity disorders are caused by excessive immune response to harmless environmental antigens (Kay, 2000). One explanation for the increased incidence of these conditions in industrialized countries is the so-called hygiene hypothesis, which states that the overresponsiveness of the immune responses to environmental antigens in allergy is the result of improved hygiene standards and, consequently, an insufficient exposure to antigen early in life (Brooks et al., 2013). Also, a number of studies have suggested that aberrant microbial colonization of the gastrointestinal tract and, especially, reduced or adult-type *Bifidobacterium* sp. early in life are associated with an increased risk for the development of atopic and allergic diseases (Björkstén et al., 2001; Ege et al., 2011; Kalliomäki et al., 2001; Kirjavainen et al., 2001, 2002; Watanabe et al., 2003). This provides the rationale for attempts to reduce the risk of, prevent, or even treat these conditions by administration of bifidobacteria.

13.6.1 Atopic Dermatitis

By far the most trials on interventions in atopic and allergic diseases involving bifidobacteria were performed in patients with AD, also termed atopic eczema. Some of these trials aimed at improving severity of the symptoms by providing preparations containing bifidobacteria directly to the patients. In a small trial, children with atopic eczema during breast-feeding were weaned to a formula containing *B. lactis* BB-12 (or a probiotic *Lactobacillus* sp.) and after 2 months of treatment ($3-8 \times 10^{10}$ CFU per day) children showed significantly improved symptoms of eczema compared to the placebo group (Isolauri et al., 2000). By contrast, dietary supplementation of infants with eczema (age 3–6 months) with *B. lactis* CNCM I-3446 (1×10^{10} CFU daily) for 3 months did not improve disease-relevant parameters over placebo (Gore et al., 2012). In adults given either 2×10^{10} CFU of *B. breve* strain YY per day or a placebo for 8 weeks, objective AD severity scores were significantly reduced in the probiotic group compared with the placebo group (Yoshida et al., 2010).

Another approach taken by different groups is the attempt to increase colonization of the newborn by administering bifidobacteria prenatally to the mother. In some of the clinical trials, bifidobacteria were then also administered to the infant after birth. For example, in a placebo controlled trial (total n = 474) comparing different probiotics, *B. animalis* subsp. *lactis* HN019 was administered daily at a dose of 9×10^9 CFU to pregnant women from week 35 of gestation until 6 months after birth and their children received the same strain from birth until the age of 2 years (Wickens et al., 2008). However, no significant effect on the prevalence of atopy and eczema was observed. These results were confirmed by a second trial with the same strain and dose reporting only a slight improvement of atopic sensitization and eczema by the treatment over placebo and the effect was restricted to two groups of patients with polymorphisms in some of the genetic susceptibility loci including the genes for Toll-like receptors 4 and 7, human β -defensin, and interleukin 4 receptor (Marlow et al., 2015; Morgan et al., 2014). In a similar setup, a mix of *B. breve* M-16V and *B. longum* BB536 (total daily dose: 1×10^{10} CFU) or placebo was administered to 130 mothers prenatally for 1 month before delivery and to their children after birth for 6 months and the risk of developing AD was reduced significantly in the treatment group (Enomoto et al., 2014).

13.6.2 Allergic Rhinitis

Singh et al. (2013) conducted a trial in a total of 20 patients with allergic rhinitis during the peak of the pollen season who were administered *B. lactis* NCC2818 (one daily dose of 4×10^{9} CFU) or placebo for 8 weeks. In the treat-

ment group, total nasal symptom scores and percentages of activated CD63 expressing basophils were significantly lower after 4 weeks. In line with this, stimulated blood lymphocytes secreted lower levels of Th-2 cytokines IL-5 and IL-13, typically associated with allergic reactions. Three further trials focused exclusively on allergic rhinitis caused by the particular antigen Japanese cedar pollen. In all three trials *B. longum* BB536 was administered at different doses, in different matrices and for different periods of time and significant improvements in subjective or objective symptom scores for rhinorrhea, ocular, or composite scores were reported (Xiao et al., 2006a,b, 2007).

13.7 INFLAMMATORY DISORDERS OF THE GASTROINTESTINAL TRACT

Besides infectious inflammation, there are several, sometimes chronic, inflammatory conditions of the gastrointestinal tract that are not associated with a particular pathogen, for example, celiac disease, IBD, and IBS. These diseases are characterized by a hypersensitivity of the gastrointestinal mucosa and immune system and/or aberrant and excessive immune response to (harmless) dietary or microbial antigens (Longstreth et al., 2006; Sollid and Jabri, 2013; Xavier and Podolsky, 2007). In almost all cases, a dysbalanced microbiota is associated with disease and often levels of bifidobacteria are reduced (Cotter, 2011; Marasco et al., 2016; Rajilić-Stojanović et al., 2011). Based on these findings and the fact that some bifidobacteria exert antiinflammatory properties *in vitro* and murine models of these diseases (Di Giacinto et al., 2005; Grimm et al., 2015; Jeon et al., 2012; Kayama et al., 2012; Preising et al., 2010; Veiga et al., 2010), several trials were conducted aiming at prevention or treatment of these diseases by supplementation with bifidobacteria.

13.7.1 Celiac Disease

B. infantis strain NLS was tested in a trial with 22 adult patients who receiving two capsules daily containing either 2×10^9 CFU or placebo (Smecuol et al., 2013). While intestinal permeability was not changed in either group, treated patients reported a significant improvement in digestion, reflux and constipation, and serum levels of disease-related autoantibodies were reduced. A similar trial was conducted in 33 children that were newly diagnosed for celiac disease and were treated with *B. longum* CECT 7347 (1×10^9 CFU daily for 3 months) or placebo (Olivares et al., 2014). In the treatment arm of the trial, increase in body size was significantly greater and total, as well as human leukocyte antigen DR-specific T lymphocytes, a subpopulation associated with celiac disease, were significantly reduced. In a slightly larger trial, 49 children with celiac disease on a gluten-free diet were randomized into 2 groups receiving either a supplement containing a mix of 2 *B. breve* strains (BR03 and B632; total daily dose 2×10^9 CFU) or placebo for 3 months (Klemenak et al., 2015). Throughout the treatment phase, serum levels of TNF- α were reduced in the treatment group, indicating an antiinflammatory effect. This effect, however, disappeared after treatment was stopped.

13.7.2 Inflammatory Bowel Disease

IBDs are chronic gastrointestinal disorders characterized by relapsing and remitting inflammation of the gastrointestinal mucosa (Xavier and Podolsky, 2007). The two principal types of IBD are ulcerative colitis and Crohn's disease. Both forms share most of the symptoms but differ in the extent and anatomic location of the tissues affected by inflammation. The two randomized, placebo-controlled trials included in this chapter were performed in ulcerative colitis patients. In one trial, patients were supplemented with 1×10^{10} CFU of viable *B. infantis* 35264 per day or placebo for 6 weeks and treated patients had reduced plasma levels of C-reactive protein and IL-6, suggesting a systemic antiinflammatory effect (Groeger et al., 2013). Of note, very encouraging results were obtained in a noncontrolled open label trial in which 14 patients refractory to standard treatment received daily doses of *B. longum* BB536 (2–3 × 10¹¹ CFU) for 24 weeks and, at the end of the trial period, 12 of 14 patients' disease activity score was markedly decreased and in 10 patients clinical remission was achieved, that is, they entered the nonactive phase of disease (Takeda et al., 2009).

13.7.3 Irritable Bowel Syndrome

IBS is a group of multifactorial, complex intestinal disorders characterized by a range of very diverse, sometimes opposing symptoms including chronic abdominal pain, bloating and altered stool frequency, constipation, diarrhea, or both, with alternating episodes. To date, there are no validated genetic, biochemical, or physiological markers for

13.8 CONCLUDING REMARKS

IBS. Thus, diagnosis is basically based on exclusion of other organic diseases. Affected persons are classified into different patients subgroups according to the ROME criteria for diagnosis of functional gastrointestinal disorders (Chang and Talley, 2011; Longstreth et al., 2006; Palsson et al., 2016). There are a number of reports on subgroup-specific alterations in the gastrointestinal microbiota that, in most cases, involve a reduction in bifidobacteria (Gerritsen et al., 2011; Jeffery et al., 2012; Kerckhoffs et al., 2009; Rajilić-Stojanović et al., 2011; Rigsbee et al., 2012).

O'Mahony et al. (2005) compared a malted milk product as vehicle for either *B. infantis* 35624, a *Lactobacillus* sp. strain, or placebo in a trial with 77 adult IBS patients. Bacteria were administered at daily doses of 1×10^{10} CFU for 8 weeks. During the treatment phase, patients in the *B. infantis* 35624 group had significantly lower composite disease scores according to two systems (Likert and VAS) compared to the placebo group. Also, individual scores for abdominal, pain/discomfort, bloating/distension were significantly lower. This was accompanied by a normalization of the IL-10/IL-12 ratio indicative of a resolution of type 1 helper T cell-driven inflammatory processes. The same group performed another, larger, trial with *B. infantis* 35624 in female IBS patients (n = 362) and tested different doses in capsules versus placebo (Whorwell et al., 2006). Interestingly, only a daily dose of 1×10^8 CFU (given for 4 weeks) yielded a significant improvement in composite symptom score, abdominal pain, bloating, bowel dysfunction, incomplete evacuation, straining, and passage of gas compared to placebo. The lower dose (1×10^6 CFU), as well as the higher dose (1×10^{10} CFU) proved to be ineffective. The lack of an effect with the higher dose, effective in the previous trial, was explained by formulation problems in the capsules, which appeared to dissolve incompletely, resulting in subeffective doses.

The effect of a 4-week treatment with *B. lactis* DN-173010 (daily dose of 1.25×10^{10} CFU in a fermented milk product) was tested by Agrawal et al. (2009). In IBS patients that received the *B. lactis* preparation, overall severity of the symptoms, as well as gastrointestinal transit and distension improved significantly compared to placebo-treated patients. Similar results were obtained in another trial with 122 patients receiving capsules containing either 1×10^9 CFU of *B. bifidum* MIMBb75 or placebo daily for 4 weeks (Guglielmetti et al., 2011). Patients in the treatment arm of the study showed significantly lower global disease scores, as well as significantly improved scores for pain/discomfort, distension/bloating, urgency, and digestive disorder.

In summary, all studies included in this chapter report significant improvements in disease symptoms and quality of life for patients, irrespective of the strain used for treatment. However, the classification of patients into subgroups with different disease symptoms makes it difficult to define universal, meaningful, and clinically relevant improvements for all patients. Only one study addressed this problem by mentioning that an analysis of the effects based on the IBS subtype was not provided (Whorwell et al., 2006). However, the authors acknowledge that case numbers in some subgroups were too small for analysis and indicate that the effects may be higher in the IBS-D subgroup of patients. In further studies this needs to be addressed by either performing trials in defined subgroups of patients or sufficient case numbers to allow analysis of subgroups retrospectively.

13.8 CONCLUDING REMARKS

Collectively, existing clinical data on bifidobacteria suggests that their use is safe in all settings tested as none of the published studies reporedt adverse effects or an increase in mortality or other complications. However, the results of clinical trials obtained so far are very heterogeneous. Treatments or preventive regimes that include single bifidobacterial strains have been tested in healthy subjects and a wide range of different clinical conditions. Moreover, a large number of different bifidobacteria have been tested in different settings. However, heterogeneity goes far beyond differences in strains and clinical conditions. On the one hand different strains of the same species have been tested for effects in the same group of patients (Table 13.1). On the other hand, widely used commercial strains have been tested in various clinical settings with, sometimes, opposing readouts and opposing or inconsistent results (Table 13.2) giving the impression of the search for the magic bullet that cures everything.

In none of the conditions addressed earlier, the available clinical data uniformly supports the use of bifidobacteria (or probiotics). Consequently, metaanalyses that have tried to assess the effect of bifidobacteria have not provided conclusive answers as to whether bifidobacteria in general or specific strains are effective in treatment or prevention of a specific condition. This is not surprising because effects caused by bifidobacteria (and probiotics in general) are almost exclusively strain specific. Nevertheless, individual studies indicate that some strains may be an alternative or supplementary treatment for some clinical conditions. However, further clinical studies with higher case numbers are required to definitely show efficacy of a particular strain. Moreover, instead of testing commercially available strains in a seemingly random manner in small-scale clinical trials more thorough *in vitro* and preclinical investigations might help to identify suitable strains for different clinical conditions.

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13. CLINICAL SIGNIFICANCE OF BIFIDOBACTERIA

TABLE 13.1 Noncomprehensive List of Clinical Trials of Commercial Bifidobacterium sp. Strains Used in D	Different Clinical Settings
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Strains	Targeted conditions/clinical settings	References				
B. lactis BB-12	Growth of healthy infants	Holscher et al. (2012); Weizman and Alsheikh (2006); Ziegler et al. (2003)				
	Prevention of illness	Hojsak et al. (2015); Merenstein et al. (2011)				
	Immune activation in healthy individuals	Bakker-Zierikzee et al. (2006); Holscher et al. (2012); Kekkonen et al. (2008); Ouwehand et al. (2008); Rizzardini et al. (2012)				
	Narcotizing enterocolitis, very low birth weight	Mihatsch et al. (2010); Stratiki et al. (2007)				
	Infectious diseases, diarrhea	Chouraqui et al. (2004); Taipale et al. (2011); Weizman et al. (2005)				
	Gastrointestinal function	Eskesen et al. (2015); Pitkala et al. (2007)				
B. lactis Bi-07	Prevention of illness	West et al. (2014)				
	Gastrointestinal function	Favretto et al. (2013)				
	Immune activation in healthy individuals	Maneerat et al. (2013); Paineau et al. (2008)				
B. lactis HN019	Gastrointestinal function	Waller et al. (2011)				
	Immune activation in healthy individuals	Arunachalam et al. (2000); Prescott et al. (2008)				
	Atopy/allergy	Marlow et al. (2015); Morgan et al. (2014); Wickens et al. (2008)				
B. animalis DN-173 010	Gastrointestinal function	Guyonnet et al. (2009a,b); Marteau et al. (2013)				
	Irritable bowel syndrome	Agrawal et al. (2009)				
B. longum BB536	Immune activation in healthy individuals	Namba et al. (2010); Rizzardini et al. (2012)				
	Critically ill patients	Akatsu et al. (2013); Kondo et al. (2013)				
	Atopy/allergy	Enomoto et al. (2014); Xiao et al. (2007, 2006a,b)				
	Inflammatory bowel disease	Takeda et al. (2009)				

 TABLE 13.2
 Noncomprehensive List of Clinical Trials on Interventions Using Bifidobacteria With Comparable Setups but Conflicting Results

Targeted conditions/strains	References	Findings				
Gastrointestinal symptoms						
Bifidobacterium animalis DN-173 010	Guyonnet et al. (2009a,b)	Significant improvements of self-reported gastrointestinal discomfort				
	Marteau et awl. (2013)	No significant improvement				
Immune activation in infants						
B. lactis BB-12	Holscher et al. (2012)	Significantly increased total sIgA				
	Bakker-Zierikzee et al. (2006)	No effect on fecal sIgA				
Infectious diseases						
B. lactis BB-12	Weizman et al. (2005)	Reduced and shortened episodes of fever, no differences in respiratory infections				
	Taipale et al. (2011)	Reduced episodes of respiratory infections				
Diarrhea						
B. lactis BB-12	El-Soud et al. (2015); Weizman et al. (2005)	Reduced and shortened episodes of diarrhea				
	Chouraqui et al. (2004)	No significant effect on diarrhea				

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14

Honeybee-Specific Bifidobacteria and Lactobacilli

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14.1 INTRODUCTION

Natural products and unexplored ecological niches encompass interesting groups when searching for alternative tools against infections. Symbionts have been described as yet unexploited sources as they are already shaped to defend their host by producing bioactive compounds and therefore can withstand resistance development. Twelve years ago we discovered bacterial symbionts within honeybees as a novel microbiota composed of collaborating lactobacilli and bifidobacteria. The symbionts were shown to be an indispensable key in honey production and present alive in all honeybees and only in their freshly produced honey around the world. To our knowledge, this is one of the greatest and most conserved symbiotic microbiota ever found in a single organism that scientifically explains why honey has been used against infections. Thus, the topic of our chapter concerns this novel microbiota and future applications as alternative tools to fight infections.

14.2 HONEYBEES

The honeybee *Apis mellifera mellifera* was classified by the Swedish "father of modern taxonomy" Carl von Linné in 1758, but honeybees are as old as flowers, which have been dependent on pollination for millions of years. Honeybees have been very important throughout history, providing us with both honey and pollination services, but yet to come, perhaps even more important, a source of alternative to antibiotics when bacterial resistance is becoming a global problem. From flowers, honeybees gather (forage) nectar and pollen, essential food sources for the whole honeybee colony from which they produce honey and bee bread.

14.3 HONEYBEE FOOD

Honeybees produce honey by foraging flower nectars. During the flight, nectar is temporarily stored in the honeybee honey stomach (Fig. 14.1). The honey stomach is a muscular enlargement of the oesophagus that can expand to a large volume. It is delimited by a structure called the proventriculus, which ensures that the nectar is never contaminated by the contents of the ventriculus (midgut), the functional stomach of honeybees. In the hive the nectar is transferred mouth-to-mouth to house bees that take it into the honeycomb for the evaporation of liquid to make honey both for seasonal storage and for feeding larvae. Nectar becomes honey when honeybees have let most of the water in it evaporate. When the honey is ripened, the honeycomb cells are sealed with wax capping, ensuring that the contents are preserved for years.

Bee bread is produced from bee pollen, which is composed of pollen, flower nectar, and secretions from the honeybee's salivary glands. Bee pollen is collected by foraging bees and packed into cells of the brood comb by house bees that eventually seal it with a drop of honey. After 2 weeks of natural fermentation, bee bread is produced and

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FIGURE 14.1 This picture shows a full honey stomach with nectar from flowers, from a dissected honeybee.

preserved for months. It is used in the hive both for seasonal storage and for feeding the queen and larvae either directly as it is but also after it has been processed to Royal Jelly by house bees.

The honeybee food sources and the food they produce are excellent nutrition for microorganisms as well. Nectar and pollen possess microorganisms as mold, yeast, and bacteria that are transported to flowers by visiting insects and wind. Inside a beehive, in a honeybee colony, the temperature is 35°C, in which foodstuffs are spoiled very rapidly and long before honeybees' foods are produced and safely stored.

14.4 THE HONEY STOMACH MICROBIOTA

In the year 2005, beekeeping of a hobbyist (Tage Kimblad) was successfully integrated in research performed by Tobias Olofsson and Alejandra Vásquez at Lund University, Sweden. It was a microorganism inventory project of a nature reserve that led to the conclusion that flowers sometimes are contaminated by beneficial bacteria originating in insects from when blooming is initiated. A theory arose that perhaps honeybees collect such beneficial bacteria when foraging and that they ended up in the hive and combs with nectar and bee pollen. The theory was only partly correct and our discovery was made, which showed that beneficial bacteria do end up in honey during production in the hive but honeybees themselves inoculate their honey with them in order to standardize their food production.

Inside the honey stomach of honeybees, Olofsson and Vásquez (2008) found a large microbiota composed of nine species of *Lactobacillus—L. kunkeei* Fhon2, *L. apinorum* Fhon13, *L. mellifer* Bin4, *L. mellis* Hon2, *L. melliventris* Hma8, *L. kimbladii* Hma2, *L. helsingborgensis* Bma5, *L. kullabergensis* Biut2, and *L. apis* Hma11, and four species of *Bifidobacterium—B. coryneforme* Bma6, *B. asteroides* Bin2, and probably two novel *Bifidobacterium* spp. (Hma3 and Bin7) (Olofsson et al., 2014a,b). One of the discovered lactobacilli, the type strain *Lactobacillus kunkeei* YH-15, was originally isolated (Huang et al., 1996) from damaged grapes in wine production in California. It strongly inhibited yeasts and was thus described as a spoilage organism of wine (Edwards et al., 1998). However, it is likely that *L. kunkeei* was transferred to the damaged grapes by honeybees themselves, while searching for a sugar source (Bae et al., 2006; Huang et al., 1996), especially because beekeeping is widespread in California because of pollination services for almond and citrus fields. Scardovi and Trovatelli (1969) detected, among other bifidobacteria, two of the honey stomach bifidobacterial species from honeybees, *B. asteroides* and *B. coryneforme*, but the sampling was performed from the entire

gastrointestinal tract with no separation of the honey stomach from the intestines. Therefore, it was believed that these bifidobacteria originated from the intestines or hindgut. The other two bifidobacteria, *Bifidobacterium* sp. Hma3 and *Bifidobacterium* sp. Bin7, are currently under a novel species description since they vary both in phenotype and genotype from the type strains *B. coryneforme* and *B. asteroides*.

Investigations by Vásquez et al. (2012) demonstrate how this microbiota is acquired and maintained within honeybees, where it is built up gradually by trophallactic exchange with nestmates. The microbiota remains viable within bees and are added in high concentrations to their food products as the symbionts detach from their niche, the honey stomach. The fact that this microbiota is transferred to the honeybees' food, as essential standardization and preservation steps, points out that these symbionts' most important functional niche is actually the honey stomach and not their intestines. The honey stomach microbiota is often in contact with oxygen. Sun et al. (2015) showed that bifidobacteria originating from honeybees could have been enriched with specific gene sets, suggesting host-specific adaptation. Beespecific genes are strongly associated with respiratory metabolism and are potential in helping those bacteria adapt to the oxygen-rich environment in honeybees. Their data display that the ancestor of bifidobacteria most likely colonized and adapted to bees during the early history of evolution and then could have spread and colonized humans.

Our further investigations of the hitherto described honeybee species have confirmed the theory that honeybees worldwide carry this specific microbiota of lactobacilli and bifidobacteria in their honey stomach and that the microbiota is added to their food products, honey, and bee bread (Olofsson and Vásquez, 2008; Vásquez et al., 2009). Since honeybees globally have been separated for many millions of years, but still carry a very similar honey stomach microbiota, we have demonstrated additional support that honeybees and these specific bacterial symbionts have been adapting to each other for millions of years.

14.5 TRADITIONAL MEDICINE HONEY

Honey has been a mysterious food used in traditional medicines since ancient times but its healing effects on wounds and on sore throats have never been satisfactorily or scientifically explained. Research on honey has increased recently (White et al., 2005) due to the escalating resistance to antibiotics globally, but the knowledge of honey's mode of action has still been attributed to its osmolarity, acidity, hydrogen peroxide content (White et al., 1963), characteristics of nectar (Taormina et al., 2001), and the presence of as yet unidentified components (Molan, 2001). Furthermore, different honey types vary considerably in their antibacterial activity (Lusby et al., 2005). Almost all the analyzed honeys from those investigations have been purchased in cans from beekeepers or purchased as medical grade honey; this is honey that is used for medical purposes. The only common feature of such honey, including medical grade ones, is that not a single one of them contain any viable member of the honey stomach microbiota (Olofsson and Vásquez, 2008; Olofsson et al., 2014a,b). Before beekeeping, when honey was harvested by honey hunters from wild honeybee colonies in high trees or along cliffs, these beneficial bacteria were the most important common feature (Vásquez et al., 2012). Therefore, it was before beekeeping, as we know it today, that honey got its reputation as a traditional medicine, a possible precursor of today's antibiotics.

14.6 THE POTENTIAL OF THE HONEY STOMACH MICROBIOTA: PRESENT AND FUTURE RESEARCH

The obvious future focus of our research, after the discovery of this symbiotic bacterial microbiota, had to be on their potential to fight against harmful bacteria affecting humans, animals, and honeybees. Since the discovery we have been studying their antimicrobial properties by delineating the mechanisms of action behind these qualities. In addition, we have studied different fields of applications where these symbionts can be applied to combat infections.

14.6.1 Mechanisms of Action

Lactobacillus and *Bifidobacterium* are known producers of biomolecules with antimicrobial action, such as organic acids, bacteriocins, antimicrobial proteins, and volatiles. Thus, we have conducted studies to investigate production of bioactive molecules by the honey stomach microbiota to understand how they interact with each other and with their host, the honeybees (Table 14.1). As expected, all the 13 strains produce organic acids including lactic, acetic, and formic acids at varying degrees (Olofsson et al., 2014a,b). In addition, the metabolite production gives us an insinuation on the different roles every one of them plays in their niche and during honeybee food production.

Bacterial species	Strains	Free fatty acids (3-OH Fas)				Volatiles						Other interesting metabolites			
Lactobacillus		C 10:0	C 12:0	C 14:0	C 16:0	Benzene	Toluene	<i>n</i> -Octane	Ethyl-benzene	Xylene	<i>n</i> -Nonane	2-Heptanone	H_2O_2	Bacteriocins and Lysozymes	Extracellular proteins (unknown function)
L. kunkeei	Fhon2	+	+		++					+				+	+++
L. apinorum	Fhon13	+++	+++	+	+				+	+		+		+	+
L. kimbladii	Hma2						+	+		+	+		+		
L. melliventris	Hma8						+	+		+	+		+		
L. helsingborgensis	Bma5						+	+		+	+		+	+	
L. kullabergensis	Biut2						+	+		+	+		+		+
L. apis	Hma11						+	+		+	+		+		
L. mellifer	Bin4					+			+						+
L. mellis	Hon2						+	+		+	+			+	+
Bifidobacterium															
B. asteroides	Bin2	+	+				+	+		+	+				+
<i>Bifidobacterium</i> sp.	. Bin7				+		+	+		+	+				
Bifidobacterium sp.	. Hma3						+	+		+	+			+	+
B. coryneforme	Bma6							+							

TABLE 14.1 Overview of the Hitherto Known Metabolite Production from the Different Members of the Honey Stomach Microbiota

Adapted from Butler, E., Alsterfjord, M., Olofsson, T.C., Karlsson, C., Malmström, J., Vásquez, A., 2013. Proteins of novel lactic acid bacteria from Apis mellifera: an insight into the production of known extracellular proteins during microbial stress. BMC Microbiol. 13, 235; Olofsson, T.C., Alsterfjord, M., Nilson, B., Butler, E., Vásquez, A., 2014. Lactobacillus apinorum sp. nov., Lactobacillus mellifer sp. nov., Lactobacillus mellis sp. nov., Lactobacillus mellifers sp. nov., Lactobacillus helsingborgensis sp. nov., and Lactobacillus kullabergensis sp. nov., isolated from the honey stomach of the honeybee Apis mellifera. Int. J. Syst. Evol. Microbiol., 64, 3109–3119; Olofsson, T.C., Butler, E., Markowicz, P., Lindholm, C., Larsson, L., Vásquez, A., 2014. Lactic acid bacterial symbionts in honeybees—an unknown key to honeys antimicrobial and therapeutic activities. Wound J. Int.

For further understanding of the honey stomach microbiota's future applications and synergistic abilities it was necessary to sequence their genomes. Among other results, the genomes demonstrated a very high level of gene content diversity within all the lactobacilli and bifidobacteria, particularly in terms of metabolic functions and surface structures (Ellegaard et al., 2015). Together, these results indicate niche differentiation within strains and phylotypes, suggesting that the honeybee honey stomach microbiota is more complex than previously thought (Tamarit et al., 2015). The large genomic material is to be of specific future interest for our understanding of the bacterial community and their symbiosis with the honeybee for years to come.

In addition, we found it important to ensure that a topical application on humans, including the viable members of the honey stomach microbiota in standardized concentrations, cause no symptoms and not affect commensal bacterial flora, nor produce any inflammatory response. The results were satisfactory and will be of relevance for future applications.

14.6.2 Human Pathogens

For decades different types of store-bought honey, including medical grade honey that have been tested against human pathogens with no overall consistent results. Rather low effects have been demonstrated against some of the known pathogens but repeated tests have often failed to confirm these results (Moore et al., 2001).

In vitro studies of human clinical wound pathogens were initially performed measuring the antimicrobial activity by using dual culture overlay assays. The honey stomach microbiota as a group and as individual members were tested against severe wound pathogens, such as methicillin-resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and vancomycin-resistant Enterococcus among others. We demonstrated a strong antimicrobial activity from each of the 13 individual strains and also a synergistic effect, which counteracted all the tested pathogens (Butler et al., 2014; Olofsson et al., 2014a,b). The effect was significantly stronger compared to when only honey without viable bacteria was used, but most importantly the effect was consistent for all pathogens tested.

14.6.3 Hard-to-Heal Horse Wounds

Due to safety considerations we decided to perform animal prehuman tests but only on authentic and already troublesome chronic wounds with no other solutions left. Two pilot products were developed: a wound dressing and a spray. The wound dressing contained sterilized natural Swedish honey mixed with the viable honey stomach microbiota in a known and high concentration. The spray contained nutrients and the viable bacteria in the same concentration as the dressing.

Ten horses were included in a proof-of-concept study monitored in collaboration with a veterinary surgeon (Olofsson et al., 2016). The horses had individual records of hard-to-heal wounds lasting between 1 and 4 years. Our obtained results were remarkable: all horses started to heal soon after the first application and the wounds were completely healed in less than 20 days. Furthermore, the formulations inhibited all pathogens in vitro, as well as in vivo. Despite no negative control in this study, the results pointed out a possible and powerful candidate for a topical treatment of hard-to-heal wounds in horses. Consequently, the results gave us confidence to continue with a seemingly good candidate for topical treatments on humans.

14.6.4 Chronic Human Wounds

Due to the positive results on equine wounds a multicenter proof-of-concept study was performed, including patients suffering from chronic leg ulcers. Our results demonstrated that healing was successfully initiated, but due to yet unpublished results of this recent study further details must be excluded at present (submitted to *Annals of Clinical Microbiology and Antimicrobials*).

14.6.5 Inhibition of Bovine Cow Mastitis Pathogens

In addition to human and equine pathogen isolates, bovine mastitis isolates from different cows were tested against the mixture of the 13 strains using a dual-culture overlay assay (Piccart et al., 2016). Growth of all tested mastitis pathogens, including antimicrobial resistant ones, was inhibited. Normally, antibiotics are used for this kind of infection but the antibacterial effect of the honey stomach microbiota could be a future promising alternative for the treatment and prevention of bovine mastitis.
14.6.6 Protection of Honeybees and Their Food

As a microbiologist one can hypothesize that the honeybee microbiota is as important for honeybees as beneficial commensals are for humans and animals. We therefore have investigated the honey stomach symbionts' significance for honeybees. Bacterial infections in honeybees are mainly caused by two different pathogens. American foulbrood disease affects the honeybee larvae and is caused by *Paenibacillus larvae*. It leads to huge economic losses because the infested colony crashes. It is most efficiently mastered by prophylactic use of antibiotics when it is allowed. The use of antibiotics is prohibited in Europe. In Sweden, as an example, it is mandatory to burn the inside of the hive and to sterilize all beekeeping equipment after an outbreak. The second infection, caused by another bacterial pathogen (*Melissococcus plutonius*) is called European foulbrood disease, which also threatens the health of the entire colony.

We have performed studies in vivo (not field studies) on both pathogens using reared larvae and both studies showed promising results (Forsgren et al., 2010; Vásquez et al., 2012). The in vitro tests, using dual culture overlay assays, were very clear-cut showing an overall inhibition of both pathogens by the honey stomach microbiota. More results of the antibacterial effect against these honeybee pathogens are awaiting publication.

Nectar and pollen, the sources behind the vital honeybee food, honey, and bee bread, are very vulnerable to spoilage by other microorganisms, which act as stressors to the honey stomach microbiota (Butler et al., 2013). In three different studies our results show that this microbiota is essential for initial spoilage defense and for the production and preservation of these two foods (Olofsson and Vásquez, 2008; Vásquez and Olofsson, 2009; Vásquez et al., 2012). Thus, this microbiota is a key to preserve honeybee health worldwide.

14.6.7 Future Applications

Antibiotics are weapons used by microorganisms like bacteria and molds to protect their niche and nutrients. Antibiotics are most often used as a single substance, consumed in high concentration. The substance is extracted from its producing microorganism and therefore static, with no ability to cope with microorganisms trying to develop resistance. Therefore, most antibiotics end up useless when antibiotic resistance has developed by other microorganisms.

The honey stomach microbiota is composed of 13 different bacteria, with a very unique property, they collaborate, and each and every one can produce different antibacterial substances when needed and in the right amounts. They have been evolutionary, shaped to defend their niche and honeybees to combat all microbial threats introduced by nectar and pollen foraging. Hence, it seems like a better idea to apply this microbiota alive and together in any future applications because they are able to produce not only one antimicrobial substance but also hundreds in the required concentrations. By using them alive in different applications they will also be able to cope when other microorganisms encountered try to develop resistance. Perhaps it is time for us to apply what nature has developed already and with no alterations in order to get sustainable alternatives to antibiotics when treating human and animal infections.

14.7 CONCLUSIONS

The honey stomach microbiota shows a promising future as a possible alternative to antibiotics fighting or preventing human, animal, and honeybee infections. Apart from that, our research has revealed that these lactobacilli and bifidobacteria possess antimicrobial and therapeutic properties and is in symbiosis with each other and with its host, the honeybee, to secure their health.

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Genetic Manipulation and Gene Modification Technologies in Bifidobacteria

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15.1 INTRODUCTION

Bifidobacteria comprise one of the major members of the human intestinal microbiota and are widely known to exhibit a variety of health benefits to the host (Chapters 12, 13, 17, 18 this book; Tojo et al., 2014). In particular, issues regarding the mechanisms of bifidobacteria survival in the intestine and their interactions with the host organisms and other intestinal microbiota members have begun to be clarified over the past decade. Notably, the progress of this clarification has been boosted by various factors. For example, the recent advancements in next-generation sequencing technology have enhanced the genomic sequencing analysis of bifidobacteria such that 52 full-length genomic sequences of genus *Bifidobacterium* have been determined [Genomes Online Database (GOLD)] (Reddy et al., 2015) since the first complete genome sequence of *Bifidobacterium* longum NCC2705 (Schell et al., 2002). These genomic sequences have accordingly become a cornerstone of the molecular genetic study of bifidobacteria. Furthermore, omics technologies represented by transcriptome and metabolome analysis have also progressed, and the available range of omics technology not only includes cultured bifidobacterial cells but also extends to the functional analysis of bifidobacteria in mouse models. For example, gnotobiotic mice harboring bifidobacteria (Ishikawa et al., 2011; O'Connell-Motherway et al., 2011) have been used to clarify bifidobacterial function in the intestine (Sugahara et al., 2015).

Functional analysis of specific genes is also necessary to clarify the mechanisms of bifidobacteria function. Gene modification systems are generally necessary to clarify gene function; however, in bifidobacteria, the development of such systems had not been addressed until recently. Conversely, over the past decade, several gene modification techniques have been developed in this genus. In this chapter, we initially introduce transformation in bifidobacteria as a fundamental technology. Subsequently, we review the current gene modification technologies; a targeted gene mutagenesis system and a transposon mutagenesis system, as well as a heterologous gene expression system as technologies for the functional characterization of genes in bifidobacteria. Finally, we would like to address future perspectives regarding the potential application of these gene modification technologies.

15.2 TRANSFORMATION OF BIFIDOBACTERIA

Transformation is an essential technology and a starting point for the functional analysis of genes in bacteria. Many *Escherichia coli–Bifidobacterium* shuttle vectors have been developed as tools for transformation. In bifidobacteria, transformation methodologies have mainly employed electroporation although the efficiency of this procedure has limited its application in various *Bifidobacterium* species/strains. A main cause of the issue is the presence of

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restriction-modification systems in bifidobacteria. Several procedures, such as methylation of the shuttle vectors for the purpose of overcoming these systems will be introduced in this section. In addition, a conjugative transfer system has also recently been established that has become an alternative transformation tool.

15.2.1 Escherichia coli–Bifidobacterium Shuttle Vectors

Shuttle vectors are frequently used to transform an objective bacterial host strain. These are basically constructed using a replicative plasmid in the host strain consisting of plasmid-derived regions required for plasmid replication combined with an *E. coli* replicon and an antibiotic resistance gene. In bifidobacteria, an *E. coli–Bifidobacterium* shuttle vector pRM2 was constructed for the first time in 1994 by combining the commercial plasmid pGEM-5Zf(+) and an enterococcal spectinomycin-resistance (Sp^R) gene with a *B. longum* cryptic plasmid, pMB1 (Missich et al., 1994). The pRM2 vector was successfully introduced into *B. longum* B2577 by an electroporation method with an efficiency of approximately 10^2 colony-forming units (CFU)/µg DNA (Missich et al., 1994) and a variety of *E. coli–Bifidobacterium* shuttle vectors have since been constructed (reviewed by Fukiya et al., 2011; Guglielmetti et al., 2013).

Functionality of the *E. coli–Bifidobacterium* shuttle vectors is determined by several important factors: (1) bifidobacterial replicons and (2) antibiotic resistance genes.

15.2.1.1 Bifidobacterial Replicons

Replicons from a variety of *Bifidobacterium* plasmids, especially from pMB1, pTB6, pMG1, and pBC1, have been utilized for shuttle vector construction (reviewed by Sun et al., 2012). Segregation stability, structural stability, and host range of the bifidobacterial replicon are important considerations for shuttle vector construction. In particular, maintaining a high-segregation stability and structural stability of the plasmid in bacterial cells is essential for stably retaining the plasmid of interest in the transformed cells. Notably, the stability of plasmids within lactic acid bacteria has been reported to be higher in theta-replicating plasmids than in rolling-circle replication plasmids (Kiewiet et al., 1993). In contrast, the segregation and structure of a variety of shuttle vectors have been reported to be stable in bifidobacteria regardless of the replication type (Álvarez-Martín et al., 2007, 2008, 2013; Cronin et al., 2007; Klijn et al., 2006; Lee and O'Sullivan, 2006; Matsumura et al., 1997; Shkoporov et al., 2008a).

Host range of the bifidobacterial plasmid is important to construct a versatile replicative plasmid for use in a number of bacterial species. To date, a number of bifidobacteria plasmids have been shown to be replicative across multiple species of *Bifidobacterium* (Álvarez-Martín et al., 2008, 2013; Argnani et al., 1996; Cronin et al., 2007; Dominguez and O'Sullivan, 2013; Grimm et al., 2014; Osswald et al., 2015; Rossi et al., 1997, 1998; Ruiz et al., 2012a). In particular, pMDY23-based shuttle vectors have been shown to replicate in a very wide range of *Bifidobacterium* species; specifically, these vectors can replicate in nine different species and subspecies including 15 strains in total (Berger et al., 2010; Grimm et al., 2014; Osswald et al., 2015; Ruiz et al., 2012a). Notably, pNZ8048, which is known as a broad host range plasmid, and its derivatives have also been successfully replicated in bifidobacteria even though it does not utilize the standard bifidobacterial plasmid replicon (Landete et al., 2014; Serafini et al., 2012; Watson et al., 2008).

15.2.1.2 Antibiotic Resistance Genes

The antibiotic resistance gene is used as a marker for specific selection of the transformant of interest. Thus, *Bifidobacterium* strain used as a host for the transformation should indicate sensitivity to the antibiotics and the antibiotic resistance should be conferred by an expression of the antibiotic resistance gene in the strain. To date, several antibiotic resistance genes have been used in bifidobacteria: chloramphenicol (Cm) and erythromycin resistance genes originating from *Staphylococcus aureus* (Rossi et al., 1996), the Sp^R gene derived from *Enterococcus faecalis* (Grimm et al., 2014; Matsumura et al., 1997; Missich et al., 1994), an ampicillin resistance gene derived from the pBluescript vector (Grimm et al., 2014), and a tetracycline resistance gene derived from *B. longum* (Álvarez-Martín et al., 2007, 2008, 2013; Flórez et al., 2006). The antibiotic susceptibility of *Bifidobacterium* strains should therefore be investigated prior to their use as a transformation host strain because the susceptibility to these antibiotics is frequently species- or strain-specific (Delgado et al., 2005; Flórez et al., 2006; Grimm et al., 2014; Kheadr et al., 2007).

Successful expression of the antibiotic resistance genes in bifidobacteria (except for the endogenous tetracycline resistance gene) indicates that the heterologous promoter of the respective antibiotic resistance gene is functional in bifidobacteria. However, the degree of the expression from these promoters may sometimes cause a problem depending on the purpose of the experiment. Most of the described antibiotic resistance genes are recognized as being useful when contained within the shuttle vectors. Strong expression is not necessarily required for conferring antibiotic resistance because the copy number of the genes is high when they are propagated in the shuttle vector. In contrast, when an antibiotic resistance gene is used as a selection marker for chromosomal gene mutagenesis, strong

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expression of the gene is necessary because it must confer the antibiotic resistance from a single copy of the gene within the chromosome. As such, the Cm resistance gene was found to be unable to confer Cm resistance when the gene was integrated into the chromosome (i.e., one copy/chromosome), likely owing to a low level of gene expression (our unpublished results). To the best of our knowledge, only the Sp^R gene and the tetracycline resistance gene have been reproducibly demonstrated to confer sufficient resistance to allow selection at the single copy/chromosome level. Expression of other antibiotic resistance genes should therefore be reinforced by using highly active promoters, as well as by an adaptation of a codon usage of the genes to that of bifidobacteria to create highly selective and versatile antibiotic resistance genes for bifidobacteria.

15.2.2 Electroporation in Bifidobacteria: Issues in Transformation Efficiency and Their Solutions

Most of the current transformation methods in bifidobacteria employ an electroporation methodology. In general, *Bifidobacterium* cells are cultured in a medium containing high concentrations of carbohydrates, such as sucrose and fructooligosaccharides to prepare electrocompetent cells for increasing transformation efficiency. Cell washing/suspension buffers containing a high concentration of carbohydrates are also used (Argnani et al., 1996; Rossi et al., 1997; Serafini et al., 2012). In addition, conditions related to incubation time and the electric pulse have also been examined for further improvement of the transformation efficiency (Argnani et al., 1996; Matsumura et al., 1997; Rossi et al., 1997; Serafini et al., 2012). However, the transformation efficiency of bifidobacteria is generally low; specifically, the median efficiency is reported to be approximately 10³ CFU/µg plasmid DNA by electroporation (Fukiya et al., 2011).

The primary reasons for the low transformation efficiency of bifidobacteria are considered to comprise the oxygen sensitivity and the presence of restriction-modification systems in these bacteria. Electrocompetent cells are generally exposed to oxygen both prior and subsequent to the application of an electrical pulse. Therefore, the viable cell count of bifidobacteria is expected to decline during this period. To overcome this problem, Park et al. (2003) added an oxygen scavenger, Oxyrase (Oxyrase Inc., Mansfield, OH), to the cell incubation buffer after the electric pulse. As a result, the transformation efficiency of *B. longum* subsp. *longum* MG1 obtained using the *E. coli–Bifidobacterium* shuttle vector pBES2 increased 100-fold (Park et al., 2003).

The restriction-modification system is generally composed of two types of DNA-binding enzymes: a restriction endonuclease that cleaves dsDNA by recognizing a specific nucleotide sequence and a methyltransferase that introduces methylation in a target base by recognizing the basically same nucleotide sequence as the corresponding restriction endonuclease. Bifidobacteria are thought to harbor multiple genes encoding restriction-modification systems across the genome. Consistent with this supposition, since the 1980s, the activity of restriction endonucleases and methyltransferases targeting various recognition sequences have been identified experimentally (Table 15.1). Notably, the recognition sequences of these enzymes appear to differ by strain or species. These restriction-modification systems are considered to decrease the transformation efficiency of bifidobacteria by degrading foreign DNA (such as plasmids) introduced into the cells. Therefore, to achieve efficient gene manipulation in bifidobacteria, it is essential to avoid or decrease the effects of the restriction-modification systems.

Recently, various trials for improving the transformation efficiency in bifidobacteria by avoiding the restriction modification system have been undertaken. The main strategy has been the removal or methylation of the restriction sites in the target DNA molecule (i.e., plasmid vectors), both of which aim to avoid recognition by the restriction endonucleases of the host strain. As an example of the former strategy, Kim et al. (2010) revealed that an isoschizomer of SacII, termed BloMGI (Roberts et al., 2015), exists in *B. longum* subsp. *longum* MG1. The *E. coli–Bifidobacterium* shuttle vector pYBamy59 was accordingly modified by removing the SacII recognition sites or by subjecting it to an in vitro methylation reaction. Notably, the resulting transformation efficiency of *B. longum* subsp. *longum* MG1 obtained using the modified pYBamy59 construct increased 8- to 15-fold compared to that achieved using the unmodified vector (Kim et al., 2010).

Modification of plasmid vectors by methylation is conducted by propagation of the vectors in *E. coli* expressing the methyltransferase gene(s) from a *Bifidobacterium* strain of interest (Brancaccio et al., 2013; O'Connell-Motherway et al., 2009, 2014; Yasui et al., 2009). Yasui et al. (2009) have identified that the two types of type II methyltransferase genes are present in the genome of *B. adolescentis* ATCC 15703^T. Introduction of a methylated plasmid pKKT427, established by propagation in *E. coli* expressing both of these genes, resulted in an increased transformation efficiency from 1–3 (by nonmethylated DNA) up to a maximum of 4×10^5 CFU/µg plasmid DNA in this strain (Yasui et al., 2009). A similar strategy has been applied to *B. breve* UCC2003, *B. animalis* subsp. *lactis* CNCM I-2494, *B. bifidum* S-17, *B. longum* subsp. *longum* NCIMB 8809, and *B. longum* 35624 (Brancaccio et al., 2013; O'Callaghan et al., 2015; O'Connell-Motherway et al., 2009, 2014; Schiavi et al., 2016).

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15. GENETIC MANIPULATION AND GENE MODIFICATION TECHNOLOGIES IN BIFIDOBACTERIA

Enzymes	Strains	Recognition sequence (5'-3') ^a	References
Endonuclease			
BadI	Bifidobacterium adolescentis LVA1	CTCGAG	Skrypina et al. (1988)
BbeAI	B. breve S50 (ATCC 15698)	GGCGCC	Roberts et al. (2015)
BbeI	B. breve YIT4006	GGCGC/C	Khosaka et al. (1982)
BbiDI	B. bifidum NCIMB 41171	5hmC (10-13/9-11) ^b	Borgaro and Zhu (2013); Roberts et al. (2015)
Bbf7411I	B. bifidum 7411	TCCGGA	Skrypina et al. (1988)
BbfI	B. bifidum LVA3	CTCGAG	Skrypina et al. (1988)
Bbi24I	B. bifidum S-24	A/CGCGT	Roberts et al. (2015)
BbiI	B. bifidum YIT4007	CTGCAG	Khosaka and Kiwaki (1984b)
BbiII	B. bifidum YIT4007	GR/CGYC	Khosaka and Kiwaki (1984b)
BbiIII	B. bifidum YIT4007	CTCGAG	Khosaka and Kiwaki (1984b)
R1.BbrUI (BbrI)	B. breve UCC2003	GGCGCC	O'Connell-Motherway et al. (2009); Roberts et al. (2015)
BbrUII (BbrII)	B. breve UCC2003	GTCGAC	O'Connell-Motherway et al. (2009); Roberts et al. (2015)
BbrUIII (BbrIII)	B. breve UCC2003	CTGCAG	O'Connell-Motherway et al. (2009); Roberts et al. (2015)
BinI	B. longum subsp. infantis 659 (ATCC 25962)	GGATC (4/5)°	Khosaka and Kiwaki (1984a)
BinSI	B. longum subsp. infantis S76e (ATCC 15702)	CCWGG	Khosaka et al. (1983)
BinSII	B. longum subsp. infantis S76e (ATCC 15702)	GGCGC/C	Khosaka et al. (1983)
Bla7920I	B. lactentis (B. longum subsp. infantis) 7920	TCCGGA	Skrypina et al. (1988)
BloHI	B. longum E194b (BloH)	R/GATCY	Hartke et al. (1996); Roberts et al. (2015)
BloHII	B. longum E194b (BloH)	CTGCA/G	Hartke et al. (1996); Roberts et al. (2015)
BloMGI	B. longum subsp. longum MG1	CCGCGG	Kim et al. (2010); Roberts et al. (2015)
BthI	B. thermophilum RU326 (ATCC 25866)	CTCGAG	Sakurai and Kosaka (1985)
BthII	B. thermophilum RU326 (ATCC 25866)	GGATC	Roberts et al. (2015)
R.BanLI	B. animalis subsp. lactis CNCM I-2494	RTCAGG	O'Connell-Motherway et al. (2014); Roberts et al. (2015)
R.BanLII	B. animalis subsp. lactis CNCM I-2494	GGWCC	O'Connell-Motherway et al. (2014); Roberts et al. (2015)
Methyltransferase			
BBIF0710	B. bifidum S17	CTCGAG	Brancaccio et al. (2013); Roberts et al. (2015)
M.BadAI	<i>B. adolescentis</i> ATCC 15703^{T}	GATC	Roberts et al. (2015); Yasui et al. (2009)
M.BadAII	<i>B. adolescentis</i> ATCC 15703 ^T	CCNGG	Roberts et al. (2015); Yasui et al. (2009)
M.BanLI	B. animalis subsp. lactis CNCM I-2494	RTC <u>A</u> GG	O'Connell-Motherway et al. (2014)
M.BanLII	B. animalis subsp. lactis CNCM I-2494	GGW <u>C</u> C	O'Connell-Motherway et al. (2014)
M.BbrUI (M.BbrI)	B. breve UCC2003	GG <u>C</u> GCC	O'Connell-Motherway et al. (2009); Roberts et al. (2015)

TABLE 15.1	Currently Reported	Restriction Endonuclease	s and Methyltransferases	in Bifidobacteria
	/ 1		/	

Enzymes	Strains	Recognition sequence (5'-3') ^a	References
M.BbrUII (M.BbrII)	B. breve UCC2003	GTCGAC	O'Connell-Motherway et al. (2009); Roberts et al. (2015)
M.BbrUIII (M.BbrIII)	B. breve UCC2003	CTGC <u>A</u> G	O'Connell-Motherway et al. (2009); Roberts et al. (2015)
M.BlmNCI	B. longum subsp. longum NCIMB 8809	GATN(5)TGCC	O'Callaghan et al. (2015)
M.BlmNCII	B. longum subsp. longum NCIMB 8809	CCWGG	O'Callaghan et al. (2015)
M.BlmNCIII	B. longum subsp. longum NCIMB 8809	TCGG <u>C</u> CGA	O'Callaghan et al. (2015)
BBL306_0231	B. longum subsp. longum CCUG 30698	CTGC <u>A</u> G	O'Callaghan et al. (2015)
BBL306_1028	B. longum subsp. longum CCUG 30698	CGGG <u>A</u> G	O'Callaghan et al. (2015)
BBL306_1753	B. longum subsp. longum CCUG 30698	GC <u>A</u> N(9)TGC	O'Callaghan et al. (2015)
M.BloAI	B. longum subsp. infantis ATCC 15697^{T}	GG <u>C</u> GCC	Roberts et al. (2015)
RM.BloAII (BloAII)	<i>B. longum</i> subsp. <i>infantis</i> ATCC 15697 ^T	GAGG <u>A</u> C	Roberts et al. (2015)

TABLE 15.1 Currently Reported Restriction Endonucleases and Methyltransferases in Bifidobacteria (cont.)

"Slash indicates the identified cleavage site of the endonucleases. Underline indicates the identified bases methylated by the methyltransferases.

^b5hmC: 5-hydroxymethylcytosine; the cleavage site is variable; it will be digested between nucleotide bases from 10 to 13 bases downstream of 5hmC.

'GGATC (4/5) indicates that the cleavage site is variable: it will be digested between nucleotide bases 4 or 5 bases downstream of the recognition sequence (5'-GGATC-3').

In recent years, methylation sites and recognition sequences of methyltransferases in bacteria have been identified and estimated in a genome-wide fashion by using single-molecule real-time (SMRT) DNA sequencing (Pacific Biosciences of California, Inc., Menlo Park, CA) (Fang et al., 2012; Furuta et al., 2014; O'Callaghan et al., 2015; O'Connell-Motherway et al., 2014; Powers et al., 2013). The SMRT sequencing methodology identifies every nucleotide that is newly incorporated into the DNA chain in a real-time manner and can acquire the kinetics information of their uptake in the process of extending the DNA strand using DNA polymerase (Davis et al., 2013). Because incorporation of the nucleotide into the extended strand is delayed only when the pairing base in a template DNA strand is methylated, methylation sites can be distinguished from nonmethylated sites in the bacterial genome based on the kinetics information. In addition, the nucleotide sequence recognized by methyltransferases can be estimated by in silico analysis of the nucleotide sequence information near the methylation sites (Davis et al., 2013). In bifidobacteria, recognition sequences of methyltransferases and methylation sites have been identified in B. animalis subsp. lactis CNCM I-2494, a widely used probiotic strain, and B. longum subsp. longum NCIMB 8809 using the SMRT sequencing technique and this information has been applied to increasing the transformation efficiency of these strains (O'Callaghan et al., 2015; O'Connell-Motherway et al., 2014). As the recognition sequence for a restriction endonuclease and a corresponding methyltransferase is generally the same, the use of a plasmid with removed or methylated recognition sequences of bifidobacterial restriction endonucleases will also likely increase the transformation efficiency of a variety of *Bifidobacterium* strains of interest.

15.2.3 Conjugational Transfer System: An Alternative Tool for Transformation

Conjugational transfer refers to a phenomenon in which DNA, such as a plasmid is transferred in the form of single-stranded DNA from a donor to a recipient bacterium through the contact between different kinds of bacterial cells. Bacterial transformation by using the conjugational transfer system has been applied in many bacterial species, including *Actinobacteria* (Gormley and Davies, 1991; Schäfer et al., 1994). In bifidobacteria, Shkoporov et al. were the first to attempt development of the conjugational transformation system in 2008, although they obtained only marginal performance (Shkoporov et al., 2008a) and no reports on the development of the conjugational transfer system in bifidobacteria had since been presented. Recently, however, Dominguez and O'Sullivan (2013) have successfully transferred plasmids from *E. coli* to a variety of *Bifidobacterium* strains using the conjugational transfer system. One critical point for their success is that they conducted the conjugation experiments in an aerobic rather than in an an-aerobic condition (Dominguez and O'Sullivan, 2013). Furthermore, Bottacini et al. (2015) identified a megaplasmid, pMP7017, of over 190 kbp in the full length from *B. breve* JCM 7017-199 could be transferred to several other *B. breve* and

B. longum subsp. *longum* strains (Bottacini et al., 2015). Conjugational transfer will likely be widely applied to bifidobacteria in the future because this system is simple to perform without the requirement of preparation of electrocompetent cells and is basically insusceptible to the restriction-modification systems.

15.3 HETEROLOGOUS GENE EXPRESSION IN BIFIDOBACTERIA

Heterologous gene expression is used for the functional characterization of bifidobacteria and their genes, as it allows additional abilities to be imparted in bifidobacteria. For example, reporter systems using genes that contribute to fluorescence or bioluminescence have been developed in bifidobacteria (Cronin et al., 2008, 2010, 2012a; Grimm et al., 2014; Landete et al., 2014; Montenegro-Rodríguez et al., 2015). These reporter systems represent an important technology for elucidating the molecular mechanisms of the intestinal colonization and the health-promoting effects of bifidobacteria, as they are useful to observe the growth and dynamics of the bifidobacteria ex vivo or in vivo, as well as in vitro. Heterologous gene expression has also been used to increase the ability of bifidobacteria to adhere to intestinal epithelial cells and their stress tolerance (Gleinser et al., 2012; He et al., 2012a; Watson et al., 2008). In addition, *Bifidobacterium* strains expressing heterologous proteins, such as an antigen or a prodrug-converting enzyme have been applied to live vaccines and cancer therapy (Fukiya et al., 2012; Guglielmetti et al., 2013; Sun et al., 2012). Collectively, the heterologous gene expression system can be considered an important genetic manipulation system that contributes to the elucidation of the fundamental nature of bifidobacteria and their application in foods and pharmaceuticals. Notably, strict regulation of gene expression in bifidobacteria is necessary to construct a sophisticated heterologous gene expression system according to the purpose of the study. Therefore, the accumulation of information on factors involved in the regulation of gene expression, represented most predominantly by the promoter and ribosome-binding site (RBS), is important.

15.3.1 Factors for Regulating Heterologous Gene Expression: Promoters

Promoters comprise DNA regions that are essential to initiate the transcription of a gene and to control gene expression. Generally, in bacteria, the promoter region contains two types of promoter motifs, -35 sequences and -10 sequences. These motifs are present in regions of approximately 35 and 10 bp upstream, respectively, from the transcription start site of the gene. In bifidobacteria, many promoter sequences have been identified and their -35 and 10 motifs have been predicted. However, consensus sequences have not yet been reported.

In addition to estimating the consensus sequences, functional analysis of various bifidobacterial promoters is also important. In particular, sufficient understanding of their activities and induction conditions in bifidobacteria and in *E. coli* is considered to be important. Promoter activity in bifidobacteria is one of the most critical functions for effective heterologous gene expression. Promoters with both low and high activity are required depending on the purpose of the study. In addition, an understanding of the induction conditions of the promoter is essential to effect strict control of gene expression. Furthermore, promoters that exhibit low activity in *E. coli* are suitable for the stable cloning of heterologous genes because an adverse effect on the growth of *E. coli* sometimes occurs when the cloned heterologous gene is highly expressed.

To analyze the function of these bifidobacterial promoters, promoter-reporter assay systems are frequently utilized. At present, the β -glucuronidase gene *gusA* (*uidA*) is most frequently used as a reporter gene (Álvarez-Martín et al., 2012a,b; Cronin et al., 2007, 2012b; Fanning et al., 2012; Grimm et al., 2014; Klijn et al., 2006; Maze et al., 2007; Ruiz et al., 2012b; Sangrador-Vegas et al., 2007; Sun et al., 2014; Ventura et al., 2005; Zomer et al., 2009). Genes encoding arabinofuranosidase, β -glucosidase, α -galactosidase, anaerobic green fluorescent protein, and luciferase have also been employed in the reporter system, and the functions of the bifidobacterial promoters have been elucidated (Cronin et al., 2008; Montenegro-Rodríguez et al., 2015; Ruiz et al., 2012b; Sakanaka et al., 2014; Wang et al., 2012; Youn et al., 2012). In particular, a reporter system using luciferase genes enables the measurement of promoter activity even in vivo. Therefore, this system is especially useful for analyzing the function of the promoter of bifidobacteria in the intestinal tract (Cronin et al., 2008).

In particular, the analysis of promoter function using these reporter systems has commonly indicated that a promoter of the bifidobacterial gene encoding a glyceraldehyde-3-phosphate dehydrogenase (P_{gap}) exhibits high activity in bifidobacteria (Grimm et al., 2014; Klijn et al., 2006; Sakanaka et al., 2014; Shkoporov et al., 2008b; Sun et al., 2014). Notably, the activity of bifidobacterial promoters has been found to not necessarily be consistent between bifidobacteria and *E. coli* (Sakanaka et al., 2014; Wang et al., 2012). These results suggest that the specificity of promoter recognition in *Bifidobacterium* may not be exactly the same as that in *E. coli*. However, despite these recent achievements,

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studies comparing the activity of various bifidobacterial promoters and, in particular, inducible promoters, are limited (Cronin et al., 2008; Grimm et al., 2014; Klijn et al., 2006; Sakanaka et al., 2014; Sun et al., 2014; Wang et al., 2012). Therefore, the accumulation of information on the functions and motifs (i.e., -35 sequences and -10 sequences) of

Therefore, the accumulation of information on the functions and motifs (i.e., -35 sequences and -10 sequences) of bifidobacterial promoters is necessary to establish more sophisticated heterologous gene expression systems in this organism. In terms of the identification of the promoter motifs, comprehensive characterization of the transcription start sites by a high-throughput sequencing of 5'-rapid amplification of cDNA ends library will be a promising way (Matteau and Rodrigue, 2015).

15.3.2 Factors for Regulating Heterologous Gene Expression: RBS

Studies investigating factors involved not only in the transcriptional efficiency of the gene (such as promoters and transcription terminators) but also in the translational efficiency are currently in progress. The translation process includes several steps: initiation, elongation, and termination of translation and the recycling of ribosomes. Among these, He et al. (2012b) have focused on improving the translation initiation efficiency in bifidobacteria. To improve the efficiency of the expression of a target gene, it is necessary to understand the optimal sequence of the RBS and the optimal distance between the RBS and the start codon. They have identified the optimal RBS sequence in bifidobacteria, including *E. coli* (5'-AGGAGG-3') by in silico analysis. Furthermore, the optimal distance between the RBS and the start codon has been experimentally shown as being 5 bp in *B. longum* subsp. *longum* 105-A (He et al., 2012b). Currently, analysis of the translation mechanisms is limited but is expected to gain importance when considered with respect to heterologous gene expression.

15.4 GENE MUTAGENESIS SYSTEMS IN BIFIDOBACTERIA

To elucidate the molecular mechanisms of intestinal colonization and the health-promoting effects of bifidobacteria, development of a gene mutagenesis system is essential. Gene mutagenesis systems, which can inactivate genes present in the genome, are extremely useful as an analysis system for gene functions because the effects of gene inactivation can be directly reflected in the phenotype. The gene mutagenesis systems mainly used in bacterial genetics comprise targeted gene mutagenesis and transposon mutagenesis systems. Gene mutagenesis can be achieved by utilizing molecular genetic tools that were introduced in the previous sections of this chapter. In this section, we will outline the gene mutagenesis systems that have been developed in bifidobacteria and their use in the gene function analysis of this organism.

15.4.1 Targeted Gene Mutagenesis System in Bifidobacteria

The targeted gene mutagenesis system currently utilized in bifidobacteria includes techniques for inactivating the function of the target gene using homologous recombination. Several *Bifidobacterium* strains, such as *B. longum* subsp. *longum* NCC2705 have been suggested to exhibit a low homologous recombination frequency because of the lack of *recBCD* genes in these strains, which are responsible for the major homologous recombination pathways of prokaryotes (Guglielmetti et al., 2013; Schell et al., 2002). Recently, the frequency of homologous recombination in bifidobacteria has been experimentally investigated using *B. longum* subsp. *longum* 105-A (Sakaguchi et al., 2012). In this strain, the use of homologous regions spanning 1.0–3.0 kbp led to the observation of an adequate frequency of homologous recombination is possible by using *Bifidobacterium* strains that demonstrate a relatively high transformation efficiency (over 10^4 CFU/µg DNA).

A mutagenesis system for targeting bifidobacteria genes was first reported in 2008 (O'Connell-Motherway et al., 2008). Since then, a variety of targeted gene mutagenesis systems have been developed (Fig. 15.1) (Arigoni and Delley, 2008; Fukuda et al., 2011; Hidalgo-Cantabrana et al., 2015; Hirayama et al., 2012; O'Connell-Motherway et al., 2009; Sakaguchi et al., 2012, 2013). The most widely used system is based on a single-crossover recombination (Table 15.2). This mutagenesis system can inactivate the target gene by inserting a plasmid into the interior of the gene via homologous recombination between the homologous regions on a nonreplicating plasmid and on the target gene (Fig. 15.1A). Because this technique is capable of introducing a mutation in a gene by only the single-crossover recombination, the generation of mutants is relatively easy. Conversely, this technique has a disadvantage that the genotype of the mutants might be returned to that of the wild type by homologous recombination, as well as because





FIGURE 15.1 Schematic representation of the targeted gene modification technologies in bifidobacteria. Target genes for modification are indicated as open arrows. Antibiotic resistance genes are represented as filled squares marked by R. In (B–D), theoretically two types of target gene alleles can be generated in both the first crossover and the second crossover. However, for simplicity, only the scheme yielding desired mutant is described. (A) Single-crossover plasmid insertion. Nonreplicative targeting vector harboring the internal region of the target gene (represented as open squares marked by X) is used for the first crossover. Homologous recombination between the internal region in the vector and the chromosomal target gene results in splitting the target gene. (B) Double-crossover allelic exchange. Nonreplicative targeting vector harboring the 5' and 3' regions of the target gene (represented as open squares marked by X and Y), between which an antibiotic resistance gene is inserted, is used for the first crossover. The first crossover occurs between the homologous regions (in this case X), resulting in the insertion of the targeting vector into the target gene. If the second crossover, which occurs only at a low frequency, proceeds between the homologous regions different from those used in the first-crossover recombination (in this case Y), the desired gene disruption mutant will be generated. (C) Double-crossover markerless gene deletion. The procedure is similar to that indicated in (B). However, a nonreplicative plasmid vector that harbors 5' and 3' regions of the target gene (represented as open squares marked by X and Y) without inserting an antibiotic resistant gene is used for the first crossover. The second crossover can occur in the first-crossover integrants at a low frequency. Markerless gene deletion mutant can be generated when the second crossover occurs as described in (B). (D) Markerless gene deletion by increasing a frequency of the second crossover. The procedure is similar to that indicated in (C). In this method, a conditional replication vector lacking a bifidobacterial plasmid replication protein RepA gene (*ArepA*) and harbors 5' and 3' regions of the target gene (represented as open squares marked by X and Y) is used for the first crossover. Subsequently, a repA+ plasmid harboring repA gene is introduced into the first-crossover integrant. The supply of RepA from the repA+ plasmid can initiate a replication of the chromosome-integrated conditional-replication vector, which leads to high frequency of the second crossover to avoid growth defects due to the interference of normal chromosome replication. In consequence, markerless gene deletion mutant can be generated. This figure is a modified version of the one that appears in our review article (Fukiya et al., 2012), with the copyright holder's permission.

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Strains	Mutagenized genes	Putative functions	Gene mutagenesis methods	References
Bifidobacterii	um animalis subsp. lac	tis	· ·	
NCC2818	tetW	Tetracycline resistance gene	Markerless deletion by DCR	Arigoni and Delley (2008)
DSM 10140	Balat_1410	Hypothetical membrane- anchored protein for EPS biosynthesis	Markerless deletion by DCR	Hidalgo-Cantabrana et al. (2015)
B. breve				
BR-A29	FL-SBP	ABC transporter solute-binding protein for fucosyllactose transport	Plasmid insertion by SCR	Matsuki et al. (2016)
JCM 7017	B7017_1848	Alcohol dehydrogenase	Plasmid insertion by SCR	Bottacini et al. (2014)
JCM 7017	pMP7017_0199	Surface protein	Plasmid insertion by SCR	Bottacini et al. (2015)
NCFB 2258	B2258_0392 (galG)	β -Galactosidase GH 42 family	Plasmid insertion by SCR	O'Connell et al. (2013b)
NCFB 2258	B2258_1268	Oleate hydratase	Plasmid insertion by SCR	O'Connell et al. (2013b)
UCC2003	Bbr_0106 (<i>cldE</i>)	Cellodextrin-binding protein	Plasmid insertion by SCR	Pokusaeva et al. (2011)
UCC2003	Bbr_0123 (<i>apuB</i>)	Extracellular type II amylopullulanase	Plasmid insertion by SCR	O'Connell-Motherway et al. (2008, 2009)
UCC2003	Bbr_0133 (<i>tadA</i>)	Type II/IV secretion system protein for Tad pilus assembly	Plasmid insertion by SCR	O'Connell-Motherway et al. (2011b)
UCC2003	Bbr_0161 (nanK)	N-Acetylmannosamine kinase	Plasmid insertion by SCR	Egan et al. (2014a)
UCC2003	Bbr_0164 (<i>nanB</i>)	ABC transport system solute- binding protein	Plasmid insertion by SCR	Egan et al. (2014a)
UCC2003	Bbr_0165 (<i>nanC</i>)	ABC transport system permease protein	Plasmid insertion by SCR	Egan et al. (2014a)
UCC2003	Bbr_0168 (nanA)	N-acetylneuraminate lyase	Plasmid insertion by SCR	Egan et al. (2014a)
UCC2003	Bbr_0173 (nanR)	GntR-type transcriptional repressor	Plasmid insertion by SCR	Egan et al. (2015)
UCC2003	Bbr_0221 (bfeU)	High-affinity iron permease	Plasmid insertion by SCR	Christiaen et al. (2014)
UCC2003	Bbr_0223 (bfeB)	Conserved hypothetical membrane-spanning protein	Plasmid insertion by SCR	Christiaen et al. (2014)
UCC2003	Bbr_0417 (galC)	Solute binding protein	Plasmid insertion by SCR	O'Connell-Motherway et al. (2011a)
UCC2003	Bbr_0420 (galG)	β -Galactosidase GH 42 family	Plasmid insertion by SCR	O'Connell-Motherway et al. (2011a)
UCC2003	Bbr_0422 (galA)	Endogalactanase	Plasmid insertion by SCR	O'Connell-Motherway et al. (2009, 2011a)
UCC2003	Bbr_0430	Priming glycosyltransferase	Plasmid insertion by SCR	Fanning et al. (2012)
UCC2003	Bbr_0441	Glycosyltransferase	Plasmid insertion by SCR	Fanning et al. (2012)
UCC2003	Bbr_0527 (gosD)	Sugar permease protein	Plasmid insertion by SCR	O'Connell-Motherway et al. (2013)
UCC2003	Bbr_0529 (gosG)	β -Galactosidase GH 42 family	Plasmid insertion by SCR	O'Connell-Motherway et al. (2013)
UCC2003	Bbr_0541 (<i>luxS</i>)	S-ribosylhomocysteine lyase	Plasmid insertion by SCR	Christiaen et al. (2014)
UCC2003	Bbr_0651	Conserved hypothetical secreted protein	Plasmid insertion by SCR	Fouhy et al. (2013)

TABLE 15.2 Bifidobacterial Genes Inactivated by Targeted Gene Mutagenesis

(Continued)

TABLE 15.2	Bifidobacterial Genes Inactivated by Targeted Gene Mutagenesis (cont.)

Strains	Mutagenized genes	Putative functions	Gene mutagenesis methods	References
UCC2003	Bbr_0838	Bile-inducible membrane protein belonging to the major facilitator superfamily	Plasmid insertion by SCR	Ruiz et al. (2012b)
UCC2003	Bbr_0849 (atsR2)	Transcriptional regulator, ROK family	Plasmid insertion by SCR	Egan et al. (2016)
UCC2003	Bbr_0851 (<i>atsT</i>)	Carbohydrate transport protein	Plasmid insertion by SCR	Egan et al. (2016)
UCC2003	Bbr_0852 (atsA2)	Sulfatase	Plasmid insertion by SCR	Egan et al. (2016)
UCC2003	Bbr_1247 (<i>nagA2</i>)	N-acetylglucosamine-6-phosphate deacetylase	Plasmid insertion by SCR	Egan et al. (2014a)
UCC2003	Bbr_1318 (serR)	Response regulator	Plasmid insertion by SCR	Álvarez-Martín et al. (2012b)
UCC2003	Bbr_1320 (serU)	Serpin-like protein	Plasmid insertion by SCR	Álvarez-Martín et al. (2012b)
UCC2003	Bbr_1419 (<i>rbsA</i>)	Ribose transport system ATP- binding protein	Plasmid insertion by SCR	Pokusaeva et al. (2010)
UCC2003	Bbr_1551 (<i>lacS</i>)	Galactoside symporter	Plasmid insertion by SCR	O'Connell-Motherway et al. (2013)
UCC2003	Bbr_1552 (<i>lacZ</i>)	β -Galactosidase GH 2 family	Plasmid insertion by SCR	O'Connell-Motherway et al. (2013)
UCC2003	Bbr_1586	Aminoglycoside phosphotransferase	Plasmid insertion by SCR	Fouhy et al. (2013)
UCC2003	Bbr_1587 (<i>lnbP</i>)	Lacto-N-biose phosphorylase	Plasmid insertion by SCR	Egan et al. (2014b)
UCC2003	Bbr_1683 (<i>phoP</i>)	Response regulator	Plasmid insertion by SCR	Álvarez-Martín et al. (2012a)
UCC2003	Bbr_1742 (<i>fucP</i>)	L-Fucose permease	Plasmid insertion by SCR	Egan et al. (2014b)
UCC2003	Bbr_1833 (<i>lacZ7</i>)	β-Galactosidase	Plasmid insertion by SCR	Egan et al. (2014b)
UCC2003	Bbr_1856 (melE)	α-Galactosidase/raffinose synthase	Plasmid insertion by SCR	O'Connell et al. (2013a)
UCC2003	Bbr_1857 (<i>melD</i>)	α-Glucosidase	Plasmid insertion by SCR	O'Connell et al. (2013a)
UCC2003	Bbr_1860 (melA)	Sugar ABC transporter solute- binding protein	Plasmid insertion by SCR	O'Connell et al. (2013a)
UCC2003	Bbr_1863 (melR2)	LacI-type transcriptional regulator	Plasmid insertion by SCR	O'Connell et al. (2014)
UCC2003	Bbr_1864 (melR1)	LacI-type transcriptional regulator	Plasmid insertion by SCR	O'Connell et al. (2014)
UCC2003	Bbr_1867 (<i>rafB</i>)	Raffinose-binding protein	Plasmid insertion by SCR	O'Connell et al. (2013a)
UCC2003	Bbr_1868 (<i>rafR</i>)	Repressor open reading frame kinase-type regulator	Plasmid insertion by SCR	O'Connell et al. (2014)
UCC2003	Bbr_1869 (<i>rafA</i>)	α-Galactosidase	Plasmid insertion by SCR	O'Connell et al. (2013a)
B. longum subs	sp. longum			
105-A	BL105A_0887 (<i>pyrE</i>)	Orotate phosphoribosyl transferase	Markerless deletion by DCR	Sakaguchi et al. (2012, 2013)
105-A	BL105A_1470 (<i>lnbX</i>)	Lacto-N-biosidase	Plasmid insertion by SCR	Sakurama et al. (2013)
105-A	BL105A_1707 (<i>xynF</i>)	Extracellular exo-xylanase	Allelic exchange by DCR	Sakaguchi et al. (2012)
105-A	BL105A_1898 (aga)	α-Galactosidase	Markerless deletion by DCR	Hirayama et al. (2012)
35624	<i>pgt</i> ₆₂₄	Priming glycosyl transferase for EPS production	Plasmid insertion by SCR	Schiavi et al. (2016)

TABLE 15.2 Bifidobacterial Genes Inactivated by Targeted Gene Mutagenesis (cont.)

	Mutagenized			
Strains	genes	Putative functions	Gene mutagenesis methods	References
NCIMB 8809	arfB	α-L-Arabinofuranosidase	Plasmid insertion by SCR	O'Callaghan et al. (2015)
NCC2705	BL0033 (fruE)	Sugar ABC transporter solute- binding protein	Allelic exchange by DCR	Fukuda et al. (2011); Sakaguchi et al. (2012)
NCC2705	BL0108	Serine protease inhibitor	Markerless deletion by DCR	Arigoni and Delley (2008)

ABC, ATP-binding cassette; DCR, double-crossover recombination; α -Gal, α -galactosidase; SCR, single-crossover recombination.

the whole plasmid, including the homologous regions, remains in the genome of the mutant. To overcome this drawback and to maintain stability of the mutated gene, a mutagenesis system using double-crossover recombination is necessary.

Gene mutagenesis systems by the double-crossover recombination are roughly divided into an allelic exchange system and a markerless gene deletion system (Fig. 15.1B–D). The allelic exchange system replaces the target gene in the genome with the selection marker gene, such as an antibiotic resistance gene via the double-crossover recombination (Fig. 15.1B). Thus, the mutant strain will exhibit a resistant phenotype to the antibiotics used, facilitating selection of the mutants. Furthermore, as the plasmid-derived sequences including the homologous regions other than the antibiotic resistance gene are removed from the genome, no possibility exists for the reoccurrence of homologous recombination. Therefore, the genotype of the mutant strain can be stably maintained. Gene mutagenesis using this system has been applied in bifidobacteria by Fukuda et al. (2011) and Sakaguchi et al. (2012, 2013) (Table 15.2). In particular, Sakaguchi et al. (2012, 2013) have successfully introduced mutations in genes by utilizing a temperature-sensitive plasmid pKO403 and a bidirectional selection marker gene, *pyrE* (Sakaguchi et al., 2012, 2013).

In the aforementioned gene mutagenesis systems, the introduction of a mutation in the gene of interest is always accompanied by the incorporation of residual foreign nucleotide sequences (e.g., plasmid sequences or antibiotic resistance genes) in the genome of the mutants. This may lead to several problems in generating and characterizing the resultant mutants. The first issue is that the insertion of the foreign nucleotide sequence may affect the expression level of the gene(s) adjacent to the target gene. This phenomenon, called polar effects, leads to difficulty in clarifying whether the cause of the phenotypic change of the mutants is due to the target gene mutation or derived from the change in the expression level of the gene(s) surrounding the target gene. Notably, the occurrence polar effects have been suggested in some bifidobacterial studies (Álvarez-Martín et al., 2012b; Sakurama et al., 2013). The second issue is that the number of mutations that can be introduced into the genome is limited to the number of available antibiotic resistance genes. In the gene mutagenesis systems described earlier, each successive gene mutagenesis will result in an additional antibiotic resistance gene being retained in the genome. However, as currently the antibiotic resistance genes that demonstrate sufficient resistance ability at the copy number of 1 copy/ chromosome to be useful in gene mutagenesis studies are limited to the Sp^R gene and tetracycline resistance gene, it would be difficult to produce multiple-gene mutants of three or more using the aforementioned gene mutagenesis systems.

Thus, a markerless gene deletion system is necessary to avoid these issues. This system can introduce a deletion of the target gene without leaving a residual foreign nucleotide sequence. Therefore, this system can assess the function of a target gene very accurately and the number of mutations in the genome does not depend on the number of available antibiotic resistance genes. In bifidobacteria, markerless gene deletion systems have been reported by several research groups (Arigoni and Delley, 2008; Hidalgo-Cantabrana et al., 2015; Hirayama et al., 2012; Sakaguchi et al., 2012, 2013) (Fig. 15.1C, D and Table 15.2). In particular, Hirayama et al. (2012) have succeeded in the introduction of a markerless gene deletion in *B. longum* subsp. *longum* 105-A by increasing the frequency of the second crossover (Fig. 15.1D). This increased frequency has been achieved by inducing the initiation of replication of a condition-al-replication vector, which lacks a gene encoding the plasmid replication protein RepA and had been integrated into the target gene by the first-crossover recombination. The subsequent introduction of an *E. coli–Bifidobacterium* shuttle vector harboring a *repA* gene supplies RepA in the first-crossover recombinants. The RepA then initiates the replication of the conditional replication vector in the target gene and interferes with the ordinal chromosome replication. Therefore, the frequency of the second crossover may be increased by the cell to remove the conditional replication vector (and, simultaneously, cause a deletion in the target gene), and thus avoid the severe growth defect owing to the interference with chromosomal replication (Hirayama et al., 2012).

As shown in Table 15.2, the majority of the target genes of the mutants generated to date contribute to carbohydrate assimilation. Functional analysis of genes that contribute to carbohydrate assimilation is essential to understand colonization mechanisms of the bifidobacteria in the intestinal tract where nutrient (carbohydrate) availability is constantly changing. In addition, Bbr_0430, a gene involved in the production of extracellular polysaccharides (Fanning et al., 2012), *luxS*, involved in the production of the autoinducer-2 (Christiaen et al., 2014), and *tadA*, which encodes an ATPase responsible for the pilus assembly of the type IVb tight adherence pili (O'Connell-Motherway et al., 2011b), have also been identified as genes that contribute to the intestinal colonization of conventional mice. Furthermore, the fructose binding protein FruE, a component of the ATP-binding cassette transporter for fructose, was revealed to contribute to the enhanced production of acetate by fructose assimilation through the use of gene mutagenesis as well (Fukuda et al., 2011). Notably, the production of acetate via FruE-containing ATP-binding cassette transporter function in vivo has been shown to prevent the murine mortality consequent to *E. coli* O157:H7 infection (Fukuda et al., 2011).

Currently, *Bifidobacterium* species that have successfully undergone targeted gene mutagenesis are limited to *B. breve*, *B. longum* subsp. *longum*, and *B. animalis* subsp. *lactis*, which exhibit relatively high transformation efficiency. Among these, *B. breve* UCC2003 represents the most frequently used strain for targeted gene mutagenesis (Table 15.2). Currently, these systems have not been applied to strains with low transformation efficiency. To overcome this situation, efforts for the development of improved and efficient targeted gene mutagenesis systems and toward increasing the transformation efficiency of *Bifidobacterium* species/strains are required.

15.4.2 Transposon Mutagenesis System in Bifidobacteria

The transposon mutagenesis system consists of a method for introducing insertional mutations into genes in various places in the bacterial genome of interest by transposing a transposable element. Thus, the transposon mutagenesis system can readily produce a variety of mutants by single transposition experiments. As examples, transposon mutants could be produced at the efficiency of 10^5 and 10^6 CFU/µg of plasmid DNA harboring the transposon in *Corynebacterium glutamicum* R (Suzuki et al., 2006) and *Rhodococcus erythropolis* JCM 3201 (Sallam et al., 2006), both belonging to the phylum *Actinobacteria*. Thus, using a "library" comprising numerous transposon mutant strains, it is possible to identify genes that contribute to a particular phenotype in vitro and in vivo. Furthermore, in recent years, the relative abundance of individual mutants in the library can be comprehensively measured by subjecting the PCR-amplified DNA of the transposon-insertion regions to next generation sequencing (van Opijnen and Camilli, 2013). Applying this technique, it has become possible to identify genes that contribute to a particular phenotype and decrease of the individual mutants. Accordingly, transposon mutants genesis system can be considered to represent one of the most powerful systems for gene function analysis.

In the bacterial transposon mutagenesis system, a Tc1/mariner type transposable element *Himar1*, derived from a horn fly, and the bacterial transposon Tn5 have been widely used (Choi and Kim, 2009; Picardeau, 2010). In bifidobacteria, Ruiz et al. (2013) have developed the first transposon mutagenesis system by applying the Tn5-based EZ::TN transposome (Epicentre Biotechnologies, Madison, WI, USA). According to their report, transposon mutants of *B. breve* UCC2003 have been produced at up to 1554 \pm 192 CFU in one round of the transposition introduction experiment. Furthermore, using this approach, a transposon mutant library of *B. breve* UCC2003 containing 20,000 mutants has been constructed. This mutant library is considered to represent a valuable contribution to gene function analysis of *B. breve* UCC2003. In addition, this transposon mutagenesis system has been applied to another strain of *B. breve*, NCFB 2258 (Ruiz et al., 2013).

Transposon mutagenesis systems using the insertion sequence (IS) elements derived from closely related species or strains of the target bacteria have also been developed in a variety of bacterial species (Camacho et al., 1999; Licandro-Seraut et al., 2012; Sallam et al., 2006; Suzuki et al., 2006). The IS element comprises a transposable element with a simple structure and many kinds of IS elements have been found to date from a large number of bacteria and archaea genomes. In general, IS elements require a catalytic enzyme for transposition, termed a transposase (Tpase). The Tpase gene(s) are located between the inverted repeat (IR) sequences at both ends of the element. Tpase recognizes both IRs and then transfers the element to a different location of the genome through the recognition of the target sequence (generally several base pairs). The application of IS elements to transposon mutagenesis requires efficiently transposable IS elements without a target sequence preference.

IS elements from bifidobacteria have not yet been applied to the transposon mutagenesis system despite being frequently found in their genome (Fukiya et al., 2011). One reason is that the functions of these IS elements, such as their transposition activity and selectivity, have not been sufficiently analyzed. In addition, direct and indirect evidence of transposition had been reported in only a small number of IS elements from bifidobacteria (Fukiya et al., 2010;

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González-Vara et al., 2003; Lee et al., 2008). Therefore, the isolation and characterization of novel IS elements from bifidobacteria are important for the development of IS-mediated transposon mutagenesis systems of bifidobacteria. Notably, an IS3-family element IS*Blo11* has been recently identified in *B. longum* subsp. *longum* 105-A in our laboratory by trapping transposable elements in an IS-trap vector harboring *sacB*, which confers sucrose sensitivity to *E. coli* (Sakanaka et al., 2015). IS*Blo11* transposes at a frequency of 10⁻⁵ and transposes into nonconserved target sequences (3 or 4 bases) in an *E. coli*-F-plasmid conjugation system. These characteristics of IS*Blo11* appeared to be suitable for transposon-mediated mutagenesis. Therefore, the development of a transposon mutagenesis system using IS*Blo11* has been conducted by our group in *B. longum* subsp. *longum* 105-A and has resulted in successful transposition at comparable transposition frequency to that of the Tn5-based transposome system in *B. breve* UCC2003 (Sakanaka et al., 2015). The findings indicate that irrespective of the system utilized, application of the transposon mutagenesis system in a variety of *Bifidobacterium* species/strains serves as a promising means for the comprehensive characterization of gene functions.

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As described in this chapter, the development of genetic manipulation systems in bifidobacteria has progressed considerably during the recent decade. Targeted gene mutagenesis and transposon mutagenesis have become possible in highly transformable model strains, such as *B. breve* UCC2003 and *B. longum* subsp. *longum* 105-A. A future challenge is to apply these systems to other strains, such as has been demonstrated in a research strategy reported by O'Connell-Motherway et al. (2014). This strategy was composed of two steps: (1) identification of methyltransferase genes and their recognition sequences in the strain of interest through methylome analysis, using the SMRT system; and (2) heterologous methylation of the gene-mutagenesis vectors in *E. coli* strains expressing the identified methyltransferase genes to increase the transformation efficiency by avoiding the restriction of the vectors (O'Connell-Motherway et al., 2014). The efficacy of this strategy has been demonstrated in a probiotic strain *B. animalis* subsp. *lactis* CNCM I-2494 and *B. longum* subsp. *longum* NCIMB 8809 (O'Callaghan et al., 2015; O'Connell-Motherway et al., 2014) and is inferred to be applicable in a variety of additional strains, although this remains to be validated.

Together, these findings strongly suggest that the targeted gene mutagenesis will be widely applied to various *Bifidobacterium* species. In addition, further attempts will be necessary to develop the other gene mutagenesis systems as well. Although the markerless gene deletion system has overcome the problem of a limited variation of selection markers, development of a recycling system of the selection markers is necessary to achieve their continuous use. Use of site-specific recombination systems, such as FLP/*FRT* (Datsenko and Wanner, 2000) or Cre/*loxP* (Fukiya et al., 2004; Lambert et al., 2007), which are utilized in other bacterial species for selection marker recycling, might represent one such possibility. Furthermore, a genome editing system using CRISPR/Cas9, which has been applied in various organisms in recent years (Mougiakos et al., 2016), is expected to be applied to bifidobacteria in the near future.

Furthermore, the development of new systems of transposon mutagenesis is likely to be promoted, which could be applied to a variety of *Bifidobacterium* species/strains. Identifying genes that contribute to a particular phenotype using the transposon mutant library is a powerful forward-genetic screening method, minimizing the difficulty in identifying genes that contribute to the phenotype of bifidobacteria at least in vitro. A remaining hurdle is to identify genes involved in the function of bifidobacteria in vivo (i.e., in the host intestine) as well. As described in the Section 15.4.2, several transposon-insertion sequencing methods have been developed (van Opijnen and Camilli, 2013). These systems can identify the transposon insertion regions of several tens to hundreds of thousands of cells at a time. Therefore, application of these systems to bifidobacteria will likely overcome the hurdle of functional analysis in vivo. For example, *Bacteroides thetaiotaomicron* genes involved in survival/fitness in the intestinal tract of mice have been identified through use of this system (Goodman et al., 2009). The same system has been further applied to other *Bacteroides* species to clarify the responses of each *Bacteroides* species in the gut microbiota to dietary changes (Wu et al., 2015). As bifidobacteria are currently at a stage where such analysis is applicable, genes involved in the function of bifidobacteria in vitro/in vivo are expected to be comprehensively identified in the near future.

In conclusion, bifidobacteria not only have been known as a beneficial intestinal bacterium but also have become one of the representative intestinal bacteria in terms of the genome- and gene-level analysis through the accumulation of efforts over the past decade. The development of genetic manipulation techniques in bifidobacteria serves as a very informative precedent for other intestinal bacteria. Therefore, bifidobacteria represent a likely model for the future functional analysis of genes and genomes of other intestinal bacteria.

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Production of Probiotic Bifidobacteria

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16.1 INTRODUCTION

As the readership of this book knows, probiotics are defined as live microorganisms that, when administered in sufficient amounts, confer a health benefit on the host (Hill et al., 2014). In order to document health benefits, intervention studies have to be performed in the target host; for bifidobacteria, this is usually humans. In order to be able to perform an intervention trial, investigational products, that is, probiotic biomass, have to be produced. It is therefore important that already in an early stage attention is paid to the production of probiotics. In contrast to in vitro and small-scale animal studies, human intervention studies require fairly large amounts of probiotic material. The production of this material and the subsequent downstream processing is more challenging than one might anticipate in comparison to lab-scale culture of bifidobacteria. Before even considering a human intervention study, the identity and safety of the strain needs to be evaluated and confirmed. The production needs to provide sufficient yield in reasonable time. Furthermore, the culture medium should be free from components potentially hazardous to human consumption. Downstream processing needs to provide material sufficiently stable for a human study. For subsequent commercialization, these criteria become even more important. There may be a wish to remove certain allergens from the culture medium. The yield has to remain high and viability losses small over a 24-month storage at different temperatures and humidity to provide commercial feasibility. For commercialization, also the regulatory framework becomes crucial. This chapter describes these and other topics related to the production of probiotics in general and bifidobacteria in particular.

16.2 SAFETY

The safety of *Bifidobacterium* species for human consumption has been investigated over many years and with focus on similar criteria to address safety as noted in regional regulatory requirements (Ishibashi and Yamazaki, 2001; Miquel et al., 2015; Saarela et al., 2002). In accordance with regulatory allowance, safety evaluation is dependent on the category of the intended use bifidobacteria fall into, as previously introduced (Sanders et al., 2010). While it is well understood that traditionally fermented foods containing bifidobacteria have been consumed by humans for thousands of years, a careful introduction of microbial cultures with substantially increased dosage should include thorough safety evaluation (Choi et al., 2005; Merenstein et al., 2015). For probiotics in general, safety assessment has been discussed and proposals, some specific to bifidobacteria species, have previously been recommended (Pariza et al., 2015; Sanders et al., 2010). Pariza et al. (2015) proposed a decision tree-based approach composed of 13 questions for new food and dietary supplement applications using microbial cultures. Taxonomic identification is paramount to safety, and evaluation is often characterized by genome sequencing of individual strains for unambiguous classification of the correct genus and species. The Food and Drug Administration (FDA) recently communicated methods for microbial identification of probiotic strains, and demonstrated inconsistencies in commercial probiotics classification as of utmost importance to safety concerns (Patro et al., 2016). Genome sequencing is a powerful tool

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in evaluating safety of bifidobacteria, allowing for investigation into the genetic potential therein. While no *Bifidobacterium* species has ever been reported to contain toxigenic or pathogenic strains, a survey of genetic annotations that encode for virulence genes and known toxin sequences can eliminate concern that these genes may have been acquired through horizontal gene transfer (HGT) (Pariza et al., 2015).

The presence of transferable antibiotic resistance genes should also be considered. In order to address the question of transferability of antibiotic resistance, it is best to define the two types of resistance. Intrinsic resistance reflects an organism's ability to thrive in the presence of an antimicrobial agent, is not horizontally transferable, and poses no risk of passing resistance onto other bacteria (Mathur and Singh, 2005). Acquired resistance is defined as a mutation in the genome that confers the resistance, or the acquisition of a gene that provides the same effect. Bifidobacteria have been reported to have both intrinsic and acquired resistances to many classes of antibiotics, only some of which are known to be transferable (Nawaz et al., 2011; Zhang et al., 2011). There are three identified mechanisms of HGT in bacteria: natural transformation, conjugation, and transduction. While some bifidobacteria species have these abilities and some do not, strain-level differences using genome sequence can be evaluated in order to determine if HGT is likely (Ouoba et al., 2008).

Antibiotic resistance has been previously documented to be transferable on plasmids, transposases, and phage (Aires et al., 2007; Colomer-Lluch et al., 2011; Wang et al., 2006); however, the most commonly reported resistance in bifidobacteria is tetracycline, having both intrinsic and acquired resistance reported (Chopra and Roberts, 2001; Gueimonde et al., 2010). The presence of a *tetW* gene that is immediately downstream of a transposon has been identified in all sequenced strains belonging to *B. animalis* subsp. *lactis* to date. Furthermore, the sequence of this gene is 100% conserved and contains similar GC content as the remaining genome, suggesting long-term association. The ability of *B. animalis* subsp. *lactis* strains to transfer tetracycline resistance was evaluated by Gueimonde et al. (2010) and no transfer of resistance to *B. animalis* subsp. *lactis* strains or any of the three other species evaluated in the in vivo experiment was observed. To date, there has not been any evidence that the *tetW* gene that is cotranscribed in tandem with this transposase has any ability to transfer resistance, and therefore poses no known risk. Additionally, through comparative genomics of public genomes of *B. animalis* subsp. *lactis*, the overall genomic plasticity of the species is extremely stable, where plasmid association has only been indicated in four species to date (*B. longum*, *B. globosum*, *B. asteroids*, *B. indicum*), and evidence of HGT between strains of the same species is rare (Wei et al., 2012).

Finally, very few reports have surfaced documenting negative effects of bifidobacteria, and have been exclusive to vulnerable populations, such as those with compromised immune function and infants (Sanders et al., 2010; Van Den Nieuwboer et al., 2014). Special safety assessment would be necessary for critically ill, compromised, or under-developed groups (Yamasaki et al., 2012). In addition to the safety considerations already listed, strict manufacturing controls and processing should be used to eliminate contamination of allergenic ingredients, such as dairy for prod-ucts indicated for sensitive populations (Moneret-Vautrin et al., 2006).

While no guarantees exist for the safety of all strains belonging to *Bifidobacterium*, determination of risk can be a time-consuming but important task. Specific assessment of commercial strains of bifidobacteria intended for human consumption needs to be completed via the many considerations outlined here and as required by regulation.

16.3 PRODUCTION

The general production process is similar between lactobacilli and bifidobacteria, Fig. 16.1. The media mix comprises water, carbohydrate source, nitrogen source, salts, and vitamins that are heat-treated and transferred to the main fermentor. The main fermentor is inoculated with a seed culture and the fermentation growth continues with base control until optimal harvest. Choice of growth temperature, base control pH, and base utilized for control impacts the final product performance and characteristics and depends on the organism cultured. The material is separated to obtain a concentrated cell mass and a cryoprotectant is added prior to freezing. The freeze-drying stage removes water and allows for longer storage. Each stage is optimized for the particular strain that is being produced and can impact the robustness of the product and its ability to recover upon rehydration. Strains that are very genetically similar can have different nutritional or processing considerations; optimization is therefore strainspecific. The practicality of *Bifidobacterium* fermentation for the purposes of biomass production is dependent on the right combination of predicted genetic and observed traits. Moreover, the variance of *Bifidobacterium* genetics within the genus is somewhat correlated to different media formulations. While it has been reported that the predominant manufactured species *B.animalis* subsp. *lactis* is genetically monomorphic, with greater than 99.9% identity to all reported genomes of the same subspecies, perhaps surprisingly the production conditions for this group of strains vary (Loquasto et al., 2013). This underscores the importance of a highly controlled manufacturing process



FIGURE 16.1 Schematic representation probiotic bacterium production.

for strain-specific production requirements needed for both the tracking of strains through the process, as well as reducing the potential of genetic drift. Small genetic changes can make a tremendous impact on phenotype even when there is gross homology of metabolic potential, perhaps because carbohydrate fermentation is so varied across *Bifidobacterium* species. While it is a known feature of *Bifidobacterium*, and perhaps even a taxonomic identifier, that all species can utilize the so-called bifidus pathway to produce lactic and acetic acid, the carbon source fermentation varies not only at the species level, but also at the strain level (Pokusaeva et al., 2011). Furthermore, at the fermentation level, differences can be observed in cell morphology, presence of viscosity, and fermentation growth times. All the stages within the production process should be considered to obtain optimal freeze-dried cell count and stability.

Various strains may have different sensitivities to:

- raw materials,
- seed handling and preparation protocols,
- ultrahigh temperature equipment,
- bioreactor configuration and agitation,
- atmosphere,
- separation,
- freezing survival, and
- freeze-drying survival.

Production needs therefore to be optimized for each particular strain and while strains within the same species may give hints on what to focus on, each strain has specific requirements and sensitivities.

16.4 STABILITY

The stability of bifidobacteria is dependent on a number of factors, which include species, strain, fermentation media, protective agents, and drying processes. It seems that some *Bifidobacterium* species are inherently more stable than others. That inherent stability can sometimes be further enhanced by processing parameters; however, certain species do not respond well to commercial-scale fermentation no matter how much optimization work is attempted.

In general, bifidobacteria stability is highly influenced by water activity, packaging as it relates to moisture (for low water activity products)/oxygen (for refrigerated products) barrier, and interaction with other ingredients when the cells are actively metabolizing. For probiotic dietary supplements and other applications using freeze-dried cultures, water activity will have the greatest impact on cell survival over the shelf life of the product. It is important to maintain low water activity to keep the cells in a live, but metabolically inactive state. For bifidobacteria in high water activity foods, such as fresh, fermented dairy, their metabolism is slowed by the refrigerated storage conditions, but consideration should be given to interaction with ingredients, such as fruit and enzymes and pH conditions, which could result in accelerated cell death.

When comparing the stability of culture concentrates (single-strain freeze-dried material) of four different *Bifidobacterium animalis* subsp. *lactis* strains the rate of cell loss is very similar (Fig. 16.2). These four strains have different



FIGURE 16.2 Stability of *Bifidobacterium animalis* ssp. *lactis* strains as culture concentrate. Performance measured every 3 months after storage at 25°C and <20% relative humidity.



FIGURE 16.3 Stability of *Bifidobacterium animalis* ssp. *lactis* strains in capsules. Performance measured every 3 months after storage at 25°C and <20% relative humidity.

optimized production conditions specific to the strain. Once those same four strain materials are put into a more stressful environment (a blend with microcrystalline cellulose in a capsule and packaging with a poorer barrier to moisture), the difference between them becomes more obvious (Fig. 16.3). *Bifidobacterium animalis* subsp. *lactis* Bi-07 is known to be more sensitive to application environments than the other three strains, having other distinct phenotypic characteristics despite its similarity in genetic sequence to the other strains (Stahl and Barrangou, 2012).

16.5 REGULATORY

Microencapsulation has been employed as a means to improve probiotic stability, both during storage and intestinal passage. Various techniques have been used to create the capsule: extrusion- and emulsion-based techniques, fluid bed, spray drying, hybridization, aerosol impinging, and electrospinning. The different techniques use different materials for the encapsulation matrix, such as alginate, whey and other proteins, pectin, carrageenan, carboxymethyl cellulose, chitosan (Martin et al., 2015; Sarao and Arora, 2017). While microencapsulation may improve the stability of probiotics, they often also involve an additional or otherwise suboptimal processing step. Thus, a tradeoff needs to be made on live counts at the start of the process and a potential improved stability throughout shelf life. Encapsulation techniques need to be optimized for the particular strain in question; so, no standard solution exists. Furthermore, microencapsulation is not a panacea for all stability issues that probiotics may have. Other techniques should be explored as they may provide more appropriate solutions for improving stability.

16.5 REGULATORY

Despite the long history of use in foods of live microbial organisms, the category of many of these same microorganisms as probiotics equates to a status of "regulatory ambiguity" in many parts of the world. The advent of the widely accepted definition of the term "probiotic" by the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO, 2001) covers the theme of probiotics. However, because of the varying degrees of recognition of health-promoting properties of these bacterial strains, and the confusion between live microbial cultures used for fermented foods and those that provide a health benefit (probiotics), there is cause for concern (Hill et al., 2014). As clearly stated previously, lactic acid bacteria, including *Bifidobacterium* and *Lactobacillus*, have been recognized as those genera with a number of species used as cultures for food preparation and preservation, and also play a key role in gastrointestinal health and function (Picard et al., 2005; Salminen et al., 1998a). However, the mere presence in food and the gut alone does not define the noteworthy aspect of these lactic acid–producing bacteria, nor does it categorize them as probiotics. Numerous species of bifidobacteria have been well characterized and clearly demonstrated to provide health benefits, hence, recognized as a "probiotic." A global consensus of how to define and categorize probiotics using common fundamental requirements is lacking, contributing to this state of regulatory ambiguity (Arora, 2015).

Currently, regulatory categories of probiotics can vary greatly across the globe, as they are independently determined by the country and corresponding regulatory body. However, most often the aspect of intended use determines the regulatory category that probiotics, including bifidobacteria, fall into and which regulations apply. The requirements for each category of application (intended use) will vary from jurisdiction to jurisdiction; however, a broader categorization of product types can most often be captured if the intended use is defined. The simplest and most straightforward intended use would be that of *an article used for food*, whereby the probiotic would be added to the food as an ingredient. This would require a demonstration of safety of the probiotic strain according to the specific country's requirements.

Bifidobacterium species have historically been considered safe and suitable for human consumption with several published studies addressing their safety (Aguirre, 1993; Gasser, 1994; Salminen et al., 1998b). *Bifidobacterium* species have been included among the many microorganisms intentionally added to food that should be regarded as safe based on European Food Safety Authority (EFSA)'s comprehensive assessment of safety. A list of qualifying microorganisms was compiled to represent those that meet the criteria of Qualified Presumption of Safety (QPS) and do not raise safety concerns (European Parliament, 2006). This QPS list has been updated frequently and, to date, there are five species of *Bifidobacterium* listed among the Gram-positive, nonsporulating bacteria. The most recent update indicates no safety concerns, and so the listing of species of *Bifidobacterium* remains unchanged in the 2013 QPS update (EFSA Panel on Biological Hazards, 2016).

Bifidobacterium species have a long history of safe use when consumed as part of dairy food and supplement products, with eight *Bifidobacterium* species listed in the IDF *Inventory of Microorganisms with a Documented History of Use in Food* (Mogensen et al., 2002). A more recent IDF inventory—*Safety Demonstration of Microbial Food Cultures in Fermented Food Products*—provides an update to the aforementioned inventory of microbial species, taking a global perspective versus the original focus of European fermented foods. The updated inventory lists a reorganization of the *Bifidobacterium* species included, with seven species of *Bifidobacterium* listed with two subspecies of *B. animalis* listed (Bourdichon et al., 2012).

There are a number of *Bifidobacterium* species that have been submitted to the FDA as GRAS (generally recognized as safe) notifications, where the *FDA has no questions*. This assessment of GRAS status with no questions recognizes a product as safe based on publicly available information within the submission details of intended use and the

statutory basis for the GRAS determination, and the general knowledge of safety of this product confirmed by qualified experts. A variety of strains within species of *Bifidobacterium* have been granted GRAS status by the FDA in a number of food types, including general foods and infant formula.

If the intended use is to *supplement the diet*, probiotics would normally be delivered in a format, such as a capsule, tablet, or powder, outside of the realm of conventional food, and are referred to with different terms globally—natural health products, dietary supplements, complimentary medicines, and food supplements, to list a few. Supplementing the diet most often indicates that there is a belief that this ingredient provides a benefit to health in some way, and these benefits must be supported by data. For probiotics, the intended effect of supplementation on the normal structure and/or function of the body (i.e., to maintain health) is most often recognized as acceptable in the food/dietary supplement world, where there is no indication of a "reduction of risk," or of intention to cure, treat, mitigate, diagnose, or prevent disease. For example, supplementation with distinct strains of *Bifidobacterium animalis* subsp. *lactis* have been demonstrated in numerous studies to support both immune (Ashraf and Shah, 2014) and digestive health (Miller et al., 2016) in healthy individuals, not considered diseased. These types of studies confirm the proposed health benefits of *Bifidobacterium* species for maintaining ideal gastrointestinal health and, in turn, promotion of maintenance of health.

In the United States, the FDA allows for statements of benefit to the structure or function of the body to accompany the product if the statements are truthful and not misleading. No preapproval process is necessary. However, adequate substantiation must be available to support the aforementioned benefit. Dietary supplement manufacturers follow current good manufacturing practice specifically designed for dietary supplements (Federal Food Drug and Cosmetic Act, 2007c), which differentiate these ingredients from those manufactured using current good manufacturing practice for human foods (Federal Food Drug and Cosmetic Act, 2007d).

There are a few countries with regulatory recognition of probiotic strains that may be used to supplement the diet. Health Canada not only recognizes specific genera and species as safe for use, they go so far as to list the general health-promoting (nonstrain specific) properties of listed probiotics for use in foods in regard to their contribution to healthy gut microbiota (Health Canada, 2016) and also as medicinal ingredients in natural health products (NNHPD, 2015). The Italian Ministry of Health has been a long-standing proponent of the benefit of true probiotics as able to "support intestinal flora balance," with the QPS as reference for safety at the species level (Italian Ministry of Health, 2013). The Australian government has also constructed a list of acceptable probiotic strains as approved for use as active ingredients in listed medicines (Therapeutic Goods Administration, 2016), which includes a number of species of *Bifidobacterium*. Currently in India, the Food Safety and Standards Authority of India (FSSAI, 2015) has put together a notification of draft regulation, where a list of probiotic strains providing a physiological benefit has been proposed. While this is draft regulation, this is a very promising step forward in the global recognition of the category and the benefit of probiotics. There are a number of countries with specified lists of probiotic strains generally recognized as health promoting, all containing common species of *Bifidobacterium* within.

There is a niche category where the intended use is to address the unique needs of individuals with specific medical conditions and distinct nutritional requirements that cannot be met by the diet alone. *Foods for special medical purpose* (FSMPs) (Australia New Zealand Food Standards Code, 2016; China Food and Drug Administration, 2016a; EFSA, 2015), also known as *medical foods* (Federal Food Drug and Cosmetic Act, 2007d) is a unique category where products can be marketed with claims specific to the dietary management of the disease or condition of interest. These products are formulated specifically for a patient with distinct nutrient needs, with the intent to manage a disease or condition. This category of intended use (FSMPs and medical foods) addresses specific medically determined nutrient requirements, where unambiguous claims can be made, but falls under the broader category of foods. So, it seems to be a nice categorization for those products that lie outside of the realm of drugs but recognized to help individuals with unique conditions manage their disease through ingestion of these product types.

Lastly, the intended use of those products with the intention to *cure, treat, mitigate, diagnose, or prevent disease,* are categorized as drugs or drug-like products. In the U.S., the category of probiotics with any of these drug-like intended uses, would result in the product category of a *biologic* (Federal Food Drug and Cosmetic Act, 2007a,b). This category is distinct from that of a probiotic used to supplement the diet, promoting benefits to the structure or function of the body; probiotics as biologic products must clearly demonstrate the beneficial effect of the product on the specific disease or condition targeted. Clinical trials specifically designed for and approved within the investigational new drug process are required to appropriately demonstrate the clinical benefit (Hoffman, 2008). The products are manufactured using current Good Manufacturing Practice for Drugs (US Food and Drug Administration, 2016).

Not unlike the US drug approval process, EFSA has rigorous requirements to indicate that a product provides a *reduction of risk* of a disease or condition under Article 14(1)(a) (European Parliament, 2006) of a disease or condition. A great body of scientific evidence is required, where numerous clinical trials must be completed, clearly demonstrating the beneficial effect of the product on the specific disease or condition targeted. Recently, EFSA (2016) has put together guidance on the aspect of health claims on the topics of gastrointestinal and immune health, which are most often the benefits seen with probiotic organisms. This will hopefully provide a positive opportunity for the recognition of health benefits associated with specific strains of probiotic microbes in countries within the European Union, in the categories of "reduction of risk" or even a "function health claim."

Health Canada currently takes an interesting approach in considering reduction of risk and disease-oriented health claims, where probiotics are considered within the category of Natural Health Products. This risk-based approach sets efficacy requirements for disease/condition categories of low, medium, or high risk, and considers health claims as general, by health effect, and by health condition. This system provides a very organized approach for product manufacturers to determine what is appropriate to present as evidence in support of the condition risk and the proposed benefit (Health Canada, 2012). In turn, there are numerous probiotic products commercially available as Canadian Non-Prescription and Natural Health Products that promote the benefits of probiotic micro-organisms for therapeutic use, with approved health claim language, including species of *Bifidobacterium* (Health Canada, 2012).

16.6 CONCLUSIONS

Production of good-quality probiotics, including bifidobacteria, is of prime importance already in the early stages of documenting efficacy and is paramount for successful commercialization. Production of probiotics is more than just growing biomass. In order to be able to guarantee quality, stability, and safety of the product strain-specific process development is needed. Many properties affect the growth and stability of bifidobacteria; these properties should therefore be considered early in the development of new probiotic strains. Likewise, the continuously evolving regulatory environment in various countries around the world needs to be taken into consideration early and followed continuously.

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Prebiotics, Probiotics, and Synbiotics: A Bifidobacterial View

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17.1 INTRODUCTION

The three terms used in the title of this chapter are used differently and have different effects on scientific databases. The term most widely used in PubMed, at the end of 2015, was *probiotics*, with a little more than 14,000 occurrences, *prebiotic* was the second most used term with less than 3,000 and "synbiotics" around 700. As a curiosity, *Bifidobacterium* alone scored about 6000 records. The combinations of the four terms (*Bifidobacterium* and either probiotics or prebiotics or synbiotics) produced an output of about 2500 papers for the first combination, rather less than 1000 for the second and around 150 for the third. This bibliographic overview strongly suggests that whereas our understanding concerning the genus *Bifidobacterium* and the beneficial probiotic bacteria is supported by a solid scientific literature, less is known about the interaction between bacteria, such as bifidobacteria and substrates they are able to ferment in a specific way.

As the terms *probiotics, prebiotics,* and *synbiotics* imply a beneficial action of bifidobacteria toward human beings and other animals, this chapter will begin with an overview of the definitions of these terms and the regulations controlling the health claims related to the beneficial action.

Beneficial activity can be exerted upon healthy or unhealthy individuals, therefore the chapter will review available data on the efficacy of bifidobacteria strains when administered to both groups of human beings, having in mind that the same strain, when used in ill subjects, has to comply with the safety regulations for pharmaceutical products.

17.2 DEFINITIONS USED IN SCIENTIFIC RESEARCH AND REGULATIONS

Let us analyze the definitions of probiotics and prebiotics.

Probiotics is a mixture of Latin (*pro* = for, in favor of) and Greek (*bios* = life) and is not from the Greek language alone as sometimes reported (*pro* in ancient Greek means "before"), and was originally intended to identify substances beneficial for the growth of microorganisms (Hamilton-Miller et al., 2003), a function nowadays attributed to "prebiotics." The definition of probiotics was then reshaped to identify "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance" (Fuller, 1989) and after a long series of small variations evolved into the Food and Agriculture Organization/World Health Organization (FAO/WHO) definition of "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001; Hill et al., 2014).

The appearance of the term "prebiotics" (this one entirely derived from Greek and meaning "before life") dates back to 1995 (Gibson and Roberfroid, 1995) to identify a "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health." In this case the meaning of the word is "before life," whatever the origin may be of the prefix "pre." A definition proposed by FAO in the final report of a FAO Technical meeting (held in Rome in 2007) states as follows: "A prebiotic is a non-viable food component that confers a health benefit on the host associated with modulation of the microbiota" (Pineiro et al., 2008). This definition was recently criticized (Bindels et al., 2015)

The Bifidobacteria and Related Organisms. http://dx.doi.org/10.1016/B978-0-12-805060-6.00017-X Copyright © 2018 Elsevier Inc. All rights reserved. with the claim that it "does not require the prebiotic to be fermented or metabolized by the gut microbes, and therefore does not distinguish among substances that modulate gut microbiota composition solely through an inhibitory action. As a consequence, antibiotics would be prebiotics according to this definition." However, in this challenging note authors seem to ignore that antibiotics are pharmaceuticals and then they cannot be identified as food components.

Not so well defined is the term *synbiotics*, also proposed by Gibson and Roberfroid (1995) in the same paper where they define the prebiotic concept: "By combining the rationale of pro- and prebiotics, the concept of synbiotics is proposed to characterize some colonic foods with interesting nutritional properties that make these compounds candidates for classification as health-enhancing functional food ingredients." However, this concept has not been fully elucidated, as was pointed out by Schrezenmeit and De Vrese (2001): "The term *synbiotic* is used when a product contains both probiotics and prebiotics. Because the word alludes to synergism, this term should be reserved for products in which the prebiotic compound selectively favors the probiotic compound. In this strict sense, a product containing oligofructose and probiotic bifidobacteria would fulfill the definition, whereas a product containing oligofructose and probiotic bifidobacteria would not. However, one might argue that synergism is attained in vivo by ingestion of lactobacilli on the one hand and promotion of indigenous bifidobacteria on the other hand." Therefore, there is an ambiguity in the definition that is still to be solved.

Definitions are then the starting point to also describe the mechanisms of action of probiotics and prebiotics, which are deeply different:

- Probiotics efficacy could be explained by a direct action on the host (i.e., the gut-associated lymphoid tissue) and it is not necessarily related to changes in the gut microbiota composition.
- Prebiotics efficacy, on the contrary, has to be mediated by beneficial alterations of the gut microbiota composition.

It is surprising that even the scientific world seems to have some confusion on this matter; for example, Kristensen et al. (2016) carried out a systematic analysis on randomized clinical trials evaluating the effects of probiotics on the overall structure of the fecal microbiota of healthy adults, including the number of species that are present, the evenness (distribution of species within the populations), and whether the study participants receiving probiotics had changes in gut bacteria that were different from placebo groups. Authors concluded that probiotics were unable to modify the overall composition of the gut microbiota of healthy subjects, but this is not what is expected from probiotics in this specific (healthy) state; for example, why would it be positive to alter an healthy ecosystem? In this case probiotics strains have simply to "colonize" or "persist" in the gut, a function that is supported by a full range of published clinical trials (for a recent review concerning bifidobacteria, see Grimm et al., 2014).

Definitions are always necessary but they are always a source of problems, as the recent debate in the European Union dealing with the relationship between the health claims legislation and the use of the terms probiotics and prebiotics, has pointed out. It is necessary to keep in mind that the 2002 FAO/WHO definition of probiotics is playing a role of reference document for a large number of Regulatory Agencies all over the world; many of them refer to this definition in establishing national rules to manage probiotic food and health claims (FAO/WHO, 2002). The regulatory view on *probiotics* implies that this term means food or food supplements containing a certain quantity of viable bacterial strains, identified at the strain level and with a potential to confer, if administered in the right amount, health benefits to the target host. This last part of the definition is causing trouble in the European Union, as the European Commission has classified the words "probiotics" and "prebiotics" as "health claims," according to Regulation 1924/2006. In the guidance released in 2007 for the application of this regulation, the Commission states: "A claim is a health claim if in the naming of the substance or category of substances, there is a description or indication of a functionality or an implied effect on health, Examples: 'contains antioxidants' (the function is an antioxidant effect); 'contains probiotics/prebiotics' (the reference to probiotic/prebiotic implies a health benefit). Equally, claims which refer to an indication of a functionality in the description of a nutrient or a substance (for instance as an adjective to the substance) should also be classified as a health claim. Examples: 'with prebiotic fibres' or 'contains prebiotic fibres'" (European Commission, 2007).

At the moment, as no claims have been positively evaluated by the European Food Safety Authority (EFSA) and then approved by the Commission, in many member states of the European Union the use of the word "probiotic" in the label, advertisements, and so forth of foods/food supplements containing beneficial bacteria has been banned.

In the later sections of this chapter literature supporting the beneficial use of bifidobacteria will be reviewed, leaving to readers the decision about the real efficacy of these bacteria.

We may note that in different regulatory environments, such as the Canadian one, four nonstrain specific claims have been granted to seven species of bifidobacteria (Health Canada, 2009), as reported in Table 17.1.

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TABLE 17.1	Nonstrain-Specific	Claims by Heal	th Canac	la Since 2	009
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Eligible bacterial species	Acceptable nonstrain-specific probiotic claims for food
Bifidobacterium adolescentis Bifidobacterium animalis subsp. animalis; Bifidobacterium animalis subsp. lactis (synonym: B. lactis) Bifidobacterium bifidum	 Probiotic that naturally forms part of the gut flora. Provides live microorganisms that naturally form part of the gut flora. Probiotic that contributes to healthy gut flora. Provides live microorganisms that contribute to healthy gut flora.
Bifidobacterium breve	
Bifidobacterium longum subsp. infantis comb. nov. Bifidobacterium longum subsp. longum subsp. nov.	

In Switzerland the Federal Office of Public Health (FOPH, https://www.bag.admin.ch/bag/en/home. html?_organization=317) approved two health claims dealing with reduction of intestinal transit time due to the action of bifidobacteria; the first one deals with a specific fermented milk with a strain of *B. animalis* and states "Activia contributes to digestive comfort by reducing transit time and bloating" while the second one has been granted to *B. lactis* HN019 (DuPont–Danisco) as a food/food supplement ingredient. It is important to note that, although these species names are still widely used in product labels, in 2004 *B. animalis* was reclassified as *B. animalis* subsp. *animalis* subsp. nov. and *B. lactis* as *B. animalis* subsp. *lactis* subsp. nov. (Masco et al., 2004).

As regards prebiotics, EFSA has assessed several applications for prebiotics-related claims and one of them was positively evaluated. In an opinion released in 2014 (EFSA, 2015) fructooligosaccharides (FOS) from inulin were acknowledged to be able to exert a reduction of postprandial glycaemic responses. The opinion applies to nondigestible carbohydrates (e.g., nonstarch polysaccharides, resistant oligosaccharides, and resistant starch), which should replace sugars in foods or beverages to obtain the claimed effect. Reduction of postprandial glycaemic index was believed by the EFSA panel to be a beneficial physiological effect as consumption of nondigestible carbohydrates results in reduced insulinaemic responses compared with the consumption of sugars.

In 2015 EFSA also assessed in a positive way the relationship between the consumption of "native chicory inulin" and maintenance of normal defecation by increasing stool frequency. Moreover, in this approval EFSA also states that, to obtain the claimed effect, 12 g of "native chicory inulin" should be consumed daily, which is definitely a relevant amount of inulin (EFSA, 2015).

Curiously, none of the applications dealing with the core activity of prebiotics, that is, the beneficial manipulation of the ratio among different bacterial groups inhabiting the gut, received a positive evaluation.

The puzzling difference between the European approach and what we may define as "the rest of the world" approach could be identified in the opposite position on the role played by the administration of beneficial bacteria to healthy individuals.

In the task of evaluating claims under Regulation (EC) 1924/2006, the EFSA (2009) states that: "Increasing the number of any groups of bacteria in not in itself considered as beneficial. The Panel considers that no evidence has been provided that...'enhance levels of beneficial microflora' are beneficial to human health. The Panel considers that 'support a balanced/beneficially affect intestinal microflora' in the context of decreasing potentially pathogenic intestinal microorganisms might be beneficial to human health."

According to this approach, the intestinal colonization by probiotics without a concurrent reduction on potentially pathogenic intestinal microorganisms does not represent a health effect as referred to in Article 2, paragraph 2, point 5 of Regulation (EC) 1924/2006.

It is also assumed that there is not any indication about a "healthy" or "well-balanced" composition of the intestinal microbiota. On this basis a number of applications has received a negative outcome.

On the contrary, Health Canada (as an example) has approved four claims (Table 17.1) stating that a healthy gut flora does exist and that a probiotic action could be simply the ingestion of some bifidobacteria. Health Canada also provides indications on what are called the "conditions of use": "A serving of stated size of a product should contain a minimum level of 1.0×10^9 CFU of one of the eligible microorganism(s) that is (are) the subject of the claim."

To conclude this overview, it should be noted that the New Zealand Ministry for Primary Industries (2015) has published a document on the "Global Regulatory Environment of Health Claims on Foods" that summarizes the presence of health claims for bifidobacteria in a range of countries.

In China the preapproved claim is: "Regulating gastrointestinal tract flora, facilitating digestion, facilitating defecation."

In Singapore (http://www.ava.gov.sg/) prebiotics health claims have been detailed more than in any other country. There are claims for prebiotics as a generic descriptor of a category of substances "Prebiotic promotes the growth of good Bifidus bacteria to help maintain a healthy digestive system" but also substance-specific claims for inulin "Inulin helps support growth of beneficial bacteria/good intestinal flora in the gut"; "Inulin helps increase intestinal bifidobacteria and helps maintain a good intestinal environment but also Inulin helps in calcium absorption" and for oligofructose "Oligofructose stimulates the bifidobacteria, resulting in a significant increase of the beneficial bifidobacteria in the intestinal tract. At the same time, the presence of less desirable bacteria is significantly reduced."

In regard to probiotics, the Singapore Authority states that a probiotic bacterium: (1) helps to maintain a healthy digestive system, (2) helps in digestion, (3) helps to maintain a desirable balance of beneficial bacteria in the digestive system, and (4) helps to suppress/fight against harmful bacteria in the digestive system, thereby helping to maintain a healthy digestive system.

In the following sections a wealth of information will be provided about the beneficial actions of bifidobacteria; the challenge in upcoming years will be to earn a European health claim.

In regard to the safety of probiotic bifidobacteria, if the strain belongs to a species with a long history of safe use in the human food chain, and if its intended use will be for "otherwise healthy" people (this is the FAO/WHO definition for the food/food supplement use of probiotics), the only additional requirement is the absence of transferable antibiotic-resistant genetic determinants. The long history of safe use of bifidobacteria provides a solid body of knowledge on their safety as food ingredients by healthy consumers, but nothing is granted about their use in pathological conditions; this is the reason why here we insist in discriminating between the need of different safety assessments for "food use" or for "pharma use" of bifidobacteria.

It is unfortunate that part of the scientific and clinical world has paid little if any attention to this relevant point; clinical trials have been conducted that don't pay enough attention to the safety assessment of the specific strains used in that specific pathological setting.

Since 2007 EFSA (http://www.efsa.europa.eu) has compiled a list of bacterial species with a "Qualifies Presumption of Safety" (QPS); this list contains the five following species of bifidobacteria: *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, and *B. longum*. We have used the names as reported in the QPS list, which is a quite restricted number when compared to the existing species of this genus. Therefore, the use in the European Union of species of bifidobacteria not included in the QPS list would require a specific safety dossier.

While adverse events for Lactobacillus probiotics have been reported, mainly for subjects in critical conditions, such as immunocompromised patients (Doron and Snydman, 2015), there are very few reports specifically addressing negative outcomes of bifidobacteria. B. dentium, which has never been used in probiotic preparations, is the species endowed with pathogenic potential as it was isolated from dental caries (but there is not a specific cause-effect link), and from lower respiratory tract specimens (Ventura et al., 2009). It has been isolated from a case of peritonsillar abscess but in a mixed population together with several other anaerobes (Civen et al., 1993). One culture of *Bifidobacterium* sp. was among other anaerobic bacteria reported in a recent study on anaerobes in pleural empyema (Boyanova et al., 2004). B. longum and B. breve are occasionally found in clinical materials. Sepsis caused by B. longum after acupuncture therapy, and neonatal meningitis caused by *B. breve*, has been reported (Nakazawa et al., 1996). It is also worthwhile to note that on the rare occasions when bifidobacteria are recovered from patients with sepsis, they are one of the components of a mixture of other anaerobic bacteria and patients are usually in critical conditions. Moreover, in most of the reports dealing with bifidobacteria isolated from the bloodstream of patients consuming probiotics containing bifidobacteria, when a clear identification was performed, it turned out that the probiotic strain was not involved. As an example, in a recent report (Avcin et al., 2015) a case of sepsis caused by bifidobacteria in a child with Philadelphia chromosome-positive acute B-cell lymphoblastic leukemia was described. In this patient, who was accustomed to consuming a probiotic product containing *B. longum*, abdominal symptoms coincided with two blood cultures that yielded bifidobacteria isolates; however, taxonomic identification of these cultures allowed to allot them to *B. breve* species, possibly translocated from the gut of the patient. However, even if the occurrence of adverse effects caused by the consumption of bifidobacteria is rare (a recent literature search in Pubmed database done by authors, updated at March 2015, retrieved only 21 cases of human bacteremia due to bifidobacteria, it seems worthwhile to point out again that in extreme conditions safety cannot be taken for granted; as an example, Bertelli et al. (2015) documented two cases of *B. longum* subsp. *infantis* bacteremia in preterm newborns receiving probiotics. Identification of the isolates has been achieved by comparative genomics, confirming their homology with the strain contained in the probiotic product.

On the contrary there is a long list of published reports (van den Nieuwboer et al., 2014, 2015a,b) in which the safety of bifidobacteria has been shown in children, immunocompromised subjects, and others. We may then conclude that *Bifidobacterium* spp. are safe, even if they are from time to time associated with infections; as a precautionary principle, however, a special attention is to be paid to their use in preterm neonates or critically ill patients. Turning to prebiotics safety it becomes clear that very little information is available quite surprisingly, in contrast to the abundant literature on the safety of probiotics. Most of the published data are confined to prebiotic use in infant nutrition (López-Velázquez et al., 2013; van den Nieuwboer et al., 2014) and they show a lack of adverse effects when prebiotics are used, even in very early infancy. However, animal model studies (Mennitti et al., 2014, 2015) suggested in a first paper that 10% supplementation with oligofructose during pregnancy and lactation of dams reduced body weight, body weight gain, and length in the offspring. In a second study the same research group concluded that supplementing the dam's diet with 10% of oligofructose during pregnancy and lactation (independent of hydrogenated vegetable fat addition) contributes to the increased proinflammatory status of 21-day-old offspring, possibly through the activation of the TLR4 (toll-like receptor 4) pathway. These results are controversial (Agostoni et al., 2004) but taken together with the note that "modern community-wide molecular approaches have revealed that even the established prebiotics are not as specific as previously assumed" (Bindels et al., 2015), it seems prudent to suggest that investigation on prebiotics safety could be an interesting area of future research. For example, it is well known that during the first few days of prebiotic administration, several subjects complain about the insurgence of meteorism and flatulence (Cummings et al., 2001b) but bifidobacteria, which are believed to be the specific target for prebiotics, are totally unable to produce gas. This lack of selectivity also threatens some of the definition of prebiotics as Bindels et al. (2015) also pointed out: "Overall, studies indicate that shifts induced by current prebiotic carbohydrates are not as selective as previously assumed (probably due to functional redundancy among gut inhabitants and cross-feeding), which means that the current prebiotic definition, if strictly adopted, would exclude virtually all carbohydrates."

It is necessary to point out that the FAO (2007) definition is the only one in which (on purpose, as one of the authors of this chapter was also among the experts convened by FAO for the technical meeting) the selectivity criterion for the term *prebiotic* has been removed. All of the preceding discussion is intended to reinforce the suggestion for further research efforts to establish the real action and therefore the safety profiles of prebiotics.

As far as we know, specific reports for the safety assessment of synbiotics are extremely limited (van den Nieuwboer et al., 2014, 2015a,b) and unable to add more evidence to what has been reported for probiotics and prebiotics.

17.3 CLINICAL EFFECTIVENESS OF PROBIOTICS, PREBIOTICS, AND SYNBIOTICS IN OTHERWISE HEALTHY PEOPLE

The use of probiotics and prebiotics as either dietary supplements or foods may represent a strategic approach to maintain general health and prevent disease in healthy people of all ages. Although it is true that bifidobacteria can be generally considered safe in this large category of people, it is also true that their efficacy needs to be proven in large and methodologically rigorous clinical trials. Indeed, the most difficult challenge for probiotic research is providing proof of efficacy in healthy subjects in which cellular or biochemical parameters fluctuate only within a certain narrow, physiological range. In this section, we will examine the available evidence supporting or undermining prophylactic administration of pro- and prebiotics, focusing on key health issues common with individuals in three different age groups, namely infants, adults, and the elderly. It is important to stress that the overwhelming majority of probiotic studies have investigated the effects of mixtures of various strains belonging to different species. For the purpose of this section, only trials using *Bifidobacterium* strains alone, or multispecies mixtures in which *Bifidobacterium* spp. represent a major constituent, will be taken into account.

17.3.1 Probiotics, Prebiotics, and Synbiotics in Healthy Infants

The infant's immune system is immature at birth and early exposure to microbial antigens via the gastrointestinal tract has a profound influence on development and proper functioning not only during infancy but also throughout life. It is well established that breast feeding provides maximum health benefits for infants, resulting in a lower incidence of infections and a lower risk of immune-related disorders compared with formula-fed babies. The proliferation of human milk banks for preterm babies proves how important human milk is considered to be. Feeding is a major factor driving the configuration of the gut microbiota of newborn babies and a plethora of studies show that the gut microbiota of breastfed infants is actually dominated by bifidobacteria, representing up to 91% of fecal bacteria (Harmsen et al., 2000). The fecal levels of bifidobacteria in breastfed babies is associated with the oligosaccharide content of human breast milk (Zivkovic et al., 2011), which represents a growth factor for *Bifidobacterium* species, such as *B. breve* and *B. longum* subsp. *infantis*, possessing the capability to ferment these carbohydrates
(Ruiz-Moyano et al., 2013; Sela et al., 2008). Furthermore, milk oligosaccharides provide a direct defense action by several mechanisms including antibacterial activity and preventing pathogen adhesion to epithelial cells (Pacheco et al., 2015). Human milk oligosaccharides and the establishment of a gut microbiota enriched in bifidobacteria thereby concur in supporting intestinal barrier function and modulating immunity (Chichlowski et al., 2012; Wickramasinghe et al., 2015). While human breast milk is unique in its composition and function, the supplementation of infant formulas with probiotics and/or prebiotics can positively affect the health status during infancy by modulating the composition of infant gut microbiota to resemble that of breastfed babies. A number of studies suggest that probiotic supplements containing bifidobacteria may increase intestinal bifidobacteria populations and reduce potentially harmful enterobacteria and clostridia both in preterm and full-term infants (Langhendries et al., 1995; Li et al., 2004; Mohan et al., 2006; Underwood et al., 2013; Wu et al., 2015).

Most data regarding the prebiotic effects in formula-fed babies have been published so far for a 9:1 mixture of galactooligosaccharides (GOS) and long-chain inulin or fructooligosaccharides (FOS). Several studies provided consistent evidence for the bifidogenic effect of the mixture of GOS and FOS (Costalos et al., 2008; Moro et al., 2002) and the observed increase in bifidobacteria correlated with increase in metabolic activity (Bakker-Zierikzee et al., 2005; Knol et al., 2005). In many studies the most important beneficial effect linked to the mixture of GOS and FOS at levels of 0.4–0.8 g/100 mL of formula was the improvement of stool characteristics, such as stool consistency and stool frequency (Bisceglia et al., 2009; Costalos et al., 2008; Moro et al., 2002; Scholtens et al., 2014). An increase in stool frequency was also found with several combinations of GOS/FOS and probiotics (Chouraqui et al., 2008; Puccio et al., 2007; Vlieger et al., 2009).

Inulin and oligofructose supplementation in infant formula has also been demonstrated to promote a microbiota composition and stool frequency and characteristics in newborns closer to those of breastfed infants (Closa-Monasterolo et al., 2013; Veereman-Wauters et al., 2011). Pectin-derived acidic oligosaccharides (pAOS) have gained attention as functional components for infant formula but the findings on the effects on the microbial ecology of infants are still inconsistent and need further investigations (Fanaro et al., 2005; Magne et al., 2008). More recently, also polydextrose (PDX) has been investigated in combination with GOS (1:1 ratio at a level of 4 g/L) in healthy-term infants and produced soft stools and a bifidogenic effect closer to breast milk (Ashley et al., 2012; Scalabrin et al., 2012). Similar effects have been reported for β -palmitate (Schmelzle et al., 2003; Yao et al., 2014; Yaron et al., 2013) but additional studies are needed to confirm the evidence of clinical benefits.

Bifidobacterium spp. has been mostly studied, alone or in combination with other species, in the prevention of infectious and diarrheal diseases in healthy pediatric populations. In a multicenter, double-blind, controlled study, 90 healthy infants received a milk formula supplemented with B. lactis Bb 12 (10⁸ CFU/day) but the incidence of diarrhea was not significantly different from controls (Chouraqui et al., 2004). In the similar study by Weizman and Alsheikh (2006) in full-term healthy infants aged less than 4 months, the authors compared milk-based formula supplemented with either B. lactis Bb12, L. reuteri ATCC 55730, or a probiotics-free formula. Infants in childcare who were fed a formula supplemented with *B. lactis* Bb12 had fewer and shorter episodes of diarrhea compared with controls, but the effect was more pronounced for L. reuteri. Prebiotics, alone or associated with probiotics, may also assist in reducing the risk of serious infections in infants, but no definitive conclusion on their efficacy can be drawn on the basis of the available literature. In a randomized controlled trial, infants receiving 0.4 g/100 mL GOS/FOS for 12 months had a lower incidence of acute diarrhea and respiratory tract infections compared with controls (Bruzzese et al., 2009). In a very recent trial on 365 healthy-term infants, formula containing GOS alone produced beneficial changes in fecal microbial composition and fecal characteristics but no changes in the incidence of infections (Sierra et al., 2015). Sazawal et al. (2010) investigated the effects of milk supplemented with *B. lactis* HN019 (1.9×10^7 CFU/ day) and galactooligosaccharides (2.4 g/day) in children aged 1–3 years. They observed an effect of synbiotic on diarrhea but only in children younger than 24 months and an overall preventive effect on pneumonia and severe acute lower respiratory infection. In out-of-home child-care, administration of a yogurt drink containing 5×10^9 CFU/ serving, B. lactis Bb12 and 1 g of inulin significantly reduced days of fever in healthy children 12–48 months of age (Ringel-Kulka et al., 2015).

Several probiotics have been shown to possess immunomodulatory properties but very few studies have been conducted to evaluate the putative effects on vaccine antibody responses in infants. Soh et al. (2010) explored specific IgG antibody responses to Hepatitis B (HepB) vaccination in infants receiving *B. longum* BL999 and *L. rhamnosus* LPR but the probiotic showed only a trend toward improving HepB surface antibody responses. Mullié et al. (2004) used a formula not containing viable bacteria but containing bifidogenic factors generated through a fermentation process involving *B. breve* C50 and *Streptococcus thermophilus*. In infants receiving poliovirus vaccination, the formula was able to increase fecal bifidobacteria and antipoliovirus IgA titers, with antibody titers correlating with bifidobacteria levels. In a very recent study by Wu et al. (2015), administration of *B. longum* BB536 improved Th1 immune response

by enhancing the secretion of cytokine IFN-γ but had no significant effects in terms of enhancing the antibody titer after hepatitis B (HepB), poliomyelitis (Polio), and diphtheria, tetanus, and pertussis (DTP) vaccinations. It has to be noted that in 2002 *B. longum*, *B. infantis*, and *B. suis* were unified into the single species *B. longum*, and recognized as distinct biovars, namely "longum," "infantis," and "suis," which were lately established as subspecies (Mattarelli et al., 2008).

A common problem in otherwise healthy infants is represented by colic, that is, a condition associated with excessive crying over a regular period during the day that commonly occurs between the ages of 2 weeks and 4 months. Most of the studies on the use of probiotics to prevent and/or manage infantile colic rely on either *L. rhamnosus* (now *L. casei* subsp. *rhamnosus*) LGG or *L. reuteri* or associations of these strains with *Bifidobacterium* spp. The study by Weizman and Alsheikh (2006) found no differences in episodes of crying per day or in daily crying scores between groups administered formula supplemented with *B. lactis* Bb12 or a probiotics-free formula at 1 month. Conversely, the study of Saavedra et al. (2004) indicated that formulas supplemented with *B. lactis* Bb12 and *S. thermophilus* reduced frequency of colic or irritability; anyway, the study relied on parental reporting of clinical signs and related symptoms, and this represents a major limitation. As far as colic is concerned, a study using GOS-supplemented formula-fed infants (0.4 g/100 mL) displayed lower amounts of *Clostridium* spp., higher count of *Bifidobacterium* spp., and a lower incidence of colic with respect to the control group (Giovannini et al., 2014).

A number of clinical probiotic trials evaluated bifidobacteria administration to prevent allergic diseases in infants although most of the studies in this area of research are focused on multistrain probiotics. Several *Bifidobacterium* strains were included in these studies, including *B. bifidum* BGN4, *B. lactis* AD011, *B. lactis* Bb12, *B. lactis* HN019, *B. breve* M-16V, and *B. longum* BB536. Controlled trials showed that probiotic supplementation decreased the incidence of atopic dermatitis while providing scarce clinical evidence for probiotics in the prevention of other allergic disease, such as asthma or food allergy (Dotterud et al., 2010; Enomoto et al., 2014; Kim et al., 2010; Niers et al., 2009; Soh et al., 2009; Wickens et al., 2008). Overall, these studies demonstrated that both prenatal and postnatal probiotic treatments are necessary for an effective prevention of eczema. In addition, in the study of Wickens et al. (2008), comparing the effects of two probiotic interventions, *L. rhamnosus* HN001 but not *B. animalis* subsp. *lactis* HN019 significantly reduced eczema incidence compared with placebo, suggesting the strain-specificity of clinical effect of probiotic bacteria. These results were confirmed in a synbiotic study by Kukkonen et al. (2007). In this large study, 1223 pregnant women carrying children at high risk for allergy were administered a probiotic preparation including *B. breve* Bb99 together with lactobacilli and propionibacteria; their newborn infants received the same probiotics plus 0.8 g/day galacto-oligosaccharides. Probiotic treatment displayed no overall preventive effect on allergic diseases but significantly reduced eczema and IgE-associated eczema.

A few studies have described the beneficial effects of prebiotics on infant eczema. In babies at high risk of atopy, supplementation of formula with a GOS/FOS mixture (0.8 g/100 mL) was associated with a significantly higher number of fecal bifidobacteria and a reduced incidence of atopic dermatitis (Moro et al., 2006). The protective effect extended beyond the feeding period of 6 months until 2 years of life (Arslanoglu et al., 2008). A GOS-/FOS-/pAOS-containing formula resulted effective in reducing atopic dermatitis up to the first year in infants with low risk of atopy, as well (Grüber et al., 2010).

As far as probiotics are concerned, it is important to point out that almost all *Bifidobacterium* strains added to infant formula have been so far isolated from infant fecal samples or fermented food. Breast milk seems to be a source of beneficial bacteriain itself and clinical studies on strains isolated from breast milk should be expected since they could represent a promising option for the improvement of infant formulas (Arboleya et al., 2011).

17.3.2 Probiotics, Prebiotics, and Synbiotics in Healthy Adults

The community structure of human gut microbiota develops during the first 2 years of life when it reaches a diverse and well-balanced composition that remains relatively stable in healthy individuals throughout adult-hood (Costello et al., 2009). The abundance of bifidobacteria within the human gut decreases with age and it has been found that in healthy adult humans their proportion is around 4%–5% of total fecal microorganisms (Eckburg et al., 2013; Mueller et al., 2006). A key concept when dealing with possible applications of probiotics to beneficially modulate the gut microbial ecology in adult populations is that a well-established microbial community is likely to offer resistance to alterations induced by foreign probiotic strains. Evidence supporting this hypothesis comes from studies conducted in healthy adults, which failed to demonstrate that administration of *Bifidobacterium* strains can induce significant changes in the composition of the intestinal microbiota, as highlighted in the recent metaanalysis (Kristensen et al., 2016) cited earlier. Conversely, a wide range of nondigestible carbohydrates have so far been tested in adults for potential bifidogenic activity. Among these, most studies have demonstrated the ability of fructans, such

as inulin and FOS to increase the bifidobacteria numbers in the gut of healthy adults (Bouhnik et al., 2007; Costabile et al., 2010; Gibson, 1999; Kolida and Gibson, 2007; Kruse et al., 1999; Ramirez-Farias et al., 2009; Tuohy et al., 2001; Whelan and Judd, 2005). Studies in humans have demonstrated that fecal bifidobacteria levels are stimulated by consumption of GOS as well (Bouhnik et al., 1997; Depeint et al., 2008). The bifidogenic nature of lactulose (10 g/day) (Bouhnik et al., 2004; Tuohy et al., 2002), xylooligosaccharides (XOS) (Finegold et al., 2014; Yang et al., 2015), and polydextrose (Jie et al., 2000) has also been exploited in human studies but clinical trials demonstrating health-promoting activities are still scarce.

The positive effects of Bifidobacterium probiotics have been well documented in healthy adult people with regard to the improvement of bowel habits or transit time. One of the largest clinical intervention study showed that *B. animalis* subsp. *lactis* Bb12 (1 or 10×10^9 CFU/day) provides relevant benefit on defecation frequency in healthy subjects with low defecation frequency and abdominal discomfort (Eskesen et al., 2015). Other strains, such as B. lactis HN019 (Waller et al., 2011), B. lactis DN-173010 (Guyonnet et al., 2009; Marteau et al., 2002; Yang et al., 2008), B. animalis subsp. lactis BS01 (Del Piano et al., 2010), and B. lactis Bi-07 (Favretto et al., 2013) displayed analogous beneficial effects on gut discomfort conditions, such as constipation in a series of randomized controlled studies but some others (B. animalis subsp. lactis Bf-6) did not (Merenstein et al., 2014). Notably, fermented milk containing B. bifidum YIT 10347 has the potential to provide health benefits by alleviating gastric symptoms in adult volunteers (Gomi et al., 2015). In one study *B. lactis* HN019 has also been tested in association with *L. acidophilus* NCFM and polydextrose and the synbiotic yogurt was able to significantly shorten colonic transit time (Magro et al., 2014). Polydextrose (4, 8, or 12 g/ day) alone has also been reported to positively influence stool consistency, reduce oro-fecal transit time and improve bowel function in healthy individuals (Hengst et al., 2009; Jie et al., 2000). A very few studies investigated the effect of consumption of synbiotic preparations on gastrointestinal function in healthy adults and the results are hardly comparable due to the differences in the combinations of probiotic strains and prebiotic compounds. Yogurt with probiotics (B. lactis Bb12, L. acidophilus La5, L. casei CRL431) and 4 g inulin did not significantly alter gastrointestinal transit time in healthy adults (Tulk et al., 2013). Another preparation containing three *Bifidobacterium* strains (*B. bifidum*, *B.* longum, and B. infantis) and three Lactobacillus strains (L. acidophilus, L. casei, L. lactis) and FOS proved effective in increasing stool frequency and improving stool consistency, thereby improving constipation (Jayasimhan et al., 2013). Likewise, dietary supplementation with a synbiotic composed of fructooligosaccharides with Lactobacillus and Bifidobacterium improved evacuation parameters and constipation intensity in constipated women (Waitzberg et al., 2013). A few human trials have addressed the capacities of *Bifidobacterium* strains to modulate immune responses in healthy adults. The study in a group of young adult women in South India showed that the probiotic yoghurt containing B. lactis Bb12 significantly increased fecal excretion of secretory immunoglobulin A (Kabeerdoss et al., 2011). The study of West et al. (2014) provided evidence of clinical benefit using *B. lactis* Bl-04 to reduce respiratory illness in healthy active subjects. The consumption of B. longum SP 07/3, B. bifidum MF 20/5, L. gasseri PA 16/8 (5 \times 10⁷ CFU/ tablet) during at least 3 months positively influences the total symptom score, the duration of common cold episodes, and days with fever (de Vrese et al., 2005). Two studies evaluated various doses of *B. lactis* Bb12 in combination with L. paracasei subsp. paracasei 431. Christensen et al. (2006) found no significant improvement in the clinical outcomes related to immune function in young healthy adults supplemented with increasing doses of the probiotic preparation. Rizzardini et al. (2012) found that probiotic supplementation significantly increases antigen-specific immune responses in healthy individuals receiving an influenza vaccination. A total of 244 healthy subjects traveling to high and medium risk destinations for travelers' diarrhea consumed 10 g/day of FOS or placebo for 2 weeks prior to travel and 2 weeks during travel, episodes of diarrhea, stool frequency and consistency showed no differences between the two groups (Cummings et al., 2001a). In a similar study in 159 healthy volunteers, 5.5 g/day of B-GOS resulted in significant differences between the prebiotic and the placebo group in the incidence and duration of travelers' diarrhea (Drakoularakou et al., 2010).

Two studies were conducted on a specific combination of long-chain inulin and oligofructose. In the first one, the authors demonstrated that β 2-1 fructans had a bifidogenic effect in healthy middle-aged human subjects but did not alter immune responses examined in the absence of an immune challenge (Lomax et al., 2012). The same research group carried out a second study with clinical outcome being response to seasonal influenza vaccination (i.e., exogenous immune challenge); here a higher antibody response to the H3N2-like strain of the vaccine and an enhanced IgG1-specific antibody response were found (Lomax et al., 2015). B-GOS administration (5.5 g/day) in overweight adults increased the numbers of bifidobacteria, improved blood and fecal inflammatory markers (i.e., C-reactive protein [CRP] and calprotectin [CLP], respectively) and increased secretion of fecal sIgA. Furthermore, there were significant effects on some metabolic syndrome markers, namely insulin, total cholesterol, and triglycerides (Vulevic et al., 2013). The effect on immune function was assessed also for XOS (8 g/day) in combination or not with *B. lactis* Bi-07 in healthy adults. XOS alone significantly reduced expression of CD16/56 on natural

killer T cells and IL-10 secretion, while XOS and Bi-07 supplementation reduced the expression of CD19 on B cells (Childs et al., 2014).

It has been suggested that ingestion of fructans may have an impact on lipid metabolism and cholesterol/ triglyceride levels, but the results of such investigations in humans are somewhat conflicting. Brighenti et al. (1999) found that daily consumption of a rice-based ready-to-eat cereal containing 18% inulin significantly reduced plasma total cholesterol and triglycerides. This reduction was inversely related to the numbers of fecal bifidobacteria. On the contrary, inulin did not appear to alter cholesterol and triglycerides in the small study of Kruse et al. (1999). In another small study in 8 healthy subjects, 10 g inulin/day reduced plasma triacylglycerol concentrations but not plasma cholesterol concentrations (Letexier et al., 2003). No effect on plasma triglycerides concentrations and hepatic lipogenesis was detected in normolipidemic individuals consuming 10 g/day inulin/FOS administered for 6 months (Forcheron and Beylot, 2007).

Finally, there is limited evidence for a prebiotic effect on stimulating absorption of several minerals and to improve mineralization of bone. Daily consumption of 8–9 g/day short- and long-chain inulin-type fructans significantly increased calcium absorption and enhanced bone mineralization in young adolescents (Abrams et al., 2005) but this result was not confirmed by Martin et al. (2010). In women with low iron status, 20 g/day inulin showed a positive trend in iron absorption, although this effect did not reach statistical significance (Petry et al., 2012).

17.3.3 Probiotics, Prebiotics, and Synbiotics in Healthy Elderly People

Old age is characterized by relevant but not yet well-established transformations in the composition and activity of the human gut microbiota. These changes reflect the impact of physiologic and behavioral modifications naturally related to ageing that result in a progressive decline in general health and well-being of seniors. Several findings suggest a decrease in the abundance and species diversity of bifidobacteria in the human gut (Woodmansey et al., 2004; Zwielehner et al., 2009) although some studies pointed out that this decrease becomes consistent only in the very last part of human life (Biagi et al., 2012; Rajilić-Stojanović et al., 2009). There is a general consensus among researchers on the age-related increase in facultative anaerobes including enterobacteria, enterococci, streptococci, staphylococci, many of which constitute opportunistic pathogens (Mueller et al., 2006; Woodmansey et al., 2004). The putative correlation between age-related changes in the structure and functions of the intestinal microbiota and greater vulnerability to disease supports a potential role for dietary intervention targeting the gut microbiota to promote health in aging populations. There is some evidence that administration of several *Bifidobacterium* strains and prebiotics augmented levels of bifidobacteria and reduced those of potentially harmful bacteria in the fecal microbiota of healthy elderly (Ahmed et al., 2007; Bartosch et al., 2005; Gopal et al., 2003; Lahtinen et al., 2009).

In older people, age-related decline in health and altered life style (e.g., changed dietary habits, reduced physical activity) often lead to alteration in bowel function resulting in symptoms, such as abdominal pain, diarrhea, bloating, and constipation. Evidence from human studies suggests that Bifidobacterium-based probiotics could provide help to improve bowel function and counteract constipation. In a clinical trial, administration of a fermented oat drink with B. longum strains 2C and 46, as well as B. lactis Bb12 showed efficacy in normalizing bowel movements of institutionalized elderly people (Pitkala et al., 2007). Long-term ingestion (16 weeks) of B. longum BB536 not only augmented bifidobacteria in the microbiota but also modulated bowel movements and normalized defecation frequency in elderly patients receiving enteral feeding (Kondo et al., 2013). As for healthy adults, some prebiotics have been observed to improve bowel function in the elderly, as well. Furthermore, the well-established capacity of prebiotics, such as inulin and FOS to stimulate the growth of bifidobacteria is of particular relevance to elderly people since a decrease in intestinal bifidobacteria content has been reported among age-related changes in the gut microbiota. In a randomized double-blind, parallel study carried out in constipated elderly individuals, administration of 20 g/day inulin stimulated the growth of bifidobacteria and produced a laxative effect (Kleessen et al., 1997). Four-week 8 g/day short-chain FOS (scFOS) ingestion increased fecal bifidobacteria counts and excretion of cholesterol in fecal samples of healthy elderly subjects (Bouhnik et al., 2007). FOS (10 g/day; 4 weeks) increased fecal bifidobacteria proportions and this was associated with an increase in frequency of spontaneous defecation and wet fecal mass, as well as decreases in plasma TBARS and cholesterol in constipated nursing-home elderly residents (Yen et al., 2011).

In the elderly, age-related deterioration of physiological functions coincides with a decrease in immunological competence, a process known as immunosenescence, which is associated with a chronic, low-grade inflammatory status. The decline in innate and adaptive immune function may contribute to increase in both the frequency and severity of infections, as well as poor vaccine responses. Although only a very few studies have been conducted on the potential of probiotics/prebiotics to modulate immune system in elderly individuals, preliminary results are encouraging. *B. lactis* HN019 has gained much attention with regard to immune-stimulant activity among healthy elderly

people and significant positive effects have been documented. Its administration at doses of 1.5×10^{11} CFU/day enhanced levels of interferon- α and increased polymorphonuclear cell phagocytic capacity (Arunachalam et al., 2000). Lower doses (5×10^9 CFU/day) enhanced the proportions of total, helper, and activated T cells in the peripheral circulation, as well as cellular immune function and leukocyte phagocytosis (Gill et al., 2001). A clinical trial by Ouwehand et al. (2008) showed that administration of *B. longum* strains 2C and 46 resulted in significant changes in the abundance of certain *Bifidobacterium* species that correlate with plasma levels of cytokine TNF- α and the regulatory cytokine IL-10. Long-term administration (12 weeks) of *B. longum* BB536 to elderly subjects receiving enteral tube feeding, provided efficacy in increasing the numbers of bifidobacteria in intestinal microbiota and maintained NK cell activities, as compared with the placebo group (Akatsu et al., 2013).

Most prebiotic studies in elderly subjects targeting immune function have focused on oligosaccharides. In a single treatment study by Guigoz et al. (2002) the bifidogenic effect of FOS was confirmed together with a decreased phagocytic activity of granulocytes and monocytes, and decreased expression of IL-6 mRNA in peripheral blood monocytes. B-GOS administration to healthy elderly people for 10 weeks resulted in positive effects on both the microflora composition and the immune response, including increased phagocytisis, NK activity and IL-10 (Vulevic et al., 2008). The results of a very recent study show that enteral administration of a formula containing two types of prebiotics (bifidogenic growth stimulator and galactooligosaccharide) improved intestinal microbiota by augmenting *Bifidobacterium* counts and maintained high antibody titers in elderly subjects vaccinated against influenza (Nagafuchi et al., 2015).

As far as synbiotics are concerned, a relevant study is that of Ouwehand et al. (2009) in which healthy elderly subjects consumed a combination of lactitol and *L. acidophilus* NCFM for 2 weeks. Fecal *Bifidobacterium* spp. levels significantly increased following the intervention, as well as prostaglandin E2 fecal levels, suggesting improvement of mucosal function. More recently, short-term synbiotic use comprising a probiotic *B. longum* strain and a prebiotic mixture of inulin and oligofructose proved effective in improving the composition of colonic bacterial communities and significantly reducing TNF- α in older people (Macfarlane et al., 2013).

17.4 THERAPEUTIC USE OF PROBIOTICS, PREBIOTICS, AND SYNBIOTICS IN GASTROINTESTINAL DISEASE

The perturbation of the intestinal microbiota composition, known as dysbiosis, has been described in several gastrointestinal diseases where it plays an important role in driving the inflammatory responses. For example, recent studies reported that the risk of irritable bowel syndrome (IBS) increases significantly after contracting different types of infectious gastroenteritis, thus demonstrating that alterations in the ecology of the gut microbiota either directly or indirectly lead to disease.

A reduced abundance of bifidobacteria is often involved in dysbiosis of the gut microbiota. As an example, in ulcerative colitis (UC) and inflammatory bowel disease (IBD), the unbalanced microbiota was characterized by a reduction of *Dialister invisus*, of an unidentified species of *Clostridium* cluster XIVa, *B. adolescentis*, and *Faecalibacterium prausnitzii* accompanied to a higher presence of *Ruminococcus gnavus* (Joossens et al., 2011). Bifidobacteria reduction was also described in the gut microbiota of celiac patients in which also *Clostridium histolyticum*, *C. lituseburense*, and *F. prausnitzii* were less abundant compared to healthy subjects (De Palma et al., 2010). The authors concluded that the decrease of bifidobacteria and the higher proportion of Gram-negative bacteria in celiac patients could contribute to increasing the risk of contracting the disease in predisposed individuals. A decrement of *B. longum* and *B. pseudocatenulatum* was also described in autistic children even if the total bifidobacteria content did not reach significant differences compared to a control group (Finegold et al., 2010).

The inflammatory state of the host may be due to either an unbalanced microbiota or to a nonspecific inflammation status that can lead to a shift in the gut microbiota composition that become the driving factor of a more specific inflammation. Several studies reported that the inflammation status in gastrointestinal diseases is a physiological condition linked to the unbalanced gut microbiota. Actually, the direct relationship between the inflammation and the perturbed intestinal composition has still to be assessed. The major question that has to be satisfied is, which is first actor in the development of those pathological conditions: inflammation or the altered intestinal microbiota? Probiotics have been often proposed as an overall therapeutic option to mitigate some symptoms of gastrointestinal diseases. Probiotics are highly regarded by both medical staff and patients for several reasons: they are considered safe, without contraindications, and furthermore, they represent, in chronic diseases, a useful alternative to the traditional medications that often become less effective due to prolonged administration.

Probiotic bacteria can play an important role to counteract the inflammatory status by means of direct or indirect mechanisms (Lescheid, 2014). For that reason, numerous studies deal with the use of probiotics as a way to prevent

or treat several diseases, including the allergic ones. In all cases the lion's share is made by the lactobacilli, while the number of clinical trials in which the probiotic properties of bifidobacteria are evaluated, for therapeutic uses, is quite limited. Moreover, the supportive evidence for treating diseases with probiotics, and in particular with bifidobacteria strains, can be arduous mainly for the heterogeneity of the studies in terms of the number of strains, daily doses, age of patients, scores of clinical parameters, and so forth.

Furthermore, mixtures of probiotic strains and species are often used (Chapman et al., 2011; Timmerman et al., 2004) to exploit the possible synergistic or cumulative effects of different strains to reach a higher treatment efficacy. Nevertheless, the clinical trials in which mixtures of strains are evaluated do not allow the speculation of direct correlation between efficacies of the treatment with specific probiotic microbial properties.

Considering that certain resilient bacteria exert a proinflammatory action, whereas others are able to mitigate the inflammatory responses, the modulation of gut microbiota in favor of the bacteria with beneficial effects could represent a way to prevent or treat some diseases. On the basis of this consideration prebiotics, such as inulin and oligofructose can represent a way to improve the microbial balance in the microbiota and studies on these specific products showed an increase in counts of bifidobacteria and lactobacilli in healthy children (Macfarlane et al., 2006).

The aim of the section is to provide an overview of randomized controlled trials in human studies of the major gastrointestinal diseases or disorders in which probiotic, prebiotic, and synbiotic agents are used as an adjuvant therapy in their treatments. For the reasons mentioned earlier, it will cite, with some exceptions, only studies in which the effectiveness of single bifidobacteria strain alone or in combination with prebiotics was evaluated.

17.5 IRRITABLE BOWEL SYNDROME

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the gastrointestinal tract, comprising UC and Crohn's disease (CD), whereas IBS is not associated to any inflammatory process and it can be defined as a functional gastrointestinal disorder. Then, these two terms, used often as synonyms, identified two different chronic conditions since IBS and IBD are characterized by different causes and treatments. As regards the IBS, whose causes remain unknown, it has been reported that symptoms can be triggered by specific foods due to a high rate of food intolerance of these patients (Hayes et al., 2014; Monsbakken et al., 2006). Foods more correlated to symptoms include wheat, grains, some vegetables, milk products, fatty foods, spicy foods, coffee, and alcohol (Böhn et al., 2013; Hayes et al., 2014; Monsbakken et al., 2006). To confirm the effects of diet, the dietary FODMAP approach is considered effective for the management of patients with these functional gut symptoms. The term FODMAP is the acronym for fermentable oligo-, di-, and monosaccharides and polyols (Gibson and Shepherd, 2010). FODMAPs are largely distributed in the diet since they included fructose, lactose, fructans, galactans, and polyols. These highly fermentable substances can contribute to the development of some gut symptoms. Reduction of these substances in the diet (low FODMAP diet) has been reported to reduce symptoms in IBS (Gibson and Shepherd, 2010). On the other hand, a reduction of FODMAP can have negative effects on colonic microbiota composition since growth of related healthy bacteria can be inhibited. Indeed, a low FODMAP diet, evaluated in a randomized study, reduced the abundance of Bifidobacterium spp. compared to subject group under habitual diet (Staudacher and Whelan, 2016). Moreover, in this study an improvement of IBS symptoms was reported.

The impact of low FODMAP on gut microbiota has been investigated recently by Halmos et al. (2015). A low FODMAP diet was compared to a high FODMAP diet in both IBS patients and healthy subjects. The 21 days on the diet were followed by a period of follow-up in which all subjects returned to their usual diet and for a second, shorter period under low or high FODMAP diet. Microbiota composition analysis revealed that *Bifidobacterium* spp. abundance was not significantly different in the two groups of subjects, while diversity in butyrate-producing bacteria was found in relation to the high FODMAP diet. Moreover, another effect of the low FODMAP diet was the reduction of total bacterial abundance. Surprisingly, no modification on fecal short chain fatty acid level was detected in the differences in FODMAP diet groups. The different results obtained from these two trials can be eventually explained by differences in FODMAP components in habitual (UK and Australian) diets. A reduction of bacteria with positive effects on health was reported and for this reason Halmos et al. (2015) suggested a more prudent approach to the restricted diet in the longer term and lower FODMAPs restriction to obtain symptom mitigation without strongly perturbing the gut microbiota. In the gut microbiota of IBS patients the decrease of bifidobacteria abundance is a common feature and their abundance is negatively correlated to symptoms. Then bifidobacteria supplementation during the FODMAP restricted diet may be an interesting approach for optimizing clinical management of this gastrointestinal disorder.

The effectivenesses of *L. salivarius* UCC4331 and *B. infantis* 35624 to mitigate, the cardinal symptoms of IBS have been compared (O'Mahony et al., 2005). The *B. infantis* strain was able to produce a more remarkable reduction of the

symptoms scores overall, in particular, those associated to pain and discomfort. On the contrary, groups of patients treated with this strain did not have strong beneficial effects on bowel transit and fecal consistency. Moreover, the administration of *B. infantis* 35624 ameliorated levels of IL10 and IL12 cytokines normally altered in IBS patients. *L. salivarius* UCC4331 was not effective from this point of view. These results were confirmed later by Whorwell et al. (2006); in their study *B. infantis* 35624 was administered in an encapsulated form to prevent any damage to the probiotic preparation during transit through the stomach. In this clinical trial three different doses were evaluated (10^6 , 10^8 , and 10^{10} CFU/day). The best results were achieved with the 10^8 CFU/day, whereas no significant differences were found using the lowest and highest daily doses.

B. animalis DN-173 010, administered using fermented milk in a group of IBS adult patients, improved the health-related quality of life (HRQoL) discomfort score in treated and control group receiving the heat-treated yogurt (Guyonnet et al., 2007). The percentage of responders for this parameter was significantly higher in the treated group of patients.

Guglielmetti et al. (2011) evaluated the effects of the administration of *B. bifidum* MIMBb75 (10⁹ CFU/day) on subjects' global assessment of IBS symptoms in a randomized double-blind clinical trial. Moreover, significant benefit was found for pain/discomfort and distension/bloating parameters in patients receiving the probiotic preparation compared to those of placebo group. The overall responders' rate was definitely higher in the treated group (57%) compared to placebo group (21%), and these beneficial effects at the end of the study were reported by 47% of patients belonging to the treated group and by only 11% of the placebo group.

A systematic review by Brenner et al. (2009) focused on the evaluation of probiotic use in IBS, and the authors concluded that *B. infantis* 35624 could be considered effective in reducing IBS symptoms. In two clinical trials synbiotic preparations were evaluated for their therapeutic effects on IBS patients. The first study considered a mixture of *L. acidophilus*, *L. helveticus*, and *Bifidobacterium* species in a vitamin- and phyto-extracts-enriched preparation. In this study, probiotic microorganisms were not characterized at the strain level and no information was reported concerning the species of bifidobacteria administered (Tsuchiya et al., 2004). The authors reported significant effects on IBS symptoms on 80% of treated patients. In the second trial a yogurt containing high-dose *B. animalis* subsp. *lactis* Bb12, a *Bifidobacterium* enhancer, and acacia dietary fiber was administered to IBS patients. This yogurt contained also the two yogurt starter cultures (Min et al., 2012). The authors described improvements in overall IBS symptoms of treated patients compared to the control group. Recently, a systematic and metaanalysis evaluating these trials reported that no significant effects of synbiotic products were found in reducing IBS symptoms despite both studies having an individual significance (Ford et al., 2014). Moreover, these authors on the basis of their analysis report that there is only scarce evidence supporting the use of prebiotics or synbiotics in IBS.

17.5.1 Inflammatory Bowel Disease

Crohn's and UC are the most common forms of IBD, that is, chronic inflammatory disease of the gastrointestinal tract. These two diseases may share some symptoms but are distinguishable from each other for some important characteristics and for this reason we will consider them separately.

17.5.2 Ulcerative Colitis

Several trials investigating probiotic effects on UC symptoms used VSL#3 (Chapman et al., 2011; Sood et al., 2009; Tursi et al., 2010). VSL#3 is a mixture of *B. breve*, *B. infantis*, *B. longum*, *L. acidophilus*, *L. plantarum*, *L. paracasei*, *L. bulgaricus*, and *S. thermophilus*.

No clinical trials concerning effectiveness of bifidobacteria in reducing UC symptoms are available. A synbiotic product containing *B. longum* and a prebiotic was evaluated by Furrie et al. (2005). In this small study involving 18 patients, the synbiotic preparation was administered for 4 weeks and the effects of the intake were evaluated considering the inflammatory markers and the presence of mucosal microbiota in the biopsies. Reduction of the inflammatory markers was statistically significant in the treated group of patients compared with the placebo group. The treated group patients showed a significant reduction of Bacteroidaceae fecal counts and fecal pH.

Concerning clinical studies in which prebiotics were evaluated alone for their effectiveness in reducing UC symptoms, only results of a small trial have been published (Hafer et al., 2007). In this study lactulose was administered to 17 CD and 14 UC patients. The endoscopic analysis did not reveal any improvement due to lactulose intake and moreover clinical parameters were not statistically different from the control group that did not receive lactulose. The small number of patients and the lack of placebo group are important limitations of this clinical trial. However, the authors reported an improved quality of life in terms of pain, mood, and limitations in daily life.

17.5.3 Crohn's Disease

Several studies aimed to determine the impact of probiotic treatments targeting the gut microbiota in CD patients. The majority of these trials concern the use of lactobacilli, especially *L. rhamnosus* GG, and a few studies evaluated effects of *Saccharomyces boulardii* and more recently *Escherichia coli* Nissle 1917 strain.

No data are available concerning the beneficial role of bifidobacteria in the induction and/or maintenance of remission in CD, but a randomized, double-blind, placebo-controlled trial was conducted using a *B. longum* strain combined with inulin and an oligofructose (Steed et al., 2010). The strain was described as isolated from healthy rectal epithelium but no other information was provided.

The Crohn's disease activity index and histological score were significantly improved by the synbiotic treatment. Moreover, the proinflammatory TNF- α level decreased in the synbiotic group.

FOS were administered to 54 subjects for 4 weeks and the Crohn's disease activity index score was used to assess efficacy. No clinical benefit was obtained in the treated group compared with control (Benjamin et al., 2011). Another trial using lactulose as a prebiotic was performed in CD patients, as already mentioned in the earlier UC discussion (Hafer et al., 2007).

17.6 NECROTIC ENTEROCOLITIS (NEC)

Necrotic enterocolitis (NEC) is an acute inflammatory necrosis of the intestinal tract described in preterm infants in neonatal intensive care units. The administration of probiotics is particularly critical in these children because of their incomplete intestinal epithelia and not only since they can be considered immunodeficient. For these reasons particular attention, in clinical trials, is reserved for adverse events due to the administration of these microbial products.

In a recent systematic review, Aceti et al. (2015) focused on the evaluation of probiotic effects for NEC prevention in preterm infants, the authors underlined a significant effect of bifidobacteria and of probiotic mixtures. The paper published by Kitajima et al. (1997) represents a pioneer study concerning *B. breve* YIT4010 in preventing NEC in very low birthweight (VLBW) infants. This strain, in a randomized controlled trial, was able to colonize the infant gastrointestinal tract and to reduce the growth of *Enterococcus* spp. but not that of lactobacilli. In the treated infants the weight gain was significantly higher than that of the control group. No adverse events were reported.

The effects of *B. lactis* Bb12 on the gut microbiota of 69 preterm infants was evaluated by culture dependent and independent techniques (Mohan et al., 2006). The administration of this strain led to a reduced abundance of *Clostridium* spp. and *Enterobacteriaceae*. No effects were detected on the number of antibiotic resistant microorganisms. The same *B. lactis* strain was administered to 93 preterm infants to evaluate its ability in reducing nosocomial infections (Mihatsch et al., 2010). No significant differences were detected between placebo and treated groups for all the other outcomes. Recently, *B. lactis* alone or in combination with inulin was evaluated in a group of 400 VLBW infants (Dilli et al., 2015). The main effects of probiotic and synbiotic product administration can be summarized in a reduction of the rate of clinical nosocomial sepsis, rate of NEC, and a reduction in the time required to consume 150 mL/kg day of food. No effects on prevention of NEC were obtained using inulin alone.

An uncharacterized strain of *B. lactis* was described to be able to reduce the intestinal permeability in preterm infants in which the epithelial barrier of the gut is still immature (Stratiki et al., 2007). Finally, *B. bifidum* OLB6378 in very low birthweight infants was able to reduce the time to full feed.

17.7 CELIAC DISEASE

Studies concerning probiotics for celiac disease are scarce. However, several studies using in vitro and in ex vivo models have shown that bifidobacteria can have useful properties that could be exploited in view of their application to people with celiac disease. Bifidobacteria have been described as able to change the gliadin-derived pattern by in vitro intestinal digestion, attenuating the proinflammatory effects on intestinal mucosa (Laparra and Sanz, 2010).

B. infantis was able to secret bioactive compound(s) that normalize the altered permeability of the intestinal epithelium (Ewaschuk et al., 2008). Moreover, in an in vitro study, it has been reported that a strain of *B. lactis* was able to hydrolyze gliadin into small peptides reducing its toxigenic effects (Lindfors et al., 2008). On this basis, Smecuol et al. (2013) performed a clinical trial using *B. infantis* Natren Life Start (NLS) super-strain to evaluate its effects on gut permeability, symptoms, and inflammatory cytokines in adult celiac patients. Permeability did not improve but

slight effects on digestion and constipation were reported after 3 weeks. No significant differences in inflammatory cytokines were found between treated and control group.

17.7.1 Diarrheal Diseases

Several diarrheal diseases can be considered, such as the traveler's diarrhea, rotaviruses diarrhea, acute diarrhea, *Clostridum difficile*–associated diarrhea, and antibiotic-associated diarrhea. Probiotics have been described to play a role in several diarrheal diseases' prevention and control but the majority of efficacy evaluations are focused on *Lactobacillus* spp., *S. boulardii*, or mixtures of strains belonging to different genera. A systematic review (Pillai and Nelson, 2008) focused on treatment of *C. difficile* colitis underlined that evidence to support the use of probiotic simultaneously to the antibiotic administration is limited and then their use as unique treatment is not recommended. Few studies have been published in which bifidobacteria were used to treat diarrheal diseases. However, a randomized controlled trial was performed in children (from 1 to 23 months) with acute diarrhea using *B. lactis* Bb12 strain supplemented in milk formula (El-Soud et al., 2015). The authors reported a reduction in frequency and duration of *diarrhea* compared to the control group, which received the usual clinical treatment. The administration of *B. lactis* B94 plus inulin was able to reduce the diarrhea duration in children (aged 2–60 months). The acute diarrhea in these children has different causes and the synbiotic treatment showed higher efficacy in children with rotavirus diarrhea (**İşlek et al.**, 2014).

17.7.1.1 Helicobacter pylori Infection

The standard therapy allows the eradication of 60%–90% of helicobacter pylori infection in patients using a firstline therapy and the success is 70% with a second-line therapy (Zhang et al., 2015). Probiotics intake can help the eradication of *H. pylori* because of their mechanisms of action that induce both antiinflammatory and antioxidant effects (Johnson-Henry et al., 2004; Myllyluoma et al., 2007).

A recent metaanalysis underlined that the use of probiotics as adjuvant of standard therapy in patients with *H. pylori* improves its eradication rate by about 13% and reduces the rate of adverse events by approximately 41%. These results are host age-independent, but also daily dose, genera of probiotics, and time and regimen of standard therapy influence the rate of success in *H. pylori* eradication (Zhang et al., 2015). Concerning bifidobacteria application in *H. pylori* eradication, an observational clinical study reported significant amelioration of the cure rates by using *B. infantis* 2036. In this clinical trial the efficacy of probiotic administration represented an adjuvant of three different regimens of therapy. The administration of *B. infantis* 2 weeks before starting therapy increased the eradication rate to more than 90%. *B. lactis* DN-173-010, added to yogurt, was administered to 78 patients randomized into 2 different therapy regimen groups (Yaşar et al., 2010). The administration of probiotic in both groups of patients did not increase the eradication rate.

The studies mentioned so far in this section are part of a large amount of data deriving from clinical trials that has been also analyzed by statistical treatment provided by the Cochrane Reviews and metaanalyses (Table 17.2). Almost all the studies included in the Cochrane reviews available on the subject of probiotics evaluated the efficacy of probiotic mixtures in which *Bifidobacterium* strains were in association with *Lactobacillus* spp. and other species. Therefore these reviews provide no clear indications in relation to specific intervention effects of bifidobacteria for the prevention of antibiotic- and *C. difficile*-associated diarrhea, allergic disease, and food hypersensitivity and upper respiratory tract infections. Conversely, in the Cochrane review by Alfaleh and Anabrees (2014) there were several studies among those analyzed that used *Bifidobacterium* species alone. From these data it could be concluded that there is a strong indication for using bifidobacteria strains as probiotics in the prevention of severe NEC in preterm infants. Notably, the metaanalysis by Dimidi et al. (2014) revealed that the use of *B. lactis* probiotics efficaciously reduces whole gut transit and increases stool frequency in adults with functional constipation. Further, well-designed clinical trials are needed to draw generalized conclusions on the beneficial effects of *Bifidobacterium* probiotics and recommend their use in the prevention of human health disorders.

17.7.2 Metagenomic Approaches to Assess Mechanisms of Action

Deep sequencing approach of 16S rRNA gene provides the means to investigate the impact of probiotics, prebiotics, and synbiotics on the entire microbial community. A recent study in which *L. rhamnosus* GG was administrated to 12 elderly healthy people reported that the overall community composition was stable during the treatment period, confirming results of the previously works testing the same strain (Lahti et al., 2013). However, patients could be divided in three distinct groups on the basis of transcriptional analysis. Authors conclude that *L. rhamnosus* GG could

Study details	Interventions	Study population	Clinical outcomes	References
Metaanalysis	Probiotics	Infants	Atopic diseases	Zuccotti et al. (2015)
Metaanalysis	Probiotics	Infants	Infantile colic	Anabrees et al. (2013)
Metaanalysis	Probiotics	Infants	Excessive crying	Sung et al. (2013)
Metaanalysis	Probiotics	Infants	Infectious diarrhea	Szajewska and Mrukowicz (2001)
Cochrane review	Probiotics	Infants	Allergic disease and food hypersensitivity	Osborn and Sinn (2013)
Metaanalysis	Prebiotics	Infants	Physical growth and stool characteristics	Rao (2009)
Metaanalysis	Probiotics, prebiotics, and synbiotics	Infants	Physical growth, diarrhea, colic	Mugambi et al. (2012)
Cochrane review	Probiotics	Children, adult, and older people	Acute upper respiratory infections	Hao et al. (2011)
Metaanalysis	Probiotics	Children and adults	Acute respiratory infectious	King et al. (2014)
Metaanalysis	Synbiotics	Infants and children	Atopic dermatitis	Chang et al. (2016)
Metaanalysis	Probiotics	Adults	Blood lipid concentrations	Cho and Kim (2015)
Metaanalysis	Probiotics	Adults	Traveler's diarrhea	McFarland (2007)
Metaanalysis	Probiotics	Adults	Constipation	Dimidi et al. (2014)
Cochrane review	Probiotics	Children	Antibiotic-associated diarrhea	Goldenberg et al. (2013)
Cochrane review	Probiotics	Preterm infants	Necrotizing enterocolitis	Alfaleh and Anabrees (2014)
Cochrane review	Probiotics	Children and adults	Clostridium difficile-associated diarrhea	Goldenberg et al. (2013)
Cochrane review	Prebiotics	Infants	Allergy	Osborn and Sinn (2013)
Metaanalysis	Probiotics	Preterm infants	Necrotizing enterocolitis	Aceti et al. (2015)
Cochrane review	Probiotics	Children and adults	Acute infectious diarrhea	Allen et al. (2011)
Cochrane review	Probiotics	Children and adults	Clostridium difficile-associated colitis	Pillai and Nelson (2008)
Metaanalysis	Probiotics	Children and adults	Helicobacter pylori infection	Zhang et al. (2015)
Metaanalysis	Probiotics	Adults	Irritable bowel syndrome	Brenner et al. (2009)
Metaanalysis	Probiotics	Children and adults	Irritable bowel disease	Shen et al. (2014)

TABLE 17.2	Cochrane Reviews and Metaanalyses of Clinical Trials on the Efficacy of Probiotics, Prebiotics, and Synbiotics in the
	Prevention and Treatment of Selected Clinical Diseases

modulate the transcriptional response of resident microbiota leading to a functional effect (Eloe-Fadrosh et al., 2015). Multiplex pyrosequencing approach was used to investigate effects of galactooligosaccharides on healthy subject fecal microbiota. Results underlined a bifidogenic effect of this prebiotic treatment to accomplish a reduction of *Bacteroides* (Davis et al., 2011). More recently, impact of polydextrose and soluble corn fiber was evaluated by whole-genome shotgun 454 pyrosequencing in healthy adults (Holscher et al., 2015). The fiber treatments lead to shifts of several amino acid metabolism genes, peptidases, and transporters. Moreover, a reduction of the presence of butyrate-producing bacteria was revealed after fiber intake.

17.8 CONCLUSIONS

The positive role played by bifidobacteria in supporting well-being and health of humans from the very beginning of and until the last period of their life has been under scientific scrutiny since the beginning of the previous century. The preceding review is clear evidence of this still lasting and growing interest.

While the scientific evidence is nowadays robust and covering a full range of potential applications; there are, however, some technological constraints (oxygen sensitivity, low resistance to freeze drying, etc.) that hamper the use of bifidobacteria strains in probiotic preparations.

Most of the products available on the market are mixtures of bifidobacteria and lactobacilli or other beneficial bacteria, while the use of bifidobacteria only is mostly limited to infant food.

A renewed effort in improving production technology seems therefore relevant to fully exploit the beneficial potential of *Bifidobacterium*, one of the bacterial genera most friendly to humans.

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Evidence of the In Vitro and In Vivo Immunological Relevance of Bifidobacteria

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18.1 INTRODUCTION

T-cell mediated immune responses are classified as effector [mainly but not exclusively T-helper (Th1, Th2, and Th17)] and regulatory (Treg), and both responses are perfectly balanced in a healthy situation and unbalanced in the framework of several autoimmune/inflammatory diseases as well as infections (Maloy and Powrie, 2011). Intestinal microbiota help to maintain this immunological balance by training our immune system from birth; specifically bifidobacteria play a key role in this early immunological stimulation since it is one of the first colonizers of our gut. Interestingly, cell and animal models are the main pillars for the study of the mechanistic effects of bifidobacterial immunological responses. These models are the main focus of this chapter and are discussed in detail in the following sections.

18.2 IN VITRO CELL MODELS

18.2.1 Effects on Epithelial Cells

Different experimental designs have been used to study the interactions between bifidobacteria and host cells. To study the effect of bifidobacteria on the epithelial layer of our gut, enterocyte cell lines have been extensively used, mainly HT29 and Caco2, two cell lines from human adenocarcinoma tissue in which the cellular metabolism can be altered compared to physiological conditions and, therefore the results should be carefully considered before reaching solid conclusions about the immunological response. Current data suggest certain species-specificities of the immunomodulatory properties of bifidobacteria in these models (Khokhlova et al., 2012). In this regard, *Bifidobacterium breve* IPLA20004 was able to modify the expression of 12 genes involved in inflammation in HT29 cells, among which the genes of chemokine CXCL6, the chemokine receptor CCR7, and, specially, the complement component C3 were upregulated. Furthermore, the proteome of HT29 cells showed increased levels of cytokeratin-8 in the presence of *B. breve*. This suggests that *B. breve* IPLA20004 could favor the recruitment of innate immune cells to the mucosa (Sánchez et al., 2015), although it is known that different *B. breve* strains modulate gene expression in HT29 cells in a strain-specific way (Boesten et al., 2011). A different species, *B. bifidum*, has peculiar immuno-modulatory properties, probably due to the lack of an exopolysaccharide (EPS) layer on the cell surface. A transcriptional study has shown that significant changes were detected in the transcription of genes involved in innate immunosorbent

assays, suggests that this strain modulates the innate immune response of the host (Turroni et al., 2014). A different strain, *B. bifidum* LMG13195, induces a transcriptional response in HT29 characterized by an increased expression of immune mediators and, interestingly, chemotactic molecules able to recruit lymphocytes to the intestinal mucosa (López et al., 2012c). Furthermore, using a Caco-2 cell line, *B. bifidum* strains have been shown to enhance intestinal epithelial barrier function via metabolites, such as acetate (Hsieh et al., 2015).

Another *Bifidobacterium* that has received considerable attention regarding its immunomodulatory capabilities is *B. animalis* subsp. *lactis*. This subspecies is commonly used in fermented dairy products and functional foods. *B. animalis* subsp. *lactis* BI07 was able to promote the HT29 cell capacity to activate plasminogen; tumor necrosis factor alpha (TNF- α) modulated the plasminogen-mediated bacterium–enterocyte interaction, reducing the bacterial adhesion to HT29 enterocytes and enhancing migration to the luminal compartment (Centanni et al., 2012). Also, antiinflammatory effects of *B. animalis* subsp. *lactis* strain BB12, one of the most common strains included in functional dairy products were shown in stimulated Caco-2 cells, probably due to a 50 kDa proteinaceous compound that is secreted by the bacteria to the extracellular milieu (Wang et al., 2011).

It is worth mentioning that bifidobacterial growth conditions can strongly influence the interaction process with enterocytes. For instance, when *B. infantis* and *B. bifidum* are grown in human milk oligosaccharides, they cause less occluding relocalization and higher expression of antiinflammatory cytokine, interleukin-10 compared with lactose-grown bacteria in Caco-2 cells (Chichlowski et al., 2012). Thus, strain phenotype must be carefully considered in order to extract solid conclusions about the immunological effects exerted by bifidobacteria on enterocytes.

18.2.2 Effects on Peripheral Blood Mononuclear Cells (PBMCs) and Other Immune Cell Models

Peripheral blood mononuclear cells (PBMCs) have been extensively used to explore the capability of bifidobacteria to mediate immune responses. These mononuclear cells are mainly composed of lymphocytes and monocytes, although macrophages and dendritic cells (DCs) are also present. The PBMC proliferation and cytokine expression after exposure to several bifidobacterial strains belonging to different species (i.e., B. longum, B. breve, B. bifidum, and B. animalis subsp. *lactis*) have been studied (López et al., 2010). Most of the tested *B. animalis* subsp. *lactis* and *B. longum* strains induced the secretion of IFN- γ and TNF- α , in agreement with the Th1 profile, but *B. bifidum* strains induced poor secretion of these cytokines and significant amounts of IL-17, the main product of Th17 cells, suggesting potential probiotic applications for these strains. A further characterization of *B. bifidum* immunomodulatory capabilities showed that DCs stimulated with strains suggestive of Th17 responses induced DCs maturation, showed significant capability for Th17 generation and were able to generate functional regulatory T (Treg) cells, supporting a Treg/Th17 plasticity (López et al., 2011). This Treg-inducing capability seems to be located within the membrane fraction of *B. bifidum* (López et al., 2012a). Interestingly, enrichment of intestinal microbiota of people affected by Lupus Erythematosus Systemicus with the Treginducing strain B. bifidum LMG13195 showed that this modified microbiota prevented lymphocyte overactivation, thus supporting a possible therapeutic benefit of probiotics containing Treg-inducer strains in order to restore the Treg/ Th17/Th1 imbalance present in autoimmune diseases (López et al., 2016). Altogether, these compelling data point to a key role of *B. bifidum* in mediating immune responses with potential applications in inflammatory processes.

Regarding other bifidobacterial species, it has been shown that surface-associated macromolecules are one of the main mediators of immune responses. Several works point to the role of EPS as a key macromolecule mediating interactions with immune cells in *B. breve* and *B. animalis* subsp. *lactis* (Fanning et al., 2012; Hidalgo-Cantabrana et al., 2015); mechanistic studies about the role of EPS in immunomodulation are detailed in the following sections). In *B. longum*, subsp. *longum* BBMN68 strain specifically induces DCs and semimature DCs, and this has been linked to a decrease in allergic reactions (Yang et al., 2015). On the other hand, *B. longum* strains seem to be high inducers of IL-10 production and low inducers of TNF-α stimulus in PBMCs, suggesting a potential antiinflammatory role (Pozo-Rubio et al., 2011).

All these data obtained from in vitro cell models are the first indication of the capabilities of bifidobacteria to interact and trigger immune responses and it seems clear that the particular surface structure of *B. bifidum*, a species that normally lacks an EPS and so has surface-exposed immunomodulatory proteins, confers to this species a potential role in the treatment and/or prevention of immune related diseases, although further preclinical and clinical studies are needed in order to corroborate these assumptions.

18.3 IN VIVO ANIMAL MODELS

Evidence suggests that bifidobacteria are effective in the treatment or prevention of immune-associated disorders. Germ-free and conventional animals, including healthy and disease-simulated models, have been used to study the immune modulation properties of bifidobacteria; some of these in vivo studies will be described next (Fig. 18.1).



FIGURE 18.1 Main bifidobacterial immunomodulatory effects observed in animal models.

18.3.1 Effects on Healthy and Gnotobiotic Animal Models

Germ-free, or gnotobiotic, animals have been employed to check that the oral administration of different strains and species of bifidobacteria, particularly, *B. adolescentis, B. longum, B. breve*, and *B. dentium*, differentially affect host immunity at intestinal and systemic levels (Ménard et al., 2008). Immune modulation in conventionally healthy mice following administration of *B. breve* (alone or in conjunction with prebiotic oligosaccharides), *B. longum, B. adolescentis, B. bifidum*, or *B. animalis* subsp. *lactis* (alone or in combination with lactobacilli) strains has also been reported. In general, the tested strains modulate several indices of innate immunity, as denoted by the increase in phagocytosis and NK cells activity (Gill et al., 2000; Yang et al., 2009), which are likely to increase the resistance against infection by pathogens. Augmentation of adaptive immunity is also commonly observed at intestinal and spleen levels in response to bifidobacteria administration, as reflected by the activation of the Th2 immune response and Treg cells, with a concomitant increase of antiinflammatory cytokines at intestinal and serum levels (Ehara et al., 2016; Jeon et al., 2012; Salazar et al., 2014).

18.3.2 Effects on Mouse Models of Intestinal Inflammation

Induction of certain immune response could counteract the excessive inflammation, and the concomitant tissue damage, related to autoimmune and infectious disorders. In the inflammatory bowel diseases, comprising ulcerative colitis, and Crohn's disease (CD), a malfunction of the immune system is suspected. There are various (murine) chemically induced models of colitis, the dextran sulfate sodium being widely used to evaluate potential therapies to treat this condition. This murine model of intestinal inflammation has been applied to study the immune-modulating capability of bifidobacteria. Strains of B. breve, B. animalis subsp. lactis, and B. bifidum were able to diminish damage induced by dextran sulfate sodium in mice, which was mediated through an increase of Th2 and Treg, as well as through a reduction of Th1 and Th17 responses (Hidalgo-Cantabrana et al., 2016; Zheng et al., 2014); given that a disequilibrium between effector Th and Treg cells leads to impaired immune responses, the restoration of this cellular balance by orally administered bifidobacteria is consistent with the observed antiinflammatory effect. An increase in Foxp3+ expression, a key factor regulating differentiation and function of Treg cells, was commonly found across various studies (Hidalgo-Cantabrana et al., 2016; Kim et al., 2007; Philippe et al., 2011; Zheng et al., 2014), together with increases in Th2 response (Martín et al., 2016). In opposition to these studies, Duranti and colleagues recently reported that the administration of *B. bifidum* PRL2010 locally increased TNF- α (which acted as a macrophage-activating factor during Th1 response) and reduced IL-10 (an antiinflammatory cytokine) production in an in vivo colitis model; presumably, this favors a local inflammation that could initiate the dialogue with the host (Duranti et al., 2016). Interestingly, a sortase-dependent pili was identified in this strain as the subcellular fraction triggering these effects (Duranti et al., 2016). Therefore, although several bifidobacterial strains are able to elicit benefits in colitis treatment through immune modulation, the particular mechanisms mediating these effects seem to be a strain-specific trait (Srutkova et al., 2015).

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18.3.3 Effects on Pathogen-Infection Models

Bifidobacteria have been reported to enhance immune response toward an infection challenge by Salmonella or Citrobacter, ameliorating the infection outcomes in rodent models (Fanning et al., 2012; Martins et al., 2010; O'Mahony et al., 2010; Silva et al., 2004). The strain B. animalis AHC7 attenuated Salmonella induced NF-kB activation and modulated DCs regulation as well as the cytokine secretion at intestinal level, thereby contributing to attenuate the pathogen-induced inflammation (O'Mahony et al., 2010). Accordingly, another two B. animalis subsp. lactis strains provided protection against Salmonella infection, as reflected by better intestinal tissue preservation (Martins et al., 2010). Indeed, when they were administered to gnotobiotic mice, animals mono-associated with these two strains displayed higher levels of secreted IgA (a key pathogen-blocking immunoglobulin) and IL-10, which is an essential immune regulator at intestinal level (Martins et al., 2010). Besides, in an Escherichia coli O157:H7 infection mice model, B. animalis subsp. lactis HN019 and Bifidobacterium thermacidophilum RBL 71 also enhanced phagocytic ability of blood and peritoneal cells, increased IgA content in feces, as well as IgG and IgM in serum (Gagnon et al., 2006; Shu and Gill, 2001). In addition, the ileum of bifidobacteria-fed mice showed an increase in lymphoid cells and a reduction in goblet cells, thus suggesting that bifidobacteria can attenuate local inflammation (Gagnon et al., 2006). Therefore, these works denote that some bifidobacteria could protect against enteropathogens infection through immunomodulation by controlling the activation of innate immunity at intestinal and systemic levels. Recently, immunomodulation by bifidobacteria has also been studied in different genitourinary infection animal models. A mice model of intravaginal staphylococcosis evidenced that *B. longum* and *B. bifidum* strains, either administered alone or in combination with *Lactobacillus* species, increased CD3+ and CD4+ Th cells levels in the spleen, normalizing their values when the infection was reduced (Lazarenko et al., 2012). In addition, the oral administration of B. animalis subsp. *lactis* BI-07 dramatically decreased bacterial translocation and levels of inflammatory markers (IL-6, TNF- α and C-reactive protein) in anuraemic (experimental model of rats with chronic renal failure) murine model, while a reduction of infiltrated neutrophils and eosinophils in the lamina propria of jejunum and ileum was observed (Wei et al., 2014).

Regarding infections by viruses, bifidobacteria were also able to reduce rotavirus titer and infection symptoms, which correlated with an increase in the rotavirus-specific immunoglobulin levels, in the intestine of piglet and mice models (Vlasova et al., 2016). In a rat suckling model, administration of a *B. bifidum* and *B. longum* subsp. *infantis* preparation, either alone or in combination with prebiotic oligosaccharides, increased IgA, IgG, and IgM in serum and intestinal content, in association with improved clinical outcomes (Qiao et al., 2002). In influenza virus infection, *B. longum* BB536 administration, prior to viral challenge, improved immune response to this virus in a mice model; this was reflected by a marked decrease of the proinflammatory cytokines IL-6 and IFN- γ , which correlated with amelioration of *B. longum* MM-2 in mice intranasally inoculated with influenza virus decreased IL-6 and TNF- α levels, then suggesting antiinflammatory effects; an increased IFN- α and an augmentation of NK activity were correlated, as well, with a reduction in the virus proliferation (Kawahara et al., 2015).

18.3.4 Effects on Vaccination

Sekine et al. (1994) described for the first time the use of the cell wall from *B. longum* subsp. *infantis* as an adjuvant in T-cell-mediated immune responses in mice (Sekine et al., 1994); since then, several in vivo animal models have evaluated the capability of bifidobacteria to act as coadjuvants enhancing vaccination protection against infectious diseases. As an example, the rotavirus vaccination has been tested in mice and neonatal pig models previously colonized with bifidobacteria. In a gnotobiotic pig model, colonization with *B. animalis* subsp. *lactis* Bb12 and *Lactobacillus rhamnosus* GG, prior to rotavirus vaccination and subsequent virus challenge, resulted in increased CD4+ cells and MHCII (class II major histocompatibility complex)-expressing mononuclear cells and DCs, at both intestinal and systemic levels (Vlasova et al., 2013). In addition, by using the same strains and animal model, an increase in B-cells activation in the small intestine, an augmentation in IgA levels and maturated DCs, coincident with an increased IL-6 and IL-10, were detected; therefore, this is an indication of a beneficial modulation of the immune response by bifidobacteria in combination with the rotavirus vaccination (Kandasamy et al., 2014).

18.3.5 Effects on Animal Models for Allergic Disease

Several studies aimed to demonstrate the beneficial effects of bifidobacteria on allergic diseases through in vivo approaches using animal models. Allergic diseases are characterized by an inadequate Th1 to Th2 balance, mainly

involving an overrepresentation of the Th2 responses with a concomitant inability to maintain the Th1/Th2 cytokine ratio. Regarding food allergy, there are many reports using murine models with induced IgE-mediated allergy. Oral feeding with bifidobacteria, particularly strains *B. bifidum* G9-1 or *B. breve* M-16V, revealed a suppression of the IgE levels in the serum of ovalbumin-immunized mice (Inoue et al., 2009; Ohno et al., 2005). Similarly, in a mouse model of IgE-mediated hypersensitivity to whey proteins of cow's milk, the oral administration of different bifidobacterial strains (*B. breve* M-16V and *B. bifidum* BbVK3) showed a reduction in the allergic effector response (Schouten et al., 2009; Shandilya et al., 2016). All these studies support the beneficial effect of bifidobacteria on food allergies through suppression of the excessive Th2 immune response mediated by IgE production and, then, modulating the Th1/Th2 balance. For other types of allergic manifestations, such as asthma, animal models have also been used to test the effect of probiotic bifidobacteria. Feleszko et al. (2007) verified, using an allergen-induced murine model, that the use of *B. animalis* subsp. *lactis* Bb12 in combination with *Lactobacillus rhamnosus* GG inhibited the allergic sensitization and airway disease by induction of Treg activities associated with increased TGF- β production. Antiallergic effects of a strain of *B. longum* were also demonstrated in a mouse model of allergic polysensitization (Schabussova et al., 2011).

18.3.6 Effects on Other Pathologies

Other studies, investigating the cross-talk between the genus *Bifidobacterium* and the host immunity in the context of different pathological conditions, such as cancer, obesity, and coeliac disease, have been undertaken in recent years using specific animal models (Laparra et al., 2012; Moya-Pérez et al., 2015; Sivan et al., 2015). In this regard, using a gliadin-induced enteropathy model, a strain of *B. longum* showed protective effect with the attenuation of the production of inflammatory cytokines and the CD4+ T-cell mediated immune response (Laparra et al., 2012). In high-fat diet fed mice, the administration of a strain of *B. pseudocatenulatum* promoted a reduction in the obesity-associated inflammation by restoring the lymphocyte–macrophage balance (Moya-Pérez et al., 2015).

Altogether, the data presented here revealed that although there is scientific evidence supporting the immunomodulation role of bifidobacteria in animal models, the particular immune ability seems to be strain dependent and the immune effects cannot be easily extrapolated to other related strains or species.

18.4 MECHANISMS OF INTERACTION WITH THE IMMUNE SYSTEM

In a healthy situation, the different molecular interactions (see Fig. 18.2) between intestinal bacteria and the epithelial/immune cells lead to a physiological equilibrium that is known as intestinal homeostasis. In this continuous exchange of molecular information the so-called pattern recognition receptors (PRRs) play crucial roles by recognizing different components derived from gut microorganisms. PRRs are present in different intestinal cell subsets and include, among others, transmembrane receptors (Toll-like receptors or TLRs), intracellular receptors (Nod-like receptors or NLRs), C-type lectin receptors, formylated peptide receptors, RIG-like helicases, and IPAF (Sutterwala and Flavell, 2009). PRRs are able to specifically recognize, and bind, different microbial molecules that are denominated microbial-associated molecular patterns (MAMPs), including polysaccharides, extracellular proteins, DNA, and many others. The molecular pathways activated by one or several PRRs will determine the type and nature of





the immune response, and the cumulative microbial signaling through the whole life of the host, particularly during the early stages of life, will ensure that the immune system matures in an optimal manner (Tsilingiri et al., 2012). Bifidobacteria play a relevant role in the maintenance of the immunological equilibrium through their MAMPs and metabolism, and some strains have shown important immune modulatory capabilities.

MAMPs are continuously sensed by mucosal cells and, notably, by antigen presenting cells, such as DCs and macrophages. Indeed, during the last years it has been suggested that an altered response of DCs to the gut microbiota might be at the basis of IBD (Hart et al., 2004). This autoimmune disorder is a noninfectious human disease related to immunological unbalances and gut microbiota dysbiosis (Sokol et al., 2008). In this regard, an exacerbated immune response to the gut microbiota is observed, although studies in monozygotic twins also suggested the existence of a genetic predisposition (Manichanh et al., 2012). In a recent study, the microbial profiles of terminal ileum, rectum and faeces of an inception cohort for CD showed that certain species of the genus *Bifidobacterium* were significantly underrepresented, among others *B. bifidum*, *B. longum*, *B. adolescentis*, and *B. dentium* (Gevers et al., 2014).

In a normal situation, DCs recognize the MAMPs released from bifidobacteria as safe, and those released from gut pathogens as dangerous. DC response to one or another kind of microorganism will determine the type of immune responses, both effector and regulatory. It is generally accepted that commensal microbiota, by inducing Treg response, modulates the Th1/Th2 balance in a process favoring immune tolerance toward the beneficial gut microbiota (Ventura et al., 2012). Different bifidobacteria-derived MAMPs simultaneously interact with different PRRs expressed in DCs; for instance, murine bone-marrow-derived DCs model lacking NOD2 receptor produced greater amounts of the proinflammatory IL-12 and less of the antiinflammatory IL-10 in response to bifidobacteria (Weiss et al., 2011). The same tendency was found using human monocyte-derived DCs (MoDCs) showing that bifidobacteria-host interaction is largely dependent on the immune status and the genetics of the host (Zeuthen et al., 2008).

Bifidobacteria exhibit species-specific T-cell polarizing properties as evidenced by experiments in which the cytokine profiles of MoDCs challenged with inactivated bifidobacteria cells were measured (López et al., 2010). Relative levels of key cytokines of the T-cell response (IL-10, IL-17, TNF-α, among others) suggested a specific immunomodulation mechanism for each bifidobacteria species, as reported for probiotics in general (Hill et al., 2014). This fact was further confirmed by challenging immature MoDCs with different bifidobacterial strains, and then characterizing the T response induced by coculturing these conditioned MoDCs with allogeneic naïve CD4+ cells (López et al., 2011). Among the different T-cell responses induced by bifidobacteria, *B. bifidum* has a remarkable ability to induce a Treg response (López et al., 2011). Indeed, MoDCs challenged with membrane vesicles derived from *B. bifidum* LMG13195 induced the polarization of naïve CD4+ cells into Treg, as deduced from the increases in the expression of FoxP3+ regulation factor and the CD25 marker in the resulting T-cells (López et al., 2012a). Interestingly, *B. bifidum* is not only able to interact with immune cells, but also with gut epithelial cells altering the secretion of their main cytokines (IL-6 and IL-8) through a NF-kB activation pathway (Turroni et al., 2014). This provides evidence that the immune modulatory capabilities of bifidobacteria may be dependent on bacterial subcellular fractions.

18.4.1 Effect of Compounds Secreted by Bifidobacteria

The MAMPs surface structures of *Bifidobacterium* cells have been identified as key molecular effectors initiating immune modulation in the host and, accordingly, a variation on surface-exposed components in bifidobacteria evokes different immune regulation profiles and disease outcomes. Many scientific works have reported on extracellular and uncharacterized bifidobacterial-derived compounds with immunomodulatory properties. For instance, *B. breve*conditioned supernatants (i.e., supernatants from *B. breve* cultures) increased the expression of genes coding for the proinflammatory immune mediators IL-8 and TNF- α in DCs, and also restored the levels of the regulatory mediator TGF- β after DC challenge with the pathogen *Salmonella enterica* serovar Typhi (Bermudez-Brito et al., 2013). Extracellular compounds produced by *B. longum* subsp. *infantis* attenuated inflammation through a mitogen-activated protein kinase mechanism in genetically modified mice lacking the capacity to produce the antiinflammatory IL-10 (Ewaschuk et al., 2008). Finally, soluble compounds secreted by *B. breve* induced the production of IL-10, with a concomitant decrease of the proinflammatory IL-12, in MoDCs through TLR-2 signaling (Hoarau et al., 2006). There is notable suspicion that these uncharacterized compounds supporting the immune modulatory properties of *B. breve* might be proteins, although no precise sequences have been identified so far (Hoarau et al., 2008).

18.4.2 Effect of Bifidobacterial Proteins

Proteins secreted through the cell wall or extracellular proteins are important players for the interaction of bifidobacteria with the host immune system. This subset of proteins, either attached to the bacterial surface or released to the surrounding environments, may be able to interact directly with PRRs expressed on mucosal cells and therefore susceptible to be sensed (Sánchez et al., 2010). A well-known example is extracellular serpin secreted by *B. longum* subsp. *longum*. Serpin is a "serine protease inhibitor" which specifically binds and inactivates human neutrophil and pancreatic elastases, therefore contributing to gut homeostasis by limiting the effect of these proinflammatory proteases (Ivanov et al., 2006). Bifidobacteria-derived membrane vesicles are rich in certain moonlighting proteins (proteins that perform more than one function depending on the subcellular location), such as fructose-6-phosphate phosphoketolase or enolase, which may be at the basis of the immunomodulatory properties of these beneficial microorganisms, although this is a merely speculative statement, albeit one that deserves further research (Sánchez et al., 2004).

Pili synthesized by bifidobacteria are proteinaceous structures that selfassemble into filaments on the bacterial surface and that are involved in the organisms' adherence to the intestinal mucosa, participating in the persistence of this highly competitive ecosystem, but they also have immune modulating properties (Ventura et al., 2012). Depending on the bifidobacterial species concerned, some harbors gene clusters coding for Tad pili, but also other sortase-dependent pili, which differs both in structure and in the secretion mechanism (Foroni et al., 2011). The heterologous expression of *B. bifidum* PRL2010 pili in *Lactococcus lactis*, trigger a TNF- α response and reduced IL-10 production in the murine cecum mucosa, evidencing its role in initiating the dialogue with the immune cells at intestinal level (Turroni et al., 2013).

Another surface-protein influencing T-cell response is TgaA, a type of peptidoglycan hydrolase, from *B. bifidum*, which is able to induce MoDC activation and IL-2 production (Guglielmetti et al., 2014). IL-2 is one of the main cytokines supporting Treg proliferation, which is characterized by the presence of CD25, the T-cell receptor for IL-2, and necessary together with the transcription factor FoxP3+ for expanding the Treg response (Zelante et al., 2012).

18.4.3 Effect of Bifidobacterial Exopolysaccharides

EPS are carbohydrate polymers, extracellularly located, that surround bifidobacterial surfaces and play a role in immune modulation (Hidalgo-Cantabrana et al., 2014). It is supposed that these polymers act as MAMPs, interacting with yet-unknown PRRs and participating in the molecular cross-talking and host immunomodulation, although the precise molecular mechanisms remain to be elucidated (Hidalgo-Cantabrana et al., 2012). Evidences on the immunomodulatory potential of EPS are deduced from the observed changes in immune effector production. EPS produced by *B. longum* BCRC 14634 induced production of the antiinflammatory cytokine IL-10 on basal J77A.1 macrophages, and prevented release of the proinflammatory cytokine TNF-α after challenging these cells with lypopolysaccharide, which is a usual inflammatory molecule used in this type of experiments (Wu et al., 2010). Ability of bifidobacterial EPS to modulate cytokine production patterns was confirmed with polymers isolated from eighteen bifidobacterial strains (*B. animalis* subsp. *lactis*, *B. longum*, and *B. pseudocatenulatum*), with some of them being able to decrease the antiinflammatory ratio TNF-α/IL-10 (López et al., 2012b). Using three isogenic *B. animalis* subsp. *lactis* strains producing EPS of different compositions, it was shown that the strain producing a rhamnose-rich, high-molecular weight EPS (*B. animalis* subsp. *lactis* IPLA-R1) induced IL-10 production by PBMCs, this polymer being also able to reduce TNF-α production in human colonic biopsies (Hidalgo-Cantabrana et al., 2015).

In addition to the in vitro experiments, a few scientific works using animal models have described the immunomodulatory effects of bifidobacteria-derived EPS on the host (Hidalgo-Cantabrana et al., 2014). The oral administration of *B. animalis* subsp. *lactis* IPLA-R1 strain to a cohort of Wistar rats increased the serum levels of the suppressorregulatory TGF- β cytokine and reduced the levels of the proinflammatory IL-6 in comparison to the wild-type strain (Salazar et al., 2014). The EPS layer of *B. breve* UCC2003 was essential to protect a murine model against *Citrobacter* infection, since an EPS deficient mutant did not prevent the infection and significantly enhanced production of proinflammatory cytokines IL-12, INF- γ , and TNF- α as compared to the EPS producing strain (Fanning et al., 2012). In addition, different EPS (in structure and composition) elicit differential immune responses in a colitis-induced mice model. Hence, a *B. animalis* subsp. *lactis* strain producing a "ropy"-EPS induced a higher TNF- α to IL-10 ratio in blood, a reduction of Th lymphocytes and an increase in cells expressing Foxp3+, as compared to the isogenic strain producing a nonropy EPS (Hidalgo-Cantabrana et al., 2016). These works suggest that the presence and structure of EPS conditions the bifidobacterial immune effects on the host.

18.4.4 Effect of Bifidobacterial Metabolites and DNA

Bifidobacteria possess a powerful way to induce indirect host immunomodulation through the production of acetic acid. This is achieved through the heterofermentative metabolism of hexoses, also known as bifid-shunt, which profits from the wide carbohydrate-degrading arsenal encoded in the bifidobacteria genomes to produce energy and fuel biosynthetic pathways, with the concomitant production of acetic and lactic acids (Sánchez et al., 2004). Acetic acid released in the gut by bifidobacteria is used by bacteria to produce butyric acid through their metabolism. These bacteria, mainly clostridia from Clusters IV, XIVa, and XVI, develop important metabolic functions fermenting different types of fiber and host-glycans, but are also important immune modulating bacteria (Furet et al., 2010). Butyric acid is an important immunomodulatory molecule with its own receptors in epithelial and immune cells named FFAR3 (free fatty acid receptor 3 and GPR109A) (Ahmed et al., 2009; Remely et al., 2014). From an epigenetic point of view, butyric acid acts on both DNA methylation and histone hyperacetilation, notably in the promoter region of genes involved in the control of the cellular cycle (Blottière et al., 2003). By inhibiting histone deacetylase, butyric acid acts also in the differentiation of Treg cells, increasing the expression of the Treg marker FoxP3+ (Furusawa et al., 2013).

Other metabolites produced by bifidobacteria are cofactors of enzymes catalyzing transfer of methyl groups to DNA. Among these compounds folates are key players in the maintenance of a proper epigenetic regulation (Nagy-Szakal and Kellermayer, 2011). Members of the genus *Bifidobacterium* are among the main folate producers in the intestinal microbiota, with 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, and 5,10-methylenetetrahydrofolate as the major metabolites (Mischke and Plösch, 2013). Deficiencies on folate intake due to nutritional deficiencies or intestinal microbial dysbiosis, for instance, abnormally low bifidobacteria numbers, is a condition predisposing to colorectal cancer due to global DNA hypomethylation (Crider et al., 2012).

Finally, bifidobacteria DNA is also supporting their immunomodulatory properties. Bifidobacteria possess unmethylated CpG motifs within their genomic sequence that have a specific receptor within the innate immune system of humans, the TLR9. Bifidobacterial CpG motifs have been shown to favor the Th1 response, which is focused on fighting against intracellular pathogens such as viruses (Ménard et al., 2010). An oligodeoxynucleotide derived from *B. longum* BB536 strain was linked to inhibition in IgE production in vitro and, when assayed in vivo in a murine model of type I allergic response involving injection of ovalbumin, the total and ovalbumin-specific IgE levels were lowered by the oligodeoxynucleotide, including also a decrease in Th2-specific cytokine production (Takahashi et al., 2006).

18.5 CONCLUDING REMARKS

Bifidobacteria, or their subcellular fractions, represent a huge source of immunomodulatory compounds still to be characterized. Further research describing the molecules, receptors, and molecular mechanisms of action would allow the use of selected strains to intentionally polarize T-cell response, or to manipulate the innate response in antigen-presenting cells. In addition, a rational modification of the gut microbiota, using specific bifidobacterial strains, may also allow modifying immune responses not only in inflammatory or autoimmune disorders, but in other pathologies, such as cancer.

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THE BIFIDOBACTERIA AND RELATED ORGANISMS BIOLOGY, TAXONOMY, APPLICATIONS

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The Bifidobacteria and Related Organisms: Biology, Taxonomy, Applications presents authoritative reviews covering different aspects of bifidobacteria and other genera classified with them. The book begins with some fundamental aspects of taxonomy underlining the current status of the phylum Actinobacteria, genus Bifidobacterium, and family Bifidobacteriaceae. Also included are lists of Bifidobacterium species and subspecies, which have increased greatly in number in recent years. Additionally, detailed advice is supplied for culture media and culture conditions for detection of bifidobacteria in different environments, their cultivation and their storage. The impact of genetics in bifidobacterial studies is also considered. The genus Bifidobacterium is being increasingly recognized as particularly friendly for humans, and, increasing evidence suggests, for most, if not all mammals, and possibly for other animals.

Key Features

- Provides an understanding of the major differences between bifidobacteria and lactic acid bacteria
- Provides information on the relationships between bifidobacteria and their host
- Describes 54 species and 10 subspecies of bifidobacteria and highlights the characteristics of the seven new taxa belonging to the socalled scardovial genera, describing the features of genus and species
- Explains the nutritional requirements of bifidobacteria, bifidogenic effect of particular substrates, milk oligosaccharides and carbohydrate metabolism
- Includes applications and technological considerations placed alongside the more academic matters such as nomenclature and phylogeny

About the Editors

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