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Detection and Identification of a Novel Lactic Acid Bacterial Flora Within the Honey Stomach of the Honeybee *Apis mellifera*

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Abstract This investigation concerned the question of whether honeybees collect bacteria that are beneficial for humans from the flowers that contribute to formation of their honey. Bacteria originating from the types of flowers involved, and found in different anatomic parts of the bees, in larvae, and in honey of different types, were sampled during a 2-year period. 16S rRNA sequencing of isolates and clones was employed. A novel bacterial flora composed of lactic acid bacteria (LAB) of the genera *Lactobacillus* and *Bifidobacterium,* which originated in the honey stomach of the honeybee, was discovered. It varied with the sources of nectar and the presence of other bacterial genera within the honeybee and ended up eventually in the honey. It appeared that honeybees and the novel LAB flora may have evolved in mutual dependence on one another. It was suggested that honey be considered a fermented food product because of the LAB involved in honey production. The findings are seen as having clear implications for future research in the area, as providing a better understanding the health of honeybees and of their production and storage of honey, and as having clear relevance for future honeybee and human probiotics.

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Introduction

Lactic acid bacteria (LAB) are considered to be favourable bacterial species, commonly found in healthy individuals [19] and commercially important through their use in dairy products and in probiotics (live microorganisms, which, when administered in adequate amounts, confer health benefits on the host) [8]. *Bifidobacterium* is by definition not a "true" LAB member, but because of its lactic acid production, its use in dairy products, and its known positive effects on human and animal gastrointestinal flora, it is commonly placed within this group [3]. LAB are frequently found in both flowers and as part of the bacterial flora within insects.

The honeybee *Apis mellifera* has played a central role in human welfare through history. Not only has it provided man with honey, but, more importantly, honeybees ensure the fertility of many food-producing plants through pollination. Honey is produced by honeybees from the nectar they gather. Honey is a sweet liquid composed of varying amounts of sucrose, glucose, and fructose temporarily stored in the honeybee honey stomach during flight (Fig. 1). The honey stomach is an enlargement of the oesophagus that can expand to a large volume. It ends with a structure called the "proventriculus," which ensures that the nectar is never contaminated by the contents of the ventriculus (midgut), which is the functional stomach of honeybees. In the hive the nectar is transferred mouth-to-mouth to house bees that take it to the honeycomb for the evaporation of liquid so as 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 and have added enzymes. When the honey is ripened, the honeycomb cells are sealed with wax capping [29].

Fig. 1 The full honey stomach, containing nectar from flowers, of an incoming honeybee that has been foraging. The honey stomach (a) is separated from the rest of the digestive tract at the proventriculus (b)

Honey, the mysterious food used in folk medicines since ancient times, has long puzzled people with its healing effects on wounds and on sore throats. Although research on honey has increased markedly in recent decades [28], spurred in part by mounting resistance to antibiotics, knowledge of honey's mode of action is far from complete. Honey's therapeutic properties, along with its chemical composition, including the osmolarity and acidity it possesses, can be explained in terms of the production of hydrogen peroxide by the honeybee [26], the characteristics of nectar with its flavonoid and phenolic acid content [24], and the presence of an as yet unidentified component [17]. The effect of this unidentified component, which shows substantial levels of nonperoxide antibacterial activity, is dependent both on the honeybee itself as well as the plant source [28]. Different honeys also vary considerably in their antibacterial activity [14].

Diseases in honeybees, in the form of American foulbrood disease (AFB), which is caused by *Paenibacillus larvae* [9] and colony collapse disorder (CCD) [4], lead to enormous economic losses for agriculture throughout the world, which in turn has recently led to increased honeybee research.

As a microbiologist one can hypothesize that indigenous bacterial flora is as important for honeybees as it is for humans and animals. The present study was conducted to investigate the significance of indigenous bacterial flora for honeybees. The question posed initially was whether honeybees collect bacteria that are beneficial for humans in the nectar or pollen of specific flowers, bacteria that eventually end up in their honey. The surveȳwhich was conducted concerning indigenous bacterial flora in honeybees, different types of nectar involved, honey produced and stored, and honeybee larvaētook place during a period of 2 years.

Materials and Methods

Honeybees

Bees were obtained in an apiary in the village of Jonstorp in southern Sweden from colonies maintained using standard beekeeping practices. Bacterial samplings of the honeybees (bred according to the Buckfast method), of the larvae, of flowers available to the honeybees, and of various types of fresh and stored honey were carried out during the 2-year period. Fresh honey was defined as not fully ripened 1- to 3-day old honey taken from cells that were not yet sealed with wax. The beehives were always emptied of their honey before the experiments were begun. To identify the bacteria involved and to acquire complete bacterial flora, 16S rRNA gene analysis was performed on all of the bacteria using both cloning and pure-culture techniques during the first year and using pure-culture technique alone the second year of the study.

Sampling During the First Year

A small beehive containing approximately 12,000 bees was transported to an area containing wild raspberry bushes in bloom located at Kullaberg, a nature reserve situated 10 km from Jonstorp. During the second week after this process, 10 incoming and 10 outgoing worker bees and 20 raspberry flowers were collected and sampled. The flowers were picked after they were visited by honeybees from the beehive. Samples of fresh raspberry flower honey were taken at week 4. The small beehive was then placed in the apiary in Jonstorp until the next year. During autumn 2005, honey stomachs and ivy flowers were collected and sampled. Raspberry flower honey stored for 2 and linden honey stored for 12 months were also sampled.

Sampling the Second Year

The small beehive in Jonstorp was sampled as the bees collected nectar from goat willow and oil-seed rape. The small beehive was then placed in the nature reserve for collection of raspberry flower nectar and thereafter in the apiary in Jonstorp for the collection of nectar from linden and from other flowers. A regular-sized beehive containing approximately 60,000 bees was also placed in the apiary

for a study aimed at confirming the results obtained for this smaller beehive. Approximately 10 incoming and 10 outgoing worker bees, 10 house bees, and 5 honeybee larvae (2 to 5 days old) were carefully picked by the beekeeper and were sampled on each occasion. Flowers, as well as fresh honey from a variety of different flower origins, were sampled. In addition, 5 heads, 1 hindgut, and 5 honey stomachs were obtained by aseptic excision. Only incoming bees whose honey stomachs were filled with nectar (Fig. 1) were selected for this process.

Procedure for Isolates

The flowers and bees sampled were placed in separate sterile 10-ml tubes each containing 5 ml sterile physiologic saline (0.9% w/v NaCl, 0.1% w/v Tween 80, and 0.1% w/v peptone). The anatomic samples, the fresh and stored honey, and the larvae were placed in 1.5-ml sterile microtubes each containing 0.9 ml physiologic saline. Each tube was shaken vigorously and immediately transported to the laboratory in Lund, situated 70 km from Kullaberg. Some of the samples, in tubes containing a 0.5-ml suspension, were frozen and stored at −20°C for direct 16S rDNA analysis. Pure cultures were obtained containing tryptone soy broth agar (Oxoid, Basingstoke, Hampshire, England), tomato juice agar (Oxoid), and an all-purpose medium containing Tween (Merck, Darmstadt, Germany) and Rogosa agar (Merck). The isolates were cultivated both aerobically and anaerobically at 37°C for 3 to 4 days. Ten to 30 colonies were picked randomly from each of the media involved, which contained 30 to 300 colonies each, and were subcultured to obtain pure isolates.

Genotypic Characterization

Characterization was carried out according to previous work [18]; thus, it is described only briefly here. Bacterial DNA from all of the samples was extracted and purified before polymerase chain reaction (PCR) amplification of 16S rRNA genes. In the following cloning procedure, different 16S rRNA genes from the samples were sorted and copied using competent *Escherichia coli* cells. To recover the cloned DNA, PCR amplification was carried out with universal forward and reverse M13 primers. PCR amplification of isolates was performed with universal primers ENV1 and ENV2. PCR products originating from isolates and clones were sequenced by a sequencing company using universal primers ENV1 and ENV2. For identification, these 16S rDNA sequences were searched against GenBank (National Centre for Biotechnology Information, Rockville Pike, Bethesda, MD) using the advanced BLAST similarity search option (available at http://www.ncbi.nlm.nih.gov/) and Ribosomal Database Project (RDP) II software (available at http://rdp.cme.msu.edu/). Phylogenetic analysis was done using computer software programs. Cloning bacterial 16S rRNA genes was only performed during the first year on samples from outgoing and ingoing bees, from flowers, and from not fully ripened honey when the bees were foraging on wild raspberry flowers. Cloning was also performed on raspberry honey stored for 2 and on linden honey stored for 12 months. The bacterial 16S rRNA gene sequences were deposited in GenBank using the accession numbers EF187231 through EF187250.

Phenotypic characterization of LAB isolates was analysed by Gram staining (bioMérieux, Lyon, France), catalase test (bioMérieux, Lyon, France) and L-lactic- and acetic acid production (R-Biopharm, Darmstadt, Germany) according to the manufacturers' instructions. In addition, the morphologic appearance of the isolates and their spore-forming abilities were investigated by light microscopy. Production of gas from glucose was assayed by growing the bacteria in tubes containing de man, Rogosa, Sharpe (MRS) broth (Oxoid, Basingstoke, Hampshire, England) and Durham tubes.

Results

Distribution of Isolates and Clones

A total of 398 sequences from bacteria were identified (including both clone-derived sequences and those from isolates), and all showed a similarity of >87% to type strains in RDP. One hundred twenty-four of these sequences came from clones and 274 came from isolates picked from both aerobic and anaerobic plate counts. The bacterial 16S rDNA sequences obtained from samples not originating from honeybees or their larvae are not displayed.

The results for the first year demonstrated that honeybees harboured a LAB flora dominated by the phylotype Fhon2, which is most closely related to the bacterial species *Lactobacillus kunkeei*. Large numbers of Fhon2 and lesser amounts of other LAB were isolated in viable condition from the honeybees and from fresh honey samples, but not from the flower samples, when the honeybees collected raspberry flower nectar. Other LAB, but not Fhon2, were isolated from the honey stomach but not from the flower samples when ivy was in bloom (Fig. 2).

The results obtained, which were also confirmed the second year, are summarized in Fig. 2. Analysis of the anatomic parts of the honeybees showed that the niche of the previously found LAB flora was the honey stomach (Fig. 1). As can be seen in Figure 2, honey stomach flora varies with the sources of nectar and the presence of other bacterial genera.

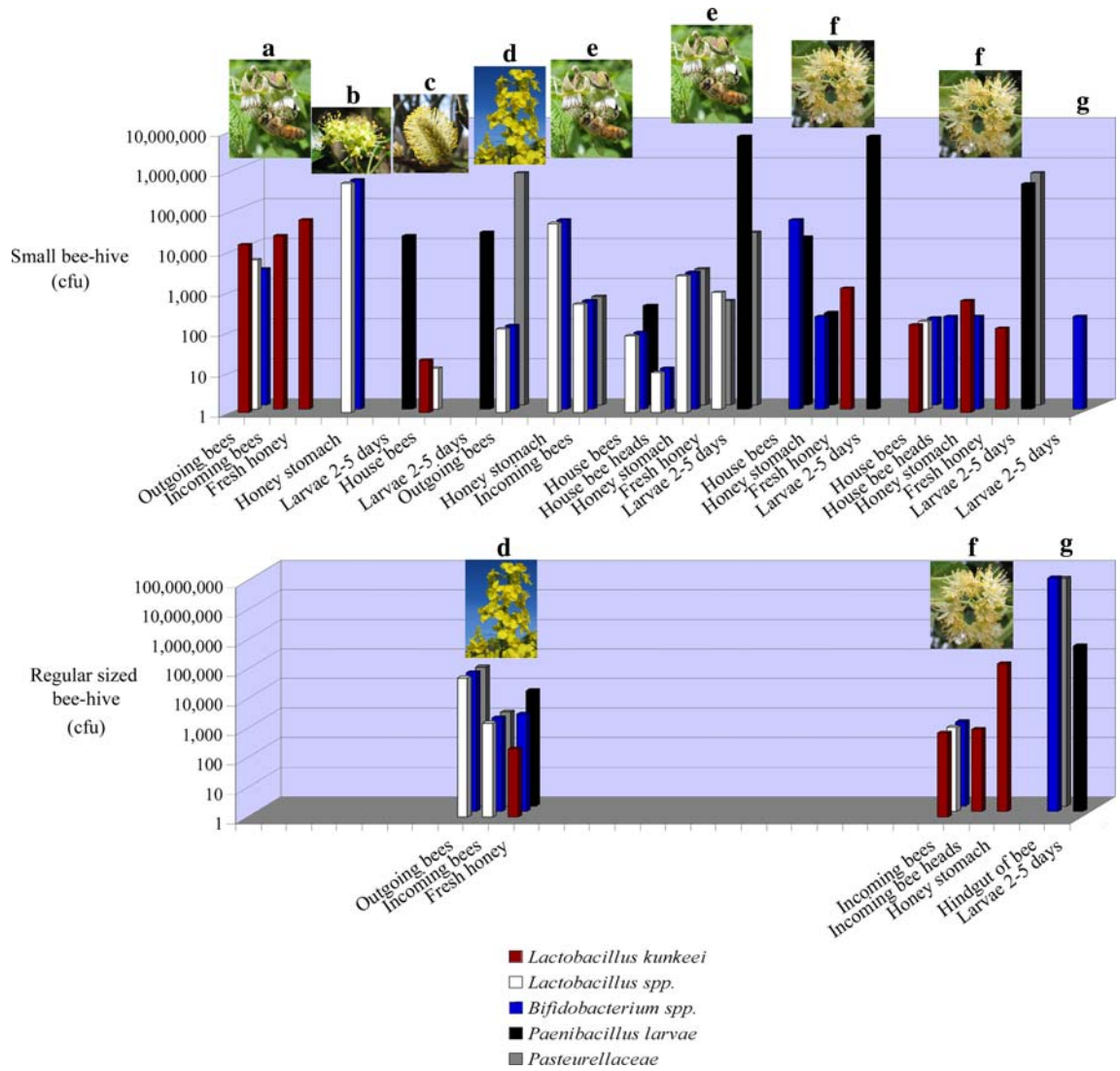

Fig. 2 Various major nectar sources and bacterial groups as found on different sampling occasions. The samplings were performed for flowers from (a) wild raspberry (*Rubus idaeus L*), summer of 2005; (b) ivy (*Hedera helix L*), autumn of 2005; (c) goat willow (*Salix caprea L*), spring 2006; (d) oil-seed rape (*Brassica napus L*), late spring 2006; (e) wild raspberry, summer of 2006; (f) linden (*Tilia x vulgaris*), summer of 2006; and (g) when the bees were fed a sugar solution, autumn of 2006. The small beehive was sampled continuously, and the regular-sized beehive served as control. The bacterial concentrations are shown in colony-forming units per honeybee, per gram fresh honey, per honey stomach, per larva, per head, and per hindgut. Species is denoted as *spp.*, which means that the genus in question is represented by at least one of the different phylotypes at each sample occasion

Bacterial Flora

The indigenous bacterial flora in the honey stomach is dominated by *Lactobacillus* and *Bifidobacterium* phylotypes (Fig. 3). Phylogenetic analysis indicated the LAB flora in the honey stomach to be composed of 10 different phylotypes, 5 of which were most closely related to the previously described species *L. kunkeei* (Fhon2), *Bifidobacterium asteroides* (Hma3, Bin7, and Bin2), and *Bifidobacterium coryneforme* (Bma6) (clusters I and III in Fig. 3 and Table 1). The other 5 phylotypes, Hon2, Hma2, Biut2, Bma5, and Hma8, were distant but most closely related to the *Lactobacillus* genus (cluster I in Fig. 3 and Table 1).

Three of the 10 different honey-stomach LAB, i.e., Fhon2, Bin2 and Hon2, were also found in fresh honey, both viable as isolates and through DNA analysis (Fig. 2). The small beehive showed large numbers of Fhon2 at the different samplings; however, when Hon2 was found in fresh honey, no Fhon2 was noted. Bin2 together with Fhon2 was only detected in fresh honey from the regular-sized beehive. Of the 3 phylotypes found in the fresh honey, Fhon2 was present in the largest amount in the first year (5×10^4 colony-forming units [CFU]/g honey) when raspberry nectar was collected. None of these 3 phylotypes were encountered in the raspberry flower honey stored for 2 months, either viable as isolates or dead as DNA traces,

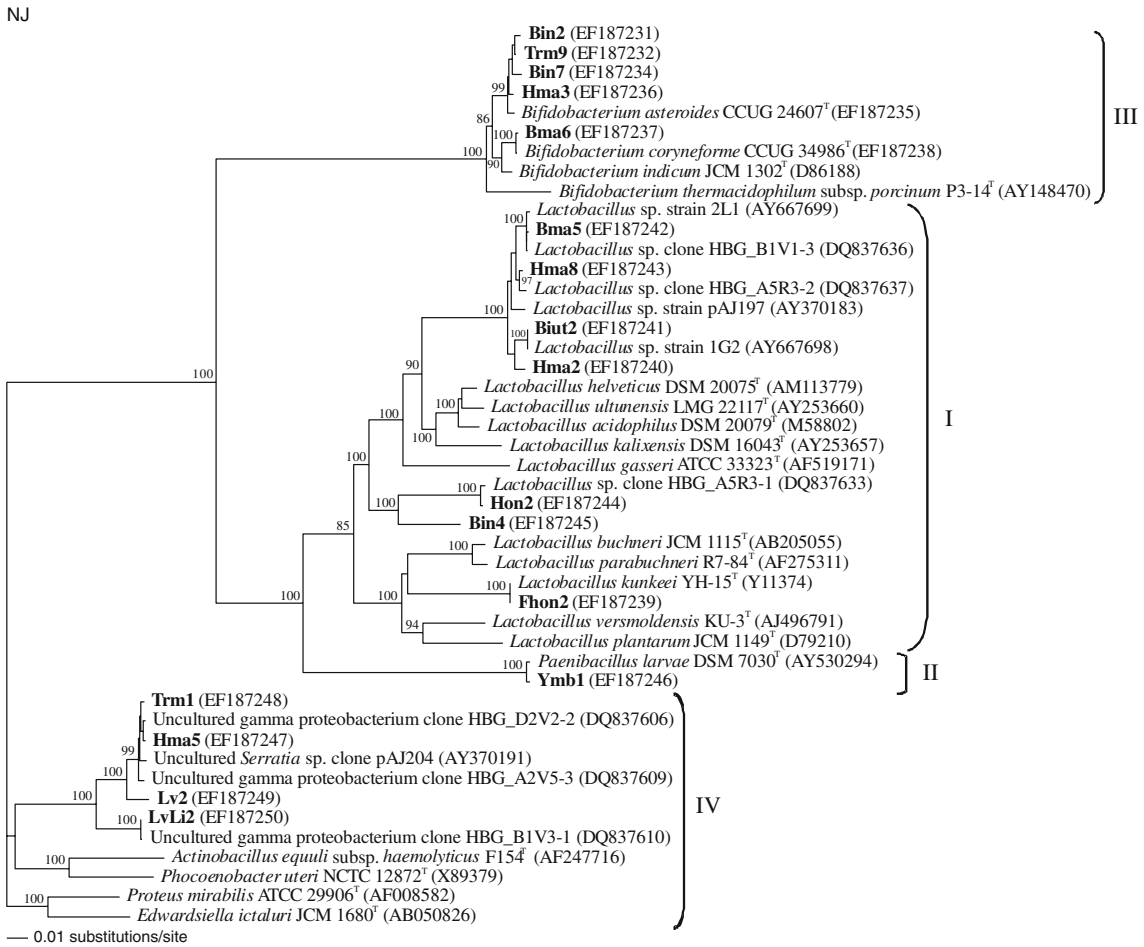

Fig. 3 A phylogenetic tree based on a distance matrix analysis of 1,350 positions in the 16S rRNA gene. Closely related type and reference strains are indicated in parentheses together with accession numbers from GenBank. Cluster I = *Lactobacillus* group; cluster II = *Paenibacillus larvae*; cluster III = *Bifidobacterium* group; and cluster IV = *Pasteurellaceae* group, which served as the out-group. The phylotypes characterised in the study are in bold print and derived from isolates, and the accession numbers are included. Bar = 0.01 substitutions/nucleotide position

although DNA traces from 1 strain (Fhon2) were found in the linden honey stored for 12 months.

Two other LAB were only found outside the honey stomach of the bees (Fig. 3): (1) phylotype Bin4, which is most closely related to the genus *Lactobacillus*, isolated from incoming bees and from house bees in the small beehive and (2) phylotype Trm9, which is most closely related to the species *B. asteroids*, and isolated from a hindgut in the regular-sized beehive (Fig. 2).

All 10 LAB phylotypes in the honey stomach were shown to be Gram positive, catalase negative, nonsporulating, and lactic acid producing. These results, together with the morphologic shapes of the bacteria, corresponded well with the characteristics of either the *Lactobacillus* or the *Bifidobacterium* genera. In addition, all of the honey-stomach LAB most closely related to the *Lactobacillus* genus were homofermentative, i.e., they do not produce gas from glucose. Furthermore, all of the bifidobacteria produced both lactic and acetic acid.

Infection with *Paenibacillus larvae*

During spring of the second year, we noted that larvae in the small beehive became infected with the pathogenous *P. larvae*, which were also detected in house bees, honey stomach, and fresh honey (Fig. 2 and cluster II in Fig. 3). Because no clinical symptoms typical of this larval disease were recorded, the beehive was employed continuously throughout the study. The numbers of *P. larvae* continued to increase with gathering of the oil-seed rape and wild raspberry nectars, but this increase ceased with gathering of the linden nectar (Fig. 2). At this point, the numbers of *P. larvae* began decreasing from 8,000,000 CFU/larva (Fig. 2) and vanished 3 weeks later without AFB developing. In addition, large numbers of 3 bacterial phylotypes (LvLi2, Lv2, and Hma5) were found in incoming and outgoing bees, in larvae, in the honey stomach, and in fresh honey during the time that the bee colony was infected with *P. larvae* (Fig. 2). These phylotypes were most closely

Table 1 Bacterial phylotypes originating from honeybees and their larvae[a]

Isolates[b]	Clones[b]	Most closely related type strain[c]	Sequence lengths and similarity[d]
Fhon2 (320–1455) [40]		*Lactobacillus kunkeei* YH-15^T (Y11374)	1455 (100.0)
	HonbakL13 (310–865) [30]	*Lactobacillus kunkeei* YH-15^T (Y11374)	865 (100.0)
Hon2 (230–1470) [15]		*Lactobacillus buchneri* JCM 1115^T (AB205055)	1470 (89.2)
Bin4 (880–1460) [2]		*Lactobacillus buchneri* JCM 1115^T (AB205055)	1460 (89.1)
Bma5 (150–1440) [1]		*Lactobacillus acidophilus* DSM 20079^T (M58802)	1440 (91.0)
Hma8 (250–1450) [10]		*Lactobacillus acidophilus* DSM 20079^T (M58802)	1450 (91.1)
Hma2 (160–1450) [8]		*Lactobacillus acidophilus* DSM 20079^T (M58802)	1450 (91.0)
Biut2 (590–1450) [6]		*Lactobacillus acidophilus* DSM 20079^T (M58802)	1450 (91.1)
Bin7 (310–1410) [20]		*Bifidobacterium asteroides* CCUG 24607^T (EF187235)	1410 (99.0)
Bin2 (210–1410) [30]		*Bifidobacterium asteroides* CCUG 24607^T (EF187235)	1410 (98.4)
Hma3 (615–1410) [8]		*Bifidobacterium asteroides* CCUG 24607^T (EF187235)	1410 (98.9)
Trm9 (305–1410) [6]		*Bifidobacterium asteroides* CCUG 24607^T (EF187235)	1410 (98.6)
Bma6 (120–1410) [2]		*Bifidobacterium coryneforme* CCUG 34986^T (EF187238)	1410 (99.6)
Ymb1 (280–1440) [15]		*Paenibacillus larvae* DSM 7030^T (AY530294)	1440 (99.6)
	Hho2MkL6 (231–407) [2]	*Paenibacillus larvae* DSM 7030^T (AY530294)	407 (100.0)
Hma5 (810–1410) [8]		*Actinobacillus equuli* subsp. *haemolyticus* F154^T (AF247716)	1410 (89.2)
Lv2 (820–1415) [7]		*Actinobacillus equuli* subsp. *haemolyticus* F154^T (AF247716)	1415 (88.1)
LvLi2 (990–1420) [5]		*Actinobacillus equuli* subsp. *haemolyticus* F154^T (AF247716)	1420 (87.8)
Trm1 (1420) [1]		*Actinobacillus equuli* subsp. *haemolyticus* F154^T (AF247716)	1420 (88.7)

[a] The identity of 16S rRNA gene sequences were generated from isolates and clones

[b] The sequence lengths are shown in parentheses, and the number of identical sequences found are shown in brackets

[c] GenBank accession numbers are shown in parentheses; taxonomic affiliation was established by comparing the sequence in the database of the Ribosomal Database Project II (http://www.rdp.cme.msu.edu/) with the entry labelled "sequence match" and the options "type" and "NCBI."

[d] The similarity to the closest type strain sequence is shown as a percentage within parentheses

related to the genera *Actinobacillus* and *Phocoenobacter*, which belong to the family *Pasteurellaceae* (cluster IV in Fig. 3 and Table 1).

The isolated bacterial genera from the small beehive were also found when sampling the regular-sized beehive (Fig. 2). The greatest number of Fhon2 were recovered from honey stomachs (5×10^5 CFU/honey stomach) at the time honeybees were collecting nectar from the linden flowers. In addition, we noted that the regular-sized beehive also became infected with *P. larvae*, although this colony also showed no clinical evidence of AFB. A fourth *Pasteurellaceae* phylotype (Trm1) was found only in hindgut, together with the *Bifidobacterium* phylotypes Bin7 and Hma3 (Figs. 2 and 3).

Discussion

The survey conducted here demonstrated that honey produced by honeybees contains LAB that originate not from flowers, as suspected, but from the honey stomach. Our results, taken as a whole, point to a novel LAB flora that was found to be composed of six *Lactobacillus* and four *Bifidobacterium* phylotypes. At least five of the phylotypes isolated from the honey stomach (Hon2, Hma2, Bma5, Biut2, and Hma8) and one from outside the honey stomach (Bin4) belong to possibly novel species most closely related to the *Lactobacillus* genus. Their sequences resembled the *Lactobacillus* genus by 89.1% to 91.1% (Table 1), which is higher than the threshold level generally used to define a species (95% to 97%) [13].

The nectar sugars probably act as inducers for the resident honey stomach flora, enhancing their numbers, with the enhancement depending on the types of flowers that the honeybees visited (Fig. 2). The bacteria are added during the process by which nectar becomes honey, which explains why we located three of them in fresh honey (Fig. 2), although it is likely that the other seven phylotypes would appear in fresh honey if more extensive sampling was conducted. In fresh raspberry honey collected in 2005 ("a" in Fig. 2), Fhon2 was found both to be viable at a concentration of 5×10^4 CFU g^{-1} fresh honey and to be present as clones, although Fhon2 was not detected by either method in the raspberry honey stored for

2 months. Nevertheless, a trace of the 16S rRNA gene was encountered when linden honey that had been stored for 12 months was sampled. Because of osmosis, neither LAB nor any other nonsporulating bacteria survive in honey, and their DNA is probably disintegrated eventually by DNAses. This is likely one reason for there having been no reports of honey LAB in previous work.

To our knowledge, this is the first time that bacteria from the genera *Lactobacillus* and *Bifidobacterium* (clusters I and III in Fig. 3) have been isolated from the honey stomach and from fresh honey. The honey stomach environment, together with the nectar that has been gathered, which are in a microaerobic state, are filled with nectar sugars and nutrients and are at a fairly optimal temperature of 35°C in the hive, regardless of the outside temperature [12]. It represents an optimal niche for the LAB that were discovered. It is important to note that in previous unpublished (the sequences are deposited in Genbank) and published work [1, 11, 21] phylotypes closely related to the LAB presented here have only been encountered in the intestines of honeybees (clusters I and III in Fig. 3). In the present study, we examined the bacterial composition of the hindgut to exclude any contamination from the honey stomach when excising it. Two of the LAB phylotypes, Bin7 and Hma3, were found sampling the hindgut (Figs. 2 and 3). It appears, therefore, that the bacteria residing in the honey stomach follow the nectar to the hindgut when the bee is feeding.

Although LAB do not survive in stored honey, it is likely that humans have been consuming viable LAB in fresh honey during and directly after honey hunts throughout human history. LAB produce such antibacterial compounds as organic acids, hydrogen peroxide, diacetyl, benzoate, and bacteriocins [25], all of which are beneficial for humans and animals [3, 19] and presumably for honeybees as well. It can be assumed that the clear antibacterial differences reported between various types of honey [14] may in part be explained in terms of the antibacterial compounds produced by LAB.

The 16S rRNA gene sequence of Fhon2, which was most frequently found in the honey stomach and in fresh honey, is identical to the sequence found in the *L. kunkeei* type strain (Fig. 3). The *L. kunkeei* type strain YH-15 was originally isolated from wine production in California. It strongly inhibited alcoholic fermentation of the yeasts *Saccharomyces bayanus* and *S. cerevisiae* [10] and was thus described as a spoilage organism [5]. Several lines of evidence indicate that *L. kunkeei* is left behind in damaged grapes by visiting honeybees [2, 10]. Whereas nectars collected have a water content of 50% to 80%, yeast can ferment fresh honey until its water content has reached the safe level of 18% [27], a process that can take several days. We thus believe that phylotype Fhon2 inhibits *Saccharomyces* species, which are the dominant yeasts causing spoilage in honey [22]. The other honey stomach LAB may have a similar function, but this hypothesis needs further investigation.

Taking all of the facts and findings presented here into account, it is feasible to believe that honey could be seen as a fermented food product [23] because of the extensive LAB flora operating on nectar sugars in the honey stomach. Another reason for regarding honey as a fermented food product is that compared with plain sugars, honey is enriched by flavour, aroma, and texture, which could be caused in part by the LAB metabolites it contains [15].

Early in spring 2006, when the wintering period came to an end, we observed a low concentration of lactobacilli and a complete lack of bifidobacteria ("c" in Fig. 2). This could be explained by the fact that neither nectar nor honey was available.

Furthermore, later in the spring, we noted that the larvae in the small beehive became infected with the pathogen *P. larvae* (Fig. 2 and cluster II in Fig. 3), which is responsible for the larval disease AFB [9]. Surprisingly, the numbers of *P. larvae* decreased from the 8,000,000 CFU/larva ("e" in Fig. 2) found at one point and vanished 3 weeks later without the larvae developing AFB.

Interestingly, large numbers of four bacterial phylotypes (LvLi2, Lv2, Hma5, and Trm1) were found during the period when both of the bee colonies were infected with *P. larvae* (Fig. 2). These bacteria were most closely related to the genera *Actinobacillus* and *Phocoenobacter*, which belong to the family *Pasteurellaceae* (Fig. 3, cluster IV) and they were found in almost the same kind of samples as *P. larvae* (Fig. 2). These four phylotypes may comprise a novel genus because their sequences only resembled the closest known taxum by 87.8% to 89.2% (Table 1), which is higher than the threshold level generally used to define a genus (91% to 95%) [13]. In previous studies, [1, 11] clones with high sequence similarities have been obtained from the guts of honey bees (cluster IV in Fig. 3), which verifies our results. It is likely that *P. larvae*, *Pasteurellaceae* phylotypes and the honey-stomach LAB flora influence each other, thus affecting their numbers. In fact, efforts have been made to combat honeybee pathogens by use of probiotics by exposing honeybees or their larvae to nonpathogenic bacteria [6, 20]. The use of *Lactobacillus* and *Bifidobacterium* species as probiotics has been found to enhance the immunity of honeybees, helping them to survive attacks of pathogens [6].

Treatment by formic, lactic, and acetic acid is widely employed by beekeepers to guard against such honeybee pathogens as *Varroa destructor* and *Nosema apis*. Organic acidsSsuch as formic acid, which is produced by bifidobacteria [16], and both lactic and acetic acid, which are produced by LAB discovered in the honey stomachare antimicrobial substances, meaning that these bacteria may be of considerable importance in protecting honeybees against pathogens. Our

results can be seen as a contribution to the development of probiotic treatments for bee colonies.

On the basis of the findings obtained, it appears that honeybees and the novel LAB flora discovered evolved in mutual dependence on one another: The LAB obtained a niche in which nutrients were available, and the honeybees were protected by the LAB from harmful microorganisms. This is also supported by the observation that symbiosis between social insect species and microbial species is common, with the two partners coevolving [7]. What we have discovered can be seen as perhaps the beginning of a new line of research in which far more extensive knowledge of honeybees health, production, and storage of honey can be achieved.

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