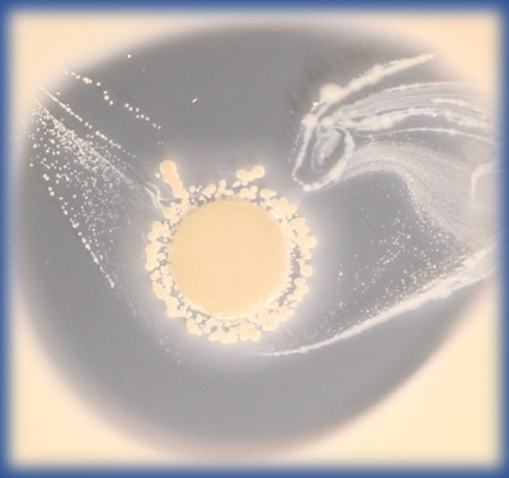

Prevention of diseases in insect farming systems

Master's Thesis

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CONTENTS

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SUMMARY

The integration of good management practice in insect production systems is important for a successful large-scale insect production. Management of diseases in insect production systems is especially important since the presence of insect pathogens generates a negative impact on the insect production. Lactic acid bacteria (*Lactobacillus kunkeei*, *Lactobacillus apinorum*; LAB mixtures) isolated from honey stomach of honey bees produce antimicrobial compounds that can inhibit the activity of different insect pathogens. Therefore, such beneficial bacteria (LAB) can be considered for use as antibiotics in the management of diseases in insect production systems. In this study, the antimicrobial activity of LAB was evaluated on three insect pathogenic bacteria; *Serratia marcescens*, *Serratia plymuthica* and *Pseudomonas aeruginosa* and promising results were obtained. Thirteen honey bee LAB strains were tested individually and in LAB mixtures. Dual culture overlay assay was used to evaluate the antimicrobial activity of LAB against the tested insect pathogenic bacteria. Pathogenicity experiments were carried out in order to know what amount of *S. marcescens* ingested kills 50% population in a given period of time. *L. kunkeei* (Fhon2), *L. apinorum* (Fhon13) and the LAB mixtures had high antimicrobial activity on the pathogenic bacteria tested. *Serratia marcescens* was non-pathogenic *per os*. However, mortality was observed after intracelomic infection.

The results of this study suggest that the use of LAB might be beneficial in large scale insect production. Next step will be to evaluate the effects of LAB strains on the insect host, *T. molitor* larvae, in combination with insect pathogenic bacteria.

Key words: bacterial disease management, insects for feed and food, insect pathogenic bacteria, honey bee lactic acid bacteria

1 INTRODUCTION

Human population is growing rapidly and with it the demand of food, especially proteins and in specific animal proteins (Hubert, 2015). The current sources of animal protein take up many resources in terms of feedstock, water and land to such an extent that it will be more difficult to provide enough protein for the demand in the near future. This challenge give the opportunity to explore new solutions, such as insects which recently have been suggested as a sustainable animal protein source (Van Huis *et al.*, 2013).

Insects as a source of protein are very interesting due to their reduced demand of resources, and at the same time they have a lower environmental footprint if compared with common livestock productions (Oonincx and De Boer, 2012). Currently, insect farming is under development switching from small scale to industrialized systems – in particular in the industrial world. However, insects farmed in intensive production systems can be more susceptible to diseases (Eilenberg *et al.*, 2015), e.g. pathogenic bacteria, which can generate a negative impact on the insect production. Therefore, in order to prevent diseases, the adoption of good management practices in insect production (GPM) is a key issue to be targeted in early stages of this development to guarantee success in large-scale insect production.

In insect farming, the main parameters to be controlled are insect density, insect diet, relative humidity and temperature (Van Huis *et al.*, 2013). However, these conditions could also favor the growth of potential insect pathogenic bacteria e.g. *Serratia marcescens, Serratia plymuthica* and *Pseudomonas aeruginosa.* The management of these types of bacteria could be a challenge if not considered on time. A possible way to manage bacteria diseases in insect rearing is by using probiotics, such as lactic acid bacteria (LAB). They have been widely reported in literature for their positive effect on the digestive system, not only for humans or animals, but more recently also reported as beneficial for insects (Bermudez-Brito *et al.*, 2012). In literature, there is a relevant group of 13 LAB which have been isolated from the honey bee and could be an

alternative to antibiotics for the management of bacterial diseases in insect farming (Vasquez *et al.*, 2012).

The evaluation of LAB effectiveness on bacterial diseases could provide novel information relevant for insect farming. In order to understand the interactions between the organisms, i.e. beneficial bacteria, insect host and insect pathogenic bacteria, individual studies must consider biological characterization. In this case, the first step is to isolate and identify beneficial bacteria that can inhibit the growth of insect pathogenic bacteria.

This thesis focuses on the development and analysis of two main topics, each one with their own aim.

The aim of the **first topic** was to investigate the inhibition effect of 13 LAB isolated from the honey bee in the management of potential bacterial diseases caused by *S. marcescens* Mm3, *S. plymuthica* and *P. aeruginosa*.

The aim of the **second topic** was to evaluate the virulence of *S. marcescens* on *Tenebrio molitor* insect using two methods of inoculation, i.e. oral and intracelomic.

This thesis is organized in the following 9 chapters: Chapter 1 is the *Introduction*. Chapter 2 is dedicated to the *Background* or literature review in relation to Insect farming systems, diseases in rearing systems, LAB, pathogenic insects and the insect, as well as the interaction between them. In this section, the reader can also find general information which can be complementary to follow the subsequent sections more easily. Chapter 3 contains the aims of this research. In the subsequent chapters 4, 5, 6 and 7 contains the experimental work, outlined as: *Materials and Methods, Results, Discussion* and *Conclusions*, respectively. Chapter 8 outlines some *Perspectives* where the future work is suggested and discussed. Finally, Chapter 9 *Appendix* includes additional material and techniques which were also developed as part of this project, and were necessary to develop the main experiments.

2 BACKGROUND

2.1 INSECT FARMING SYSTEMS

Insect farming is the practice of rearing insects in a specific place outside their natural environment where living conditions such as density, diet, relative humidity and temperature are controlled (Figure 1). Insect farming is a term recently introduced. However, the production of insects such as honeybees and silkworms dates back many years. Insect farming can be categorized in two groups, i.e. small and industrial scale. And by definition, an industrial system should produce more than one ton of fresh weight of insects per day (Van Huis *et al.*, 2013).

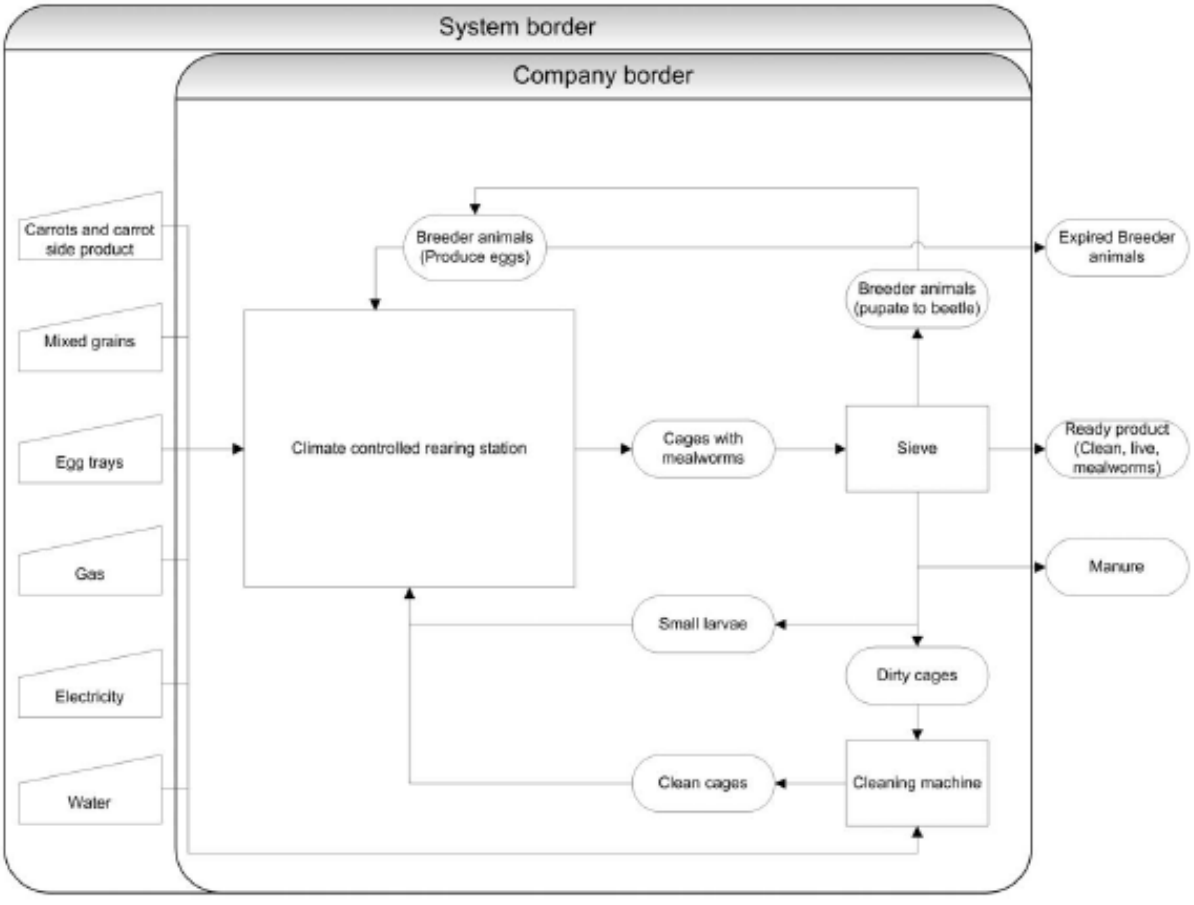


FIGURE 1. *"The mealworm production system. Flows entering the company are on the left, centrally the production steps are shown and flows exiting the system are on the right"* (Oonincx and De Boer, 2012).

A variety of products are obtained from the insects; protein, fats, chitin and dyes among other substances. Due to the high demand of protein in the world, the interest in the production of insects has increased rapidly (Van Huis *et al.*, 2013). Approximately 2000 species of insects are consumed in the world (Jongema, 2017), and most of these species are collected from nature in the tropics. For industrial purposes, 20 species are reared under the concept of insect farming and 12 of those species have high potential to be used as food and feed in the EU (Table 1) (EFSA, 2015).

Table 1. Potential insects species to be farmed in EU (EFSA, 2015)

Cientific name	Common name
Musca domestica	Common housefly
Hermetia illucens	Black soldier fly
Tenebrio molitor	Mealworm
Zophobas atratu	Giant mealworm
Alphitobus diaperinus	Lesser mealworm
Galleria mellonella	Greater wax moth
Achroia grisella	Lesser wax moth
Bombyx mori	Silkworm
Acheta domesticus	House cricket
Gryllodes sigillatus	Banded cricket
Locusta migratora migratorioides	African migratory locust
Schistocerca Americana	American grasshopper

Insect farming, as a new livestock, or mini-livestock, is a productive system that is facing many challenges while evolving in industry (Van Huis *et al.*, 2013). Some of the most important aspects in which research in insect production systems must focus are: conservation management schemes, food safety, breeding, economy, insect welfare, consumer attitudes and gastronomy, health benefits and disease management in rearing (van Huis, 2017).

2.2 DISEASES IN INSECT FARMING

Insects removed from nature and farmed in intensive systems are vulnerable to diseasescaused by pathogens. A pathogen is "A microorganism capable of producing disease under normal conditions of host resistance and rarely living in close association with the host without producing disease" " (Onstad *et al.*, 2006). The main insect pathogens groups are virus, bacteria, fungi, microsporidia and nematodes a detail information about this groups are described by Eilenberg *et al.* (2015) .

The history of the two oldest cultivated insects, *Bombyx mori* and *Apis mellifera,* indicates that diseases in insect rearing systems are a limiting factor for the production of insects. Insect pathogens affect insect production systems by reducing fitness or by killing an entire population (Eilenberg *et al.*, in press). Therefore, diseases are an important factor in monitoring and controlling insect farming systems. Some diseases and causal agents of *Bombyx mori* and *Apis mellifera* are shown in Table 2 (Eilenberg *et al.*, 2015).

Table 2. Diseases in *Bombyx mori* and *Apis mellifera* (Eilenberg *et al.*, 2015)

Insect	Bacteria	Fungi	Microsporidia	Virus
Apis mellifera	*Paenibacillus larvae*	*Ascosphaera apis*	*Nosema apis*	Acute bee paralysis virus (ABPV) (Dicistroviridae)
	Melissococcus plutonius	*Aspergillus sp.*	*Nosema ceranae*	Deformed wing virus (DWV) (Iflaviridae)
				Sacbrood virus (SBV) (picorna-like virus)
Bombyx mori	*Enterococcus faecalis*	*Beauveria bassiana*	*Nosema bombycis*	BmNPV, MNPV
	Enteroccus faecium	*Aspergillus flavus*		BmDNV-1
	Enteroccoccus mundtii	*Aspergillus oryzae*		DNV-2, DNV-3
	Serratia marcescens			BmCPV
	Proteus mirabilis			BmIFV

Production systems of insects for food and feed is an emerging industrial sector where the management conditions are focused on favoring the insects. Therefore, natural enemies are few and incidents generated by diseases are rare or even absent. In addition, the complexity of the life cycles of some pathogens reduces the list of diseases that could appear in insect

farming compared to those that occur in nature (Eilenberg *et al.*, in press). Nevertheless, diseases are expected to appear. Table 3 shows diseases in insects produced for food and feed reported in by producers in 2013.

Table 3. *"Diseases in insects produced for food and feed"* (Eilenberg *et al.*, 2015)

Insect	Fungi	Virus	Bacteria
Acheta domesticus	*Metarhizium sp.*	cricket paralysis virus (CrPV)	bacteria sp.
Tenebrio molitor	*Beauveria bassiana*	-	-
Musca dometica	*Entomophthora spp.*	-	-
Hermetia illucens	none	none	none

Management of diseases requires knowledge about the host, the pathogen, and their interaction in a specific environment. The absence of information on diseases gives an opportunity to conduct specific studies e.g. "biological and genetic characterization, phylogeny, host range, transmission, persistence, epidemic potential and safety for animals including humans" (Eilenberg *et al.*, 2015). It also allows carrying out studies on the management of environmental conditions in facilities that minimize the possibility of establishing pathogens, such as density, diet, temperature, relative humidity and photoperiod (Morales-Ramos *et al.*, 2013).

Prevention practices of diseases in rearing systems which facilitate disease management may include the following: Identification of insects that do not perform as usual. Continuously cleaning the facilities. Organizing the production system in separate batches. Maintaining genetic diversity. Restricting the use of antibiotics to urgent cases (Eilenberg *et al.*, 2015).

Other prevention practices of diseases suggested by Vilcinskas (2017) were strengthening the immune systems in insects by using of probiotics and nutritional immunology. He also suggested the adoption of good manufacturing practices (GPM) and creating international safety standards in insect farming as prevention measures.

2.3 INSECT PATHOGENIC BACTERIA

Organisms are surrounded by bacteria and insects are not an exception. Bacteria can be outside of the insect body or in the gut of the insects (Vega, 2012). Entomopathogenic bacteria belong to the Eubacteria group, which is divided into three groups according to type of cell wall: the Gram-negative bacteria, the Gram-positive bacteria and finally Eubacteria without a cell wall. Gram positive bacteria associated with insects have been studied for biological control purposes, mainly the species e.g. *Bacillus thuringinesis.* The main infection route is the oral cavity (*per os*) but infection can be vectored by other organisms or bacteria can gain entrance after injury. Among the important genera of Gram-negative bacteria associated with insects are the family Enterobacteriaceae *Serratia spp.* and *Yersinia,* the family Pseudomonadaceae *Pseudomonas spp.* and the family Coxiellaceae *Rickettsiella spp* (Vega, 2012).

Serratia spp., Photorhabdus, Xenorhabdus, Pseudomonas, Yersinia and *Paenibacillus* have the ability to produce toxins. This complex has been denominated Toxic complex (*Tc)*. Toxin production is coded in the bacterial chromosome and also in the plasmid of the bacteria. The pathogenicity of bacteria is closely related to the production of toxins.

Gram-negative bacteria have six mechanisms of mobilizing toxic proteins outside of the cell. These mechanisms have been called Type I, II, II, IV, V and VI secretion systems. They are responsible for mobilizing the toxic proteins produced by the cell through the outer membrane. Furthermore, the secretory system Type III is able to create a molecular needle which aid injection of proteins into the cytoplasm of the host. The secretory system Type VI was recently discovered in *S. marcescens*. It is the most complex of these systems and several proteins are involved (Murdoch *et al.*, 2011). The secretory pathways are one of the factors that determine the virulence of a pathogen. For example: when a secretory system is removed with genetic techniques from the pathogenic bacteria, the virulence is lost (Gerlach and Hensel, 2007).

P. aeruginosa and *S. marcescens* have been described as opportunistic and potential pathogens. An opportunistic pathogen is “A microorganism which does not ordinarily cause disease but

which, under certain conditions (e.g., impaired host immunity), becomes pathogenic". Potential pathogen: "1) A microorganism that has no method of invading or infecting a host but can multiply and cause disease if it gains entrance, for example, though a wound; potential pathogens generally grow readily in culture and do not cause specific diseases in specific hosts (modified from Bucher 1963). 2) a secondary invader". (Onstad *et al.*, 2006).

2.3.1 *Serratia spp.*

The Genus *Serratia* includes Gram-negative, rod-shaped, aerobic, non-spore-forming and flagellate bacteria and belong to the family Enterobacteriaceae. Most species are saprophytes in decayed animals or plants. However, some are considered as opportunistic pathogens. It is widely distributed and can be found in air, soil, water, plants, vertebrates and invertebrates. Some of the species most representative of this genera are *S. marcescens, S. plymuthica, S. liquefaciens, S. proteamaculans, S. grimesii, S. rubidaea, S. odorifera, S. ficaria* and *S. entomophila* (Dworkin, 2006). Their ability to produce the red pigment prodigiosin is evident from some of the bacteria. It is, however, not of diagnostic value and a character to be used merely for taxonomic identification.

The temperature for optimal growth of *Serratia sp.* is 30°C. However, *Serratia sp.* can grow at up to temperatures of 37°C. Culture media suggested for *Serratia sp*. are Nutrient broth and Luria-Bertani broth with values of pH 7.3-7.7 and 6.8-7.2 respectively (Jackson *et al.*, 2001). This might indicate that a neutral pH is the optimal condition for these bacteria to grow.

Serratia sp. has been found in healthy, diseased and deceased insects. Some orders of insects associated to *Serratia sp.* are: Orthoptera, Isoptera, Coleoptera Lepidoptera, Hymenoptera and Diptera (Grimont and Grimont, 1978). The occurrences of *Serratia sp.* in invertebrates do not necessarily cause infection. For instance, wild and healthy populations of *Ips calligraph* (Coleoptera) can carry *S. marcescens* and the presence of *Serratia sp.* in the gut of insect does not necessarily generate infection. For example, examinations of the gut flora of healthy

grasshoppers *Neombius fasciatus* revealed the presence of *S. plymuthica* as a part of their intestinal flora (Grimont and Grimont, 1978).

Lethal effects of *Serratia spp.* in insects can occur once the bacteria reach the hemocoel. The insect gut has a low level of oxygen and is thus not ideal for the proliferation of *Serratia spp.* In contrast, the hemocoel offers a nutrient-rich environment where *Serratia spp*. can multiply and generate septicemia in the insect. Consequently, they are considered potential entomopathogens (Bucher, 1960).

The pathogenicity of *Serratia spp.* is closely linked to the strain and to their production of toxic enzymes that break down the immune system of the host. Some symptoms exhibited by insects after being infected with *Serratia sp.* are: Darkening, cessation of feeding, lethargy and mortality (Jackson *et al.*, 2001).

S. marcescens produces five chitinolytic enzymes (*Chi*) which are able to degrade chitin. The molecular mass of these proteins are 57, 52, 48, 36 and 21 kilodaltons (kDa) (Fuchs *et al.*, 1986). Chitinolytic enzymes might help penetrate the foregut cuticle of insects and facilitate bacterial infection. In addition, a Serralysin-like endopeptidase with an atomic mass of 52.2 kDa, extracted from *S. marcescens* was found to have an insecticidal effect to *Phyllophaga spp.* (Pineda-Castellanos *et al.*, 2015). Chitinolytic enzymes play an important role in the virulence of *Serratia sp.* towards insects (Lysenko, 1976). Furthermore, the use of chitinases in controlling pests is an alternative to chemical pesticides (Herrera-Estrella and Chet, 1999; Pineda-Castellanos *et al.*, 2015)

Serratia entomophila and *Serratia proteamaculans* carry the plasmid pADAP 115 kb that has been related to the pathogenicity of these two bacteria in the grass Grub *Costelytra zealandica*. The pADAP plasmid contains two genes called Sep toxin complex (Tc), and Antifeeding (Apf). These genes encode for the production of toxins, and antifeeding effect (Koppenhöfer *et al.*, 2012). Three fragments of (Tc) gene named *SepA, SepB, and SepC,* encode for the production of toxic proteins with insecticidal characteristics powerful enough to generate virulence in *C.*

zealandica. However, the gene able to induce cessation of feeding is located in another part of in the plasmid pADAP (Hurst *et al.*, 2000). *S. entomophila* adheres to the foregut of the grass Grub using fimbriae and colonizes the surface of the entire gut. The cells are especially numerous around the cardiac valve (Lacey and Kaya, 2007).

2.3.2 *Pseudomonas sp.*

Pseudomonas genera is a bacterium which is rod-shaped, strictly aerobic, flagellated. Almost all species have fimbriae and pilis and belong to the family of Pseudomonadaceae. They do not form spores and do not ferment sugars. *Pseudomonas sp* is found in decomposing organic material and they play an important role in degradation. It is also found in water, flora, fauna and various environments around the world. Some of the species most representative of these genera are *P. putida, P. fluorescens, P. pseudomallei, P. mallei, P. cepacia, P. maltophila, P, picketti, P. infection, P. pneumonia, P. syringae P. entomophila, P. taiwanensis* and *P. aeruginosa* (Cabello, 2007).

Some species of this genus are pathogenic to insects. E.g. *P. entomophila* is pathogenic to *Drosophila sp. (*Vodovar *et al.*, 2005*), P. taiwanensis* is pathogenic to *Plutella xylostella, Spodoptera exigua, Spodoptera litura, Trichoplusia ni* and *Drosophila melanogaster* (Chen *et al.*, 2014) *and P. aeruginosa* is pathogenic to *Drosophila melanogaster* (D'Argenio *et al.*, 2001) and *Galleria mellonella* (Miyata *et al.*, 2003).

There are three characteristics that favor pathogenicity in *Pseudomonas spp.* First, the fimbriae and mucosae of the cell wall contribute to anchoring on the tissues of the host. Second, the production of a lipopolysaccharide-type endotoxin in the cell wall that breaks down the colonized cells. And third, the production of extracellular substances, which are toxic proteins also called toxic complex *TcA, TcB* and *TcC* (Cabello, 2007; Chen *et al.*, 2014).

P. aeruginosa is a pathogen bacterium that has mainly been found to infect insects in experiments under laboratory conditions (Krieg, 1987). This bacterium is considered a potential

pathogen and has never caused epizootics in field (Bucher, 1960). *P. aeruginosa* produces a blue pigment called pyocyanin (Lysenko, 1985).

P. taiwanensis, a novel species, can infect (*per os*) species from the orders Lepidoptera and Diptera. The toxic complex *TcC* plays an important role in the pathogenicity of *P. taiwanensis*. For example, the mortality of *P. xylostella* was 94.5% on the fifth day after oral inoculation with *P. taiwanensis* and a cell concentration of 0.5 OD_{600} (Chen *et al.*, 2014).

P. entomophila is an entomopathogenic bacterium with the ability to infect after oral inoculation various orders of insects. It is pathogenic to larvae and adults of *Drosophila* sp. It has the ability to break the cells of the stomach of the host, allowing the pathogen to enter the hemocoel. The virulence factors of this bacterium are associated with the production of insecticidal toxins, proteases, putative hemolysins, hydrogen cyanide and secondary metabolites (Vodovar *et al.*, 2006; Vodovar *et al.*, 2005).

2.4 PROBIOTICS AND LACTIC ACID BACTERIA

The concept of probiotics has been proposed by different scientists and has been subtly changed over time. At the beginning of the 20th century, Stamen Grigorov, Élie Metchnikoff and Henry Tissier were pioneers in scientifically recognizing the existence of beneficial bacteria and their ability to generate positive effects in the host organism (Ozen and Dinleyici, 2015). Subsequently, different authors have contributed to the evolution of the concept which is described in the history of probiotics (FAO/WHO, 2001). A recent definition was suggested by FAO/WHO in 2001 as "*live microorganisms which when administered in adequate amounts confer health benefit on the host*". In 2014, The International Scientific Association for Probiotics and Prebiotics approved that definition (Hill *et al.*, 2014).

The genera that compromise lactic acid bacteria (LAB) are *Lactobacillus, Lactococcus, Streptococcus, Pediococcus, Leuconostoc, Weissella, Carnobacterium, Tetragenococcus,* and *Bifidobacterium.* LAB are Gram-positive, rod-shaped or spherical and have a strong tolerance to

low pH. They grow mainly under anaerobic conditions, and they are non-spore forming. These bacteria produce lactic acid as a major end product of the carbohydrate fermentation (Klein *et al.*, 1998).

LAB are grouped into two categories. i) *Lactobacillus, Lactococcus and Streptococcus* and ii) *Bifidobacterium*. The second group differs from *Lactobacillus, Lactococcus and Streptococcus* in the content of G+C guanine plus cytosine. Bifidobacteria has >50 mol% G+C (Actinobacteria) content while the others have <50 mol% G+C content (Firmicutes). However, they belong to the group of LAB due to their production of Lactic acid (Table 4) (Coenye and Vandamme, 2003).

Table 4. Phylogenetically division of Lactic Acid Bacteria.

LAB	Groups of LAB
*Lactobacillus**	<50 mol% G+C Firmicutes
Lactococcus	
Streptococcus	
*Bifidobacterium**	>50 mol% G+C Actinobacteria

* Used commercially as probiotics

The genera of LAB currently used commercially as probiotics include *Lactobacillus* and *Bifidobacterium.* LAB are usually found in the gut of animals and in fermented food (Klein *et al.*, 1998). Probiotics have, however, been used since ancient times in the food production for humans with no one clearly understanding their benefit. Recently, probiotics have been studied with the purpose of understanding their effect on growth rates, feed intakes, nutrient digestibility, control and prevention of diseases of livestock such as poultry, pigs, and ruminants. The results of studies conducted to evaluate growth rates, feed intakes and nutrient digestibility are not consistent enough. In contrast, studies on the prevention and/or cure of diseases are a bit clearer. Thus, by using LAB it is possible to reduce the negative effects of pathogens in the host (FAO, 2016).

The mechanisms of action in probiotics are not fully clear. The positive effects of probiotics in the host is probably a combination of many interactions. Some of the mechanisms of actions are: enhancement of the epithelial barrier, increased adhesion to gut mucosa, inhibition of pathogens adhesion, competitive exclusion of pathogenic microorganisms, production of antimicrobial substances and modulation of the immune system (Figure 2) (Bermudez-Brito *et al.*, 2012).

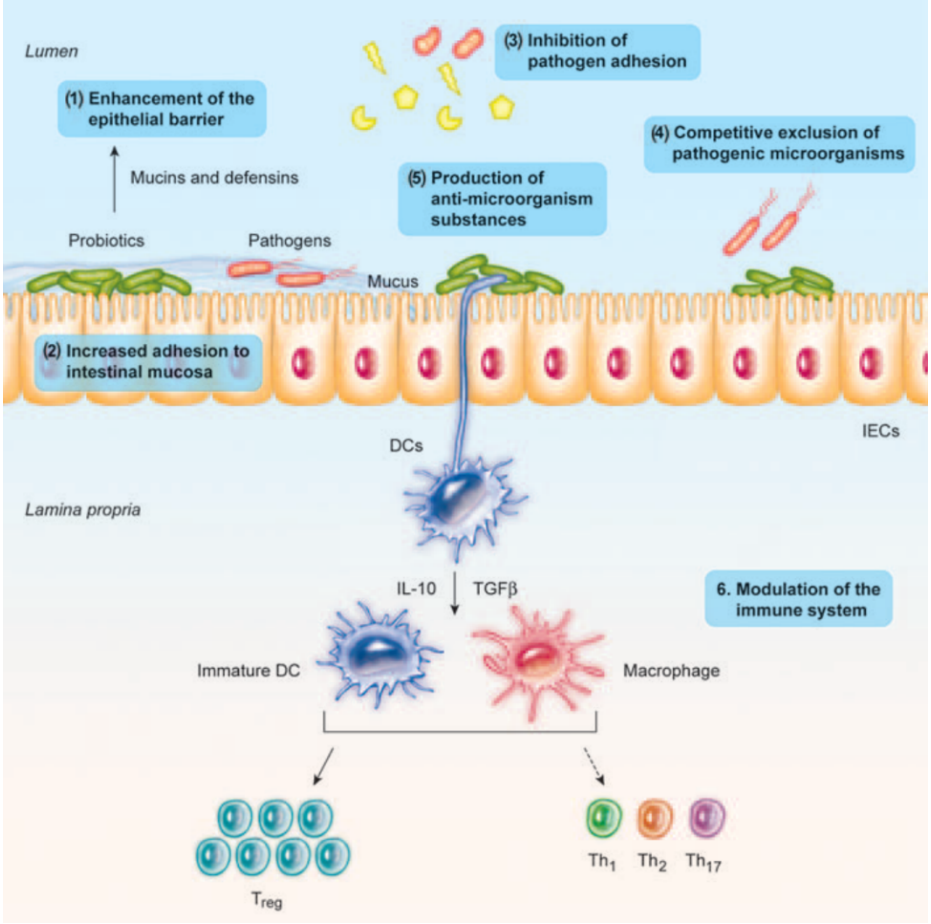


FIGURE 2. *"Major mechanisms of action of probiotics"* (Bermudez-Brito *et al.,* 2012).

Antimicrobial substances are produced by some genera of LAB which generate growth inhibition of pathogenic microorganisms. Organic acids and bacteriocin are responsible for the

antimicrobial effect. Gram negatives bacteria are inhibited by organic acids such as acetic and lactic acid. Bacteriocin is an antibacterial peptide produced mainly by gram-positive bacteria, and they usually inhibit the growth of similar or closely related bacteria (Bermudez-Brito *et al.*, 2012).

The antimicrobial activity of LAB against pathogens can be evaluated *in vitro* using a dual culture overlay technique. Dual culture overlay is a method that permits assessing the inhibition of one microorganism over another. It consists in overlapping the culture growth of two microorganisms. In the first layer, the microorganism which produces antimicrobial substances is established. The second layer contains the microorganism to be inhibited. There are expectations of an area where the inhibited microorganism is not able to grow (inhibition zone) (Butler *et al.*, 2013; Piccart *et al.*, 2016).

Honeybees collect beneficial bacteria (LAB) from the flowers and store them in their bodies. Strains of lactic acid bacteria, symbionts of honeybees *Apis mellifera*, have been isolated from the honey stomach of honeybees and identified to strain level. They are *Lactobacillus kunkeei* Fhon2*, Lactobacillus apinorum* Fhon13, *Lactobacillus mellis* Hon2, *Lactobacillus mellifera* Bin4, *Lactobacillus kullabergensis* Biut2, *Lactobacillus kimbladii* Hma2, *Lactobacillus helsingborgensis* Bma5, *Lactobacillus melliventris* Hma8, *Lactobacillus apis* Hma11, *Bifidobacterium coryneforme* Bma 6, *Bifidobacterium asteroides* Bin2, *Bifidobacterium sp.* Bin7, *Bifidobacterium sp.* Hma3 (Olofsson *et al.*, 2016b).

LAB from honeybees have a great capability of inhibiting microorganisms. They are able to inhibit pathogenic bacteria in humans, horses as well as insects. Their antimicrobial activity is due to the production of organic acids, for instance, acetic, formic and lactic acid. In addition, phylotypes *Lactobacillus kunkeei* Phon 2, *Lactobacillus apinorum* Fhon13, *B. asteroids Bin 2* and *Bifidobacterium sp.* Bin7 are able to produce fatty acids (Kabara *et al.*, 1972; Olofsson *et al.*, 2016a).

Some examples of human pathogenic bacteria inhibited by LAB from honeybees are *P. aeruginosa* and *S. marcescens (*Olofsson *et al.*, 2016b*).* These two are also potential pathogens in insects. *Mellisscoccus plutonius* (Vasquez *et al.*, 2012) , *Paenibacillun larvae* (Olofsson and Vásquez, 2008), pathogens in honeybees have also been inhibited by LAB.

The discovery of these novel bacteria and the verification of their benefits by researchers have led to the patenting and industrial commercialization of these LAB. The availability of these organisms in the market allows for potential users.

2.5 *TENEBRIO MOLITOR*

T. molitor is a cosmopolitan beetle, an important pest in stored grains and flours in several regions of the world (Ramos-Elorduy *et al.*, 2002). It belongs to the family Tenebrionidae, described by Linnaeus in 1758 within the genus Tenebrio, because it has a marginalized thorax, moniliform antennas with the last flagella widened and an oblong body.

The life cycle in *T. molitor* is divided in four stages, egg, larvae, pupa and adult (Figure 3). It is a holometabolan insect. This means that they go through a complete metamorphosis. The duration of the life cycle is a function of the temperature. Consequently, it can range from months to a couple of years. Adult females lay hundreds of oval shaped, individual white eggs coated with a sticky film. The eggs hatch, giving rise to small white larvae, which turn yellow with a brownish pigmentation in the anterior and posterior part of the body. Larvae can molt up to 20 times. Then, the pupal stage begins and individuals cease to eat. Initially, they are white and then they turn darkish yellow. Finally, the pupae transform into a white beetle that changes colors to red and then to black. At this point, they are ready to start the cycle again. Detailed information on the characteristics and duration of the 20 larval instars of *T. molitor* is given Park *et al.* (2014).

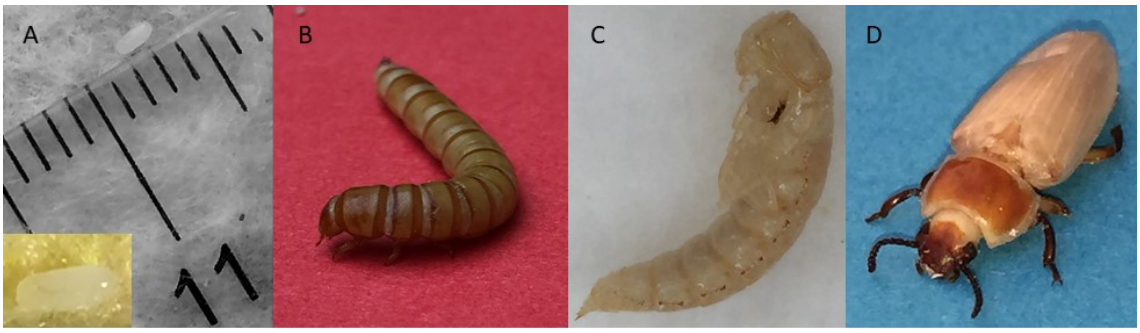


FIGURE 3. Stages of the life cycle *T. molitor* (A) Egg, (B) Larvae, (C) Pupae and (D) Adult

T. molitor has been the object of scientific studies in several areas of research. It has been widely used as a model for different ecological (Li *et al.*, 2016), physiological and biochemical studies (Beton *et al.*, 2012; Paul *et al.*, 2017). Recently, it has become very popular for animal feeding and for human consumption (Caparros Megido *et al.*, 2014; Laconisi *et al.*, 2017; Piccolo *et al.*, 2017). The content of proteins and lipids are adequate to be considered as a complement or substitute of traditional sources of protein and lipids such as fish meal and soybeans. Detailed information about the values of proteins and lipids are described in the literature (Jones *et al.*, 1972; Nowak *et al.*, 2016; Ramos-Elorduy *et al.*, 2002). The larvae contain more than 10 types of fatty acids (Paul *et al.*, 2017). Besides, it is easy to rear them on the laboratory level due to their easy adaptation to different temperatures (25-37ºC) (de Souza *et al.*, 2015).

The digestive tract of *T. molitor*, as for almost all chewing insects, is a straight tube that runs from the mouth to the anus, divided into three main regions: the stomodeum or foregut, the mesenteron or midgut, and the proctodeum or hindgut. Most of the nutrient absorption takes place in the midgut (Figure 4), which is protected by a peritrophic membrane separating the nutritional bolus from the midgut epithelium. This membrane has several functions, including i) protecting the intestinal epithelium from mechanical damage caused by hard food (in the case of insects that feed on solids, ii) protection against any chemicals that could be contained in the food, iii) protection against some pathogens. Moreover, the large size of pore the peritrophic membrane allows for passage and permits the establishment of some symbiotic bacteria (Chapman, 1998).

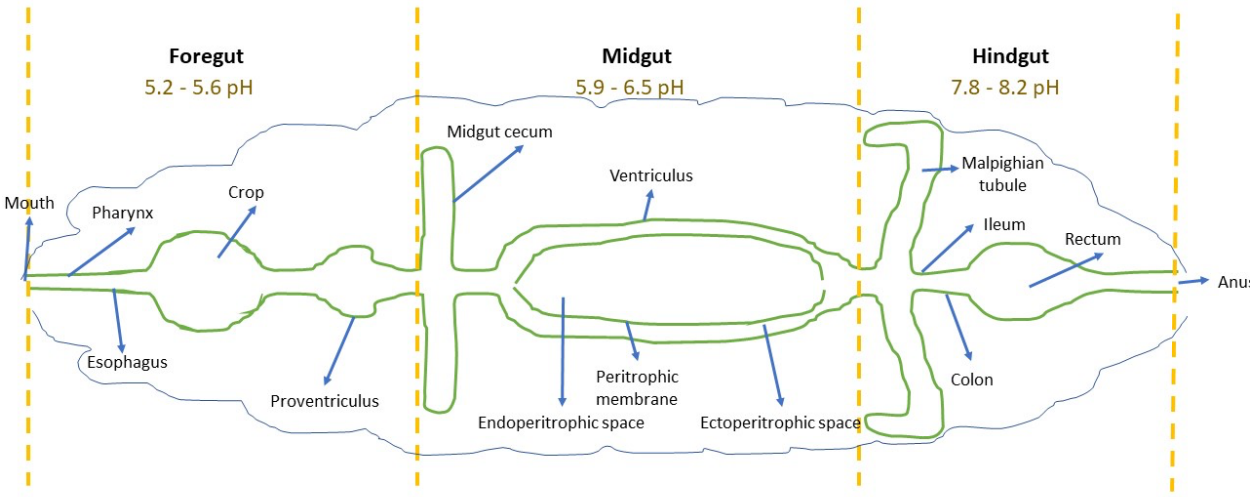


Figure 4. The insect gut. Adapted from Terra and Ferreira (2012).

Biochemical and molecular characterization of *T. molitor* guts have provided valuable information about digestive enzymes that break down macromolecules such as carbohydrates, lipids and proteins in the food digestion process (Terra *et al.*, 1985). Larvae and the adult of *T. molitor* have a great proteolytic activity in the gut, whereby several enzymes are produced in their digestion process (Chapman, 1998). The high level of diversity of the proteins in the gut of *T. molitor* is caused by the extreme gradient of pH. The anterior midgut pH is 5.2-5.6, middle part 5.9-6.5 and posterior midgut 7.8-8.2. In addition, these variations of pH indicate that they have a non-specialized digestion system giving them the advantage to be reared in a wide range of subtracts (Moreira *et al.*, 2017; Vinokurov *et al.*, 2006).

There are two big groups of proteinases in the midgut of *T. molitor* larvae. The first group, Cysteine proteinases have their activity located mainly in the anterior midgut where the predominant pH is 5.2-5.6. In contrast, the second group of Serine proteinases have a higher

activity in the posterior midgut at pH 7.8–8.2 (Vinokurov *et al.*, 2006). This particular contrast of pH is found in only a few groups of insects.

The most representative digestive proteases of these two groups in *T. molitor* are Cathepsin L-like, the major cysteine proteases, with a major activity in the anterior midgut (Beton *et al.*, 2012) and Prolyl carboxypeptidase, a serine protease present in the posterior midgut (Goptar *et al.*, 2013). Moreover, it has been found that many of the enzymes that improve digestion in this species are recycled through the peritrophic membrane by a counter-flow caused by the absorption and excretion of water in the anterior and posterior portions of the midgut respectively (Moreira *et al.*, 2017; Terra *et al.*, 1985). Another special feature of such complex digestive system is that the cells of the midgut are renewed every four days (Chapman, 1998). This indicates that *T. molitor* has a new machine to break down proteins every four days.

On the other hand, some digestion proteins increase when a pathogen is present. The expression of Serine peptidases is affected when the pathogenic bacteria *Bacillus thuringiensis* is present in *T. molitor.* As an example, a higher concentration of serine peptidases was observed in crops of *T. molitor* larvae exposed to a pathogenic strain of *B. thuringiensis* compared with the control groups. In contrast, the production of cysteine peptidases remained constant in the treated insects (Oppert *et al.*, 2012).

T. molitor produces toxic proteins or antimicrobial peptides as a protection mechanism against pathogens. Tenecin 1,2,3 and 4 are proteins expressed when a pathogen is present in the insect. The hemocytes can identify the type of the invader and induce the production of the specific toxin needed. Tenecin 1 is expressed when Gram positive or fungal pathogens e.g. *Candida alcicans* are present in the insect (Moon *et al.*, 1994). Tenecin 2 and 4 are induced when Gram negative pathogens are identified (Roh *et al.*, 2009) and (Chae *et al.*, 2012). Tenecin 3 is expressed in the presence of fungi (Lee *et al.*, 1999). However, the mechanism of the antimicrobial activity remains unknown (de Souza *et al.*, 2015).

The role of the diversity of microorganisms inhabiting the gut of *T. molitor* is far from understood. Recently, scientists have focused on gut composition and function of these communities that coexists inside the stomach of *T. molitor*. Microorganisms in the gut make beneficial contributions by enhancing the digestion processes by reducing big molecules into simple forms, thus making the digestibility of feeding materials easier while promoting immunoactivity. They can also help with the protection against enteropathogenic organisms by competition. The composition of microbial communities in the gut changes depending on the diet and rearing system conditions (Li *et al.*, 2016)**.** However, it has also been found that a novel *Spiroplasma* is one of the most abundant microorganisms in the digestive tract of healthy larvae of *T. molitor* (Jung *et al.*, 2014).

2.6 INTERACTION OF PATHOGENIC BACTERIA AND INSECTS

Insects and bacteria have co-evolved for millions of years establishing symbiotic relationships, commensalism and pathogenesis. Interaction of insects and bacterial pathogens involves a sequence of events in the insect body (Figure 5). Pathogenic bacteria usually enter through the mouth of the host and start the process of colonization and establishment, also called phase lag (Figure 6), and the production of toxins (Lacey and Kaya, 2007; Vega, 2012). Some insects, as a first mechanism of protection, contain antimicrobial compounds in the salivary fluids. For example, *Helicoverpa zea* saliva is able to minimize infectivity (Musser *et al.*, 2005). Then, food and bacteria cells move through the foregut together until they reach the midgut. The foregut, probably, provides less resistance to bacterial infections than midgut. For example, *S. entomophila* is able to adhere to the foregut in *C. zealandica* (Wilson *et al.*, 1992).

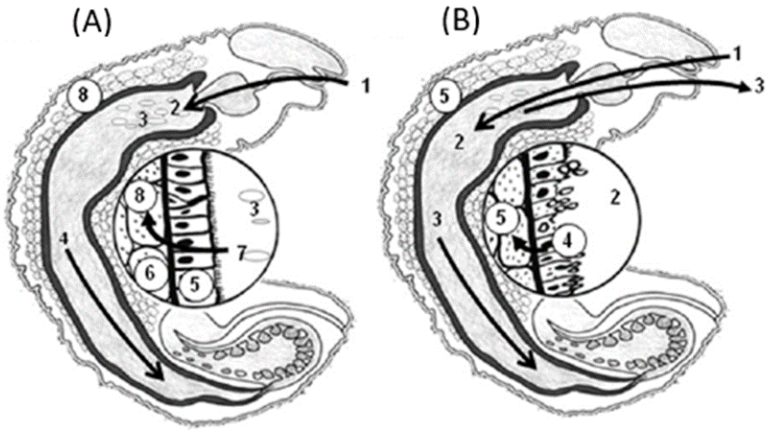


Figure 5. "*(A) Disease process of Amber disease of Costelytra zealandica. 1. Ingestion of bacteria. 2. Colonization of particulate matter and cuticular surfaces. 3. Release of Afp and Sep. 4. Cessation of feeding and gut clearance. 5. Blocking of enzyme release in the cell. 6. Fat body autoconsumption. 7. Transit of the midgut lamina. 8. Septicemia and death. (B) Disease process of Yersinia entomophaga infection of C. zealandica. 1. Ingestion of bacteria. 2. Constitutive release of Tc toxin. 3. Vomiting and purging of the gut. 4. Degradation of the midgut epithelium and invasion of the hemocoel. 5. Septicemia and death*". Adapted from Jacson *et al.,* (2012).

In contrast, the midgut has mechanical and physicochemical barriers to prevent the entrance and establishment of pathogenic bacteria. The peritrophic membrane is a great mechanical barrier of protection in the midgut. In addition, the midgut also has specific physical and chemical conditions, for example pH, ionic strength and potential redox, that can prevent or in some cases facilitate the establishment of bacteria. For instance, the physicochemical conditions of the gut in *C. zealandica* favor the infection process of *S. entomophila* (Hurst *et al.*, 2007). Furthermore, protection of insects against bacterial pathogens is enhanced by the flora present in the gut that help prevent the establishment of pathogens (Vega, 2012).

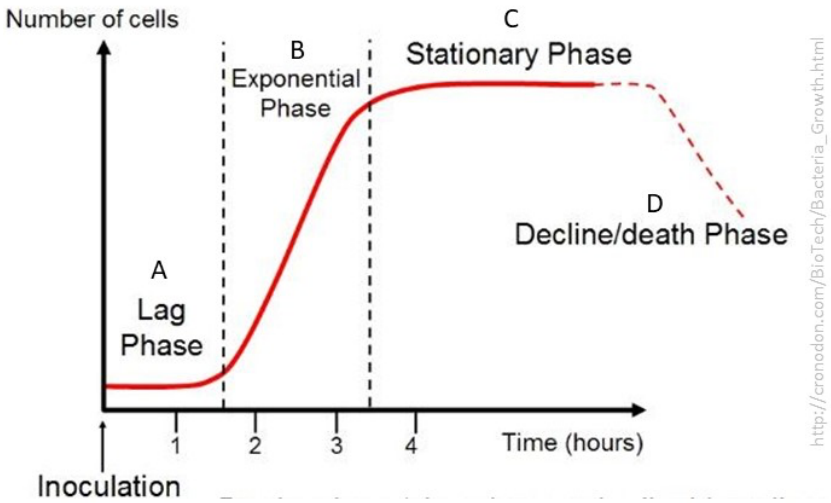


Figure 6. Phases of bacterial growth in close systems. (A) Lag (B) Log or exponential (C) Stationary (D) Death.

Successful bacteria able to produce infection are those that have overcome and entered to the hemocoel, a process described in (Figure 5). Pathogenic bacteria within the hemocoel start the exponential growth or log phase which consist in rapid proliferation by binary fission. On the other hand, it is possible that bacteria enter directly to the hemocoel of the insect after injury or vectored by another organism.

In the hemocoel, the immune system of the insects reacts to the foreign body by producing cell and humoral responses (Figure 7). They do that by phagocytizing or encapsulating the foreign body or inducing the transcription of genes for the production of antimicrobial proteins (Gillespie and Kanost, 1997). Gram-negative bacteria have a compound in the outer membrane that contains lipopolysaccharide (LPS). Hemocytes recognize LPS from gram-negative bacteria, and a chain reaction is initiated. Subsequently, the fat body starts to produce antimicrobial peptides (Vega, 2012)

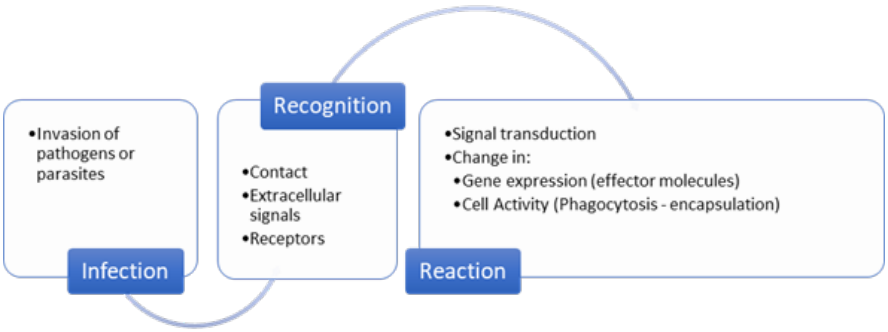


Figure 7. *"A model for insect immune responses"*. Adapted from Gillespie *et al.,* (1997).

Although, insects do not have lymphocytes or an antibody-based immune system, some studies have shown that it is possible to increase the protection system of an invertebrate to a pathogen by previous exposure to the same pathogen or other estrange body (Krams *et al.*, 2013). This protection system is known as "immune priming" (Cooper and Eleftherianos, 2017; Roth *et al.*, 2009). For example, the immune system of *T. molitor* is activated with previous exposures to nylon microfibers, and the immunological response to it is encapsulation. The encapsulation of pathogens is one of the best studied immunological responses in insects (Krams *et al.*, 2013). Hemocytes recognize the foreign organism (or object) and aggregate around them (Gillespie and Kanost, 1997). However, some pathogenic bacteria can survive in both intra- and extracellular insect tissues for subsequent reinfection. For example: *Staphylococcus aureus* applied in sub-lethal doses may remain viable in hemolymph of *T. molitor* (imago) for up to 21-28 days after infection by injection (McGonigle *et al.*, 2016).

3 AIMS OF THIS RESEARCH

General aim

In order to investigate the effect of Lactic Acid Bacteria from honeybee in the management of potential bacterial diseases caused by *S. marcescens* Mm3 and *S. plymuthica* an *P. aeruginosa*, all potential bacterial disease in *T. molitor* reared for feed and food, the following two specific aims are target in this work:

Specific aims

1) To evaluate the antimicrobial activity (*in-vitro)* of 13 strains of lactic acid bacteria from honeybees on *S. marcescens* Mm3 and *S. plymuthica* and *P. aeruginosa.*

2) To evaluate the oral and intracelomic virulence of *S. Marcescens* Mm3 towards *T. molitor* in order to determine the lethal doses. Lethal doses will then be challenged with LAB in the *in-vivo* experiments.

Hypothesis

Beneficial bacteria, lactic acid bacteria (LAB) are able to inhibit the pathogenic bacteria *S. marcescens*.

4 MATERIALS AND METHODS

4.1 BACTERIA AND INSECT

Lactic acid bacteria from honey bees

LAB from honeybees used in the experiments were isolated by the Honey Group collection, Department of Laboratory Medicine, Medical Microbiology, Lund University. The following 13 strains were used *Lactobacillus kunkeei* (Fhon2) *Lactobacillus apinorum* (Fhon13), *Bifidobacterium asteroids* (Bin2), *Bifidobacterium sp.* (Bin7), *Lactobacillus kimbladii* (Hma2), *Bifidobacterium sp.* (Hma3), *Lactobacillus helsingborgensis* (Bma5), *Lactobacillus mellis* (Hon2), *Lactobacillus kullabergensis* (Biut2), *Lactobacillus melliventris* (Hma8), *Lactobacillus apis* (Hma11), *Bifidobacterium coryneforme* (Bma6) and *Lactobacillus mellifer* (Bin4). The bacteria were obtained within an agreed collaboration between Lund University and University of Copenhagen.

Pathogenic Bacteria

S. Marcescens (Mm3) was purchased from DSMZ – the German Collection of Microorganisms and Cell Cultures GmbH. *S. plymuthica* was obtained from SOBI collection, University of Copenhagen. *P. aeruginosa* was obtained from the Lund University collection.

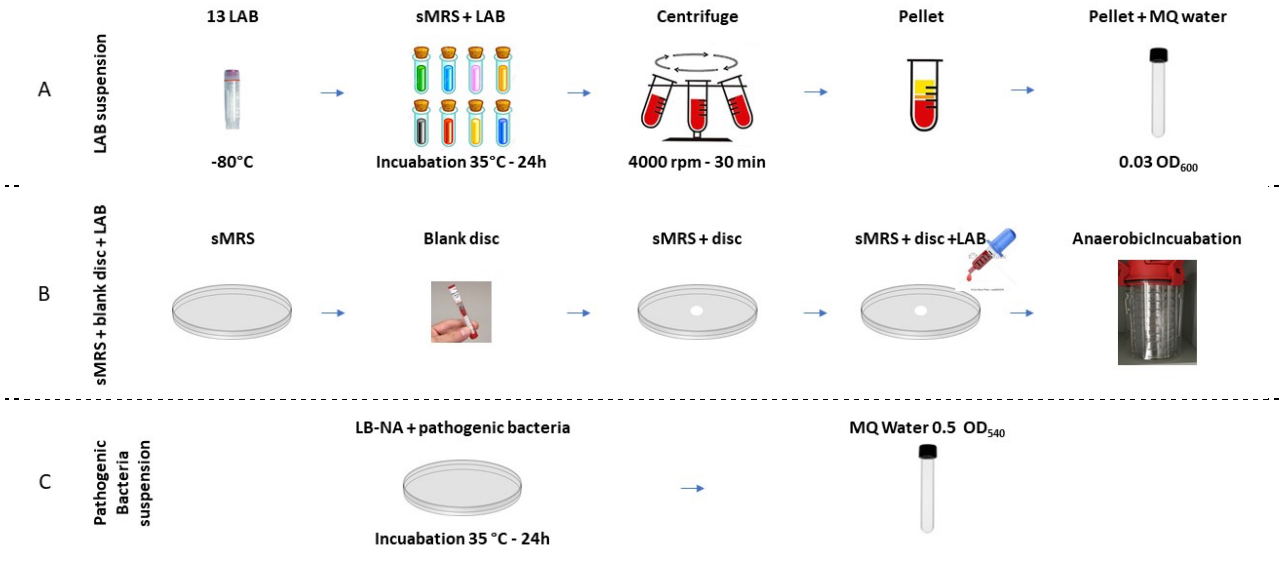
Insects

T. molitor larvae was purchased first in Avifauna Aps and then grown at University of Copenhagen for further experiments. The rearing method is reported in *Appendix* 9.1

4.2 DUAL-CULTURE OVERLAY ASSAY

Dual culture overlay assay was used to evaluate the antimicrobial activity of LAB against the tested insect pathogenic bacteria. LAB suspensions were prepared as explained in *Appendix* 9.2

on Bacterial suspension preparation. The concentrations of LAB were adjusted to 0.03 OD_{600} (Figure 8A). LAB-mixtures were prepared by blending equal volumes of a set of bacterial suspensions. Petri dishes with sMRS 1.6% agar medium were prepared and a blank disk was placed in the middle of each petri dish. LAB suspensions (10 mL) were added onto the filter disc and incubated anaerobically at 35°C for 24 h (Figure 8B). Insect pathogenic bacteria suspension was obtained as explained in the *Appendix* 9.2 Bacterial suspension preparation (Figure 8C). Cells were harvested and suspensions of 10^8 cells/ml (500mL) were prepared by mixing with their respective media (10 mL) and soft agar 0.8% at 42°C. This mix was carefully poured into petri dishes containing the discs with LAB and subsequently the petri dishes were placed in the incubator at 35°C for 24 h (Figure 8D). The inhibition zone was measured in millimeters from the middle of the disc to the edge of the inhibition zone which is the radius and doubled for the diameter. The dual-culture overlay assay methodology was implemented at the University of Copenhagen following the protocol used by Honey Group at the Department of Laboratory Medicine, Lund University.



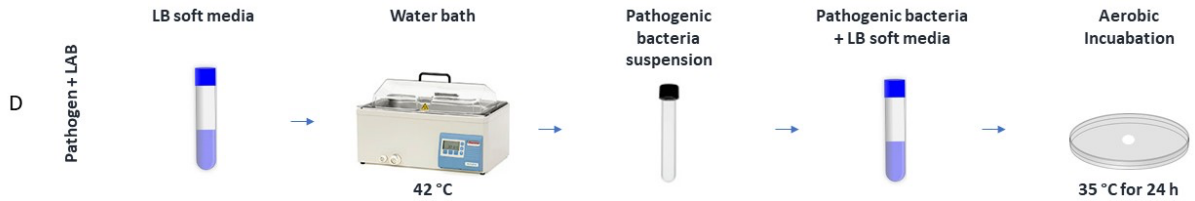


Figure 8. Dual-culture overlay assay. A) LAB suspension. B) LAB culture on solid media. C) Pathogenic bacteria suspension. D) LAB culture and pathogenic bacteria overlap.

4.3 INOCULATION OF *T. MOLITOR* WITH *S. MARCESCENS*

4.3.1 Oral inoculation

Experiments were established in order to know the amount of bacteria *S. marcescens* Mm3 ingested kills 50% of the population of *T. molitor* in a given period of time (LD_{50}). 240 middle size larvae from a 49-day-old insect rearing were selected and placed in an empty container to starve them for 24 hours before infection. Bacterial suspension was made as described in the *Appendix* 9.2 Bacterial suspension preparation. The concentration was adjusted to 10^6, 10^7, 10^8, 10^9 cells per mL. Pieces of 2x3 mm of potato were placed individually in 48-well cell culture plates. Then, 2 uL of bacterial suspension with concentrations adjusted was added to the piece of potato. This volume is equivalent on average to 4400, 44000, 440000, 4400000 bacterial cells per insect which is the dose (Table 5). Control groups received equal volume of sterile water. Starved larvae were introduced individually in the cell culture plates with the potato piece. After 24 hours, the larvae that had eaten the complete piece of potato (70% approx.) were transferred individually to a 6-well cell culture plates with 1g oats per well. Potato pieces (4x3mm) were given to each larva 3 times per week. The daily mortality percentage was evaluated during 15 days. The experiment was carried out under equal conditions of the insect rearing, which was at 28°C ±2 and 50-60% relative humidity (RH%) and repeated 3 times.

Table 5. Concentration of the suspensions used in each of the three replicates of the experiment oral

inoculation.

Concentration of the suspencion	Dose			
	Cells per insects - 2uL of suspencion			
Experiment	A	B	C	Avegare
10^6	6000	3660	3540	4400
10^7	60000	36600	35400	44000
10^8	600000	366000	354000	440000
10^9	6000000	3660000	3540000	4400000

Concentration of suspencion $10^{6.}$ in cells per mL A) 3.00, B) 1.83 and C) 1.77

4.3.2 Intracelomic inoculation

Intracelomic inoculation was performed in order to know if *S. marcescens* Mm3 was pathogenic or non- pathogenic when bacteria were injected in the hemocoel. 150 units of 56 days old larvae were selected to perform the exploratory experiment. Bacterial suspension was prepared and adjusted to concentrations of 10^3, 10^4, 10^5 and 10^6, cells per mL. 2 mL of these concentrations were injected into the larva in dorsal portion between second and third abdominal segments (figure 9A) with a 1 mL syringe and needle (0.3x13mm) with a manual injector having been previously calibrated (Figure 9B). This volume is equivalent to 11, 113, 1130, and 11320 bacterial cells per insect which is the dose. Control groups received an equal volume of sterile distillated water. Mortality percentage was evaluated during 10 days. The experiment was performed under equal conditions of the insect rearing and repeated once.

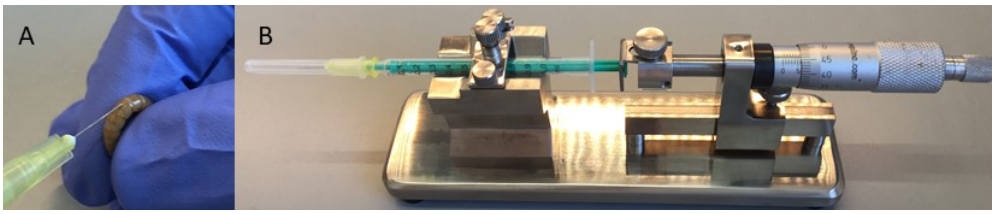


Figure 9. Intracelomic injection set.

5 RESULTS

5.1 DUAL-CULTURE OVERLAY ASSAY

This assay was run as described in Section 4.2 with 3 pathogenic bacteria and the 13 LAB selected for this study. Two sets of experiments were performed to complete the whole series. The first set, including *S. plymuthica* and *P. aeruginosa,* was tested in the laboratory of medicine at Lund University, Sweden. And the second set, including *S. marcescens,* was tested at SOBI, University of Copenhagen, Denmark.

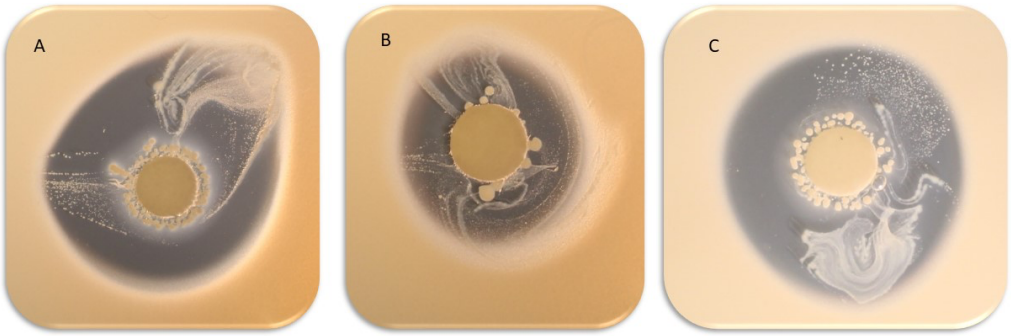


Figure 10. Dual culture overlay zones. Inhibition from honeybee LAB phylotypes A. *L. kunkeei* (Fhon2), B. *L. apinorum* (Fhon13) and C. LAB-Mixture on *S. marcescens* Mm3

L. kunkeei (Fhon2), *L. apinorum* (Fhon13), and the LAB-mixture (Figure 10) have presented the highest antimicrobial activity among the 13 strains of LAB evaluated on the pathogenic bacteria. The inhibition diameter was between 13,5 and 35 mm for the evaluated pathogenic bacteria. Figure 11 shows and compares the results obtained for this inhibition test.

Similarly, a clear inhibition zone for the pathogenic bacteria, *S. plymuthica* and *P. aeruginosa*, has been observed with 12 LAB from honeybee analyzed *L. kunkeei* (Fhon2), *L. apinorum* (Fhon13), *B. asteroids* (Bin2), *B.ifidobacterium sp*. (Bin7), *L. kimbladii* (Hma2), *Bifidobacterium sp*. (Hma3), *L. helsingborgensis* (Bma5), *L. mellis* (Hon2), *L. kullabergensis* (Biut2), *L. melliventris*

(Hma8), *L. apis* (Hma11), and *B. coryneforme* (Bma6)). The inhibition diameter for *S. plymuthica* was between 8 and 30 mm for the single LAB, and 35 mm for the LAB-mixture. Meanwhile for *P. aeruginosa*, the inhibition diameter was between 4 and 21 mm for the single LAB, and 31 mm for the LAB-mixture.

On the other hand, no inhibition was observed on *S. marcescens* Mm3 when tested with *B. asteroids* (Bin2), *Bifidobacterium sp*.(Bin7), *L. kimbladii* (Hma2), *Bifidobacterium sp.* (Hma3), *L. helsingborgensis* (Bma5), *L. mellis* (Hon2), *L. kullabergensis* (Biut2), *L. melliventris* (Hma8), *L. apis* (Hma11), *B. coryneforme* (Bma6) and *L. mellifer* (Bin4). Contrarily, *L. kunkeei* (Fhon2), *L. apinorum* (Fhon13), and the LAB-mixture showed an inhibition diameter between 13,5 and 22 mm.

It is worth noticing that none of the experiments using *L. mellifer* (Bin4) have shown inhibition to the 3 pathogenic bacteria tested.

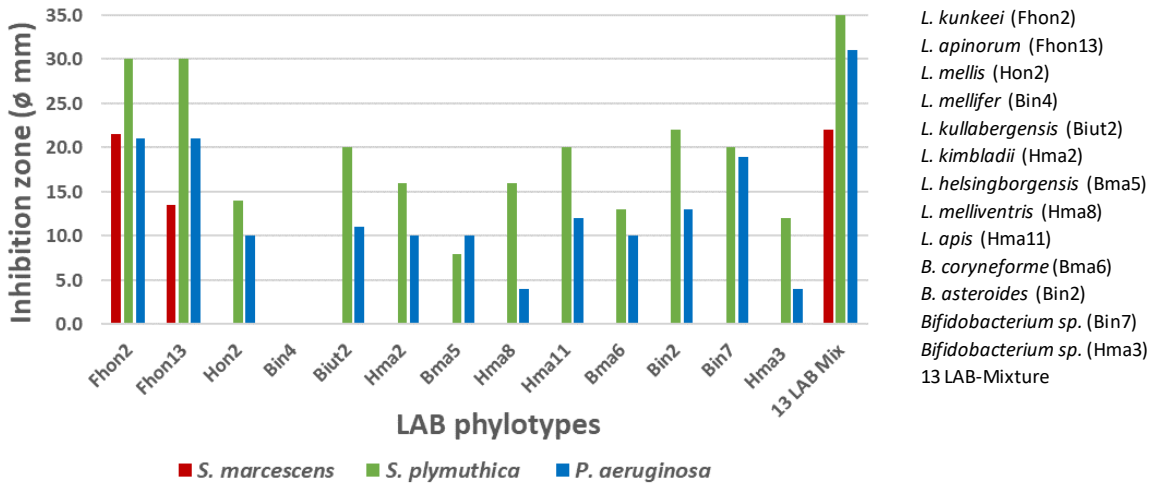


Figure 11. Inhibition zones of 13 lactic acid bacteria phylotypes (LAB) on three insect pathogenic bacteria *S. marcescens Mm3*[1], *S. plymuthica*[2] *P. aeruginosa*[2] (four[1] and two replicas[2]).

Several experiments were performed while the dual culture overlay technique was successfully established at the SOBI laboratories. The results obtained after the technique was successfully implemented at SOBI were analyzed with non-parametric statistical tests, Kruskal-Wallis test, Mann-Whitney Pairwise and Dunn post-hoc tests, since the data violated the statistical assumptions of homogeneity and normality of variance.

The Kruskal-Wallis test showed statistically significant differences between treatments ($p = 0.00008725$). The Mann-Whitney Pairwise and Dunn's post-hoc tests indicated that there were not statistically significant differences between the inhibition diameters generated by *L. kunkeei* (Fhon2) and LAB-mixture while there were differences statistically significant between *L. apinorum* (Fhon13) and both *L. kunkeei* (Fhon2) and LAB-mixture. However, statistically significant differences were observed between on the one hand *L. apinorum* (Fhon13), *L. kunkeei* (Fhon2) and the LAB- mixture and on the other hand the other single LAB evaluated.

5.2 INOCULATION OF *T. MOLITOR* WITH *S. MARCESCENS*

5.2.1 Oral Inoculation

This test was performed as described in Section 4.3.1 in order to evaluate the amount of bacteria *S. marcescens* Mm3 ingested needed to kill 50% of the population of *T. molitor* within a given time frame (LD_{50}). However, the results show that no mortality was registered in any of the doses 4400, 44000, 440000, 4400000 bacterial cells per mL or control groups.

A preliminary test was performed in order to ensure that the insects took in the full dose. The optimum size of potato to eat for a 49 days old larva was 2x3mm (70% of the population ate this piece). This potato size guaranteed that the desired number of bacterial cell was eaten.

5.2.2 **Intracelomic inoculation**

This test was performed as described in Section 4.3.2 in order to evaluate the amount of bacteria *S. marcescens* Mm3 injected needed to kill 50% population of *T. molitor* in a given time

(LD_{50}). The exploratory experiment of intracelomic inoculation shows that *S. Marcescens* Mm3 **was virulent** in doses of 11, 113, 1130, 11300 cells per insect, to *T. molitor* larva. The Larvae began to die at all doses 24 hours after infection, except in the control group. *S. Marcescens* Mm3 produced 50% of mortality on day 3 at doses of 113, 1130, 11300 cells per larva.

6 DISCUSSION

6.1 DUAL-CULTURE OVERLAY ASSAY

L. kunkeei (Fhon2), *L. apinorum* (Fhon13) and LAB-mixture consistently generated the largest zones of inhibition on different pathogenic bacteria. In our research results, *L. kunkeei* (Fhon2), *L. apinorum* (Fhon13) and LAB-mixture showed greater antimicrobial activity than the other individual LAB from honeybees. This is parallel to the results of (Olofsson *et al.*, 2016b) experiments, where *L. kunkeei* (Fhon2), *L. apinorum* (Fhon13) and LAB-mixture, generated the largest zones of inhibition on the pathogenic bacteria *S. marcescens* and *P. aeruginosa*.

In addition to the production of antimicrobial substances, it is important that LAB species are robust. *L. kunkeei* (Fhon2) and *L. apinorum* (Fhon13) show in our experiment notable characteristics such as rapid growth, resistance to changes in temperature and consistency over time when it comes to germination. This attribute of robustness, in addition to its great inhibiting effect, positions these two bacteria as potential candidates as probiotics for insect disease management. Figure 12 shows the growth kinetic of 13 LAB species in which *L. kunkeei* (Fhon2) and *L. apinorum* (Fhon13) stand out for their rapid growth over the others in the first 30 hours (Butler *et al.*, 2013)

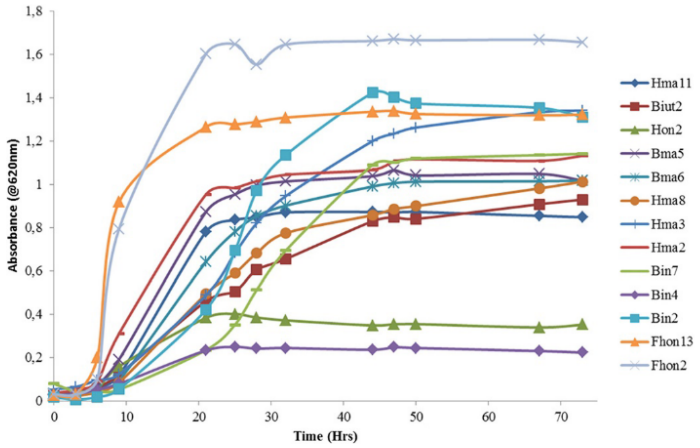


Figure 12." *Growth kinetic analysis of all 13 species of LAB 0–3 days. LAB were grown on MRS agar and changed into new MRS medium, and kinetic growth curves were measured in triplicate. All 13 LAB were measured from 0 to 72 hours at 620nanometers. This was performed to discover the different growth phases of the LAB and to determine when each enters early stationary phase*" (Butler *et al.*, 2013).

The bioactive products present in 13 LAB species complement each other. Each LAB produces a variety of antimicrobial substances such as organic acids, hydrogen peroxide, volatiles, fatty acids, among others (Olofsson *et al.*, 2016b). The use of LAB-mixture complements the actions of each of the other bacteria, generating a greater inhibition zone than when a single LAB is used. In our experiments, the LAB-mixture shows higher inhibition zones than the ones produced by single LAB. These results could be explained by the complementary effect that exists between the LAB compounds.

Furthermore, all of the single LAB, *L. kunkeei* (Fhon2), *L. apinorum* (Fhon13), *Bifidobacterium asteroids* (Bin2), *Bifidobacterium sp.* (Bin7), *Lactobacillus kimbladii* (Hma2), *Bifidobacterium sp.* (Hma3), *Lactobacillus helsingborgensis* (Bma5), *Lactobacillus mellis* (Hon2), *Lactobacillus kullabergensis* (Biut2), *Lactobacillus melliventris* (Hma8), *Lactobacillus apis* (Hma11) and *Bifidobacterium coryneforme* (Bma6) have the potential to inhibit pathogenic bacteria as show in our results, except *Lactobacillus mellifer* (Bin4). Nevertheless, *Lactobacillus mellifer* (Bin4) has shown its inhibitory potential in studies performed by (Olofsson *et al.*, 2016b), which is remarkable because it has the capacity to consistently inhibit several pathogenic bacteria.

Organic acids are mainly responsible for growth inhibition on Gram-negative bacteria. The entry of organic acids into the cells of Gram-negative bacteria lead to changes in pH that caused the death of pathogenic bacteria (Bermudez-Brito *et al.*, 2012). Three types of organic acids are produced by LAB from honeybees, acetic acid, formic and lactic (table 6). *L. kunkeei* (Fhon2), and *L. apinorum* (Fhon13) contain big amounts of total organic acids with a big participation of lactic acid in the total. However, *L. helsingborgensis* (Bma5), *L. mellis* (Hon2), *L. kullabergensis* (Biut2), *L. melliventris* (Hma8) produced values of total organic acids higher than *L. kunkeei* (Fhon2), and *L. apinorum* (Fhon13) but they did not inhibit as much as *L. kunkeei* (Fhon2).

Table 6. Organic acids produced by each of the 13 LAB.Adapted from (Olofsson *et al.,* 2016b)

		Organic acids		
Cientific name	Strain	Acetic acid	Formic acid	Lactic acid
Lactobacillus kunkeei	Fhon2	>263	>17	680
Lactobacillus apinorum	Fhon13	>327	>28	600
Bifidobacterium asteroides	Bin2	>302	>20	260
Bifidobacterium sp.	Bin7	>297	>25	420
Lactobacillus kimbladii	Hma2	>271	>16	710
Bifidobacterium sp.	Hma3	>294	>20	220
Lactobacillus helsingborgensis	Bma5	>267	>16	900
Lactobacillus mellis	Hon2	>290	>16	770
Lactobacillus kullabergensis	Biut2	>258	>14	950
Lactobacillus melliventris	Hma8	206.4	12.7	1060
Lactobacillus apis	Hma11	>306	>16	500
Bifidobacterium coryneforme	Bma6	208.2	13	260
Lactobacillus mellifer	Bin4	161.8	9.3	600

The values refered in this table are mg/sample

Lactic acid bacteria and Bifidobacteria are susceptible to lost viability. Varying degrees of difficulty in establishing LAB were observed. The difficulty levels were grouped into categories from 1 to 4 starting with the group that did not present difficulty in the establishment (Table 7). It has been reported that Lactobacillus and Bifidobacteria easily lose their viability during storage and during the freezing and thawing process (Collado, 2004; Fisher and Garczynski, 2012). Probably the degree of difficulty on culturing of LAB is associated with the loss of viability and the time of storage in our group of bacteria studied.

Table 7. Level of difficulty in the establishment of 13 LAB from honeybee.

Cientific name	Strain	Establishment difficulty level
Lactobacillus kunkeei	Fhon2	1
Lactobacillus apinorum	Fhon13	
Bifidobacterium asteroides	Bin2	2
Bifidobacterium sp.	Bin7	
Lactobacillus kimbladii	Hma2	
Bifidobacterium sp.	Hma3	
Lactobacillus helsingborgensis	Bma5	
Lactobacillus mellis	Hon2	3
Lactobacillus kullabergensis	Biut2	
Lactobacillus melliventris	Hma8	
Lactobacillus apis	Hma11	
Bifidobacterium coryneforme	Bma6	
Lactobacillus mellifer	Bin4	4

6.2 INOCULATION OF *T. MOLITOR* WITH *S. MARCESCENS*

S. marcescens Mm3, pathogenic bacteria of *Melolontha melolontha* (Ugras *et al.*, 2014) was not pathogenic to *T. molitor* when it was orally inoculated. All possible mechanisms of the *Serratia* sp. to produce infection such as the production of adhesins, *SepA SepB SepC* toxin, Serralysin-like, anti-feeding effect gene and chitinolytic enzymes, were not enough to infect *T. molitor*. Probably, the defense mechanism of *T. molitor* such as the production of serine peptidases, toxins Tenecin 2 and 4, the sharp gradient of pH or the diversity of microorganism in the gut were responsible for the mitigation of the infection.

Some examples could indicate that pathogenicity via the gut of *Serratia sp*. could be insect and strain specific. For instance, *S. entomophila* (154) and *S. proteamaculans* (2746 and 142) are specific pathogenic to *C. zealandica* (Tan *et al.*, 2006), *S. marcescens* Sm67, Sm81, Sm65, Sm89 and Sm73 are pathogenic to *Phyllophaga blanchardi* but non-pathogenic to *Spodoptera frugiperda* (Pineda-Castellanos *et al.*, 2015), *S. marcescens* Mm3 is pathogenic to *M. melolontha* (Ugras *et al.*, 2014). Still, our results show that it is not pathogenic to *T. molitor.*

In contrast, Lysenko (1985) suggests that pathogenicity could be influenced by the environment and biodiversity of micro-flora where insects are reared. Insects and bacteria can coexist, but changes in the environment could trigger the development of some of the existent bacteria and become pathogenic. The fact that the pathogenicity of some *Serratia sp* could be strain specific or influenced by the environment (Vega and Kaya, 2012) suggests that research should be performed with bacteria isolated from the studied insect and under the conditions where they present a problem, for instance an insect farming system.

The virulence generated by the doses tested in intracelomic inoculation experiments confirm that *S. marcescens* Mm3 is a "*potential pathogen, a microorganism that has no method of invading or infecting a host but can multiply and cause disease if it gains entrance*" (Bucher, 1960; Vega and Kaya, 2012) in *T. molitor* larvae.

Symptoms of pathogenicity were absent in larvae of *T. molitor* injected with pathogenic bacteria *S. marcescens* Mm3. After death, the insects presented dark pigmentation starting from the cephalic capsule and spread to the whole body (Figure 13). Thus, infections generated by *S. marcescens* Mm3 are difficult to identify with direct observation. The early diagnosis of diseases is fundamental to provide proper management of the diseased insect batch and prevent the dispersion of the pathogen.

Figure 13. Death larva post injection with pigmentation on the cephalic capsule.

Strengthening the bacterial diversity of the gut of *T. molitor* could be an option in order to minimize the negative effects of pathogenic bacteria. The coexistence of several microorganisms in an enclosed space creates competition for food and space (Li *et al.*, 2016). *T. molitor* have a non-specialized digestive tract making this possible to feed them with a variety of substrates (Moreira *et al.*, 2017; Vinokurov *et al.*, 2006). Therefore, insect diets made from industrial by-products could be a source of microorganism that increases or maintains the gut diversity while minimizing pathogen by competition (Bermudez-Brito *et al.*, 2012; FAO, 2016). For instance, this could be the use of beet molasses, potato steam peelings, spent grains and beer yeast and bread remains (Oonincx *et al.*, 2015). In any case, a prior evaluation of the by-product should be performed (Lysenko, 1985).

Enrichment of the diets with probiotics contributes to the reduction of possible epizootics. It has been shown that the addition of probiotics contributes to disease control in poultry, pigs, fish, humans (FAO, 2016) and some insects (Forsgren *et al.*, 2010; Vasquez *et al.*, 2012). Therefore, supplementation of *T. molitor* diet with appropriate LAB could be a conducive alternative for the control of potential bacterial diseases given its ability to produce antimicrobial substances (Li *et al.*, 2016).

LAB could be an alternative to antibiotics for the management of diseases in rearing systems. The intensive use of antibiotics to control diseases has forced bacteria to become resistant. Insect husbandry should not follow the same management practices that conventional livestock production has used (Van Huis *et al.*, 2013). Therefore, the search for alternative products to antibiotics is imminent. According to the report of EFSA (2015), antibiotics have not been used in the production of insects for feed and food in Europe. However, antibiotics could be used in case of an extreme urgency and for a short period in order to reduce damages to insect farming (Eilenberg *et al.*, 2015).

The premise for disease management in insect farming should be prevention. Right conditions such as population density, diet, temperature, relative humidity and genotype, should be considered in order to provide the best conditions for insects without compromising their health. Imbalances in the aforementioned factors maximize the disease risk of insects.

7 CONCLUSIONS

From the first part of this thesis, a literature review was presented with general concepts and state of the art about insect farming systems, diseases in insect farming systems, Insect pathogenic bacteria, probiotics, LAB, *T. molitor*, and interaction between pathogens and Insects. This background was the basis for the understanding and developing of the experimental part, as well as support for the results and discussion.

From the experimental part, there are 2 main conclusions from the results that are important to consider during insect rearing

Two beneficial bacteria LAB from honeybees *L. kunkeei* (Fhon2)*, L. apinorum* (Fhon13) were found to have high antimicrobial activity on the pathogenic bacteria *P. aeruginosa, S. plymuthica* and *S. marcescens*. By showing that some of the LAB strains inhibit insect pathogenic bacteria *in -vitro,* we believe that they might be beneficial for the insects and so used in large scale insect production.

S. marcescens Mm3 was not pathogenic *per-os* to *T. molitor* larvae reared under optimum conditions temperature, relative humidity, diet and density. However, *T. molitor* larvae have a high risk of infection, if *Serraria marcescens* Mm3 finds a place of entry to the hemocoel.

This work has also developed and implemented detailed procedures for different techniques used during the experiments. These techniques and methods were adapted from literature or other labs, but initially didn't exist in the Section of Organismal Biology (SOBI) at the University of Copenhagen. In this context, dual-culture overlay assay was found as a powerful technique to evaluate antimicrobial activity generated by a beneficial an organism on pathogenic organism.

8 PERSPECTIVES

The knowledge generated in this work, i.e. the inhibition capacity of LAB on pathogenic bacteria and lethal dose of pathogenic bacteria on the insects will aid the future design of in-vivo experiments where host insect is simultaneously challenged by pathogenic bacteria and the beneficial bacteria LAB.

The inhibition of growth generated by LAB could be evaluated in other pathogenic organisms affecting rearing systems e.g. *T. molitor,* which is susceptible to fungal diseases (Eilenberg *et al.*, 2015). Therefore, it could be interesting to evaluate the antimicrobial activity of LAB in this pathogenic group.

Early identification of diseases helps minimize loss of production and distribution of pathogens in a production system. Two alternatives might be valuable for identifying diseases in the early stages: i) The first strategy is the collection and analysis of insects that die from unknown causes or insects that do not perform well (Eilenberg *et al.*, 2015). ii) The second strategy is the establishment of a systematic sample comparing values obtained from the samples with pre-established values in a growth curve, which could indicate the health status of the insects. Insects with subnormal growth should be sent for analysis to the insect pathology laboratory.

Techniques can be established in the laboratory to determine the health status of insects. Insects produce antimicrobial peptides when exposed to a pathogen. These compounds can be evaluated using tests of inhibition zones. Assessments of specific compounds could also be made, for instance assessments of the production of Tenecin 1, 2, 3, and 4 which is a protein produced by *T. molitor* when pathogens, bacteria gram-positive, gram negative and fungi are identified (Chae *et al.*, 2012; Haine *et al.*, 2008; Lee *et al.*, 1999; Moon *et al.*, 1994; Roh *et al.*, 2009).

The samples entered into the laboratory for analysis will form a database of diseases for the insect industry. The information compiled by the laboratory with the accessions made by the

producers will allow for the advancement of research on disease management consisting of: I) having an inventory of pathogenic microorganisms to mass-reared insects. (ii) conducting studies on insect-specific pathogens in a production system. The prevention and identification of diseases requires qualified personnel. It is therefore necessary to consider cooperation between stakeholders to create such a facility.

9 APPENDIX

9.1 EXPERIMENTAL INSECT REARING

The insect rearing was maintained in the facilities of Section of Organismal Biology (SOBI) at the University of Copenhagen. The controlled conditions in the rearing system were temperature, relative humidity, diet, photoperiod and density. The experimental rearing system was maintained at 28°C (±2) and 50-60% relative humidity (RH%) on a diet of organic oats supplemented with chicken egg whites 1% and the photoperiod regime was 24 hours of darkness. 300 eggs of *T. molitor* approx. plus 200g of supplemented diet was introduced into a plastic container (16.5cm length × 10.5cm width × 7cm height). The diet took up the bottom three centimeters of the container. The containers were perforated in the sides and in the lid to facilitate air circulation (Figure 13). Potatoes were provided as a water resource twice a week. The initial set of larvae was purchased in Avifauna Aps, and three full cycles were obtained from this initial population. This experimental rearing system was adapted from the model used by the Hosts and Parasites Research Group, SOBI, University of Copenhagen.

Figure 13. Experimental rearing system at SOBI University of Copenhagen

An experimental healthy insect rearing system was achieved with more than 200 production units and a total of 40.000 insects approx. All stages from eggs to larvae, pupae and adults of *T. molitor* in rearing were available. Furthermore, the production units contained larvae of equal age, which is a desirable feature for experimental purposes. The duration of the stages observed in the rearing system was: one week from oviposition to hatching, then larvae the had a duration range from 10 up to 16 weeks with a peak in week 14, and finally the pupa stage lasts one week. Adults were observed during 3 weeks with the purpose of egg collection (Appendix 9.4).

9.2 BACTERIAL SUSPENSION PREPARATION

Pathogenic bacteria used in the experiments were produced in Luria Bertani broth (LB) or Nutrient Broth (NA) or Tryptic Soy Broth (TSB) under agitation at 180 rpm and incubated for 24 hours at 35°C. Bacterial cells were harvested by centrifugation at 5000 rpm for 10 minutes (Ramarao *et al.*, 2012). The pellets were washed and resuspended in sterile Milli-Q water. Another method used was to culture the pathogenic bacteria in solid medium incubated 24 hours at 35 ° C. The cells were harvested and resuspended in sterile Milli-Q water.

LAB suspensions for experiments were cultured individually in supplemented Man, Rogosa and Sharpe medium (sMRS) with 2% fructose and 0.1% L-cysteine and incubated at 35°C for 24 h. The cultures were centrifuged at 4000 rpm during 30 minutes. The pellets were washed and resuspended in sterile Milli-Q water. Bacteria that did not grow were activated by culturing them in (sMRS) and then incubated at 35°C for 72h. This procedure was repeated twice a week for bacteria that did not grow until achieving their respective germination.

Calibration curve: A calibration curve was performed for *S. Marcescens* in order to establish the relation between optical density (OD_{600}) and colony forming units (CFU/mL) (Peñuelas-Urquides *et al.*, 2013). Data was analyzed with a lineal regression model and a t-Test two-sample assuming unequal variances.

Long-term storage of bacterial cells: Pathogenic bacteria were preserved in glycerol at -80°C (Koppenhöfer *et al.*, 2012). LAB was preserved in a Hogness medium [final concentration: 36 mM K2HPO4, 13.2mM KH2PO4, 0.4 mM MgSO4, 1.7 mM Na3-citrate, 6.8 mM(NH4)2SO4, 4.4% (v/v) glycerol] also at -80°C (Birren and Lai, 1996; Werner *et al.*, 1997). Long- term storage is an important technique to ensure the availability and quality of the biological material to use in experiments. In order to guarantee sufficient bacterial stock to work during the experiment, several batches of bacteria were frozen.

The regression model indicates that both methods, that is CFU/mL counting and OD_{600} measurement of cell concentration in a suspension, have a direct correlation with a coefficient of determination of R^2=0.9566 and ρ= 0.9780. Then, CFU/mL could be estimated with OD_{600} (Figure 14).

The T-test, two-sample assuming unequal variances shows that among samples of *S. marcescens* Mm3 (N = 26), there is a low probability that the dates are randomly distributed between the two methods, number of cells measured with OD_{600} (M = 0.56, SD = 0.8756) and number of cells counted CFU/mL (M= 6.66 $x10^8$, SD = 1.04 $x10^9$), t(25) = -3.27 , $p \leq 0.05$, $CI._{99}$ 1.708, 2.0595 (p=0.003064). Therefore, there is a positive and significative relationship

between the number of cells when measured with OD_{600} and CFU/mL for *S. marcescens* Mm3 samples.

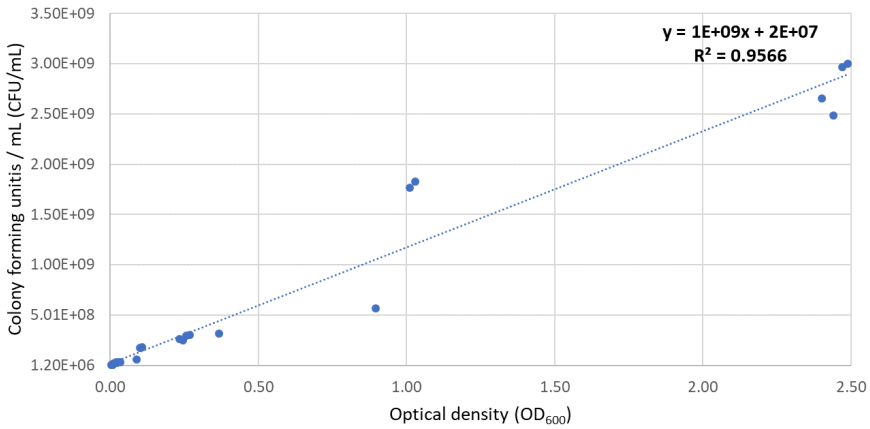


Figure 14. Correlation of *S. marcescens* Mm3 cell concentration evaluated with two methods CFU/mL counting and OD_{600} measurement.

Experiments using large sets of bacteria require that bacterial cell counts be made fast and accurately. Counting the colony forming units is the most accurate method, since the cells counted are living cells but the suspension is one-day old when it should be used. In contrast, measurements of turbidity such as optical density provide a quick result, even though errors in the estimation of cells could be made if certain precautions are not taken: i) A previous calibration curve of OD_{600} and CFU must be done **for each organism**. ii) Measurements must be done in the same growth phase of the organism. iii) Correct use of the equipment with sharp calibration is mandatory (Sutton, 2011).

Having thus obtained the equation with the linear regression where $y = 1x10^9(X) + 2x10^7$, it is possible to calculate the CFU with OD_{600} measurements. This equation can be applied only for *S. Marcescens* Mm3. Estimates of other bacteria with this equation could result in an error.

One limitation of the estimation of cells using OD_{600} is that the range measured is narrow, between 10^7 to 10^9 cells per mL. The equipment cannot read lower concentrations than these.

According to the bacteria supplier's instructions, *S. marcescens* Mm3 should be cultured on nutritive agar. In contrast, some authors suggest the use of the Luria-Bertani medium (Jackson *et al.*, 2001; Tan *et al.*, 2006). We have tested both media in our experiments without observing differences in the bacterial growth.

9.3 BIOLOGISK APV/RISIKOVURDERING FOR ARBEJDE MED INSEKT PATOGENE BAKTERIER GRUPPE 2

Skema over arbejdsprocessen **Biologisk APV/Risikovurdering for arbejde med *insekt patogene bakterier* Klassificeret i gruppe 2**									
	A **Del-opgave**	**B** **Ansvarlig** **(*udførende*)**	**C** **Arbejdsproces** *arbejdsgang,*	**D** **Risiko for eksponering**	**E** **Mulighed for eksponering ved planlagt arbejdsgang**	**F** **Processug** **Personlige værnemidler** **Affald**	**G** **Mulighed for eksponering ved uheld**	**H** **Hvad gør man ved uheld?** *Beskriv afværgeforanstaltninger*	**I** **SOP*** **APB****
GENERELT	Arbejder med biologiske agenser, Klassificeret i gruppe 2	Charlotte Fisher Annette Bruun Jensen og Jørgen Eilenberg	Medarbejder skal have nødvendig oplæring instruktion i at udføre arbejder forsvarlig. Samt orientering om RISICI af projektansvarlig	*Pseudomonas aeruginosa* For raske personer er risikoen minimal.	Minimal. Der indføres gode rutiner. General mikrobiologisk arbejde med	LAF – bænk skal benyttes under arbejdet. Handsker skal benyttes (PVC eller nitril)	Minimal	Forsøg at begrænse udslippet mest muligt. Udslippe skal opsamles og dekontamineres ved autoklavering 121 C i 20 min. eller ved tilsætning af Rodalon til slutkoncentrationen 0.5%.	

		Master og PhD studerende	(bevillingshaver) og evt. sikkerhedsgruppen. Arbejdsområde: Det er kun tilladt at arbejde med biologisk agens klasse 2 i de laboratorier der er afmærket med  Arbejdsområde (-bord) afmærkes med tilsvarende skilte. Ved inkubation, opbevaring af ting i køleskabe/fryser afmærkes	Bakterierne er opportunistisk patogene, d.v.s de forårsager infektion hos personer med nedsat immunforsvar eller anden svækkelse. *Pseudomonas aeruginosa* kan forårsage infektion, herunder øjnene, mund og åbne sår. *Pseudomonas aeruginosa* giver ofte lungebetændelse hos folk med den arvelige sygdom cystisk fibrose.	ovennævnte organism kan udgøre en fare dersom bakteriekultur, aerosoler og lign. kommer i kontact med åbene sår. Grundig håndvask og rengøring efter endt arbejde.	Kittel skal benyttes (tilknappet) Sikkerhedsbriller bruges om nødvendigt Vedr. Fodtøj se sikkerhedsforskrift. Arbejdet skal udføres, så dannelse af aerosoler begrænses mest muligt. Affald: Petriskåle og andet engangsmateriale kommes i gule tætsluttende affaldssække mærket "klinisk risikoaffald" Gule sække anbringes ved elevator og afhentes af Driftafdelingen og afleveres derefter til særlig sygehusaffald.		Redskaber, der anvendes ved opsamlingen, skal ligeledes dekontamineres og forurenede overflader aftørres med 70% ethanol elle 0,5% Rodalon. Underret straks sikkerhedsgruppen om uheldet og de forholdsregler, der er truffet. Sikkerhedsgruppen foretager journal af uheldet og evt. videre anmeldelse til relevante myndigheder, samt orienteret faggruppelederen. Ved mistanke om indtagelse eller kontakt af Klasse 2 kontaktes egen læge ved symptomer. Symptomer:	

			tingene med tilsvarende skilt.			Kulturvæske og lign. opsamles i lukkede beholdere. Affaldet dekontamineres ved autoklavering (121 C, 20 min). Glasvarer dekontamineres ved autoklavering 121 C, 20 min). Kanyler, pasteur pipette og anden skærende affald anbringes i plastdunke med skruelåg indeholdende 0.5% rodalon.		Feber, smerte, røde øjne, mave gener. Behandling er tilgængelig: En lang række antibiotika Varige mén: Ingen	
1	Isolerer ukendte insekt patogene bakterier	Charlotte Fisher Annette Bruun Jensen og Jørgen Eilenberg	Insekter med symptomer håndteres med redskaber hvis nødvendigt. Arbejdet udføres i sterilbænk med flow D328.	*Pseudomonas aeruginosa* For raske personer er risikoen minimal. Bakterierne er	Minimal. Der indføres gode rutiner. General mikrobiologisk arbejde med ovennævnte	LAF – bænk skal benyttes under arbejdet. Handsker skal benyttes (PVC elle nitril) Kittel skal benyttes	Minimal	Forsøg at begrænse udslippet mest muligt. Udslippet skal opsamles og dekontamineres ved autoklavering 121 C i 20 min. eller ved tilsætning af Rodalon til slutkoncentrationen 0.5%.	** APB for 70 % ethanol

		Master og PhD studerende	Podenål bruges til at overføre bakterier til flydende eller fast medie.	opportunistisk patogene, d.v.s de forårsager infektion hos personer med nedsat immunforsvar eller anden svækkelse. *Pseudomonas aeruginosa* kan forårsage infektion, herunder øjnene, mund og åbne sår. *Pseudomonas aeruginosa* giver ofte lungebetændelse hos folk med den arvelige sygdom cystisk fibrose.	organism kan udgøre en fare dersom bakteriekultur, aerosoler o lign. kommer i kontact med åbene sår. Grundig håndvask og rengøring efter endt arbejde. .	(tilknappet) Sikkerhedsbriller bruges om nødvendigt Vedr. Fodtøj se sikkerhedsforskrift. Arbejdet skal udføres, så dannelse af aerosoler begrænses mest muligt. Affald: Petriskåle og andet engangsmateriale kommes i gule tætsluttende affaldssække mærket "klinisk risikoaffald" Gule sakke anbringes ved elevator og afhentes af Driftafdelingen og afleveres derefter til særlig sygehusaffald. Kulturvæske og lign.		Redskaber, der anvendes ved opsamlingen, skal ligeledes dekontamineres og forurenede overflader aftørres med 70% ethanol elle 0,5% Rodalon. Underret straks sikkerhedsgruppen om uheldet og de forholdsregler, der er truffet. Sikkerhedsgruppen foretager journal af uheldet og evt. videre anmeldelse til relevante myndigheder, samt orienteret faggruppelederen. Ved mistanke om indtagelse eller kontakt af Klasse 2 kontaktes egen læge ved symptomer. Symptomer:	

						opsamles i lukkede beholdere. Affaldet dekontamineres ved autoklavering (121 C, 20 min). Glasvarer dekontamineres ved autoklavering 121 C 20 min). Kanyler, pasteur pipette og anden skærende affald anbringes i plastdunke med skruelåg indeholdende 0.5% rodalon.		Feber, smerte, røde øjne, mave gener. Behandling er tilgængelig: En lang række antibiotika Varige mén: Ingen	
2	Identifikation af insektpatogene bakterier	Charlotte Fisher Annette Bruun Jensen og Jørgen Eilenberg Master og	Ukendte bakterieisolater identificeres ved DNA metoder og eller morfologi Arbejde med isolaterne indtil DNA ekstraktionen foregår i	*Pseudomonas aeruginosa* For raske personer er risikoen minimal. Bakterierne er opportunistisk	Minimal. Der indføres gode rutiner. General mikrobiologisk arbejde med ovennævnte organism kan	LAF – bænk skal benyttes under arbejdet. Handsker skal benyttes (PVC elle nitril) Kittel skal benyttes	Minimal	Forsøg at begrænse udslipptr mest muligt. Udslippet skal opsamles og dekontamineres ved autoklavering 121 C i 20 min. eller ved tilsætning af Rodalon til slutkoncentrationen 0.5%.	** APB for 70 % ethanol

		PhD studerende	sterilbænk	patogene, d.v.s de forårsager infektion hos personer med nedsat immunforsvar eller anden svækkelse. Pseudomonas aeruginosa kan forårsage infektion, herunder øjnene, mund og åbne sår. *Pseudomonas* aeruginosa giver ofte lungebetændelse hos folk med den arvelige sygdom cystisk fibrose.	udgøre en fare dersom bakteriekultur, aerosoler o lign. kommer i kontact med åbene sår. Grundig håndvask og rengøring efter endt arbejde. .	(tilknappet) Sikkerhedsbriller bruges om nødvendigt Vedr. Fodtøj se sikkerhedsforskrift. Arbejdet skal udføres, så dannelse af aerosoler begrænses mest muligt. Affald: Petriskåle og andet engangsmateriale kommes i gule tætsluttende affaldssække mærket "klinisk risikoaffald" Gule sakke anbringes ved elevator og afhentes af Driftafdelingen og afleveres derefter til særlig sygehusaffald. Kulturvæske og lign.		Redskaber, der anvendes ved opsamlingen, skal ligeledes dekontamineres og forurenede overflader aftørres med 70% ethanol elle 0,5% Rodalon. Underret straks sikkerhedsgruppen om uheldet og de forholdsregler, der er truffet. Sikkerhedsgruppen foretager journal af uheldet og evt. videre anmeldelse til relevante myndigheder, samt orienteret faggruppelederen. Ved mistanke om indtagelse eller kontakt af Klasse 2 kontaktes egen læge ved symptomer. Symptomer: Feber, smerte, røde øjne,	

						opsamles i lukkede beholdere. Affaldet dekontamineres ved autoklavering (121 C, 20 min). Glasvarer dekontamineres ved autoklavering 121 C 20 min). Kanyler, pasteur pipette og anden skærende affald anbringes i plastdunke med skruelåg indeholdende 0.5% rodalon.		mave gener. Behandling er tilgængelig: En lang række antibiotika Varige mén: Ingen	
3	Klassificering og risikovurdering	Charlotte Fisher Annette Bruun Jensen og Jørgen Eilenberg Master og	Når bakterierne er identificeret tjekkes det om den står på listen af biologisk agens https://arbejdstilsynet.dk/da/regler/bekendtgorelser/b/biologiske-agenser-57/bilag-8-klassifikation-af-biologiske-age	*Pseudomonas aeruginosa* For raske personer er risikoen minimal. Bakterierne er opportunistisk	Minimal. Der indføres gode rutiner. General mikrobiologisk arbejde med ovennævnte organism kan	LAF – bænk skal benyttes under arbejdet. Handsker skal benyttes (PVC elle nitril) Kittel skal benyttes	Minimal	Forsøg at begrænse udslippet mest muligt. Udslippet skal opsamles og dekontamineres ved autoklavering 121 C i 20 min. eller ved tilsætning af Rodalon til slutkoncentrationen 0.5%.	

		PhD studerende	Findes bakterien i klasse 2-4 skal der udarbejdes en SOP og indberettes ifald der skal arbejdes yderlige med organismerne. Det skal desuden vurderes om organismer der ikke er på listen potentielt kan inficere mennesker. Dette sker via en litteratur søgning.	patogene, d.v.s de forårsager infektion hos personer med nedsat immunforsvar eller anden svækkelse. Pseudomonas aeruginosa kan forårsage infektion, herunder øjnene, mund og åbne sår. *Pseudomonas aeruginosa* giver ofte lungebetændelse hos folk med den arvelige sygdom cystisk fibrose.	udgøre en fare dersom bakteriekultur, aerosoler o lign. kommer i kontact med åbene sår. Grundig håndvask og rengøring efter endt arbejde. .	(tilknappet) Sikkerhedsbriller bruges om nødvendigt Vedr. Fodtøj se sikkerhedsforskrift. Arbejdet skal udføres, så dannelse af aerosoler begrænses mest muligt. Affald: Petriskåle og andet engangsmateriale kommes i gule tætsluttende affaldssække mærket "klinisk risikoaffald" Gule sakke anbringes ved elevator og afhentes af Driftafdelingen og afleveres derefert til særlig sygehusaffald. Kulturvæske og lign.		Redskaber, der anvendes ved opsamlingen, skal ligeledes dekontamineres og forurenede overflader aftørres med 70% ethanol elle 0,5% Rodalon. Underret straks sikkerhedsgruppen om uheldet og de forholdsregler, der er truffet. Sikkerhedsgruppen foretager journal af uheldet og evt. videre anmeldelse til relevante myndigheder, samt orienteret faggruppelederen. Ved mistanke om indtagelse eller kontakt af Klasse 2 kontaktes egen læge ved symptomer. Symptomer: Feber, smerte, røde øjne,	

						opsamles i lukkede beholdere. Affaldet dekontamineres ved autoklavering (121 C, 20 min). Glasvarer dekontamineres ved autoklavering 121 C 20 min). Kanyler, pasteur pipette og anden skærende affald anbringes i plastdunke med skruelåg indeholdende 0.5% rodalon.		mave gener. Behandling er tilgængelig: En lang række antibiotika Varige mén: Ingen	
4	Opformering og opbevaring	Charlotte Fisher Annette Bruun Jensen og Jørgen Eilenberg Master og	Isolater der skal bruges til vækst forsøg håndteres i sterilbænke. Isolater opformeres eller opbevares i særligt afmærkede racks, klimaskabe,	*Pseudomonas aeruginosa* For raske personer er risikoen minimal. Bakterierne er opportunistisk	Minimal. Der indføres gode rutiner. General mikrobiologisk arbejde med ovennævnte organism kan	LAF – bænk skal benyttes under arbejdet. Handsker skal benyttes (PVC elle nitril) Kittel skal benyttes	Minimal	Forsøg at begrænse udslippet mest muligt. Udslippet skal opsamles og dekontamineres ved autoklavering 121 C i 20 min. eller ved tilsætning af Rodalon til slutkoncentrationen 0.5%.	

		PhD studerende	hylder. Isolater opbevares altid i særligt afmærkede bokse.  	patogene, d.v.s de forårsager infektion hos personer med nedsat immunforsvar eller anden svækkelse. *Pseudomonas aeruginosa* kan forårsage infektion, herunder øjnene, mund og åbne sår. *Pseudomonas aeruginosa* giver ofte lungebetændelse hos folk med den arvelige sygdom cystisk fibrose.	udgøre en fare dersom bakteriekultur, aerosoler o lign. kommer i kontact med åbene sår. Grundig håndvask og rengøring efter endt arbejde. .	(tilknappet) Sikkerhedsbriller bruges om nødvendigt Vedr. Fodtøj se sikkerhedsforskrift. Arbejdet skal udføres, så dannelse af aerosoler begrænses mest muligt. Affald: Petriskåle og andet engangsmateriale kommes i gule tætsluttende affaldssække mærket "klinisk risikoaffald" Gule sakke anbringes ved elevator og afhentes af Driftafdelingen og afleveres derefert til særlig sygehusaffald. Kulturvæske og lign.		Redskaber, der anvendes ved opsamlingen, skal ligeledes dekontamineres og forurenede overflader aftørres med 70% ethanol elle 0,5% Rodalon. Underret straks sikkerhedsgruppen om uheldet og de forholdsregler, der er truffet. Sikkerhedsgruppen foretager journal af uheldet og evt. videre anmeldelse til relevante myndigheder, samt orienteret faggruppelederen. Ved mistanke om indtagelse eller kontakt af Klasse 2 kontaktes egen læge ved symptomer. Symptomer: Feber, smerte, røde øjne,	

						opsamles i lukkede beholdere. Affaldet dekontamineres ved autoklavering (121 C, 20 min). Glasvarer dekontamineres ved autoklavering 121 C 20 min). Kanyler, pasteur pipette og anden skærende affald anbringes i plastdunke med skruelåg indeholdende 0.5% rodalon.		mave gener. Behandling er tilgængelig: En lang række antibiotika Varige mén: Ingen	
5	Koncentrationsbestemmelse og foder	Charlotte Fisher Annette Bruun Jensen og Jørgen Eilenberg Master og	Ved bakterie koncentrationsbestemmelse via tællekamre tilsættes bakterierne i sterilbænke og tællekamret dekontamineres med 70% ethanol elle 0,5% Rodalon straks efter brug.	*Pseudomonas aeruginosa* For raske personer er risikoen minimal. Bakterierne er opportunistisk	Minimal. Der indføres gode rutiner. General mikrobiologisk arbejde med ovennævnte organism kan	LAF – bænk skal benyttes under arbejdet. Handsker skal benyttes (PVC eller nitril) Kittel skal benyttes	Minimal	Forsøg at begrænse udslipper mest muligt. Udslipper skal opsamles og dekontamineres ved autoklavering 121 C i 20 min. eller ved tilsætning af Rodalon til slutkoncentrationen 0.5%.	

		PhD studerende	Foderblandninger der indeholder bakterier foretages i sterilbænk.	patogene, d.v.s de forårsager infektion hos personer med nedsat immunforsvar eller anden svækkelse. *Pseudomonas aeruginosa* kan forårsage infektion, herunder øjnene, mund og åbne sår. *Pseudomonas aeruginosa* giver ofte lungebetændelse hos folk med den arvelige sygdom cystisk fibrose.	udgøre en fare dersom bakteriekultur, aerosoler o lign. kommer i kontact med åbene sår. Grundig håndvask og rengøring efter endt arbejde. .	(tilknappet) Sikkerhedsbriller bruges om nødvendigt Vedr. Fodtøj se sikkerhedsforskrift. Arbejdet skal udføres, så dannelse af aerosoler begrænses mest muligt. Affald: Petriskåle og andet engangsmateriale kommes i gule tætsluttende affaldssække mærket "klinisk risikoaffald" Gule sakke anbringes ved elevator og afhentes af Driftafdelingen og afleveres derefert til særlig sygehusaffald. Kulturvæske og lign.		Redskaber, der anvendes ved opsamlingen, skal ligeledes dekontamineres og forurenede overflader aftørres med 70% ethanol elle 0,5% Rodalon. Underret straks sikkerhedsgruppen om uheldet og de forholdsregler, der er truffet. Sikkerhedsgruppen foretager journal af uheldet og evt. videre anmeldelse til relevante myndigheder, samt orienteret faggruppelederen. Ved mistanke om indtagelse eller kontakt af Klasse 2 kontaktes egen læge ved symptomer. Symptomer: Feber, smerte, røde øjne,	

						opsamles i lukkede beholdere. Affaldet dekontamineres ved autoklavering (121 C, 20 min). Glasvarer dekontamineres ved autoklavering 121 C 20 min). Kanyler, pasteur pipette og anden skærende affald anbringes i plastdunke med skruelåg indeholdende 0.5% rodalon.		mave gener. Behandling er tilgængelig: En lang række antibiotika Varige mén: Ingen	
6	Infektion af insekter.	Charlotte Fisher Annette Bruun Jensen og Jørgen Eilenberg Master og	Håndtering af foder med bakterier eller injektion af bakterier i insekterne foretages i sterilbænk. Insekter der er blevet eksponeret for	*Pseudomonas aeruginosa* For raske personer er risikoen minimal. Bakterierne er opportunistisk	Minimal. Der indføres gode rutiner. General mikrobiologisk arbejde med ovennævnte organism kan	LAF – bænk skal benyttes under arbejdet. Handsker skal benyttes (PVC eller nitril) Kittel skal benyttes	Minimal	Forsøg at begrænse udslipper mest muligt. Udslipper skal opsamles og dekontamineres ved autoklavering 121 C i 20 min. eller ved tilsætning af Rodalon til slutkoncentrationen 0.5%.	

		PhD studerende	bakterierne opbevares efterfølgende i særlige kasser og afmærkede områder i laboratorierne/klimaskabe. Insekterne (melormene) opbevares i beholdere hvorfra de ikke kan slippe ud. Håndtering af eksponerede insekter foretages altid i sterilbænk.	patogene, d.v.s de forårsager infektion hos personer med nedsat immunforsvar eller anden svækkelse. *Pseudomonas aeruginosa* kan forårsage infektion, herunder øjnene, mund og åbne sår. *Pseudomonas aeruginosa* giver ofte lungebetændelse hos folk med den arvelige sygdom cystisk fibrose.	udgøre en fare dersom bakteriekultur, aerosoler o lign. kommer i kontact med åbene sår. Grundig håndvask og rengøring efter endt arbejde. .	(tilknappet) Sikkerhedsbriller bruges om nødvendigt Vedr. Fodtøj se sikkerhedsforskrift. Arbejdet skal udføres, så dannelse af aerosoler begrænses mest muligt. Affald: Petriskåle og andet engangsmateriale kommes i gule tætsluttende affaldssække mærket "klinisk risikoaffald" Gule sakke anbringes ved elevator og afhentes af Driftafdelingen og afleveres derefter til særlig sygehusaffald. Kulturvæske og lign.		Redskaber, der anvendes ved opsamlingen, skal ligeledes dekontamineres og forurenede overflader aftørres med 70% ethanol elle 0,5% Rodalon. Underret straks sikkerhedsgruppen om uheldet og de forholdsregler, der er truffet. Sikkerhedsgruppen foretager journal af uheldet og evt. videre anmeldelse til relevante myndigheder, samt orienteret faggruppelederen. Ved mistanke om indtagelse eller kontakt af Klasse 2 kontaktes egen læge ved symptomer. Symptomer: Feber, smerte, røde øjne,	

						opsamles i lukkede beholdere. Affaldet dekontamineres ved autoklavering (121 C, 20 min). Glasvarer dekontamineres ved autoklavering 121 C 20 min). Kanyler, pasteur pipette og anden skærende affald anbringes i plastdunke med skruelåg indeholdende 0.5% rodalon.		mave gener. Behandling er tilgængelig: En lang række antibiotika Varige mén: Ingen	

9.4 PRODUCTION OF *T. MOLITOR* EGGS

Materials:

1. Plastic container
2. Small plastic bottle
3. Cotton pads
4. Petri dish
5. Piece of net (holes of 1x1 mm)
6. Sand
7. Adults of *T. molitor*
8. Elastic band
9. Water
10. Oats
11.

Procedure:

Day 1

The egg collector

- Fill the petri dish with sand.
- Put net over the petri dish.
- Tie the net and the petri box with an elastic band.

The water drinker

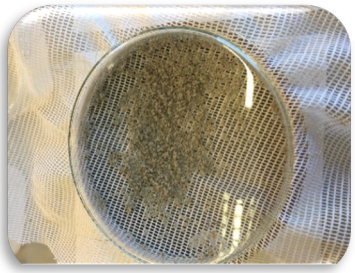
- Take the small bottle and fill it with water.
- Put the cotton pads around the edges of the bottle as shown in the image.

Insert into the plastic box:

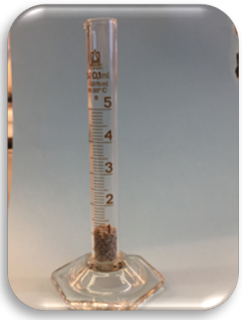
- A thin layer of oats on the bottom
- 14 adults / dm2
- The water drinker in a horizontal position
- The egg collector

Day 2

- Take the egg collector and turn it over.
- Prepare a container to collect the sand that passes through the net.
- The eggs will stick to the net.
- Remove the elastic band and the net.



- Measure the eggs in a graduated cylinder.



- Prepare a plastic container with a thin layer of oats.
- Place the eggs on top of the oats.

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