The effect of honeybee--specific Lactic Acid Bacteria on American foulbrood disease of honeybees

Lamei, Sepideh

2018

The effect of honeybee-specific Lactic Acid Bacteria on American foulbrood disease of honeybees

Sepideh Lamei
Department of Ecology
Faculty of Natural Resources and Agricultural Sciences
Uppsala
Department of Translational Medicine
Faculty of Medicine
Lund University

Doctoral Thesis
Swedish University of Agricultural Sciences
and Lund University
Uppsala/Lund 2018

Acta Universitatis agriculturae Sueciae
2018:23
Lund University Doctoral Dissertation
2018:76

Cover: Honeybee colonies located at the Swedish University of Agricultural Sciences, Uppsala, Sweden.
(photo: E. Forsgren)

ISSN 1652-6880
ISSN 1652-8220
ISBN (print version) 978-91-7760-186-9
ISBN (electronic version) 978-91-7760-187-6

Print: SLU Service/Repro, Uppsala 2018

The effect of honeybee-specific Lactic Acid Bacteria on American foulbrood disease of honeybees

Abstract

The honeybee, *Apis mellifera*, is one of the most economically important pollinators and highly valued for its honey and wax production. Managed honeybees occupy an increasingly critical role in agricultural productivity and food security. American foulbrood (AFB) is a highly contagious and destructive bacterial honeybee brood disease caused by *Paenibacillus larvae* that affects beekeeping worldwide. However, only a minority of bacteria associated with honeybees are harmful. Honeybee-specific Lactic Acid Bacteria (hbs-LAB), a defined group of beneficial bacteria inhabiting the honey crop, have strong antimicrobial properties important for honey production and honeybee health that could be exploited for combating diseases such as AFB.

The aim of this thesis was to investigate the effect of hbs-LAB on *P. larvae* and AFB, both in culture, in individual larval bioassays, and at colony level.

First we showed that the laboratory cultivation of the 13 distinct hbs-LAB was significantly improved by the addition of L-cysteine and fructose to the medium and optimized a culture-independent molecular technique for the detection and identification of the individual hbs-LAB species. Secondly the effect of the cell free supernatant, the secretome, from a culture mix of the 13 hbs-LAB species was investigated on *P. larvae* growth and associated larval mortality. The results showed that this secretome strongly inhibited the multiplication of *P. larvae* vegetative cells but that spore germination appeared to be unaffected, and that it decreased the mortality of *P. larvae* infected larvae. Finally it was shown that oral administration of hbs-LAB supplement to honeybee colonies had no influence on colony-level *P. larvae* spore levels or colony strength. Furthermore, the results showed that although the antibiotic tylosin decreased AFB symptoms in colonies, it had no effect on *P. larvae* spore levels.

In conclusion, the colony-level results do not contradict the antagonistic effects observed in individual larvae in laboratory studies, but rather suggest that supplementary administration of live bacteria may not be the most effective way to harness such effects in a useful application.

Key words: American Foulbrood, *Apis mellifera*, beneficial microbes, *Bifidobacterium*, honeybees, honeybee-specific lactic acid bacteria, *Lactobacillus*, *Paenibacillus larvae*.

Author's address: Sepideh Lamei, SLU, Department of Ecology, Box 7044, 750 07 Uppsala, Sweden/ Department of Translational Medicine, Lund University, Malmö, Sweden.

E-mail: sepideh.lamei@slu.se/ sepideh.lamei@med.lu.se

Honungsbispecifika mjölksyrabakterier och deras effekt på bakterien *Paenibacillus larvae*, en sjukdomsalstrare hos honungsbin

Sammanfattning

Honungsbiet, *Apis mellifera*, är en av de ekonomiskt mest viktiga pollinatörerna och också högt värderat som producenter av honung och vax. Odlade honungsbin intar även en allt viktigare roll i jordbruksproduktion och livsmedelssäkerhet. Sjukdomen amerikansk yngelröta, AY, orsakad av bakterien *Paenibacillus larvae* är ett exempel på en mycket smittsam och allvarlig sjukdom som påverkar biodlingen världen över. Även om vissa mikroorganismer hos honungsbin orsakar sjukdom är långt ifrån alla skadliga. Honungsbispecifika mjölksyrabakterier, hbs-LAB, som lever i binas honungsmage besitter starkt antimikrobiella egenskaper som är viktiga för honungsproduktion och bihälsa och som kan utnyttjas för att bekämpa sjukdomar som amerikansk yngelröta.

Syftet med arbetet som presenteras i den här avhandlingen har varit att undersöka de honungsbispecifika mjölksyrabakteriernas effekt på bakterien *P. larvae* i bakteriell odling, i individuella honungsbilarver och i bisamhällen.

Först visades att hbs-LAB behöver specifika näringsämnen som L-cystein och fruktos för en optimal tillväxt på artificiella medier i laboratoriet och en molekylär teknik för detektion och identifiering av de 13 hbs-LAB optimerades. Vidare undersöktes vilken påverkan den cellfria supernatanten, ”sekretomet”, producerat från en blandkultur av de 13 arterna av hbs-LAB hade på tillväxten av *P. larvae* och den resulterande larvdödligheten. Resultaten visade att sekretomet starkt hämmar uppförökningen av *P. larvae* utan påverkan på sporernas germinering, och att det minskar dödligheten hos infekterade bilarver. Slutligen visade det sig att tillskott av hbs-LAB i bifoder inte hade någon påverkan på vare sig spormängder av *P. larvae*, symptom av AY eller bistyrkan i bisamhället. Dessutom visade resultaten att även om antibiotikumet tylosin minskar symptom på AY, har det ingen effekt på mängden sporer i bisamhället.

Sammanfattningsvis kan man säga att den uteblivna effekten av att fodra bisamhällen med hbs-LAB inte motsäger de effekter som utan tvivel kan ses i enskilda honungsbilarver. Det visar snarare att det behövs mer forskning och utveckling för att kunna utnyttja sådana effekter i användbara applikationer inom biodlingen.

Nyckelord: Amerikansk yngelröta, antimikrobiell effekt, *Apis mellifera*, *Bifidobacterium*, honungsbin, honungsbispecifika mjölksyrabakterier, *Lactobacillus*, *Paenibacillus larvae*.

Författarens adress: Sepideh Lamei, SLU, Department of Ecology, Box 7044, 750 07 Uppsala, Sweden/ Department of Translational Medicine, Lund University, Malmö, Sweden.

E-mail: sepideh.lamei@slu.se/ sepideh.lamei@med.lu.se

Dedication

To everyone who supported me in this adventure!

The one who falls and gets up is so much stronger than the one who never fell.
Roy T. Bennett

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Lamei S**, Hu YOO, Olofsson TC, Andersson AF, Forsgren E and Vásquez A* (2017). Improvement of identification methods for honeybee-specific Lactic Acid Bacteria; future approaches. *PLOS One* 12(3), e0174614.

II **Lamei S***, Stephan JG, Riesbeck K, Nilson B, de Miranda JR and Forsgren E. The secretome of honeybee-specific lactic acid bacteria inhibits *Paenibacillus larvae* growth (submitted manuscript).

III Stephan JG[#], **Lamei S**[#], Pettis JS, Riesbeck K, de Miranda JR and Forsgren E*. The effect of honeybee-specific lactic acid bacteria supplement on the levels of *Paenibacillus larvae* in experimentally American foulbrood infected honeybee colonies (manuscript).

Papers I-III are reproduced with the permission of the publishers.

* Corresponding author
[#] Contributed equally

The contribution of Sepideh Lamei to the papers included in this thesis was as follows:

- Principal author. Designed the study together with the supervisors. Performed the laboratory work, the data processing, the statistical analysis and the interpretation of the results. Produced the figures/tables and wrote most of the text, with editorial assistance from the co-authors.

- Principal author. Designed the study together with supervisors. Performed the laboratory work, the data processing and the interpretation of the results. Coordinated the production of the manuscript and wrote most of the text, with editorial assistance from the co-authors.

- Co-principal author. Designed the study together with supervisors. Performed the laboratory work, the data processing and the interpretation of the results. Coordinated the production of the manuscript and wrote most of the text, with editorial assistance from the co-authors.

Abbreviations

AFB	American Foulbrood
MRS	De Man, Rogosa & Sharpe (medium)
sMRS	Supplemented MRS
nsMRS	non-supplemented MRS
LAB	Lactic Acid Bacteria
hbs-LAB	Honeybee-specific Lactic Acid Bacteria
MALDI-TOF MS	Matrix-assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry
PAMPs	Pathogen-Associated Microbial Patterns
qPCR	Quantitative Polymerase Chain Reaction
rep-PCR	Repetitive element PCR
ERIC	Enterobacterial Repetitive Intergenic Consensus
PM	Peritrophic Matrix

1 Honeybees

The Western honeybee, *Apis mellifera*, is the most economically valuable insect pollinator globally. An estimated 35 % of human food consumption depends on insect pollination and over 90 % of those crops rely on honeybee pollination services (Klein et al., 2007). Even though the honeybee is economically valuated for its services and products used by humans, the honeybee's role in sustaining natural plant biodiversity as an ecosystem service provider is immeasurable (Potts et al., 2010).

Honeybees are eusocial insects living in perennial colonies with overlapping generations, cooperative brood care and a reproductive division of labor. The colony consists of three castes: female worker bees which can number between 15000-50000 depending on the time of year with a peak in the summer and a dearth in the winter; a few hundred male drones usually present in the spring; and one reproducing female queen bee (Winston, 1991).

The honeybee nest cavity maintains a relatively constant temperature and humidity providing an ideal environment for parasites and pathogens. Due to the close contact of individuals via casual contacts or trophallaxis, numerous and diverse opportunities for pathogen transmission are possible. Honeybee parasites and pathogens need to be successful at multiple levels within the honeybee colonies in order to reproduce and disperse to new hosts. The first step requires successfully infecting an individual at which point the pathogen must then be able to infect additional individuals to assure a sufficient parasite load within the colony. Finally, the pathogen must successfully gain access and infect new colonies (Forfert et al., 2015).

Studies show that the genes involved in the innate immunity of honey bees, compared to other insects, are reduced to one third (Evans et al., 2006; Weinstock et al., 2006). Instead, honeybees rely largely on colony level adaptive pathogen resistance mechanisms for disease defense, *e.g.* social immunity (Cremer et al., 2007). The cooperation of individuals within the colony results in a social immunity mitigating disease transmission. This type of defense can

consist of behavioral, physiological and organizational adaptations to prevent the entrance, establishment, and spread of disease within the colony. Since there is often a difference between individual and colony level virulence of honeybee pathogens, interactions at both levels needs to be considered. An inverse relationship between the individual- and colony level virulence for the two principal genotypes of *P. larvae* has been observed. The more virulent genotype at the individual level (ERIC-II) kills the larvae before they are capped, such that they can be easily detected and removed by the bees resulting in a reduced virulence at the colony level. The reverse is true for *P. larvae* of ERIC-I genotype. The lower virulence at the individual level allows infected larvae to be capped, escaping early detection and removal by the bees. Therefore, infection with *P. larvae* of ERIC-I genotype has a longer time for spore production, contributing to the higher colony level virulence (Genersch et al., 2005; Poppinga and Genersch, 2015). Recently researchers focused on the role of beneficial microorganism and their role as an additional defense in honeybees. The results presented in **Paper II** and **Paper III** show that any promising results of the effects of hbs-LAB on pathogens at the individual level in controlled laboratory experiments do not necessarily translate directly into an effective treatment for honeybee colonies.

2 Beneficial microorganisms

The unpredictable nature and complex biosynthetic capabilities of beneficial microorganisms have made them interesting candidates for resolving particularly difficult problems in life sciences. The various ways in which microorganisms have been used over the past years to advance medical technology, human and animal health (Rashid and Sultana, 2016), food processing, food safety and quality (Bernardeau et al., 2006; Rattanachaikunsopon and Phumkhachorn, 2010), genetic engineering, environmental protection, agricultural biotechnology (Zhou et al., 2009) provide a most impressive record of achievements.

Lactic Acid Bacteria (LAB) are Gram-positive, non-spore forming beneficial bacteria that have been key components of microbiology research and practice since the 19th century. They were first described as milk-souring organisms due to the sour milk that arose from their production of lactic acid (Stiles and Holzapfel, 1997). Lactic acid bacteria belong to a biologically defined group rather than a taxonomically identified group including the genera *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*. Properties such as nutritional, environmental and adhesive adaptations have provided LAB with the ability to adapt to different environments. They are found in food matrices such as dairy products, meat, vegetables, sourdough bread and wine but also in human mucosal surfaces such as the oral cavity, the vagina and the gastrointestinal tract (Dicks and Botes, 2010; Florou-Paneri et al., 2013; Fraga et al., 2008; Gobbetti and Corsetti, 1997).

LAB are closely associated with food and feed processing and production, and have a Generally Recognized As Safe (GRAS) status, which define them as safe to use as probiotics for human and animal consumption (Florou-Paneri et al., 2013; Rashid and Sultana, 2016). Probiotics are defined as “live microorganisms which, when administered in sufficient quantities, have a demonstrable positive effect on the host/recipient, and their administration is not

associated with negative effects on organisms or the environment" (FAO/WHO, 2002).

In this thesis *Bifidobacteria* have been included in the LAB definition due to their similar origin, their production of organic acids and common use by the food and biotech industries (Makarova and Koonin, 2007). However, *Bifidobacteria* are quite diverse compared to other LAB members, since their genomes have a much higher GC content (GC DNA content 42-67%, other LAB members: GC DNA content <53%), belong to the phylum Actinobacteria and use a different metabolic pathway called the "bifid shunt" (Felis and Dellaglio, 2007; Yin et al., 2005).

2.1 The honeybee microbiome

Social insects such as honeybees provide unique resources for the bacterial microbiome, thanks to *e.g.* the high number of individuals within a colony, the food sharing and the close relationship between colony members. Not surprisingly, some bacterial species associated with honeybees, such as *P. larvae*, are clearly pathogenic, but most species have never been associated with any disease symptoms and their impact on the honeybee is largely unknown (Rokop et al., 2015). Gut bacteria are transmitted and shared by the colony members through oral-fecal transmission, oral trophallactic interaction, consumption of stored pollen or bee bread, interactions with older bees within the hive and contact with hive material during the early adult stage (Kwong and Moran, 2016; Martinson et al., 2012; Powell et al., 2014).

The adult honeybee gastrointestinal tract is divided into three major organs (crop, midgut and hindgut) (Figure 1), providing different functions in the catabolism and absorption of food and different environment for bacterial symbionts.

The honey crop is an enlargement of the esophagus which is not involved in digestion but is a storage bag for transporting nectar from the flower to the hive and to share liquid nutrition with other nest mates (Crailsheim, 1988). Although the temperature of the honey crop is fairly constant and it is rich of sugars, the conditions favoring microbial growth (*e.g.* pH, osmotic conditions, the availability of proteins and micronutrients) may fluctuate widely. The honey crop is therefore not an optimal niche for microbial growth but likely acts as a selective filter allowing relatively few bacteria, such as species within *Lactobacillaceae* and *Bifidobacteriaceae, Entrobacteriaceae* and *Acetobacteraceae* to flourish (Corby-Harris et al., 2014a, 2014b; Killer et al., 2014; Olofsson and Vásquez, 2008). Lactic acid bacteria have peculiar characteristics like the ability to tolerate and multiply in acidic pH, to produce

organic acids like lactic and acetic acid, and to metabolize sugars (Olofsson et al., 2014a; Stoyanova et al., 2012). Such features explain the effectiveness of these bacteria in colonizing the honey crop. The research presented in this thesis focus on 13 hbs-LAB species; nine *Lactobacilli* and four *Bifidobacteria* (Olofsson et al., 2014a; Olofsson and Vásquez, 2008).

Three major bacterial phyla dominate the honeybee gut microbiome; Proteobacteria (including *Gilliamella, Frischella*, and *Parasaccharibacter*), Firmicutes (e.g., *Lactobacillus sp.* Firm-4 and Firm-5), and Actinobacteria (such as *Bifidobacterium*). The hindgut is colonized by a large microbial community including *Gilliamella apicola, Snodgrassella alvi*, *Frischella perrara*, *Lactobacillus* Firm-4 and *Lactobacillus* Firm-5 and *Bifidobacterium asteroides* (Kwong and Moran, 2016). Although other bacteria may occasionally be present, the mentioned phyla represent bacterial families that seem specifically adapted to life within the honeybee host.

The maintenance of this stable and distinct microbial community depends on the nutrition, the hive environment (Anderson et al., 2013; Zhao et al., 2018), the social interactions (Mattila et al., 2012) and age (Hroncova et al., 2015; Martinson et al., 2012) of honeybees. This microbial community is a highly dynamic and adaptive system whose composition is subject to seasonal trends (Corby-Harris et al., 2014a; Hroncova et al., 2015; Ludvigsen et al., 2015).

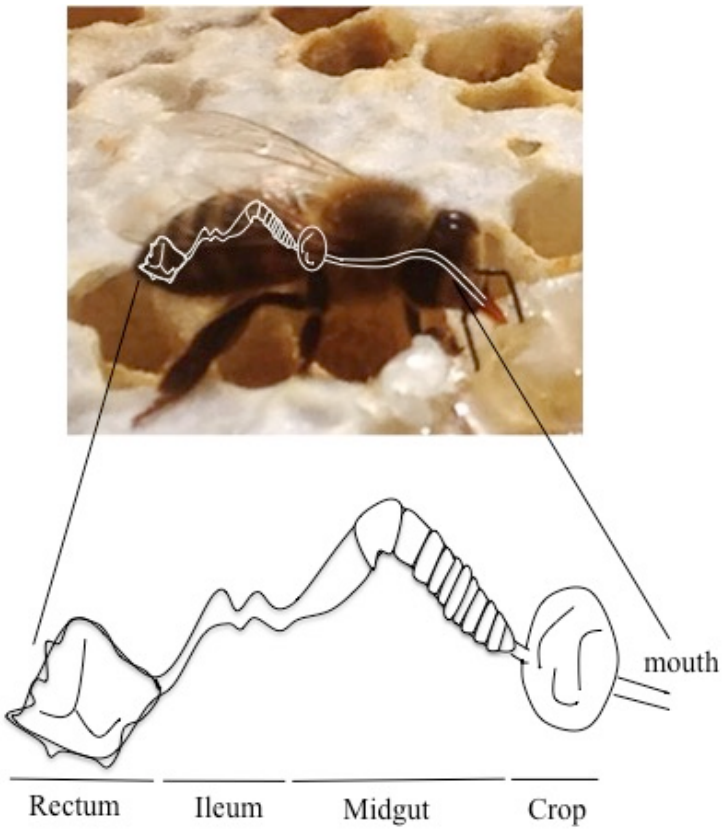

Figure 1. Honeybee digestive tract. © Sepideh Lamei

2.2 Honeybee-specific lactic acid bacteria

Honeybee-specific lactic acid bacteria (Table 1) have been isolated from the honey crop (Figure 2) (Killer et al., 2014; Olofsson et al., 2014a; Olofsson and Vásquez, 2008). However, other researchers also isolated these bacteria from the gut of *A. mellifera* (Corby-Harris et al., 2014a; Hroncova et al., 2015; Killer et al., 2014), pollen and bee bread (Martinson et al., 2012; Vásquez and Olofsson, 2009), royal jelly (Asama et al., 2015), flowers and hive environment (Neveling et al., 2012; Tajabadi et al., 2013), floral nectar (Anderson et al., 2013) and grape juice (Edwards et al., 1998).

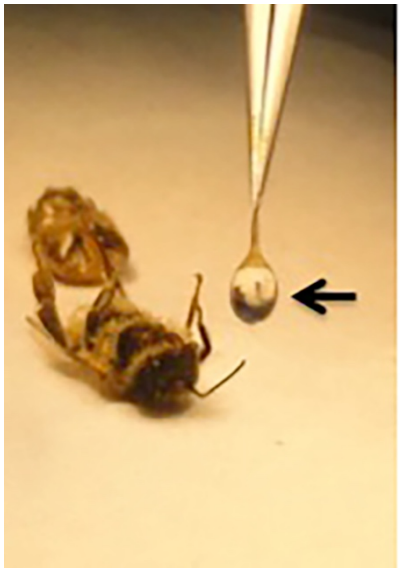

Figure 2. The arrow shows the honey crop of a honeybee. © Sepideh Lamei

All hbs-LAB species are Gram-positive, non-spore forming and non-motile facultative anaerobic bacteria that produce lactic acid. Most of them also lack catalase, except *B. coryneforme* Bma6N and *B. asteroides* Bin2N. The hbs-LAB can grow at a wide range of temperatures, (15-50 °C) and acidity (pH 3-12). These robust traits may be due to their tough membrane which contains a thick peptidoglycan layer (Olofsson et al., 2014a). The bacteria are found in other sub-species of *Apis* as well as in stingless bees (Meliponini) in both Europe and America (Vásquez et al., 2009).

The abundance of hbs-LAB species varies depending on season, honeybee health and floral resources. It was observed that *L. kunkeei*, the most dominant species across all *Apis* sub-species, is abundant during spring and summer in the honey crop, honey, pollen and bee bread but nearly absent during winter (Corby-Harris et al., 2014a; Olofsson and Vásquez, 2008; Vásquez et al., 2012). Previously, *L. kunkeei* was described as a fructophilic LAB which needs external electron acceptors such as fructose, pyruvate and oxygen for glucose metabolism (Endo, 2012; Endo et al., 2012). The isolation of this species from the bee hive environment (Neveling et al., 2012), fermented wine (Edwards et al., 1998) and flowers indicates its niche adaptation (Vásquez et al., 2012). Moreover, the niche adaptation characteristic has been confirmed by a study showing that *L. kunkeei* has lost some genes and protein families resulting in a relatively small genome compared to other closely related species. From 268 lost protein families with assigned functions, 15% have affected the carbohydrate metabolism and

transport indicating that they may have evolved to deal with shifts from nutritionally poor to rich environments (Tamarit et al., 2015).

The sequencing of the hbs-LAB genomes has revealed the presence of numerous phosphotransferase systems involved in the uptake of sugars, particularly in *L. apis, L. helsingborgensis, L. melliventris, L. kimbladii, L. kullabergensis* (Ellegaard et al., 2015). Moreover, hbs-LAB express large putative cell-surface proteins of unknown function (Ellegaard et al., 2015; Kwong et al., 2014). Many accessory genes related to carbohydrate transport and storage have been detected in the genomes of *L. kunkeei* (Tamarit et al., 2015) and some of the other hbs-LAB (Ellegaard et al., 2015). Considering the carbohydrate-rich diet of honeybees (e.g. nectar, honey), it is not surprising that the hbs-LAB have evolved to thrive on this resource and are all able to metabolize glucose and fructose, the main constituents of nectar and honey (Lamei et al., 2017; Olofsson et al., 2014a).

2.2.1 Hbs-LAB metabolism and metabolites

The symbiotic roles of gut bacteria are mostly based on metabolic interactions with their host (Flint et al., 2017). Therefore, studying the metabolism of the gut microbiota is the key to understanding how the microbiome's effects on the host are mediated.

Lactic acid bacteria are generally chemotrophic and need nutrient rich media including carbohydrates and amino acids for growth. These bacteria have been classified as homo-fermentative, obligate or facultative hetero-fermentative (Gänzle, 2015; Kandler, 1983). Environmental conditions affect bacterial metabolism and the honey crop is both microaerophilic and carbohydrate rich (Winston, 1991; Zhao et al., 2018). The metabolic pathways can therefore be used to characterize the hbs-LAB functions.

Homo-fermentative LAB catabolize one mole of glucose in the glycolysis (Embden-Meyerhof) pathway to yield two moles of pyruvate under conditions of excess glucose and limited oxygen. This process yields two moles of ATP per glucose consumed (Pot et al., 1994; Rattanachaikunsopon and Phumkhachorn, 2010). All of the lactobacilli used in this study except *L. kunkeei* and *L. apinorum* are homo-fermentative bacteria (Table 1)(Olofsson et al., 2014a). *Lactobacillus helveticus, Streptococcus thermophilus, Lactobacillus acidophilus* are some examples of LAB of other species in this group (Gänzle, 2015).

Hetero-fermentative LAB utilize glycolysis, but unlike homo-fermentative microorganisms, these bacteria possess phosphoketolase pathways to ferment hexoses. Theoretically, end products like CO_2, lactate and ethanol are produced in equimolar quantities from the catabolism of one mole of glucose (Pot et al.,

1994). Obligate hetero-fermenting bacteria (e.g., *L. kunkeei* and *L. apinorum*) (Table 1) lack aldolase and must divert the flow of carbon through a different series of reactions, the pentose phosphate or phosphoketolase pathway. Facultative hetero-fermenting bacteria (*e.g.*, *Lactobacillus plantarum*) utilize hexoses through the homo-fermentative pathway, but they also possess an inducible phosphoketolase with pentoses acting as inducers (Gänzle, 2015; Pot et al., 1994).

Bifidobacteria degrade hexose sugars through a particular metabolic pathway, termed the ‘‘bifid shunt’’, where the fructose-6-phosphoketolase enzyme, a taxonomic marker for the family of Bifidobacteriaceae, plays a key role. Carbohydrate metabolic abilities may vary considerably between bifidobacterial strains (Pokusaeva et al., 2011).

Organic acids such as acetic acid and lactic acid are the main end metabolites produced by LAB during fermentation, and that is also the case for hbs-LAB. The antimicrobial effect of the organic acids produced is due to the decrease in pH, making the conditions acidic and unfavorable for many pH sensitive pathogens. The high concentration of organic acids (succinate, acetate and propionate) in the gut of bees colonized by LAB confirms that acid fermentation is a major metabolic activity of the bee symbiotic bacteria, as was previously inferred from functional genomic analyses (Zheng et al., 2017).

Lactobacillus kunkeei Fhon2, *L. apinorum* Fhon13 and two *Bifidobacteria* (Bin2N and Bin7N) produce free fatty acids. *Lactobacillus apinorum* Fhon13, *Lactobacillus melliventris* Hma8 and *Lactobacillus kimbladii* Hma2 produce 2-heptanone: a known honeybee alarm pheromone (Olofsson et al., 2014b). 2-heptonone also acts as a local anesthetic that paralyses *Varroa* mites and wax moth larvae when bitten by honeybees (Papachristoforou et al., 2012). Hydrogen peroxide, which has a broad-spectrum inhibition against a variety of bacteria, is another antimicrobial substance produced by five of hbs-LAB (*L. helsingborgensis* Bma5, *L. apis* Hma11, *L. melliventris* Hma8, *L. kimbladii* Hma2 and *L. kullabergensis* Biut2) (Olofsson et al., 2014b). It was also shown that when hbs-LAB were subjected to molecules associated with Pathogen-Associated Molecular Patterns (PAMPs), such as lipopolysaccharide, lipoteichoic acid and peptidoglycans, they produce extracellular proteins, including enzymes, DNA chaperones, lysozymes, putative bacteriocins and proteins with completely unknown functions. Some of these proteins, such as the putative bacteriocin and helveticin-J homolog produced by *L. helsinborgensis* Bma5, could contribute further to the antimicrobial properties of these bacteria (Butler et al., 2013).

Table 1. hbs-LAB used in this project.

Species	Strain	Originally isolated from	Fermentation type
L. kunkeei	Fhon2N	Fermented grapes	Obligate heterofermenter
L. apinorum	Fhon13N	*A. m. mellifera**	Obligate heterofermenter
L. mellis	Hon2N	*A. m. mellifera**	Homofermenter
L. mellifer	Bin4N	*A. m. mellifera**	Homofermenter
L. apis	Hma11N	*A. m. mellifera**	Homofermenter
L. helsingborgensis	Bma5N	*A. m. mellifera**	Homofermenter
L. melliventris	Hma8N	*A. m. mellifera**	Homofermenter
L. kimbladii	Hma2N	*A. m. mellifera**	Homofermenter
L. kullabergensis	Biut2N	*A. m. mellifera**	Homofermenter
B. asteroides	Bin2N	*A. m. mellifera**	F6PPK**
B. asteroides	Bin7N	*A. m. mellifera**	F6PPK**
B. asteroides	Hma3N	*A. m. mellifera**	F6PPK**
B. coryneforme	Bma6N	*A. m. mellifera**	F6PPK**

**Apis mellifera mellifera*

**Fruktose 6 phosphate phosphoketolase pathway

2.2.2 Roles of hbs-LAB

It has been speculated that the hbs-LAB microbiota constitutes the first line of defense against potential brood pathogens acquired from the floral environment and that this plays an important role in the production of bee bread; a nutrient source composed of bee pollen, nectar and secretions from the bee's salivary glands that honeybees use to feed their larvae (Vásquez and Olofsson, 2009).

Bee pollen is collected by foraging bees and is transported back to the hive in the pollen baskets on the hind legs of the foragers (Herbert and Shimanuki, 1978). Nectar is also collected from flowers by foraging bees that transport it back to the hive in their honey crop or honey stomach. The pollen is packed into the brood comb cells and sealed with a drop of nectar that is regurgitated by the bees and contains hbs-LAB from the honey crop (Vásquez and Olofsson, 2009). After two weeks, the stored pollen is chemically changed through natural fermentation by the different microorganisms. It has been proposed that honeybees would be predicted to consume older stored pollen after a period of maturation to obtain maximal nutritional benefits from the bacterial fermentation. However, other studies suggest that bees preferentially consume freshly deposited stored pollen in favor of older stored pollen (Anderson et al., 2014; Carroll et al., 2017; Herbert and Shimanuki, 1978). Antifungal activity has recently been suggested for *L. kunkeei* but not for *L. helsingborgensis* found in stored pollen (Janashia et al., 2018).

Inhibitory effects from hbs-LAB on honeybee bacterial pathogens have been demonstrated convincingly, by multiple labs (Forsgren et al., 2010a; Killer et al., 2014; Vásquez et al., 2012). Previous work in our group demonstrated that the oral administration of hbs-LAB decreased the proportion of *P. larvae* infected larvae in larval bioassay (Forsgren et al., 2010a). The same hbs-LAB were orally administered to honeybee larvae challenged with *Melissococcus plutonius*, the infective agent of European foulbrood, showing that the larval mortality was significantly reduced in groups treated with the hbs-LAB mixture under laboratory conditions (Vásquez et al., 2012). The mechanisms behind these inhibitory effects were investigated further in **Paper II** of this thesis.

3 Honeybee bacterial diseases

Like most insects and livestock, honeybees are subject to many diseases and pests. Two firmicute bacteria, *Paenibacillus larvae* and *Melissococcus plutonius*, are the primary causative agents of American foulbrood (AFB) and European foulbrood (EFB) respectively, and these two are the sole formalized bacterial diseases of honeybee larvae (Animal and plant health agency, 2017; Genersch, 2010). American foulbrood is one of the major pathogenic threats to beekeeping as it is highly contagious, capable of killing infected colonies causing large economic losses worldwide. This thesis therefore focuses on the effect of hbs-LAB on AFB and its causative agent.

3.1 American foulbrood disease

In 1906, an American microbiologist isolated and classified the etiological agent of American foulbrood, AFB (White, 1906). The correct classification of the causative agent of AFB was not trivial and it took several attempts until it was eventually correctly classified as *Paenibacillus larvae* (Genersch et al., 2006), a highly contagious and destructive bacterial honeybee brood pathogen affecting beekeeping worldwide (Genersch, 2010).

American foulbrood only affects honeybee larvae while adult honeybees are resistant to the infection. Young larvae ingest *P. larvae* spores with the food provided by the nurse bees. The spores germinate in the midgut and the resulting vegetative cells rapidly proliferate. Once an abundant threshold level of vegetative cells accumulates within the larval midgut, proteases, collagenases, chitinase, and toxins are believed to allow *P. larvae* to breach the midgut epithelium via degradation of the Peritrophic Matrix (PM) of the larvae (Antúnez et al., 2011a, 2011b, 2009; Djukic et al., 2014; Genersch, 2010). When nutrients become scarce, the *P. larvae* population undergoes sporulation.

Paenibacillus larvae is a spore-forming (Figure 3), Gram-positive bacterium (Genersch et al., 2006; Heyndrickx and Vos, 1996) for which 4 distinctly different genotypes have been defined using repetitive element PCR (rep-PCR) and Enterobacterial Repetitive Intergenic Consensus (ERIC) primers (Genersch et al., 2006; Genersch and Otten, 2003). All four genotypes differ in several phenotypic characteristics, most importantly in virulence which the difference between individual level and colony level virulence and the role of social immunity has in driving this difference. Epidemiological studies show that the genotypes most frequently isolated from AFB diseased colonies are ERIC-I and ERIC-II, while ERIC-III and ERIC-IV are historical isolates deposited to culture collections in the 1950s and never yet been reported from bee colonies with typical symptoms of AFB. ERIC-I and -II are thus the most important genotypes with respect to infection of honeybee larvae. The genotype-specific differences in virulence between *P. larvae* ERIC-I and -II correspond to the time it takes to kill infected larvae. Members of *P. larvae* ERIC-II kill larvae relatively quickly, with a LT_{100} (the time it takes the pathogen to kill 100% of the infected hosts) of approximately seven days while strains of *P. larvae* ERIC-I genotype kill larvae relatively slowly (LT_{100} approximately 12 days). The difference in virulence at the individual larval level also influences the virulence at the colony level (Genersch et al., 2006, 2005; Rauch et al., 2009). Virulence in individual larvae is negatively correlated to virulence at the colony level due to the hygienic behavior of the honeybees (Rauch et al., 2009). *Paenibacillus larvae* ERIC-II genotype cause larval mortality before cell capping making it easier for the bees to detect and clean out the larval corpse, while the bees have difficulties to detect dead infected larvae with ERIC-I genotypes in capped cells, causing higher mortality.

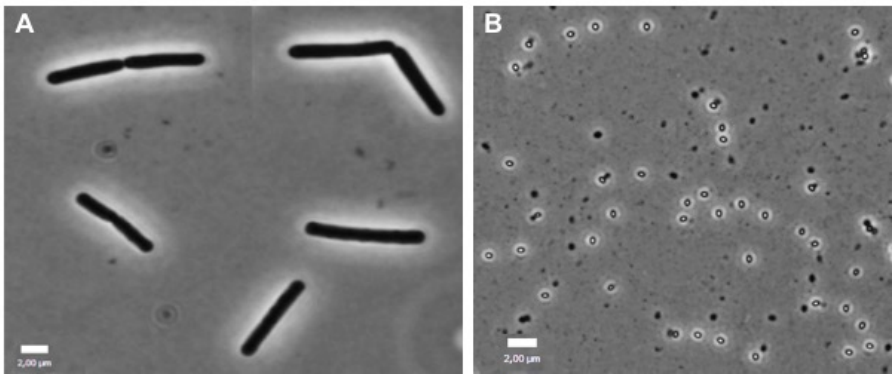

Figure 3. Paenibacillus larvae. (A); vegetative cells. (B): spore cells. © Ewa Bukowska-Faniband, Sepideh Lamei

3.1.1 AFB symptoms

Typical symptoms in colonies suffering from clinical AFB disease are irregular brood capping with capped and uncapped cells scattered irregularly across the brood frames. The caps are dark, sunken and often punctured (Figure 4), emitting a foul odor. Larval remains can be drawn out to approximately one inch using a matchstick, often referred to as the "ropiness" test (Figure 5). The ropy stage dries down to a hard scale (foulbrood scale), tightly adhering to the lower cell wall. These scales are highly infectious since they contain millions of spores which drive disease transmission within and between colonies (Genersch, 2010). It has been shown that some colonies can maintain large number of spores over several seasons without any clinical symptoms of AFB being manifested (Fries et al., 2006).

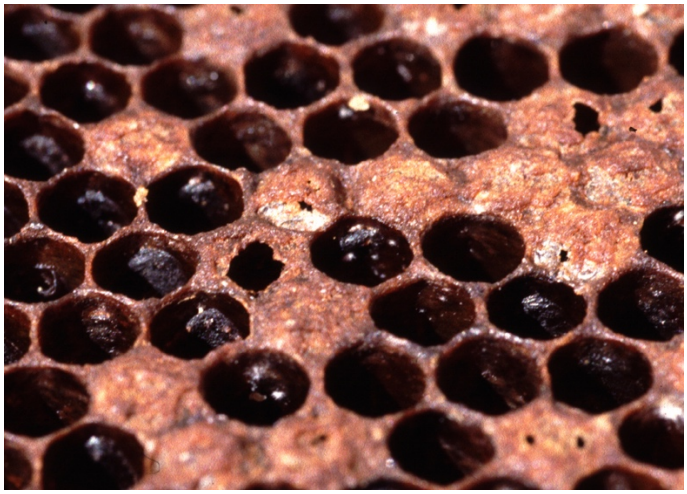

Figure 4. Dark, sunken and often punctured caps are the symptoms of American foulbrood. © Preben Kristiansen

3.1.2 AFB transmission

American foulbrood has been considered the paradigm of a highly virulent honeybee disease which can be transmitted within colonies by nurse bees and between colonies by robbing, drifting, beekeeper-mediated transmission through the transfer of contaminated frames between colonies.

The spread of *P. larvae* within the honeybee colony occurs mainly through nurse bees feeding the larvae contaminated feed. These bees not only feed the larvae but they also clean out brood that died from AFB, thus contaminating the larval food with bacterial spores from their mouth parts. Empty brood cells

which have contained larvae that died of AFB can re-infect a new larva even if the bees clean out the cells, through the spores remaining in the dried scale.

The natural spread of *P. larvae* between colonies occurs through drifting and robbing bees carrying the bacterial spores. In addition to these "natural" routes of transmission are the much more important common beekeeping practices like the transport and reuse of contaminated hive material and brood frame movement. All material that has been in contact with diseased colonies is potentially infectious if it has not been properly sanitized. Honey and brood frames are the pieces of equipment that are most frequently swapped between hives and are therefore a major operational risk for the spread of the disease within a beekeeping operation. Honey can contain large numbers of bacterial spores and is a well-known source of infection (Fries et al., 2006; Fries and Camazine, 2001; Genersch, 2010). The colony level distribution of *P. larvae* spores differs significantly depending on the intra-colony origin of spores. Furthermore, the bacterial spore loads in adult bees are closely linked to the mortality of honeybee larvae while there is no significant relationship between spore loads in honey and the larval mortality in the colony (Lindström et al., 2008). Swarming bees from infected colonies also carry *P. larvae* spores but the chances for developing disease symptoms is low if the bees are shaken onto clean wax foundation (Fries et al., 2006).

3.1.3 AFB diagnosis methods

Detection of AFB in the field is based on the appearance and recognition of clinical symptoms of the disease, the "ropiness" test of diseased larvae (Figure 5) and through commercial immune-chromatography-based test kits (Vita-Europe). The diagnosis can be confirmed in the laboratory by the detection of *P. larvae* spores from larval remains (de Graaf et al., 2006; Hansen and Brødsgaard, 1999). To further verify the presence of *P. larvae*, material from suspected larvae/pupae, adult bees, honey, wax and debris can be cultivated on artificial bacteriological media and suspected bacterial colonies can be identified using biochemical or molecular techniques such as PCR (Alippi et al., 2004; de Graaf et al., 2006; Forsgren and Laugen, 2014; Lindström and Fries, 2005). It has been shown that culture-based techniques are more accurate than PCR for detecting *P. larvae* in clinically diseased colonies and that the prognostic value of bacterial colony counts from bee samples is superior to colony counts from debris (Forsgren and Laugen, 2014).

Since different colonies express different degrees of hygienic behavior for cleaning out diseased and dead larvae, some colonies can contain high levels of *P. larvae* spores on bees or in the honey without showing any clinical symptoms

(sub-clinical levels of infection). Sub-clinical occurrence of *P. larvae* can be detected sensitively by culturing samples of adult bees both in individual colonies and at the apiary level using composite samples of bees (Lindström and Fries, 2005).

Figure 5. Ropiness test. Larval remains can be drawn out to approximately one inch using a matchstick. © Preben Kristiansen

3.1.4 AFB control methods

American foulbrood is a statutory notifiable disease within the European Union and disease incidence has to be reported to the competent authorities and control measures carried out according to the laws of the country (Lodesani and Costa, 2015). Such control measures include the burning of infected colonies and hive material. However, not all colonies are destroyed upon the detection of disease symptoms. In some countries the legislation allows for the use of "the shook swarm method", where all the adult bees are shaken onto new wax foundation and the brood frames are burned. It is very important that hive equipment from diseased colonies is sterilized carefully considering that the spores of *P. larvae* are extremely resilient and can stay infectious for decades (Genersch, 2010).

Current European legislation does not allow beekeepers to use antibiotics since there is no maximum residue limit (MRL) set for the antibiotic substances used to control AFB (tylosin, tetracycline, oxytetracycline and streptomycin).

Antibiotics can therefore not be used legally, since there is a zero tolerance limit to antibiotic residues in honey (Reybroeck et al., 2012). In countries outside the European Union such as the USA, Canada and Argentina, antibiotics are used routinely, and often prophylactically, as a strategy to control AFB. However, several problems are associated with the extended use of antibiotics: (i) antibiotics are not effective against the infectious bacterial spores. They therefore only suppress clinical symptoms but do not cure AFB; (ii) chemical residues can persist in honey affecting its quality and safety for human consumption (Reybroeck et al., 2012); (iii) the use of antibiotics may lead to the emergence of resistant *P. larvae* strains (Evans, 2003; Lodesani and Costa, 2015; Murray and Aronstein, 2006).

Recent discoveries of the strong antagonistic effect of bacterial microbiota on the infectivity and pathogenicity of *P. larvae* (Forsgren et al., 2010b; Janashia and Alaux, 2016; Killer et al., 2014; Maggi et al., 2013; Sabaté et al., 2012), breeding for disease resistant honeybees (Evans, 2004; Spivak and Reuter, 2001) and treatment with natural antimicrobial substances like essential oils of various plants (de Almeida Vaucher et al., 2015; Fuselli et al., 2009) have identified possible new approaches for AFB control. In this thesis, we focused on the antagonist effect of hbs-LAB on *P. larvae* and their potential as a health promoting supplement for honeybee colonies.

4 Aims

The overall goal of this thesis is to gain deeper understanding on the role of hbs-LAB in honeybees and the effect of these bacteria on the honeybee pathogen *Paenibacillus larvae.* The specific aims of this project are:

I. Improve identification methods for detection and identification of hbs-LAB.

II. Determine the effect of hbs-LAB secretome on the growth of *P. larvae* genotypes ERIC-I and ERIC-II and the mortality of infected larvae.

III. Investigate the effect of oral administration of hbs-LAB supplement and tylosin on spore levels of *P. larvae*, AFB symptoms and colony strength.

5 Methods

5.1 Culture-dependent techniques

Identification is the first step in establishing the bacterial etiology of a particular disease. It includes the procedures and techniques used to correctly identify bacteria. Traditional methods of bacterial identification rely on phenotypic identification of the causative organism such as culturing on plates or in broth in addition to biochemical methods.

5.1.1 Traditional culturing and growth conditions

A true understanding of the physiology of the hbs-LAB and the role they play in their ecological niches and in honeybee health is based on an in-depth understanding of their mode of action and/or their metabolism. Investigating such properties requires artificial media for optimal bacterial growth. The successful transition of bacteria from the *in vivo* to the *in vitro* environment requires that nutritional and environmental growth requirements can be adequately met (Hayek and Ibrahim, 2013).

In this thesis 13 hbs-LAB consisting of 9 *Lactobacillus* and 4 *Bifidobacterium* species specified in the introduction (Table 1) were grown in Man, Rogosa & Sharpe (OXOID, England) broth (MRS) supplemented with 2% fructose (Merck, Sollentuna, Sweden) and 0.1% L-cysteine (Sigma-Aldrich, Stockholm, Sweden) (sMRS) at 35°C. In **Paper I**, we used a turbidimetric assay to follow the growth curves of the hbs-LAB in various supplemented media. This study showed that hbs-LAB require supplements such as fructose and L-cysteine rather than glucose for optimal growth under laboratory conditions (Figure 6). The optimized growth conditions was also crucial for the downstream identification of hbs-LAB by MALDI-TOF MS (**Paper II**) and for testing the

antimicrobial activity of the bacteria in agar well diffusion assays (**Paper II**). The agar well diffusion assay has been widely used for evaluation of antimicrobial activity, especially for biologically derived compounds. In this test, we tested antimicrobial activity of the Cell Free Supernatant (CFS) from a mixture of the 13 hbs-LAB of incubated together for 18 h, *i.e.* the secretome of a complex LAB community, containing both individual and cross-metabolized bacterial products. The molecular weight and hydrophobicity of a substance may affect its diffusion properties, and thus the outcome of an agar well diffusion test (Valgas et al., 2007). Since the possible antimicrobial substances (and their physico-chemical properties) in the CFS of the hbs-LAB mixture were unknown, the agar well diffusion assays were complemented by "turbidimetric assays" to investigate the inhibition activity of the CFS on the spore and vegetative cells of *P. larvae* (**Paper II**). This technique provides information of the growth pattern of *P. larvae* as well as any effect from antimicrobial compounds causing a delayed lag phase or a reduced growth rate compared to controls.

Paenibacillus larvae genotypes ERIC-I (CCUG 48979) and ERIC-II (CCUG 48972) were cultured on MYPGP agar (Mueller-Hinton broth, Yeast extract, Potassium phosphate, Glucose and Pyruvate) plates (Nordström and Fries, 1995) and incubated at 35°C with 5% CO_2 for 72 h. To prepare spore suspensions with defined concentrations, *P. larvae* genotypes ERIC-I and ERIC-II were cultured on MYPGP agar and incubated at 35°C with 5% CO_2 for 3 weeks. Bacterial colonies were suspended in sterile 0.9% saline and a total microscopic count of the spores was made in a Helber Bacteria Counting Chamber (Hawksley) using a phase-contrast light microscope (400x, Reichert, Austria) (Figure 3). To prepare suspensions of vegetative cells, *P. larvae* spores were inoculated in MYPGP broth and incubated at 35°C with 5% CO_2. After 24-30 h incubation, the samples were checked for *P. larvae* cells by phase contrast microscope (Figure 3) (Forsgren et al., 2008).

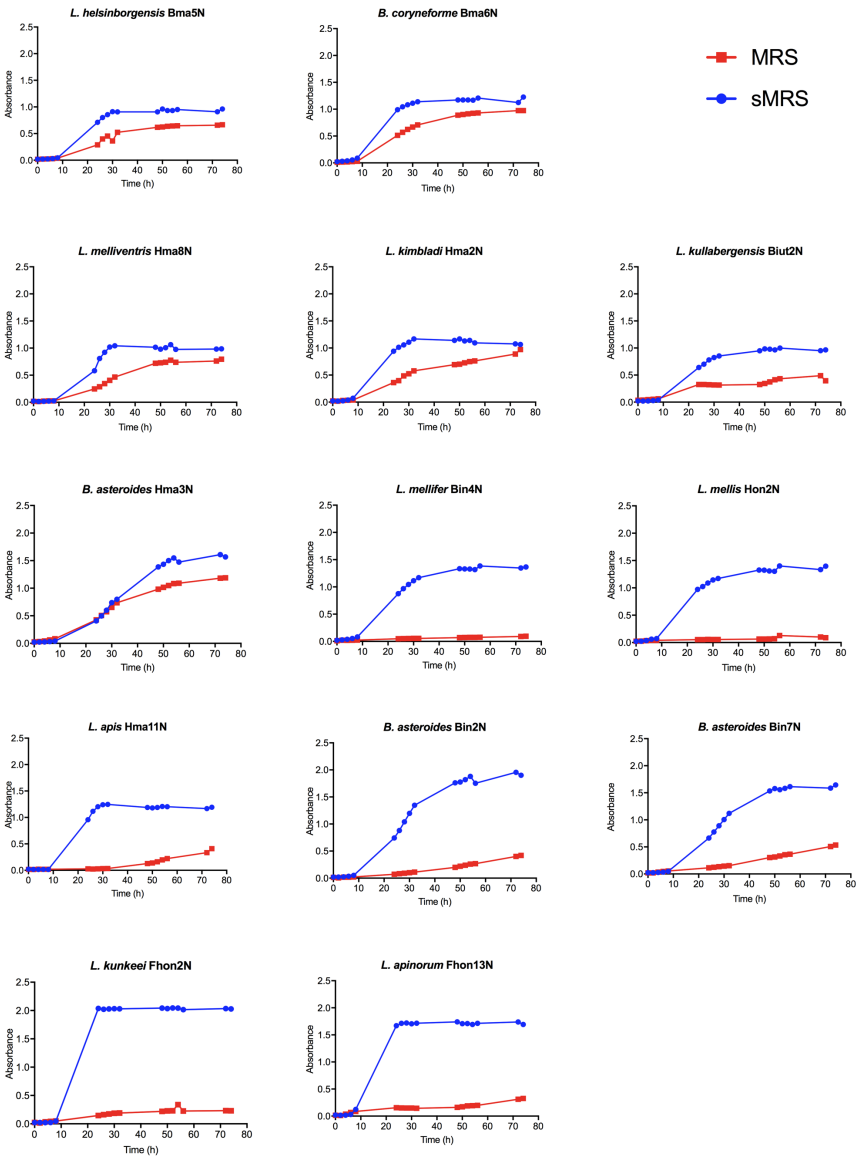

Figure 6. Growth curves of hbs-LAB in MRS (red) with supplemented-MRS (blue) at measured OD (λ 600 nm).

5.1.2 Matrix-Assisted Laser Desorption/ Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry (MS)

Mass spectrometry is an analytical technique which offers the possibility of an accurate, rapid, inexpensive identification of microorganisms, such as bacteria, isolated in clinical microbiology laboratories without the need for morphological phenotyping and laborious biochemical testing (Singhal et al., 2015).

MALDI-TOF MS can examine the profile of proteins detected directly from intact bacteria. This technique, based on relative molecular masses, is a soft ionization method allowing desorption of peptides and proteins from whole cultured microorganisms. Ions are separated and detected according to their molecular mass and charge. This approach yields a reproducible spectrum for a given bacterial strain within minutes, consisting of a series of peaks corresponding to m/z ratios of ions released from bacterial proteins during laser desorption (Wunschel et al., 2005).

A number of published studies have demonstrated the accuracy of MALDI-TOF MS for the identification of a broad spectrum of bacteria including *Lactobacilli* and *Bifidobacteria* (Anderson et al., 2014; Buneśová et al., 2014; Foschi et al., 2017). Although there are culture independent applications for samples consisting of blood, urine and cerebrospinal fluid (DeMarco and Burnham, 2014; Murray, 2012; Segawa et al., 2014), the limiting factor of this technique is still the requirement for culturing of isolates prior to testing. Moreover, it is recommended that MALDI-TOF MS should be used in combination with other molecular methods such as 16S rRNA sequencing to differentiate closely related species (Carbonnelle et al., 2011; Clark et al., 2013).

The process of sample preparation for the identification of microbes by MALDI-TOF MS depends upon the source from which it was isolated or on the chemical nature of the constituents of its cell wall. Studies have shown that a preparatory extraction of microbes with formic acid (FA) reportedly increases the ability of MALDI-TOF MS to identify Gram-positive bacterial species (Dubois et al., 2010; Mellmann et al., 2008) while this extra preparation is not necessary for Gram-negative bacteria (Eddabra et al., 2012; Stephan et al., 2011). Formic acid was used prior to the addition of the matrix on top of each sample for the detection and identification of hbs-LAB (**Paper II**).

5.2 Culture-independent techniques

Culture-dependent bacterial identification approaches have shown limitations in terms of recovery rate: the set of obtained isolates may not always truly reflect the proportion of microbial composition of the sample that can be cultured. Lactic acid bacterial species occurring at low frequencies are often out-competed

in vitro by numerically more abundant microbial species, making isolation of the less abundant bacterial species difficult. As a result, ecological studies increasingly rely on culture-independent methods, usually based on DNA or RNA sequences, for analyzing the composition of complex bacterial samples (Temmerman et al., 2004).

5.2.1 16S rRNA Illumina Miseq sequencing

With the advent of the Sanger chain termination sequencing method in 1977, scientists were able to sequence DNA in a reliable, reproducible manner (Sanger et al., 1977). 16S ribosomal RNA (rRNA) sequencing is a commonly used amplicon sequencing method to identify and compare the number of bacteria present within a given sample (Chakravorty et al., 2007). Chain termination sequencing has been largely replaced by synthesis-based sequencing methods, which are better suited for automation, high throughput capacity, accuracy and cost-effectiveness, with the Illumina platform currently considered the gold standard in microbial diversity analysis. In principle, the concept behind Illumina sequencing is similar to older sequencing methods by the incorporation of fluorescently labeled nucleotides during DNA synthesis and fluorophore excitation of millions of fragments in parallel (Degnan and Ochman, 2012; Sinclair et al., 2015).

Until recently, a significant challenge of the Illumina sequencing of 16S rRNA gene amplicon has been to accurately identify true biological sequence variants while excluding amplicon sequencing errors (Callahan et al., 2015; Edgar, 2013; Eren et al., 2015). A common way to study microbial communities is to cluster similar amplicon reads. This masks most amplification errors but at the same time removes some true biological variants (Edgar, 2013; Kozich et al., 2013). In **Paper I**, we present how we successfully retrieved the 16S rRNA gene sequences for the 13 hbs-LAB strains using Illumina amplicon sequencing and the Divisive Amplicon De-noising Algorithm (DADA2), an open-source software package, for modeling and correcting Illumina-sequenced amplicon errors. Sample composition is inferred by dividing amplicon reads into partitions consistent with the error model (Online Methods). DADA2 is reference-free and applicable to any genetic locus. The DADA2 R package implements the full amplicon workflow: filtering, de-replication, sample inference, chimera identification, and merging of paired-end reads. Researchers have compared DADA2 to four other algorithms (Online Methods): UPARSE, an OTU-construction algorithm with the best published false-positive results; MED, an algorithm with the best published fine-scale resolution in Illumina amplicon data; and the popular mothur (average linkage) and QIIME (uclust) OTU

methods. These comparisons show that DADA2 is more accurate than the other methods for resolving fine-scale variation and outputs fewer incorrect sequences than the best OTU method. The precision of DADA2 improves downstream measures of diversity and dissimilarity and could potentially allow amplicon methods to probe strain level variation (Callahan et al., 2015).

5.3 Hbs-LAB experimental design

We tested the effect of hbs-LAB on *P. larvae* in individual honeybees (**Paper II**) and at honeybee colony level (**Paper III**).

In **Paper II**, larval exposure bioassays were used to assess the inhibitory activity of the hbs-LAB CFS on *P. larvae* pathogenesis in honeybee larvae. First instar worker larvae from honeybee colonies located at the Swedish University of Agricultural Sciences, Uppsala, Sweden were grafted with a Chinese grafting tool (Bienenzuchtgeräte, Graze, Weinstadt, Germany) into individual wells of 48-well tissue culture plates, each containing 10 µL of pre-warmed diet (Aupinel et al., 2005). Each bioassay included technical controls for assessing the background larval mortality. Bioassays with >15% background mortality were excluded from the data. The experimental larvae were infected with $1x10^4$ *P. larvae* spores per larvae: an infectious dose that produces sufficient larval mortality (about 70%) to observe the remedial effect of hbs-LAB treatment on larval mortality (Forsgren et al., 2010b). Different experimental groups were fed larval diet supplemented with either the CFS (3 µL of reconstituted CFS in 10 µL diet) or a mixture of live 13 hbs-LAB cell suspension ($1x10^7$ bacteria per 10 µL diet) for 6 consecutive days, with increasing amounts of daily diet (10 µL, 10 µL, 20 µL, 30 µL, 40 µL and 60 µL food/larva/day, respectively). Control bioassays assessing any direct effect of live hbs-LAB on uninfected larvae were all negative. The larvae were maintained in an incubator at 35ºC with a relative humidity of 96%. The larvae were checked daily for mortality before feeding. Dead larvae were removed, the gut content streaked onto MYPGP agar for the cultivation of *P. larvae* with the identity of the bacterial colonies confirmed by PCR (Dobbelaere et al., 2001). Each bioassay was repeated three times for both *P. larvae* genotypes (ERIC-I and II), with a total of 30 larvae per replicate, except for the *P. larvae*-infected experimental group treated with live hbs-LAB bacteria, which was replicated twice.

The effect of oral administration of hbs-LAB supplement and tylosin were investigated further on *P. larvae* spore counts and clinical symptoms and to explore the relationship between colony size and the disease determination on honeybee colonies in USA (**Paper III**). Forty honeybee colonies headed by queens of the same age from a single queen breeder were established in March

2014 using 1.2 kg packages of honeybees in an isolated apiary in Beltsville, MD, USA. The colonies were placed in an apiary with a history of AFB, and the frames and boxes used came from colonies that were managed with AFB in previous studies. All colonies in this apiary would have had some exposure to AFB, but to insure exposure, all colonies were given a standard spore suspension as described below. The colonies were fed three times with a 1:1 sucrose: water solution to help establish the colonies and promote growth of adult bee populations. The colonies were assessed and inspected on April 23th for the presence of AFB and AFB severity rated as noted below. The colonies were assigned to the treatment groups using a stratified random design, *i.e*. colonies were ranked according to AFB severity, divided into groups of four down the ranking, with subsequent random treatment assignment within each ranking group, with each colony in each ranking group receiving one of the following treatments, in a double-blinded fashion: (1) antibiotic (tylosin), (2) hbs-LAB placebo, (3) hbs-LAB supplement, (4) no treatment. The supplement was administered on two occasions, April 23-25th and May 7-9th 2014, right after the sampling in these months. All 40 colonies in the AFB apiary were inoculated on May 1st 2014 with a suspension of *P. larvae* spores to boost the onset of AFB. Colonies were sampled, assessed and inspected for AFB symptoms at 6 occasions between April and August 2014. Adult honeybees were sampled monthly from each colony between April and August 2014. An additional sampling was done in May, resulting in a total of six sampling occasions. Approximately 200 adult honeybees per sample were collected from the brood chamber in a small cardboard box. The honeybee samples were stored at -20°C until further analysis. The level of AFB was determined by screening of each frame and making a visual inspection of the brood (immature larval and pupal honeybees) for evidence of infection. AFB is easy to identify in the field as the signs of disease are diagnostic and include partially uncapped cells, foul odour and the presence of abnormal looking brood within cells. Samples of diseased cells were taken and laboratory test conducted for positive identification to confirm the field diagnoses. Each frame with brood was rated for AFB infection on a 0–3 scale. A zero was assigned if there were no visible signs of disease, a one indicated fewer than 10 diseased cells, a two indicated 11–100 diseased cells, and a three indicated more than 100 diseased cells. This rating system creates a composite score that can increase with increasing severity or can be divided by the number of frames of brood to give an average infection rating per brood frame.

6 Results and discussion

6.1 Detection and identification of hbs-LAB (Paper I)

The access to adequate techniques is of key importance for the identification of the honeybee microbiome and for answering research questions regarding their role in honeybee health.

In **Paper I,** traditional culturing and 16S rRNA Illumina sequencing were used as complementary techniques for the detection and identification of hbs-LAB. In order to find a culture media optimal for the growth of the 13 hbs-LAB species, we tried to mimic the hbs-LAB natural environment and growth requirements. We investigated which supplements are essential for optimal growth of hbs-LAB and confirmed that the addition of L-cysteine and fructose is crucial for their growth (Butler et al., 2016, 2013; Lamei et al., 2017), likely associated with their natural habitats being honey crops, honey (Olofsson and Vásquez, 2008; Vásquez et al., 2012) and bee bread containing foraged nectar and pollen (Vásquez and Olofsson, 2009) rich in amino acids and sugars. Nectar consists mainly of sucrose and its mono-saccharides fructose and glucose but the exact composition of sugars differs between continents, seasons and floral sources (Nicolson and Thornburg, 2007). Consequently, hbs-LAB have had to adapt to carbohydrate-rich diets. We observed however, that only two hbs-LAB species (*L. kunkeei* and *L. apinorum*) need fructose for growing under laboratory conditions. Interestingly, *L. kunkeei* has previously been classified as an obligate fructophilic bacteria (Endo et al., 2012; Neveling et al., 2012).

16S rRNA Illumina sequencing was used in **Paper I** as a complementary, molecular-based technique for hbs-LAB identification. The difficulty with 16S rRNA gene sequencing for hbs-LAB identification is that these bacteria have high sequence similarity and very high similarity scores, requiring very fine resolution analyses to separate individual species. This is discussed in **Paper I**

where it is concluded that differentiate the bacteria to the strain level using only 16S rRNA amplicon data is difficult. The high similarity in the 16S rRNA gene has been observed in other honeybee gut bacteria such as *Gilliamella apicola*, *Snodgrasella alvi* (Engel et al., 2014) and two *Lactobacillus* spp. strains, wkB8 and wkB10 (Kwong et al., 2014). It has been shown recently that deep sequencing of short amplicons of 16S rRNA genes (the V4 region) is insufficient to identify *S. alvi* at the strain level (Ellegaard et al., 2015). Using Illumina sequencing and a new software to correct for sequencing errors, DADA2, we were able to identify and separate all 13 hbs-LAB (**Paper I**, Figure 7). It has been shown that DADA2 is more accurate and has a higher resolution than the other OUT constructing methods such as UPARSE (Callahan et al., 2015). We observed multiple but highly similar sequence types in four of the Lactobacilli strains (Biut2N, Hma2N, Hma8N, Fhon13N) likely due to multiple copies of their 16S rRNA gene (Ellegaard et al., 2015). In *Escherichia coli*, the use of multiple rRNA operons has been shown to facilitate shifts from poor to rich growth conditions (Condon et al., 1995). Thus, possessing multiple copies of the 16S rRNA gene could indicate that hbs-LAB strains may have evolved under selection for rapid growth following shifts from nutritionally poor to rich environments.

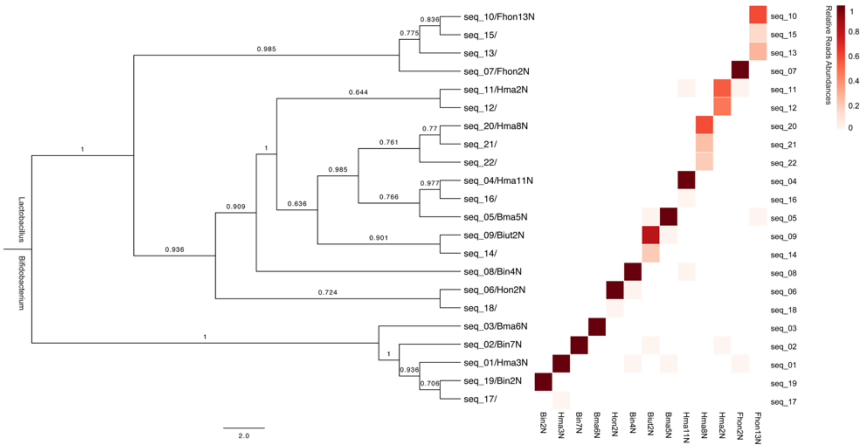

Figure 7. **Phylogenetic tree based on multi-alignment and abundance distribution of hbs-LAB 16S rRNA sequences inferred in this study (Paper I).** The dendrogram illustrates the phylogenetic relationship of the 22 sequence types. Leaf labels marked with hbs-LAB strain names indicate sequences identical to the reference 16S rRNA sequence. The relative abundance of sequence types for each strain is shown in the heat map (each column represents one strain).

6.2 The inhibitory activity of hbs-LAB on *P. larvae* growth in artificial media and in honeybee larvae (Paper II)

The controlled and limited use of antibiotics in the EU and the emergence of antibiotic-resistant *P. larvae* strains have stimulated the search for feasible alternatives to antibiotics, including beneficial microorganisms, for treating or preventing AFB disease. So far, the inhibitory effects against *P. larvae* of bacteria such as *Bacillus* (Alippi and Reynaldi, 2006; Minnaard and Alippi, 2016), *Lactobacillus* (Forsgren et al., 2010a; Killer et al., 2014) and *Enterococci* (Audisio et al., 2011) have been tested under laboratory conditions. Many researchers have tried to identify and purify the active substances responsible for the antimicrobial activity of these bacteria.

It has been shown that individual members of the hbs-LAB microbiota produce metabolites with antimicrobial activity (Butler et al., 2013; Olofsson et al., 2014b). One of the hypothesis of this thesis was that a combination of the 13 hbs-LAB species was most likely to be effective against *P. larvae* since all species co-exist in the honey crop and possibly cross-metabolize secreted compounds and produce a richer and more diverse set of metabolites and antimicrobial peptides (McNally et al., 2014; Olofsson and Vásquez, 2008). The antimicrobial activity of the secretome produced by a mixed culture of the 13 hbs-LAB was tested on *P. larvae* growth and infectivity in honeybee larvae (**Paper II**). A strong inhibition by the CFS was noticed on the growth of *P. larvae* genotypes ERIC-I and ERIC-II resulting in clear and sharp inhibition zones in agar well diffusion assays. The results were confirmed by *in vivo* larval exposure bioassays showing that CFS feed supplement decreased larval mortality caused by *P. larvae* infection. The observed positive effect on larval survival was even higher than when larval feed was supplemented with live hbs-LAB (Figure 8). The results of this study clearly show that the hbs-LAB produce end excrete antimicrobial substances which inhibit the multiplication of *P. larvae* and have the ability to prevent infection and death in honeybee larvae.

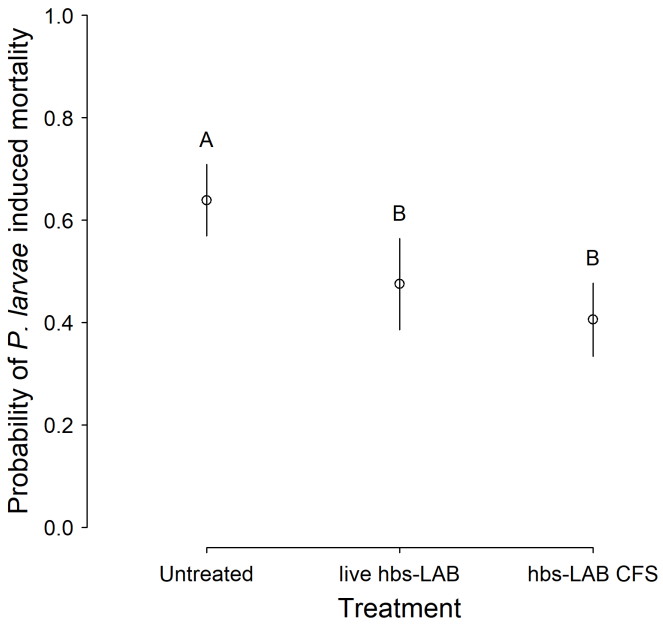

Figure 8. **Inhibitory effect of hbs-LAB cell-free supernatant on *P. larvae* in larval bioassays (Paper II).** Probability of mortality for *P. larvae*-infected honeybee larvae treated with hbs-LAB cell-free supernatant (CFS), relative to untreated (negative control) and live hbs-LAB-treated (positive control) *P. larvae*-infected larvae. Symbols show predicted marginal means with CIs and different lower case letters indicate significant differences (Tukey contrast; $P<0.01$).

The next step was to assess which stages in *P. larvae* germination and growth were affected (**Paper II**). The *P. larvae* growth curve is characterized by an initial rapid vegetative growth resulting in sharp peak followed by a decline in cell density as the bacterial culture enters the sporulation phase (Figure 9). The generation times of ERIC-I and ERIC-II genotypes were about 48.4 min and 18.7 min, respectively. The growth peak of spore-initiated cultures was delayed by ~32 h (ERIC-I) and ~24 h (ERIC-II) relative to vegetative cell-initiated cultures, corresponding to the time the spore cells needed to germinate before the vegetative cell growth starts. As a result of the addition of the hbs-LAB secretome, growth peak initiated from either *P. larvae* spore or vegetative cell is entirely absent and the growth curves reached far lower final cell densities. The hbs-LAB secretome clearly impeded bacterial proliferation and perhaps also the triggers for sporulation (Figure 9). This is likely to be the primary reason for the enhanced survival of *P. larvae*-infected larvae treated with the hbs-LAB secretome.

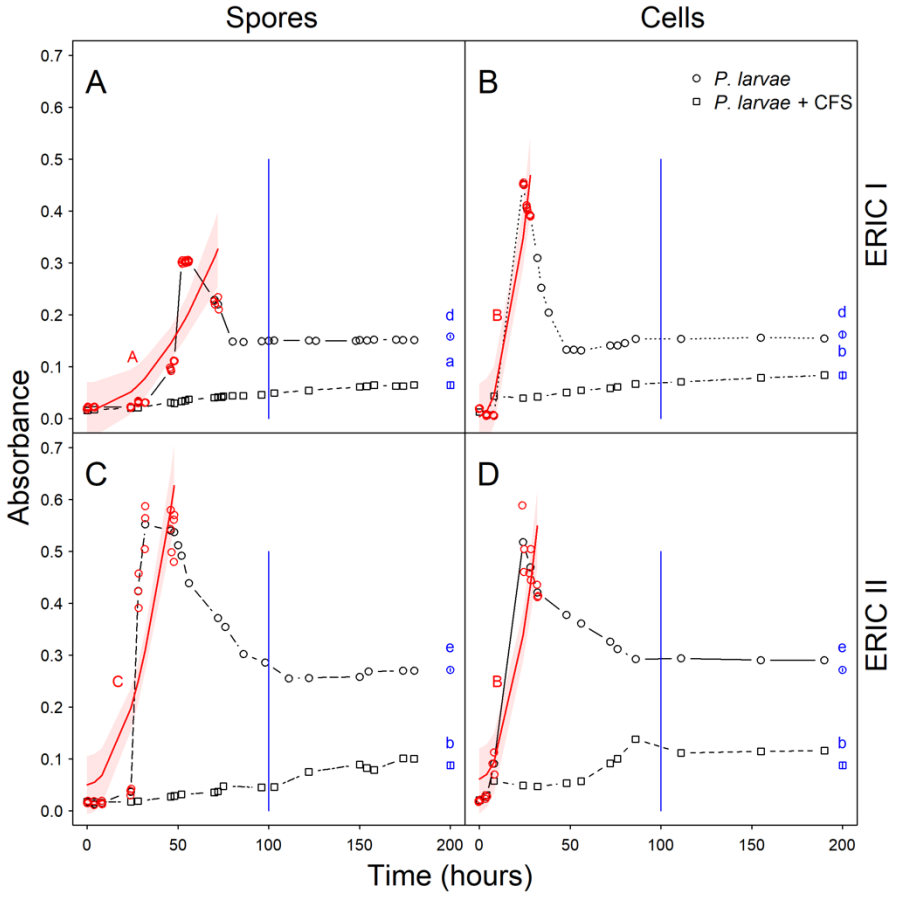

Figure 9. **Inhibitory effect of hbs-LAB CFS on *P. larvae* growth in broth.** Growth curves (black symbols and black lines) of *P. larvae* in MYPGP broth supplemented with hbs-LAB cell-free supernatant (*P. larvae* + CFS) compared to controls (*P. larvae*). Bacterial growth was reflected by increasing turbidity and measured by the optical density at 600 nm (OD_{600}). The growth curves were initiated with either *P. larvae* ERIC-I spores (A), *P. larvae* ERIC-I vegetative cells (B), *P. larvae* ERIC-II spores (C), or *P. larvae* ERIC-II vegetative cells (D). In blue: Data right from the vertical lines indicate the subset that was used to compare both genotypes (ERIC-I/II) and growth stage (spores/cells) combination in the stationary phase. The symbols show predicted marginal means ± CIs (CIs are very small) and different lower case letters indicate significant differences (Tukey contrast; $P<0.01$). In red: The strength of the growth increase of *P. larvae* was compared among all four combinations. Circles show the original subset and lines indicate model predictions with bootstrapped confidence limits. Different upper-case letters indicate significantly different slopes (Tukey contrast; $P<0.05$).

Individual hbs-LAB secrete a wide variety of antimicrobial substances and each species exudes its own unique set of metabolites (Olofsson et al., 2014b). Organic acids are some of the main metabolites produced by LAB. The

antagonistic effect of *Lactobacillus johnsonii*, *L. brevis* (Mudroňová et al., 2011) and *L. johnsonii* (Audisio et al., 2011) was mainly due to acidification, pH: 4.0 - 4.5, generated by organic acids (Audisio et al., 2011). Moreover, the spores of *Bacillus cereus* were more resistant to organic acids than the vegetative cells, with the resistance furthermore affected by pH (Wong and Chen, 1988). In our study (**Paper II)** however, the pH of MYPGP broth inoculated with the hbs-LAB secretome was in the pH range of 5-6. The hbs-LAB supernatant probably contains antimicrobial substances other than organic acids such as proteins and peptides contributing to the observed inhibitory effect. Alippi and Minnaard recently isolated a bacteriocin-like compound secreted by *B. cereus*, which has antimicrobial activity against *P. larvae* genotypes ERIC-I and ERIC-II (Minnaard and Alippi, 2016). Other research groups isolated active antimicrobial substances against *P. larvae* such as Iturin A, fatty acids (Benitez et al., 2012), lipopeptides and surfactin (Sabaté et al., 2009). Future research should investigate the effects of individual hbs-LAB against *P. larvae* and fractionation studies of the secretome produced by the hbs-LAB species, both in isolation and in co-culture, coupled with proteomic and metabolomic analyses, to identify the active antimicrobial substances produced.

6.3 The effect of hbs-LAB on *P. larvae* spore counts, AFB symptoms and honeybee colony strength (Paper III)

The final questions addressed in this project was whether applying hbs-LAB as a feed supplement to honeybee colonies could prevent elevated *P. larvae* spore levels and AFB symptoms or have a general positive effect on colony health (**Paper III**).

The results showed that neither treatment with supplemental hbs-LAB, nor the antibiotic, tylosin, had any effect on *P. larvae* spore levels (Figure 10). The application of the hbs-LAB supplement had no effect on colony strength measured as frames of bees and brood. However, treatment with the antibiotic tylosin suppressed AFB symptoms (**Paper III**). This confirms that antibiotics such as tylosin only affect the vegetative form of bacteria but not the spores. Even if there are no reports of tylosin-resistant *P. larvae* isolates to date and the antibiotic is still effective on vegetative bacteria, the spores remain unaffected and infective for many years which may lead to reoccurrence of AFB in a colony, apiary or a beekeeping practice (Genersch, 2010; Oldroyd et al., 1989).

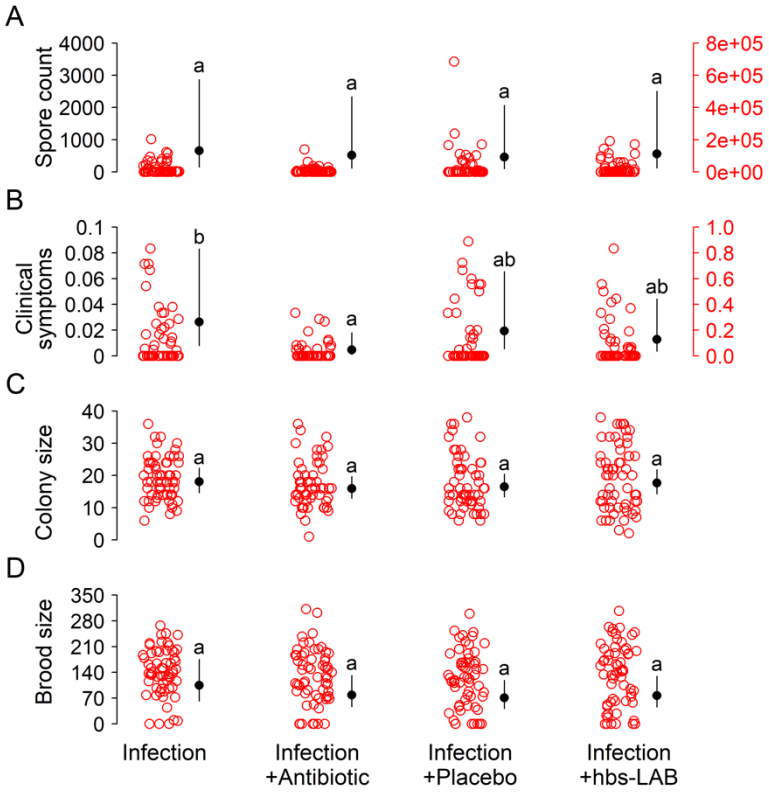

Figure 10. **The effect of Tylosin and hbs-LAB on American foulbrood and colony strength.** Shown are American foulbrood spore counts (colony forming units per bee) from adult bees and symptoms (probability of AFB score with respect to maximal score for each colony) depending on the four treatments (Infection = American foulbrood infection; Antibiotics = Tylosin; LABc = placebo of honey-bee-specific Lactic Acid Bacteria; LAB = honey-bee-specific Lactic Acid Bacteria). Colony strength is represented by colony size (number of frame sides with bees) and brood size (number of brood). Red circles, with the respective red scale on the right side if necessary, show the original data from all 40 bee colonies on all six sampling occasions with respect to treatment. The letters indicate significant differences between predicted marginal means with confidence limits from the respective models that still included the treatment effect. Based upon the results presented in **Paper III** we can conclude that any promising results of the effects of hbs-LAB on pathogens at brood level in controlled laboratory experiments (Forsgren et al., 2010a) (**Paper II**) do not necessarily translate directly into an effective treatment for honeybee colonies.

7 Conclusions

7.1 Detection and identification of hbs-LAB

- It is imperative to use supplements such as fructose and L-cysteine to successfully isolate and grow hbs-LAB.
- Illumina amplicon sequencing of the 16S rRNA genes is a successful approach for the detection and identification of the 13 hbs-LAB to the strain level.
- As a result of the limitations in current culture-dependent and molecular-based techniques, we recommend to use them as complementary techniques for future investigations of hbs-LAB and other members of the honeybee bacterial gut microbiome.

7.2 The inhibitory activity of hbs-LAB on *P. larvae* growth in artificial media and in honeybee larvae

- *Paenibacillus larvae* ERIC-II genotype have a shorter germination time (ERIC-I: ~32 h, ERIC-II: ~24 h), a shorter generation time (ERIC-I: ~ 48.4 min, ERIC-II: 18.7 min) and a higher final cell density in MYPGP broth than *P. larvae* of ERIC-I genotype. We observed a delayed/reduced sporulation for ERIC-II (*i.e.* dominant vegetative stage) compared to ERIC-I (*i.e.* dominant spore stage).
- Honeybee-specific lactic acid bacteria produce antimicrobial substances, a secretome, which reduce larval mortality caused by *P. larvae*.
- After adding the hbs-LAB secretome, the *P. larvae* growth curve increased only slightly and very slowly reaching far lower final cell densities than in the non-supplemented media.
- The hbs-LAB secretome inhibits the vegetative growth of *P. larvae* but have no effect, no inhibition or delay, on the spore germination.

7.3 The effect of hbs-LAB on *P. larvae* spore counts, AFB symptoms and honeybee colony strength

- *Paenibacillus larvae* spore levels in honeybee colonies did not change after treatment with supplemental hbs-LAB.
- The application of the hbs-LAB supplement had no effect on colony strength measured as frames of bees and brood.
- Symptoms of AFB were lower in colonies treated with tylosin.
- Tylosin did not decrease the spore levels of *P. larvae*.

In conclusion, honeybee colonies have multiple homeostatic mechanisms for managing colony health and performance of which hbs-LAB are but a small component. This thesis does not refute the beneficial nature of honey crop bacteria as abundantly shown previously (Forsgren et al., 2010b; Killer et al., 2014; Olofsson et al., 2014b; Vásquez et al., 2012), but it does show that translating this knowledge into a useful application is not straightforward and requires careful consideration of the social character and the natural homeostatic mechanisms governing health, microbiome diversity and function in honeybee colonies (Alberoni et al., 2016; Engel et al., 2016).

References

Alberoni, D., Gaggìa, F., Baffoni, L., Di Gioia, D., 2016. Beneficial microorganisms for honey bees: problems and progresses. Appl. Microbiol. Biotechnol. 100: 9469–9482.

Alippi, A.M., Lopez, A.C., Aguilar, O.M., 2004. A PCR-based method that permits specific detection of *Paenibacillus larvae* subsp. *larvae*, the cause of American foulbrood of honey bees, at subspecies level. Lett Appl Microbiol 39: 25–33.

Alippi, A.M., Reynaldi, F.J., 2006. Inhibition of the growth of *Paenibacillus larvae*, the causal agent of American foulbrood of honeybees, by selected strains of aerobic spore-forming bacteria isolated from apiarian sources. J. Invertebr. Pathol. 91: 141–146.

Anderson, A.C., Sanunu, M., Schneider, C., Clad, A., Karygianni, L., Hellwig, E., Al-Ahmad, A., 2014. Rapid species-level identification of vaginal and oral lactobacilli using MALDI-TOF MS analysis and 16S rDNA sequencing. BMC Microbiol. 14: 312.

Anderson, K.E., Carroll, M.J., Sheehan, T., Mott, B.M., Maes, P., Corby-Harris, V., 2014. Hive-stored pollen of honey bees: many lines of evidence are consistent with pollen preservation, not nutrient conversion. Mol. Ecol. 23: 5904–17.

Anderson, K.E., Sheehan, T.H., Mott, B.M., Maes, P., Snyder, L., Schwan, M.R., Walton, A., Jones, B.M., Corby-Harris, V., 2013. Microbial ecology of the hive and pollination landscape: bacterial associates from floral nectar, the alimentary tract and stored food of honey bees (*Apis mellifera*). PLoS One 8: e83125.

Animal and plant health agency, 2017. Foulbrood Disease of Honey Bees and other common brood disorders. UK.

Antúnez, K., Anido, M., Arredondo, D., Evans, J.D., Zunino, P., 2011a. *Paenibacillus larvae* enolase as a virulence factor in honeybee larvae infection. Vet. Microbiol. 147: 83–89.

Antúnez, K., Anido, M., Schlapp, G., Evans, J.D., Zunino, P., 2009. Characterization of secreted proteases of *Paenibacillus larvae*, potential virulence factors involved in honeybee larval infection. J. Invertebr. Pathol. 102: 129–132.

Antúnez, K., Arredondo, D., Anido, M., Zunino, P., 2011b. Metalloprotease production by *Paenibacillus larvae* during the infection of honeybee larvae. Microbiology 157: 1474–1480.

Asama, T., Arima, T.H., Gomi, T., Keishi, T., Tani, H., Kimura, Y., Tatefuji, T., Hashimoto, K., 2015. *Lactobacillus kunkeei* YB38 from honeybee products enhances IgA production in healthy adults. J. Appl. Microbiol. 119: 818–826.

Audisio, M.C., Torres, M.J., Sabaté, D.C., Ibarguren, C., Apella, M.C., 2011. Properties of different lactic acid bacteria isolated from *Apis mellifera* L. bee-gut. Microbiol. Res. 166: 1–13.

Aupinel, P., Fortini, D., Dufour, H., Taséi, J.N., Michaud, B., Odoux, J.F., Pham-Delegue, M.H., 2005. Improvement of artificial feeding in a standard in vitro method for rearing *Apis mellifera* larvae. Bull. Insectology 58: 107–111.

Benitez, L.B., Velho, R.V., de Souza da Motta, A., Segalin, J., Brandelli, A., 2012. Antimicrobial factor from *Bacillus amyloliquefaciens* inhibits *Paenibacillus larvae*, the causative agent of American foulbrood. Arch. Microbiol. 194: 177–185.

Bernardeau, M., Guguen, M., Vernoux, J.P., 2006. Beneficial lactobacilli in food and feed: long-term use, biodiversity and proposals for specific and realistic safety assessments. FEMS Microbiol. Rev. 30: 487–513.

Buneśová, V., Vlková, E., Rada, V., Hovorková, P., Musilová, Š., Kmet, V., 2014. Direct identification of Bifidobacteria from probiotic supplements. Czech J. Food Sci. 32: 132–136.

Butler, È., Alsterfjord, M., Olofsson, T.C., Karlsson, C., Malmström, J., Vásquez, A., 2013. Proteins of novel lactic acid bacteria from *Apis mellifera mellifera*: an insight into the production of known extra-cellular proteins during microbial stress. BMC Microbiol. 13: 235–246.

Butler, È., Oien, R.F., Lindholm, C., Olofsson, T.C., Nilson, B., Vásquez, A., 2016. A pilot study investigating lactic acid bacterial symbionts from the honeybee in inhibiting human chronic wound pathogens. Int. Wound J. 13: 729–37.

Callahan, B.J., Mcmurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J., Holmes, S.P., 2015. DADA2 : High resolution sample inference from amplicon data. Nat. Methods 13: 1–14.

Carbonnelle, E., Mesquita, C., Bille, E., Day, N., Dauphin, B., Beretti, J.L., Ferroni, A., Gutmann, L., Nassif, X., 2011. MALDI-TOF mass spectrometry tools for bacterial identification in clinical microbiology laboratory. Clin. Biochem. 44: 104–109.

Carroll, M.J., Brown, N., Goodall, C., Downs, A.M., Sheenan, T.H., Anderson, K.E., 2017. Honey bees preferentially consume freshly stored Pollen. PLoS One 12: 1–21.

Chakravorty, S., Helb, D., Burday, M., Connell, N., 2007. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. J Microbiol Methods 69: 330–339.

Clark, A.E., Kaleta, E.J., Arora, A., Wolk, D.M., 2013. Matrix-Assisted laser desorption ionization-time of flight mass spectrometry: A fundamental shift in the routine practice of clinical microbiology. Clin. Microbiol. Rev. 26: 547–

603.
Condon, C., Liveris, D., Squires, C., Schwartz, I., Squires, C.L., 1995. rRNA Operon Multiplicity in *Escherichia coli* and the Physiological Implications of *rrn* Inactivation. J. Bacteriol. 177: 4152–4156.
Corby-Harris, V., Maes, P., Anderson, K.E., 2014a. The bacterial communities associated with honey bee (*Apis mellifera*) foragers. PLoS One 9: e95056.
Corby-Harris, V., Snyder, L.A., Schwan, M.R., Maes, P., McFrederick, Q.S., Anderson, K.E., 2014b. Origin and effect of Alpha 2.2 Acetobacteraceae in honey bee larvae and description of *Parasaccharibacter apium* gen. nov., sp. nov. Appl. Environ. Microbiol. 80: 7460–7472.
Crailsheim, K., 1988. Regulation of food passage in the intestine of the honeybee (*Apis mellifera* L.). J. Insect Physiol. 34: 85–90.
Cremer, S., Armitage, S.A.O., Schmid-Hempel, P., 2007. Social Immunity. Curr. Biol. 17: 693–702.
de Almeida Vaucher, R., Giongo, J.L., Bolzan, L.P., Côrrea, M.S., Fausto, V.P., Alves, C.F. dos S., Lopes, L.Q.S., Boligon, A.A., Athayde, M.L., Moreira, A.P., Brandelli, A., Raffin, R.P., Santos, R.C.V., 2015. Antimicrobial activity of nanostructured Amazonian oils against *Paenibacillus* species and their toxicity on larvae and adult worker bees. J. Asia. Pac. Entomol. 18: 205–210.
de Graaf, D.C., Alippi, a M., Brown, M., Evans, J.D., Feldlaufer, M., Gregorc, A., Hornitzky, M., Pernal, S.F., Schuch, D.M.T., Titera, D., Tomkies, V., Ritter, W., 2006. Diagnosis of American foulbrood in honey bees: a synthesis and proposed analytical protocols. Lett. Appl. Microbiol. 43: 583–90.
Degnan, P.H., Ochman, H., 2012. Illumina-based analysis of microbial community diversity. ISME J. 6: 183–194.
DeMarco, M.L., Burnham, C.A.D., 2014. Diafiltration MALDI-TOF mass spectrometry method for culture-independent detection and identification of pathogens directly from urine specimens. Am. J. Clin. Pathol. 141: 204–212.
Dicks, L.M.T., Botes, M., 2010. Probiotic lactic acid bacteria in the gastro-intestinal tract: health benefits, safety and mode of action. Benef. Microbes 1: 11–29.
Djukic, M., Brzuszkiewicz, E., Funfhaus, A., Voss, J., Gollnow, K., Poppinga, L., Liesegang, H., Garcia-Gonzalez, E., Genersch, E., Daniel, R., 2014. How to kill the honey bee larva: Genomic potential and virulence mechanisms of *Paenibacillus larvae*. PLoS One 9.
Dobbelaere, W., de Graaf, D., Peeters, J., Franciscus, J., 2001. Development of a fast and reliable diagnostic method for American foulbrood disease (*Paenibacillus larvae* subsp. *larvae*) using a 16S rRNA gene based PCR. Apidologie 32: 363–370.
Dubois, D., Leyssene, D., Chacornac, J.P., Kostrzewa, M., Schmit, P.O., Talon, R., Bonnet, R., Delmas, J., 2010. Identification of a variety of *Staphylococcus* species by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J. Clin. Microbiol. 48: 941–945.
Eddabra, R., Prévost, G., Scheftel, J.-M., 2012. Rapid discrimination of environmental *Vibrio* by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Microbiol. Res. 167: 226–230.

Edgar, R.C., 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat. Methods 10: 996–8.

Edwards, C.G., Haag, K.M., Collins, M.D., Hutson, R.A., Huang, Y.C., 1998. *Lactobacillus kunkeei* sp . nov . : a spoilage organism associated with grape juice fermentations. Appl. Microbiol. 5: 698–702.

Ellegaard, K.M., Tamarit, D., Javelind, E., Olofsson, T.C., Andersson, S.G., Vásquez, A., 2015. Extensive intra-phylotype diversity in lactobacilli and bifidobacteria from the honeybee gut. BMC Genomics 16: 284.

Endo, A., 2012. Fructophilic lactic acid bacteria inhabit fructose-rich niches in nature. Coaction 6: 6–9.

Endo, A., Irisawa, T., Futagawa-Endo, Y., Takano, K., Toit, M. Du, Okada, S., Dicks, L.M.T., 2012. Characterization and emended description of *Lactobacillus kunkeei* as a fructophilic lactic acid bacterium. Int. J. Syst. Evol. Microbiol. 62: 500–504.

Engel, P., Kwong, W.K., McFrederick, Q., Anderson, K.E., Barribeau, S.M., Chandler, J.A., Cornman, R.S., Dainat, J., de Miranda, J.R., Doublet, V., Emery, O., Evans, J.D., Farinelli, L., Flenniken, M.L., Granberg, F., Grasis, J.A., Gauthier, L., Hayer, J., Koch, H., Kocher, S., Martinson, V.G., Moran, N., Munoz-Torres, M., Newton, I., Paxton, R.J., Powell, E., Sadd, B.M., Schmid-Hempel, P., Schmid-Hempel, R., Song, S.J., Schwarz, R.S., VanEngelsdorp, D., Dainat, B., 2016. The Bee Microbiome: Impact on Bee Health and Model for Evolution and Ecology of Host-Microbe Interactions. MBio 7: e02164-15.

Engel, P., Stepanauskas, R., Moran, N. a, 2014. Hidden diversity in honey bee gut symbionts detected by single-cell genomics. PloS Genet 10: e1004596.

Eren, A.M., Morrison, H.G., Lescault, P.J., Reveillaud, J., Vineis, J.H., Sogin, M.L., 2015. Minimum entropy decomposition: unsupervised oligotyping for sensitive partitioning of high-throughput marker gene sequences. ISME J 9: 968–979.

Evans, J.D., 2003. Diverse origins of tetracycline resistance in the honey bee bacterial pathogen *Paenibacillus larvae*. J. Invertebr. Pathol. 83: 46–50.

Evans, J.D., 2004. Transcriptional immune responses by honey bee larvae during invasion by the bacterial pathogen, *Paenibacillus larvae*. J. Invertebr. Pathol. 85: 105–111.

Evans, J.D., Aronstein, K., Chen, Y.P., Hetru, C., Imler, J.-L., Jiang, H., Kanost, M., Thompson, G.J., Zou, Z., Hultmark, D., 2006. Immune pathways and defence mechanisms in honey bees *Apis mellifera*. Insect Mol. Biol. 15: 645–56.

FAO/WHO, 2002. Guidelines for the Evaluation of Probiotics in Food. London, Ontario.

Felis, G.E., Dellaglio, F., 2007. Taxonomy of Lactobacilli and Bifidobacteria. Curr. Issues Intest. Microbiol. 8: 44–61.

Flint, H.J., Duncan, S.H., Louis, P., 2017. The impact of nutrition on intestinal bacterial communities. Curr. Opin. Microbiol. 38: 59–65.

Florou-Paneri, P., Christaki, E., Bonos, E., 2013. Lactic Acid Bacteria as Source of Functional Ingredients. In: Lactic Acid Bacteria-R& D for Food, Health and

Livestock Purposes. pp. 589–614.
Forfert, N., Natsopoulou, M.E., Frey, E., Rosenkranz, P., Paxton, R.J., Moritz, R.F.A., 2015. Parasites and pathogens of the honeybee (*Apis mellifera*) and their influence on inter-colonial transmission. PLoS One 10: 1–14.
Forsgren, E., Laugen, A.T., 2014. Prognostic value of using bee and hive debris samples for the detection of American foulbrood disease in honey bee colonies. Apidologie 45: 10–20.
Forsgren, E., Olofsson, T.C., Vásquez, A., Fries, I., 2010a. Novel lactic acid bacteria inhibiting *Paenibacillus larvae* in honey bee larvae. Apidologie 41: 99–108.
Forsgren, E., Olofsson, T.C., Vásquez, A., Fries, I., 2010b. Novel lactic acid bacteria inhibiting *Paenibacillus larvae* in honey bee larvae. Apidologie 41: 99–108.
Forsgren, E., Stevanovic, J., Fries, I., 2008. Variability in germination and in temperature and storage resistance among *Paenibacillus larvae* genotypes. Vet. Microbiol. 129: 342–349.
Foschi, C., Laghi, L., Parolin, C., Giordani, B., Compri, M., Cevenini, R., Marangoni, A., Vitali, B., 2017. Novel approaches for the taxonomic and metabolic characterization of lactobacilli: Integration of 16S rRNA gene sequencing with MALDI-TOF MS and 1H-NMR. PLoS One 12: 1–18.
Fraga, M., Perelmuter, K., Delucchi, L., Cidade, E., Zunino, P., 2008. Vaginal lactic acid bacteria in the mare: evaluation of the probiotic potential of native *Lactobacillus* spp. and *Enterococcus* spp. strains. Antonie Van Leeuwenhoek 93: 71–8.
Fries, I., Camazine, S., 2001. Implications of horizontal and vertical pathogen transmission for honey bee epidemiology. Apidologie 32: 199–214.
Fries, I., Lindström, A., Korpela, S., 2006. Vertical transmission of American foulbrood (*Paenibacillus larvae*) in honey bees (*Apis mellifera*). Vet. Microbiol. 114: 269–274.
Fuselli, S.R., Maggi, M., de la Rosa, S.B.G., Principal, J., Eguaras, M.J., Fritz, R., 2009. *In vitro* antibacterial and antiparasitic effect of citrus fruit essential oils on the honey bee pathogen *Paenibacillus larvae* and the parasitic mite *Varroa destructor*. J. Apic. Res. 48: 77–78.
Gänzle, M.G., 2015. Lactic metabolism revisited: Metabolism of lactic acid bacteria in food fermentations and food spoilage. Curr. Opin. Food Sci. 2: 106–117.
Genersch, E., 2010. American Foulbrood in honeybees and its causative agent, *Paenibacillus larvae*. J. Invertebr. Pathol. 103: S10-19.
Genersch, E., Ashiralieva, A., Fries, I., 2005. Strain- and genotype-specific differences in virulence of *Paenibacillus larvae* subsp. *larvae*, a bacterial pathogen causing American foulbrood disease in honeybees. Appl. Environ. Microbiol. 71: 7551–5.
Genersch, E., Forsgren, E., Pentikäinen, J., Ashiralieva, A., Rauch, S., Kilwinski, J., Fries, I., 2006. Reclassification of *Paenibacillus larvae* subsp. *pulvifaciens* and *Paenibacillus larvae* subsp. *larvae* as *Paenibacillus larvae* without subspecies differentiation. Int. J. Syst. Evol. Microbiol. 56: 501–511.
Genersch, E., Otte, C., 2003. The use of repetitive element PCR fingerprinting (rep-PCR) for genetic subtyping of German field isolates of *Paenibacillus larvae*

subsp. *larvae*. Apidologie 34: 195–206.
Gobbetti, M., Corsetti, A., 1997. *Lactobacillus sanfrancisco* a key sourdough lactic acid bacterium : a review. Food Microbiol. 14: 175–187.
Hansen, H., Brødsgaard, C.J., 1999. American foulbrood: a review of its biology, diagnosis and control. Bee World 80: 5–23.
Hayek, S.A., Ibrahim, S.A., 2013. Current Limitations and Challenges with Lactic Acid Bacteria : A Review. Food Nutr. Sci. 4: 73–87.
Herbert, E.W., Shimanuki, H., 1978. Chemical composition and nutritive value of bee-collected and bee-stored pollen. Apidologie 9: 33–40.
Heyndrickx, M., Vos, P.D.E., 1996. Reclassification of *Paenibacillus* (formerly *Bacillus*) *pulvifaciens* (Nakamura 1984) Ash et al. 1994, a Later Subjective Synonym of *Paenibacillus* (formerly *Bacillus*) *larvae* (White 1906) Ash et al. 1994, as a Subspecies of *P. larvae*, with Emended Description. Int. J. Syst. Bacteriol. 46: 270–279.
Hroncova, Z., Havlik, J., Killer, J., Doskocil, I., Tyl, J., Kamler, M., Titera, D., Hakl, J., Mrazek, J., Bunesova, V., Rada, V., 2015. Variation in honey bee gut microbial diversity affected by ontogenetic stage, age and geographic location. PLoS One 10: 1–17.
Janashia, I., Alaux, C., 2016. Specific Immune Stimulation by Endogenous Bacteria in Honey Bees (Hymenoptera: Apidae). J. Econ. Entomol. 109: 1474–1477.
Janashia, I., Choiset, Y., Jozefiak, D., Déniel, F., Coton, E., Moosavi-movahedi, A.A., Chanishvili, N., Haertlé, T., 2018. Beneficial Protective Role of Endogenous Lactic Acid Bacteria Against Mycotic Contamination of Honeybee Beebread. Probiotics & Antimicro. Prot. 1–9.
Kandler, O., 1983. Carbohydrate metabolism in lactic acid bacteria. Antonie Van Leeuwenhoek 49: 209–224.
Killer, J., Dubná, S., Sedláček, I., Švec, P., 2014. *Lactobacillus apis* sp. nov., from the stomach of honeybees (*Apis mellifera*), having an in vitro inhibitory effect on the causative agents of American and European foulbrood. Int. J. Syst. Evol. Microbiol. 64: 152–157.
Klein, A.M., Vaissiere, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A., Kremen, C., Tscharntke, T., 2007. Importance of pollinators in changing landscapes for world crops. Proc. R. Soc. 274: 303–313.
Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., Schloss, P.D., 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the miseq illumina sequencing platform. Appl. Environ. Microbiol. 79: 5112–5120.
Kwong, W.K., Mancenido, A.L., Moran, N.A., 2014. Genome Sequences of *Lactobacillus* sp. Strains wkB8 and wkB10, Members of the Firm-5 Clade, from Honey Bee Guts. Genome Announc 2: e01176–14.
Kwong, W.K., Moran, N.A., 2016. Gut microbial communities of social bees. Nat. Rev. Microbiol. 14: 374–384.
Lamei, S., Hu, Y.O.O., Olofsson, T.C., Andersson, A.F., Forsgren, E., Vásquez, A., 2017. Improvement of identification methods for honeybee specific Lactic Acid Bacteria; future approaches. PLoS One 12: e0174614.

Lindström, A., Fries, I., 2005. Sampling of adult bees for detection of American foulbrood (*Paenibacillus larvae* subsp. *larvae*) spores in honey bee (*Apis mellifera*) colonies. J. Apic. Res. 44: 82–86.

Lindström, A., Korpela, S., Fries, I., 2008. The distribution of *Paenibacillus larvae* spores in adult bees and honey and larval mortality, following the addition of American foulbrood diseased brood or spore-contaminated honey in honey bee (*Apis mellifera*) colonies. J. Invertebr. Pathol. 99: 82–86.

Lodesani, M., Costa, C., 2015. Limits of chemotherapy in beekeeping: development of resistance and the problem of residues. Bee World 86: 102–109.

Ludvigsen, J., Rangberg, A., Avershina, E., Sekelja, M., Kreibich, C., Amdam, G., Rudi, K., 2015. Shifts in the Midgut/Pyloric Microbiota Composition within a Honey Bee Apiary throughout a Season. Microbes Environ. 30: 235–244.

Maggi, M., Negri, P., Plischuk, S., Szawarski, N., De Piano, F., De Feudis, L., Eguaras, M., Audisio, C., 2013. Effects of the organic acids produced by a lactic acid bacterium in *Apis mellifera* colony development, *Nosema ceranae* control and fumagillin efficiency. Vet. Microbiol. 167: 474–83.

Makarova, K.S., Koonin, E. V, 2007. Evolutionary genomics of lactic acid bacteria. J. Bacteriol. 189: 1199–208.

Martinson, V.G., Moy, J., Moran, N. a., 2012. Establishment of characteristic gut bacteria during development of the honeybee worker. Appl. Environ. Microbiol. 78: 2830–2840.

Mattila, H.R., Rios, D., Walker-Sperling, V.E., Roeselers, G., Newton, I.L.G., 2012. Characterization of the active microbiotas associated with honey bees reveals healthier and broader communities when colonies are genetically diverse. PLoS One 7.

McNally, L., Viana, M., Brown, S.P., 2014. Cooperative secretions facilitate host range expansion in bacteria. Nat. Commun. 5: 1–8.

Mellmann, A., Cloud, J., Maier, T., Keckevoet, U., Ramminger, I., Iwen, P., Dunn, J., Hall, G., Wilson, D., LaSala, P., Kostrzewa, M., Harmsen, D., 2008. Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry in comparison to 16S rRNA gene sequencing for species identification of nonfermenting bacteria. J. Clin. Microbiol. 46: 1946–1954.

Minnaard, J., Alippi, A.M., 2016. Partial characterization of bacteriocin-like compounds from two strains of *Bacillus cereus* with biological activity against *Paenibacillus larvae*, the causal agent of American Foulbrood disease. Appl. Microbiol. 63: 442–449.

Mudroňová, D., Toporčák, J., Nemcová, R., Gancarčíková, S., Hajdučková, V., Rumanovská, K., 2011. *Lactobacillus* sp. as a potential probiotic for the prevention of *Paenibacillus larvae* infection in honey bees. J. Apic. Res. 50: 323–324.

Murray, K.D., Aronstein, K.A., 2006. Oxytetracycline-resistance in the honey bee pathogen *Paenibacillus larvae* is encoded on novel plasmid pMA67. IBRA 45: 207–214.

Murray, P.R., 2012. What Is new in clinical microbiologymicrobial identification by MALDI-TOF mass spectrometry. J. Mol. Diagnostics 14: 419–423.

Neveling, D.P., Endo, A., Dicks, L.M.T., 2012. Fructophilic *Lactobacillus kunkeei* and *Lactobacillus brevis* isolated from fresh flowers, bees and bee-hives. Curr. Microbiol. 65: 507–515.

Nicolson, S.W., Thornburg, R.W., 2007. Nectar Chemistry. In: Nectaries and Nectar. In: Springer. pp. 215–263.

Nordström, S., Fries, I., 1995. A comparison of media and cultural conditions for identification of *Bacillus larvae* in honey. J. Apic. Res. 34: 97–103.

Oldroyd, B.P., Goodman, R.D., Hornitzky, M.A.Z., Chandler, D., 1989. The effect on American foulbrood of standard oxytetracycline hydrochloride treatments for the control of European foulbrood of honeybees (*Apis mellifera*). Aust. J. Agric. Res. 40: 691–697.

Olofsson, T.C., Alsterfjord, M., Nilson, B., Butler, È., Vásquez, A., 2014a. *Lactobacillus apinorum* sp. nov., *Lactobacillus mellifer* sp. nov., *Lactobacillus mellis* sp. nov., *Lactobacillus melliventris* sp. nov., *Lactobacillus kimbladii* sp. nov., *Lactobacillus helsingborgensis* sp. nov. and *Lactobacillus kullabergensis* sp. nov., isolated from the honey stomach of the honeybee *Apis mellifera*. Int. J. Syst. Evol. Microbiol. 64: 3109–19.

Olofsson, T.C., Butler, È., Markowicz, P., Lindholm, C., Larsson, L., Vásquez, A., 2014b. Lactic acid bacterial symbionts in honeybees–an unknown key to honeys antimicrobial and therapeutic activities. Int. Wound J. 13: 668–679.

Olofsson, T.C., Vásquez, A., 2008. Detection and identification of a novel lactic acid bacterial flora within the honey stomach of the honeybee *Apis mellifera*. Curr. Microbiol. 57: 356–63.

Papachristoforou, A., Kagiava, A., Papaefthimiou, C., Termentzi, A., Fokialakis, N., Skaltsounis, A.L., Watkins, M., Arnold, G., Theophilidis, G., 2012. The Bite of the Honeybee: 2-Heptanone Secreted from Honeybee Mandibles during a Bite Acts as a Local Anaesthetic in Insects and Mammals. PLoS One 7: e47432.

Pokusaeva, K., Fitzgerald, G.F., Van Sinderen, D., 2011. Carbohydrate metabolism in Bifidobacteria. Genes Nutr. 6: 285–306.

Poppinga, L., Genersch, E., 2015. Molecular pathogenesis of American Foulbrood: How *Paenibacillus larvae* kills honey bee larvae. Curr. Opin. Insect Sci. 10: 29–36.

Pot, B., Ludwig, W., Kersters, K., Schleifer, K., 1994. Taxonomy of lactic acid bacteria. Blackie Academic & Professional, London.

Potts, S.G., Biesmeijer, J.C., Kremen, C., Neumann, P., Schweiger, O., Kunin, W.E., 2010. Global pollinator declines: Trends, impacts and drivers. Trends Ecol. Evol. 25: 345–353.

Powell, J.E., Martinson, V.G., Urban-Mead, K., Moran, N.A., 2014. Routes of acquisition of the gut microbiota of the honey bee *Apis mellifera*. Appl. Environ. Microbiol. 80: 7378–7387.

Rashid, M., Sultana, M., 2016. Role of Probiotics in Human and Animal Health. J Prob Heal. 4: 2–4.

Rattanachaikunsopon, P., Phumkhachorn, P., 2010. Lactic acid bacteria : their antimicrobial compounds and their uses in food production. Ann. Biol. Res. 1:

218–228.
Rauch, S., Ashiralieva, A., Hedtke, K., Genersch, E., 2009. Negative correlation between individual-insect-level virulence and colony-level virulence of *Paenibacillus larvae*, the etiological agent of american foulbrood of honeybees. Appl. Environ. Microbiol. 75: 3344–3347.
Reybroeck, W., Daeseleire, E., De Brabander, H.F., Herman, L., 2012. Antimicrobials in beekeeping. Vet. Microbiol. 158: 1–11.
Rokop, Z.P., Horton, M.A., Newton, I.L.G., 2015. Interactions between cooccurring lactic acid bacteria in honey bee hives. Appl. Environ. Microbiol. 81: 7261–7270.
Sabaté, D.C., Carrillo, L., Audisio, M.C., 2009. Inhibition of *Paenibacillus larvae* and *Ascosphaera apis* by *Bacillus subtilis* isolated from honeybee gut and honey samples. Res. Microbiol. 160: 193–199.
Sabaté, D.C., Cruz, M.S., Benítez-Ahrendts, M.R., Audisio, M.C., 2012. Beneficial Effects of *Bacillus subtilis* subsp. *subtilis* Mori2, a Honey-Associated Strain, on Honeybee Colony Performance. Probiotics Antimicrob. Proteins 4: 39–46.
Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. 74: 5463–5467.
Segawa, S., Sawai, S., Murata, S., Nishimura, M., Beppu, M., Sogawa, K., Watanabe, M., Satoh, M., Matsutani, T., Kobayashi, M., Iwadate, Y., Kuwabara, S., Saeki, N., Nomura, F., 2014. Direct application of MALDI-TOF mass spectrometry to cerebrospinal fluid for rapid pathogen identification in a patient with bacterial meningitis. Clin. Chim. Acta 435: 59–61.
Sinclair, L., Osman, O.A., Bertilsson, S., Eiler, A., 2015. Microbial Community Composition and Diversity via 16S rRNA Gene Amplicons: Evaluating the Illumina Platform. PLoS One 10: e0116955.
Singhal, N., Kumar, M., Kanaujia, P.K., Virdi, J.S., 2015. MALDI-TOF mass spectrometry: An emerging technology for microbial identification and diagnosis. Front. Microbiol. 6: 1–16.
Spivak, M., Reuter, G.S., 2001. Resistance to American foulbrood disease by honey bee colonies *Apis mellifera* bred for hygienic behavior. Apidologie 32: 555–565.
Stephan, R., Cernela, N., Ziegler, D., Pflüger, V., Tonolla, M., Ravasi, D., Fredriksson-Ahomaa, M., Hächler, H., 2011. Rapid species specific identification and subtyping of *Yersinia enterocolitica* by MALDI-TOF Mass spectrometry. J. Microbiol. Methods 87: 150–153.
Stiles, M.E., Holzapfel, W.H., 1997. Lactic acid bacteria of foods and their current taxonomy. Int. J. Food Microbiol. 36: 1–29.
Stoyanova, L.G., Ustyugova, E.A., Netrusov, A.I., 2012. Antibacterial metabolites of lactic acid bacteria: Their diversity and properties. Appl. Biochem. Microbiol. 48: 229–243.
Tajabadi, N., Mardan, M., Manap, M.Y.A., Mustafa, S., 2013. Molecular identification of *Lactobacillus* spp. isolated from the honey comb of the honey bee (*Apis dorsata*) by 16S rRNA gene sequencing. J. Apic. Res. 52: 235–241.

Tamarit, D., Ellegaard, K.M., Wikander, J., Olofsson, T.C., Vásquez, A., Andersson, S.G.E., 2015. Functionally Structured Genomes in *Lactobacillus kunkeei* Colonizing the Honey Crop and Food Products of Honeybees and Stingless Bees. Genome Biol. Evol. 7: 1455–1473.

Temmerman, R., Huys, G., Swings, J., 2004. Identification of lactic acid bacteria: culture-dependent and culture-independent methods. Trends Food Sci. Technol. 15: 348–359.

Valgas, C., Souza, S.M. De, Smânia, E.F. a, Artur, S.J., 2007. Screening Methods to Determine Antibacterial Activity of Natural Products. Brazilian J. Microbiol. 38: 369–380.

Vásquez, A., Forsgren, E., Fries, I., Paxton, R.J., Flaberg, E., Szekely, L., Olofsson, T.C., 2012. Symbionts as major modulators of insect health: lactic acid bacteria and honeybees. PLoS One 7: e33188.

Vásquez, A., Olofsson, T.C., 2009. The lactic acid bacteria involved in the production of bee pollen and bee bread. J. Apic. Res. 48: 189–195.

Vásquez, A., Olofsson, T.C., Sammataro, D., 2009. A scientific note on the lactic acid bacterial flora in honeybees in the USA – A comparison with bees from Sweden. Apidologie 40: 26–28.

Weinstock, G.M., Robinson, G.E., Gibbs, R.A., Worley, K.C., Evans, J.D., Maleszka, R., Robertson, H.M., Weaver, D.B., Beye, M., Bork, P., Elsik, C.G., Hartfelder, K., Hunt, G.J., Zdobnov, E.M., Amdam, G. V., Bitondi, M.M.G., Collins, A.M., Cristino, A.S., Lattorff, H.M.G., Lobo, C.H., Moritz, R.F.A., Nunes, F.M.F., Page, R.E., Simões, Z.L.P., Wheeler, D., Carninci, P., Fukuda, S., Hayashizaki, Y., Kai, C., Kawai, J., Sakazume, N., Sasaki, D., Tagami, M., Albert, S., Baggerman, G., Beggs, K.T., Bloch, G., Cazzamali, G., Cohen, M., Drapeau, M.D., Eisenhardt, D., Emore, C., Ewing, M.A., Fahrbach, S.E., Forêt, S., Grimmelikhuijzen, C.J.P., Hauser, F., Hummon, A.B., Huybrechts, J., Jones, A.K., Kadowaki, T., Kaplan, N., Kucharski, R., Leboulle, G., Linial, M., Littleton, J.T., Mercer, A.R., Richmond, T.A., Rodriguez-Zas, S.L., Rubin, E.B., Sattelle, D.B., Schlipalius, D., Schoofs, L., Shemesh, Y., Sweedler, J. V., Velarde, R., Verleyen, P., Vierstraete, E., Williamson, M.R., Ament, S.A., Brown, S.J., Corona, M., Dearden, P.K., Dunn, W.A., Elekonich, M.M., Fujiyuki, T., Gattermeier, I., Gempe, T., Hasselmann, M., Kadowaki, T., Kage, E., Kamikouchi, A., Kubo, T., Kucharski, R., Kunieda, T., Lorenzen, M., Milshina, N. V., Morioka, M., Ohashi, K., Overbeek, R., Ross, C.A., Schioett, M., Shippy, T., Takeuchi, H., Toth, A.L., Willis, J.H., Wilson, M.J., Gordon, K.H.J., Letunic, I., Hackett, K., Peterson, J., Felsenfeld, A., Guyer, M., Solignac, M., Agarwala, R., Cornuet, J.M., Monnerot, M., Mougel, F., Reese, J.T., Schlipalius, D., Vautrin, D., Gillespie, J.J., Cannone, J.J., Gutell, R.R., Johnston, J.S., Eisen, M.B., Iyer, V.N., Iyer, V., Kosarev, P., Mackey, A.J., Solovyev, V., Souvorov, A., Aronstein, K.A., Bilikova, K., Chen, Y.P., Clark, A.G., Decanini, L.I., Gelbart, W.M., Hetru, C., Hultmark, D., Imler, J.L., Jiang, H., Kanost, M., Kimura, K., Lazzaro, B.P., Lopez, D.L., Simuth, J., Thompson, G.J., Zou, Z., De Jong, P., Sodergren, E., Csurös, M., Milosavljevic, A., Osoegawa, K., Richards, S., Shu, C.L., Duret, L., Elhaik,

E., Graur, D., Anzola, J.M., Campbell, K.S., Childs, K.L., Collinge, D., Crosby, M.A., Dickens, C.M., Grametes, L.S., Grozinger, C.M., Jones, P.L., Jorda, M., Ling, X., Matthews, B.B., Miller, J., Mizzen, C., Peinado, M.A., Reid, J.G., Russo, S.M., Schroeder, A.J., St. Pierre, S.E., Wang, Y., Zhou, P., Jiang, H., Kitts, P., Ruef, B., Venkatraman, A., Zhang, L., Aquino-Perez, G., Whitfield, C.W., Behura, S.K., Berlocher, S.H., Sheppard, W.S., Smith, D.R., Suarez, A. V., Tsutsui, N.D., Wei, X., Wheeler, D., Havlak, P., Li, B., Liu, Y., Jovilet, A., Lee, S., Nazareth, L. V., Pu, L.L., Thorn, R., Stolc, V., Newman, T., Samanta, M., Tongprasit, W.A., Claudianos, C., Berenbaum, M.R., Biswas, S., De Graaf, D.C., Feyereisen, R., Johnson, R.M., Oakeshott, J.G., Ranson, H., Schuler, M.A., Muzny, D., Chacko, J., Davis, C., Dinh, H., Gill, R., Hernandez, J., Hines, S., Hume, J., Jackson, L.R., Kovar, C., Lewis, L., Miner, G., Morgan, M., Nguyen, N., Okwuonu, G., Paul, H., Santibanez, J., Savery, G., Svatek, A., Villasana, D., Wright, R., 2006. Insights into social insects from the genome of the honeybee *Apis mellifera*. Nature 443: 931–949.

White, G.F., 1906. The bacteria of the apiary with special references to bee diseases. Tech. Ser. 14.

Winston, M.L., 1991. The Biology of the Honey Bee. Harward University press, Cambridge MA.

Wong, H., Chen, Y., 1988. Effects of Lactic Acid Bacteria and Organic Acids on Growth and Germination of *Bacillus cereus*. Appl. Environ. Microbiol. 54: 2179–2184.

Wunschel, S.C., Jarman, K.H., Petersen, C.E., Valentine, N.B., Wahl, K.L., Schauki, D., Jackman, J., Nelson, C.P., White V, E., 2005. Bacterial analysis by MALDI-TOF mass spectrometry: An inter-laboratory comparison. J. Am. Soc. Mass Spectrom. 16: 456–462.

Yin, X., Chambers, J.R., Barlow, K., Park, A.S., Wheatcroft, R., 2005. The gene encoding xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (*xfp*) is conserved among *Bifidobacterium* species within a more variable region of the genome and both are useful for strain identification. FEMS Microbiol. Lett. 246: 251–257.

Zhao, Y., Chen, Y., Li, Z., Peng, W., 2018. Environmental factors have a strong impact on the composition and diversity of the gut bacterial community of Chinese black honeybees. J. Asia. Pac. Entomol. 21: 261–267.

Zheng, H., Powell, J.E., Steele, M.I., Dietrich, C., Moran, N.A., 2017. Honeybee gut microbiota promotes host weight gain via bacterial metabolism and hormonal signaling. Proc. Natl. Acad. Sci. 114: 4775–4780.

Zhou, Q., Li, K., Jun, X., Bo, L., 2009. Role and functions of beneficial microorganisms in sustainable aquaculture. Bioresour. Technol. 100: 3780–3786.

Acknowledgements

The last 4 years of becoming a Doctor have been a challenging and exciting journey and may have been the most interesting time in my life, if it hadn´t been so eventful before that. I would like to thank the following persons for their support and encouragement during my time as PhD student. I could not have done the work for my thesis without you!

I would like to offer special thanks to **Ingemar Fries**, who, although no longer with us but his memory would be eternal.

A special thank you to my supervisors (**Eva Forsgren, Joachim R. de Miranda and Kristian Riesbeck**) for all of your tremendous support and mentorship along the way! You believed in me, fought for my rights and gave me freedom to improve my skills.

Eva, first of all, I would like to thank you for giving the chance to join your research group and to conduct my own investigations within the doctoral project. Thank you for understanding, supporting and encouraging my vision in science. Thank you for your positive and constructive feedback when I needed it, your patience and for helping me not to lose hope when things were more difficult, and always setting everything in an appropriate perspective. You were not only a supervisor but also a good friend to me. **You are my role model as a strong woman**!

Joachim, thank you for all you have taught me. You have always been there to answer my questions and took time to explain how things work despite your busy schedule. Thanks for being patient with me to work on the papers although I made a mess with your comments and editions ☺.

Kristian, thank you for accepting me in your research group although you were aware of the difficulties.

I would like to thank our collaborators and my colleagues at SLU, **Anders F. Andersson**, **Yue O. O. Hu**, **Bo Nilson, Srinivas**, **Barbara Locke Grandér**, **Jörg** and **Emilia** for their invaluable suggestions, discussions and supports. **Emilia** thank you for being my angel in the lab. **Jörg**, thank you for teaching me statistics and always taking time (although I interrupt your breakfast time) to answer my questions and explain how things work in such an educational way.

Lars Hederstedt, I want to give my heartfelt thanks for accepting me to come to Sweden and joining your research group. You changed my life!

Stefan Roos, my mentor, instructor and friend! Thank you for always listening patiently to all of my problems, dreams and sharing with me your experiences.

My past and present colleagues at Lund and Malmö University **Lennart Ljunggren**, **Ravi Danielsson**, **Lars Olsson**, **Valentina**, **Yasmin**, **Tina**, **Gjendine**, **Anson, Matteo**, **Michael**, **Clara**, **Jeanette**, **Steven**, **Helena Malmberg**, **Renee**, **Julie**, **Wondossen**, **Margareta Persson** and all of the people at Oncology and TRC department. Thank you for lovely lunch company, fika time, conversations and making it a great working environment. **Lennart** thanks for your supporting and helping me to go further in my career. **Ravi**, thanks for encouraging me to be strong and all of the dinner sessions we had. **Håkan Axelson** and **Yvonne Ceder** and **Ramin Massoumi**, thanks for giving me a nest when the storm broke my wings. **Valentina**, my Italian friend, thank you for listening to me when I was down. **Yasmin**, thank you for cheering me up with your positive attitudes.

My friends out of the lab in Sweden, **Azadeh** and **Aidin shadnejad**, **Mojgan Safi**, **Mina Davoudi**, **Saghar Khandan** and **Alex Toresson**, **Sahar Tahvili** and **Mehdi Osooli**, **Shohreh Askaripour** and **Amir Jabbari**, **Maria Falk** and **Dennis Johnsson**, **Ewa Bukowska-Faniband** and **Moosa Faniband**, **Sushma Uthappa-Schwerdt** and **Christian**, **Layla Hossein** and **Daria Majidian**, **Gabriella** and **Sonny strömberg**, **Hala Ghandour Gari**, **Ali** and **Shohreh Roodbari**, **Shima** and **Ali Mansourian**. Thank you all for all of the fun times, laughs and team up to support me at the toughest period in my life. Special thanks to **Mojgan Zareinejad** and **Patrik Jalkell** for standing by me and caring about me like a daughter. **Eile Butler**, **Sara Karlson**, **Barbar Lettiero**, **Reihaneh Zarrizi** and **Anette Mårtensson**, our chats and after works have been

essential for me in getting through difficulties! **Eile**, you are an intelligent young researcher who I always got lessons from. **Anette**, thanks for answering all of my stupid questions, teaching me the Swedish culture and supporting me in science. **Stefanie** and **Jan Hoeber**, **Fereshteh** and **Björn**, **Sara Hossein**, **Monika Welc**, **Frauke Fedderwitz** and **Anna-Karin Johansson**, you made my lonely time in Uppsala more pleasurable and you were always a good excuse to encourage me to come back to Uppsala. **Ewa Bukowska-Faniband**, my first best friend in the lonely planet, thanks for keeping all of the secrets and showing sympathy ☺. **Maria Falk**, you are a good example of a brave and creative woman for me. **You, all, are my big family in Sweden**.

My Persian friends and colleagues at home, **Dr. Farajzadeh** and **Dr. Shahcheraghi**, **Dr. Mousavi**, **Dr. Khosravi** and **Mehdinejad** and **Kaveh Ahangar** thanks for all of your encouragements. **Tayebeh,** my best friend, I learn always from you not to give up. **Nikta**, you are the nostalgia of the good time in Iran.

To my lovely family- **Mom** and **Dad**, my brothers and their lovely families; **Soheil**, **Parvaneh**, **Ali** and **Fatemeh**, **Sasan** and **Asal**, **Saeid** and **Hanieh**, **Sajad**, **Sobhan** and **Nazanin**. I never could go so far without you. **Mom** and **Dad**, you are my first teachers in life and science. Boose bar dastane zahmat keshetan mizanam ♥!

Till min svenska familj: **Mamma Gunnel**, **Pappa Sven-Arne**, **Malin** & **Fredrik**, **Matilda** och **Olivia**, **Ewa Öberg** and **Lars Göran**. Tack för att ni välkomnat mig i familjen och stöttat mig under denna tuffa tid.

Till min kärlek **Mattias**! Jag kan inte med ord nog uttrycka min tacksamhet till dig (hoppas att jag kan lära mig bättre svenska ☺). Du gav mig gränslös kärlek och fyllde mitt ensamma liv med glädje och hopp. **Jag älskar dig♥**!

Finally, from the bottom of my heart I would like to acknowledge all the people who appeared in my life and helped, supported and encouraged me. I am proud of myself not only to have a doctoral title at the end of this period, but because of having you all as my family and friends!