“The elevator to success is out of order.

You'll have to use the stairs... one step at a time.”

Joe Girard

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Bumblebee organs, a habitat for a bacterial community

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Hommelorganen, een habitat voor een bacteriële gemeenschap

Cover illustration:

Confocal microscopic image of a cross-section of the midgut of *Bombus terrestris* with bacterial FISH probes. The bacterial layer is green, the red and white circles are pollen grains in the gut lumen. © Annelies Billiet and Haidong Wang

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List of abbreviations

ANOSIM	analysis of similarities
ANOVA	analysis of variance
BCCM/LMG	Belgian Coordinated Collections of Microorganisms / Laboratory of Microbiology Gent
bp	base pairs
CBPV	Chronic Bee Paralysis Virus
CFU	colony forming units
Co	community organization
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
FAO	Food and Agriculture Organization of the United Nations
GLM	generalized linear model
GMP	Good Manufacturing Practice
kGy	kilogray (unit of ionizing radiation dose)
LAB	lactic acid bacteria
MID	multiplex identifier
MRS agar	de Man, Rogosa and Sharpe agar
NCBI	National Center for Biotechnology Information
OD	optical densitiy
OTU	operational taxonomic unit
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEP	percentage of positive edges
QIIME	Quantitative Insights Into Microbial Ecology
R	species richness
rDNA	recombinant DNA
rRNA	ribosomal RNA
SCFAs	short-chain fatty acids
SD	standard deviation
SE	standard error
SPSS	Statistical Package for the Social Sciences
SRA	Sequence Read Archive
TAE	tris-acetate-EDTA
WHO	World Health Organization, a specialized agency of the United Nations
w/v	weight per volume
w/w	weight per weight

Scope

The bumblebee (*Bombus)* is an important member of the bee genus and can be regarded as a cold adapted bee. It is often the most abundant pollinator genus for multiple host plants in temperate climates. Bumblebee rearing started in Belgium in the late eighties and its successes for pollination of greenhouse tomatoes made commercialized pollination by bumblebees a worldwide export product. Nowadays it is a competitive international market with high quality standard rearing processes. Studying insect-microbe interactions, the bumblebee forms a fascinating study object, introducing fundamental as well as applied scientific questions. The main goals of this dissertation are to evaluate the functionality of the microbial community in bumblebees (*Bombus terrestris*) and to search for beneficial applications in bumblebee mass-rearing. **Chapter 1** gives a general introduction on the bumblebee, its gut microbiota and their functionalities. Many endogenous bacteria have a mutualistic relationship with the insect host and play a role in digestion, nutrient production and pathogen protection. Knowledge about the different bacterial groups and their functionality is discussed in function of potential applications, for instance to improve the production and the health of mass-reared bumblebees.

The first three research chapters mainly investigate how the microbiota can be altered and if it has added value for bumblebee rearing. **Chapter 2** functions as a proof of concept, investigating the effect of antibiotic treatment on the gut microbiota, bumblebee colony development and reproduction. **Chapter 3** studies if administering *Lactobacillus* and *Bifidobacterium* strains to indoor-reared bumblebees could be used as an approach for sustainable mass-rearing, by assessing the colony reproduction success and the gut microbial composition. Besides the administration of bacterial strains, also diet could have an impact on the gut bacterial composition. In **chapter 4**, the effect of different sugar syrups and pollen types on the gut microbiota was studied and how this could be a tool for bumblebee breeders to alter the bacterial richness and diversity in the gut.

The next three chapters focus more on some general biological questions in relation with the bumblebee and its microbiota. Here the rather unique aspect of the bumblebee being a

social insect could have a large implication on the association between the host and its microbiota. In most insects the adults have no contact with their offspring, abandoning the eggs in a suitable habitat for the hatching larvae. In contrast, social bees like bumblebees, have a close contact with nest mates of different developmental stages and generations. This could enhance bacterial transfer and offers opportunities for direct transfer of symbionts from one generation to the next. **Chapter 5** of this PhD research describes the impact of contact with the bumblebee colony on the colonization potential of the core bacterial families in the gut of the adult bumblebee.

In previous studies on microbial communities and their association with the host, there was a strong interest in the gut microbiota. Here we widen the focus and explore internal organs for associated bacteria, as invertebrates have an open circulatory system. The aim was to investigate if the microbial patterns could be linked with certain functionalities. **Chapter 6** focusses on the ovaries of indoor-reared bumblebee queens and studies if there is a correlation between their microbial pattern and the colony start-up success of the queen. In **chapter 7**, the microbiota in the fat body of wild bumblebees is studied to investigate if the composition of microbiota in the fat body could be linked with the sampling location and the prevalence of the pathogen *Apicystis bombi*.

Finally, all data and some perspectives for future research are discussed in **chapter 8**. The new biological insights will be used to address optimization issues and potentials for bumblebee rearing.

Chapter 1.

General introduction

1.1. Bumblebee

1.1.1. Life-cycle

The insect order Hymenoptera comprises wasps, sawflies, ants and bees, including bumblebees (*Bombus* sp.). The scientific classification of *Bombus* sp. is shown in Table 1.1.

Table 1.1. Scientific classification of *Bombus* sp.

Kingdom:	**Animalia**
Phylum:	**Arthropoda**
Class:	**Insecta**
Order:	**Hymenoptera**
Family:	**Apidae**
Tribe:	**Bombini**
Genus:	***Bombus***

Among others, bumblebees and honeybees are eusocial insects, as they live together in a colony with division of labor. The single queen is responsible for the reproduction and her workers take care of the brood and forage for food. Whereas honeybee colonies are perennial and survive winter on their food stock, bumblebee colonies are annual. This means that the colony perishes at the end of the season and only the daughter-queens hibernate and survive the winter. In spring, the bumblebee queens emerge, forage and look for a suitable nest site (Goulson, 2010). The queen provisions the nest with pollen and stores nectar in a wax pot. After two weeks, she lays her first batch of eggs within a lump of pollen and covers the pollen lump with a layer of wax mixed with pollen. She incubates the eggs by sitting on top of the pollen lump and maintains a brood temperature of 30-32 °C by shivering. After 4 days, the eggs hatch and the larvae start consuming pollen and nectar. During this period, the bumblebee queen forages for pollen and nectar. It is crucial that the queen finds enough food in the proximity of the nest and that weather conditions are favorable to forage, in order to provide food for herself and her colony. The larvae have four instars, and after 10-14 days of development, they spin a silk cocoon and pupate. After 14 days, pupation is completed and the adult emerges from the cocoon. The queen continues to lay further batches of eggs. The colony grows and tasks are divided: some bumblebee workers are

responsible for food provisioning and go foraging, while other bumblebees take care of the brood by keeping the brood warm and feed the larvae (Goulson, 2010).

Depending on the bumblebee species, larvae can be fed in two different ways: in the group of the 'pocket makers', larvae graze on the fresh pollen that is put underneath the brood clump. The larvae feed collectively, until the queen pierces holes in the wax cap over the clump during later stages of larval development. From that moment, the larvae feed on the mixture of pollen and nectar which the queen regurgitates onto the larvae. For bumblebees belonging to the 'pollen-storers', the larvae build individual cells from wax and silk. They are mostly fed on regurgitated pollen and nectar, individually for most of the development. When the larvae start to pupate, they close their individual cell with silk (Goulson, 2010).

If the nest reaches sufficient size, the queen switches to the rearing of drones (male bumblebees) and new queens. Each new queen mates and forages in order to build up reserves to survive the winter. She looks for a place to hibernate until spring. The old queen and the workers of her nest die and the old nest perishes (Alford, 1975).

1.1.2. Economic value of pollinators

Pollinators have an important economic value for the pollination of crops in the field and in greenhouses. Up to 80 % of the plant species are dependent on insect pollination for fruit or seed set (Garibaldi et al., 2013). This essential ecological service results in a direct economic value of 9.5 % of the total economic value of crops that are directly used for human consumption (Gallai et al., 2009). Typical examples are almond pollination by domesticated honeybees (*Apis*) and the use of mass-reared bumblebees (*Bombus*) for the pollination of greenhouse tomatoes and sweet pepper (Losey & Vaughan, 2006; Velthuis & van Doorn, 2006), as bumblebees are very efficient pollinators due to their 'buzz'-pollination.

1.1.3. Commercial bumblebee rearing

In 1987, commercial bumblebee rearing started as dr. De Jonghe founded the company Biobest, followed by the Dutch company Koppert Biological Systems in 1988. In the early years, tens of thousands of bumblebee queens were collected from natural populations shortly after hibernation, to start up bumblebee colonies for the indoor rearing. A small portion of the produced colonies is kept for the production of males and queens (Velthuis & van Doorn, 2006). Drones and queens mate and queens are put in a small individual box at 1-5 °C to simulate hibernation (Beekman et al., 1998; Velthuis & van Doorn, 2006). The length of the hibernation of the queens is variable in order to cope with peaks in sales. Young hibernated queens are taken from the stock and placed in a nest box in a climate room with a temperature of 28 °C and a relative humidity of 60 %. The nest boxes are provided with sugar syrup (50-60 % sugar content, *w/w*) and honeybee-collected pollen. The addition of one or more bumblebee workers, males, honeybee workers, or artificial cocoons can be used to stimulate colony initiation. When colonies have reached a size of around 50 workers, the colonies are sold. Colonies can have up to 200 individuals on their peak, and then start to produce males and daughter-queens. The entire lifespan of the colony in a greenhouse is usually between 8 and 12 weeks (Velthuis & van Doorn, 2006).

The indoor-rearing production should always follow Good Manufacturing Practice (GMP) guidelines to maintain a high standard and minimize contamination risk. These measures include regular disinfection of the rearing units and material, the use of lab coats and adapted shoes or sterile shoe covers when entering the rearing units. Rearing, mating and hibernation take place indoors, thus semi-sterile conditions are possible by excluding all contact with wild bumblebees. Contaminations are prevented by providing sterile food sources like sugar syrup and the use of irradiated honeybee collected pollen. Irradiation of pollen has demonstrated a significant reduction of bee viruses (Meeus et al., 2014). There should be a screening for honeybee and bumblebee pathogens on a regular basis to ensure pathogen-free rearing. In case pathogens would be detected, immediate actions should be taken by excluding the colony from the rearing program.

1.2. Gut microbiota of bumblebees

1.2.1 The gut microbiota in corbiculate bees

Adult bumblebees (*Bombus*) and honeybees (*Apis*) harbor a characteristic gut microbiota. Their microbiota is similar on genus level, but different on species level. Bacteria described in *Apis* and *Bombus* are listed in Table 1.2. Phylogenetic analyses of 16S rRNA sequences have indicated that some bacterial phylotypes, specifically occurring in *Bombus* and *Apis*, represent a unique clade. New bacterial species, genera and even families described over the recent years, have led to new nomenclature. Examples of new genera are *Snodgrassella* (Kwong & Moran, 2013), *Gilliamella* (Kwong & Moran, 2013), *Frischella* (Engel et al., 2013) and *Apibacter* (Kwong & Moran, 2016; Praet et al., 2016). The sociality of *Apis* and *Bombus* species facilitates bacterial transmission and thus is key to the maintenance of a more consistent gut microbiota, compared to solitary bees (Martinson et al., 2011). This characteristic gut microbiota is often called the 'core' gut microbiota, which encompasses different groups of bacteria that are primarily associated with the specified host. This means that in honeybees and bumblebees, different bacteria can be regarded as core (Cariveau et al., 2014; Meeus et al., 2015). Even within bumblebee species, there can be differences in the relative abundances of gut bacteria, resulting in several enterotypes each having other dominant bacteria (Li et al., 2015a).

1.2.2 The gut microbiota in mass-reared bumblebees (*Bombus terrestris*)

In a natural situation, opportunities to pick up bacteria from the environment and the bacterial transfer between and within colonies, are broad: the foraging wild bumblebees encounter other pollinators and bacterial sources from nature, while the nest is kept clean as feces are dropped outside the nest. Contrary, within the breeding facility the opportunities for bacterial transfer are more limited. Reared bumblebees are unable to leave the nest box and bacterial transfer between colonies is only possible when queens are set up for mating to ensure the

new breeding stock. The main bacterial transfer can happen between bumblebees within the colony via regurgitated food or via a fecal route as some of the feces remain in the nest box, providing a source of bacterial transfer of the bacteria from the hindgut (*Gilliamella* and *Snodgrassella*).

The lack of contact with other wild bees and a natural environment, could represent a bottleneck for the microbiota in the indoor-reared bumblebees. Indoor-reared *Bombus terrestris* mainly harbor Betaproteobacteria (*Snodgrassella*), Gammaproteobacteria (*Gilliamella*), Firmicutes (*Lactobacillus*) and Actinobacteria (*Bifidobacterium*). This is a subset of the gut microbiota of wild *B. terrestris* and is considered as the core gut microbiota of *B. terrestris*. Most of the wild bumblebees are still dominated by the core gut bacteria, but harbor as well a more variable prevalence of non-core bacteria, such as several non-core *Lactobacillaceae*, non-core *Bifidobacteriaceae* as well as *Enterobacteriaceae* which has never been detected in reared bumblebees. The bacterial composition in the gut of wild bumblebees shows a very high variability between individuals, while the reared bumblebees show a very stable gut composition. Indoor-reared bumblebees also show a lower community diversity and community richness than wild bumblebees (Meeus et al., 2015). For research purpose, indoor-reared bumblebees are useful as a model for the microbiota of bumblebees, which allows us to study the interaction of *Snodgrassella*, *Gilliamella*, *Lactobacillus* and *Bifidobacterium*, in a setting with minimal biological variation (Meeus et al., 2015).

Table 1.2. (Part 1) Bacteria described in the gut of *Apis* and *Bombus*

PHYLUM **Class** Order *Family*	***Genus*** *species*	Other designations	Host species
PROTEOBACTERIA			
Alphaproteobacteria			
Rhizobiales			
Bartonellaceae	***Bartonella*** *apis*	Alpha 1	*Apis*
Rhodospirillales			
Acetobacteriaceae	***Parasaccharibacter*** *apium*	Alpha 2	*Bombus*
	Gluconobacter-like	Alpha 2.1	*Apis*
	Saccharibacter-like	Alpha 2.2	*Apis*
	Bombella *intestini*	Alpha 2.2	*Apis*
Betaproteobacteria			
Neisseriales			
Neisseriaceae	***Snodgrassella*** *alvi*	Beta	*Apis*, *Bombus*
Gammaproteobacteria			
Orbales			
Orbaceae	***Gilliamella*** *apicola*	Gamma-1	*Apis*, *Bombus*
	Frischella *perrara*	Gamma-2	*Apis*
	Schmidhempelia *bombi*		*Bombus*
BACTEROIDETES			
Flavobacteriia			
Flavobacteriales			
Flavobacteriaceae	***Apibacter***		
	mensalis		*Apis*
	adventoris		*Bombus*
ACTINOBACTERIA			
Actinobacteria			
Bifidobacteriales			
Bifidobacteriaceae	***Bifidobacterium***		
	asteroides	Bifido1	*Apis*
	actinocoloniiforme	Bifido1	*Bombus*
	indicum	Bifido1	*Bombus*
	coryneforme	Bifido1	*Bombus*
	bombi	Bifido2	*Bombus*
	minimum	Bifido4	*Bombus*
	bohemicum	Bifido4	*Bombus*
	commune	BifidoX	*Bombus*
	Bombiscardovia *coagulans*	Bifido3	*Bombus*

Table 1.2. (Part 2) Bacteria described in the gut of *Apis* and *Bombus*

PHYLUM **Class** Order *Family*	***Genus*** *species*	Other designations	Host species
FIRMICUTES			
Bacilli			
Lactobacillales			
Lactobacillaceae	***Lactobacillus***		
	bombicola	Lacto1-Firm4	*Bombus*
	bombi	Lacto2-Firm5	*Bombus*
	mellis	Firm4	*Apis*
	mellifer	Firm4	*Apis*
	melliventris	Firm5	*Apis*
	kimbladii	Firm5	*Apis*
	kullabergensis	Firm5	*Apis*
	kunkeei		*Apis*, *Bombus*
	apinorum		*Apis*
	apis		*Apis*
Carnobacteriaceae	***Carnobacterium***		*Apis*
Leuconostocaceae	***Fructobacillus***		*Apis, Bombus*
	Weissella		*Bombus*
	Convivina *intestini*		*Bombus*

1.3 Methods for characterization and identification of bacteria

Over the years, several techniques have been used to characterize or identify bacterial communities. Originally, culture-based methods were used, but this technique often gives an incomplete and misleading picture of microbial communities. Each bacterial species needs specific growing conditions that are sometimes difficult to determine or imitate, and therefore not all bacterial species can be cultured. Culture-dependent techniques are not able to determine the richness and diversity of the bacterial community. In the more recent years, approaches based on DNA sequencing have provided a more reliable method to describe microbial communities. Nucleotide sequence databases have enabled classification of microorganisms by use of only short fragments of sequenced DNA. The culture-dependent techniques are still useful at present to identify new bacterial species and provide long DNA sequences for these databases. This way short sequences of DNA retrieved from the culture-independent techniques, can be identified. Thus, the use of culture-dependent and culture-independent techniques is complementary to each other.

1.3.1 Culture-dependent methods

In culture-dependent methods, bacterial suspensions from environmental samples are plated out on selective or non-selective agar media. New bacterial species are described by use of their biochemical characteristics as well as their DNA sequence. The sequences retrieved from these isolates are usually long enough to determine the bacteria on species or strain level. This has enabled researchers to isolate and describe new bacterial species and genera in the honeybee and bumblebee gut (Olofsson & Vasquez, 2008; Killer et al., 2009; Vasquez & Olofsson, 2009; Vasquez et al., 2009; Killer et al., 2010a; Killer et al., 2010b; Killer et al., 2011; Endo & Salminen, 2013; Engel et al., 2013; Killer et al., 2013; Killer et al., 2014; Kwong et al., 2014; Li et al., 2015b; Praet et al., 2015a; Praet et al., 2015b; Kwong & Moran, 2016; Praet et al., 2016).

1.3.2 Culture-independent methods

The 16S ribosomal RNA (rRNA) gene is widely used to characterize the taxonomic diversity present in bacterial communities. This sequence is composed of 9 hypervariable regions interspersed with conserved regions.

Conventional PCR methodologies

Several methods can generate a fingerprint of the bacterial community, as each bacterial DNA-fragment generates separate bands on a gel. Single-Strand Conformation Polymorphism (SSCP) is based on the principle that different single-stranded DNA molecules of identical length, each have a different folding pattern, which results in a different migration speed in a gel network.

Terminal Restriction Fragment Length Polymorphism (TRFLP) uses PCR in which one of the two primers is fluorescently labeled. The PCR product is then digested with restriction enzymes. The size and the fluorescence intensity of the individual terminal fragments are measured after separation by use of capillary electrophoresis.

Here we focus on Denaturing Gradient Gel Electrophoresis (DGGE) as this technique was used in this research. DGGE uses PCR to amplify DNA fragments of identical length with a GC-rich sequence (GC-clamp) attached to one end of the double-stranded DNA amplicons. These fragments are then loaded on a gel with a chemical denaturing gradient. As the DNA is subjected to increasing denaturing conditions, the double-stranded DNA fragments melt in a step-wise zipper-like manner as it moves across an acrylamide gel (Muyzer et al., 1993).
The previous methods have been used to assess the community richness and diversity of the microbiota of several bumblebee and honeybee species (Mohr & Tebbe, 2006; Koch & Schmid-Hempel, 2011b; Koch & Schmid-Hempel, 2011a; Disayathanoowat et al., 2012; Meeus et al., 2013; Saraithong et al., 2015). With the resulting fingerprints, the samples can be grouped into clusters with similar patterns, giving more insight into the characteristics of samples. However, these methods have a strong bias towards the most abundant bacteria and besides this, one species can result in several bands and bands of several species can overlap on the gel. So, this can lead to misinterpretation of the fingerprints. A final disadvantage is the lack of bacterial sequence information. Hereto an extra procedure is needed in which bands are cut out of the gel and are Sanger-sequenced for identification, but this only works if bands did not overlap on the gel.

A more elegant solution to identify the bacterial community is by molecular cloning of the bacterial PCR product pool, and sequencing the purified plasmids. Molecular cloning is a method in which the recombinant DNA is inserted in a vector. This vector carries the DNA molecule that will replicate the DNA fragments in the host organism. The plasmid vectors are transformed into competent *Escherichia coli* cells. *E. coli* cells are grown on selective agar plates and each clone contains a plasmid which is purified and the sequence can be determined via Sanger sequencing. Among others, Martinson et al. (2011) and Meeus et al. (2013) have used this method to identify members of the bacterial community in honeybees and bumblebees.

High-throughput sequencing

High-throughput sequencing technologies have opened new avenues in microbial community analysis by providing a cost-effective way of identifying the microbial phylotypes that are present in samples. These technologies include Illumina sequencing, Roche 454 sequencing, Ion Torrent sequencing and SOLiD sequencing. The outcome is dependent on analysis parameters and the used platform and different primer sets can lead to significant taxon-specific biases (Shakya et al., 2013). In the recent years, both Illumina and 454 pyrotag methods have been used to investigate the microbiota of bees (Moran et al., 2012; Sabree et al., 2012; Billiet et al., 2015a; Billiet et al., 2015b; Li et al., 2015a; Meeus et al., 2015; Parmentier et al., 2015b).

The focus here goes to Illumina MiSeq, as this technology was used in this research. To allow a pool of multiple samples within a single run, each specific sample is amplified with a unique multiplex identifier (MID) sequence ('barcode') in the PCR primer sequence. After the PCR products are pooled in equimolar quantities, the MIDs allow to allocate each sequence to its representative sample. In this dissertation the Illumina 2x150bp - MiSeq Reagent Kit v2 was often used. This gives an output dataset of 7.5-8.5 Gb or 24-30 million paired end reads, of a 254 bp sequence (V4 region) in our protocol. Of these, 75 % or 18 to 22.5 million pass the Illumina quality control. Pooling 120 different MIDs in a single run, results theoretically in 150,000 to 187,500 reads per sample. The raw data can be analyzed using tools such as mothur (Schloss et al., 2009) or QIIME (Kuczynski et al., 2011). As short amplicons are sequenced (in our case 254 bp in Illumina MiSeq), it is only possible to determine up to the level of bacterial genus or sometimes bacterial species.

1.3. Functionalities of gut bacteria

With several new bacterial species, genera and even families described in the guts of honey bees and bumblebees over the recent years, their associated functionality is a work in progress. In the following, we discuss their potential functionalities in digestion, the production of nutrients, biofilm formation and pathogen protection.

1.3.1. Role of the gut microbiota in digestion

1.3.1.1. Digestive tract of Hymentoptera

The insect gut can be divided into three main regions: the foregut, the midgut and the hindgut (Figure 1.1).

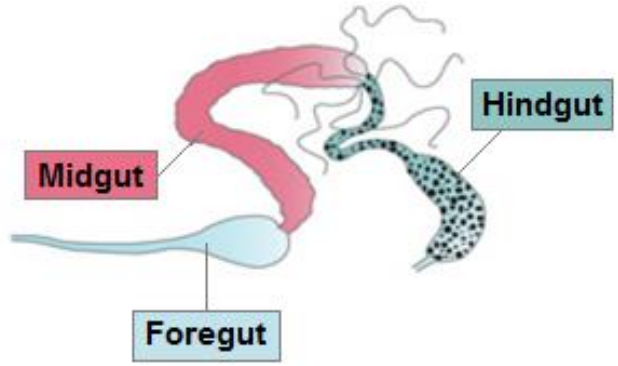

Figure 1.1. The hymenopteran gut, consisting of three main compartments: the foregut, the midgut and the hindgut. The dots in the hindgut represent the high bacterial abundance in this gut region. Figure adapted from Engel & Moran (2013).

The foregut contains the crop which mainly aids in temporally food storage when bees collect nectar to bring back to the colony (Engel & Moran, 2013). The crop contains relatively few bacteria (Martinson et al., 2012), mainly lactobacilli and bifidobacteria, forming a biofilm attached to the crop (Olofsson & Vasquez, 2008; Vasquez et al., 2012). These bacteria are acidotolerant, producing organic acids. This low pH reduces the colonization of acid sensitive bacteria in the digestive tract. The midgut is the primary site of digestion and absorption. The epithelial cells in the midgut secrete a peritrophic matrix and this divides the midgut into the endo- and exoperithrophic space (Shao et al., 2001). In bees, it is produced along the midgut

epithelium (Type I) and it is continuously replaced as it is shed (Teixeira et al., 2015). The peritrophic matrix protects the epithelial cells from mechanical damage by food particles. It is punctuated by small pores that form a barrier for most microorganisms (infection barrier) and large toxin molecules (chemical protection), while it allows the passage of enzymes and small molecules from digested food (Shao et al 2001). Microorganisms are usually in the endoperitrophic space (Engel & Moran, 2013). This part of the gut has a neutral pH (Lehane & Billingsley, 2012). The midgut contains relatively few bacteria, with most bacteria concentrated at the distal region where it joins the hindgut (Martinson et al., 2012). The hindgut functions in water resorption and is also a site of nutrient absorption (Chapman et al., 2013). The hindgut is divided into two compartments: the anterior ileum and the rectum, which is a sac-like structure in which the feces are held before defecation. The hindgut harbors 95 % of the bacteria in the gut of the honeybee. The ileum in honeybee guts mainly harbors *Snodgrassella* and *Gilliamella*, while in the rectum of the honeybee a large fraction of *Lactobacillus* was detected along with *Snodgrassella*, *Gilliamella* and *Bifidobacterium* (Martinson et al., 2012; Moran, 2015).

1.3.1.2. Digestion of pollen and nectar

In order to discuss the role of the gut bacteria in digestion one needs to know what kind of components are present in the diet of the bumblebee. Honeybees and bumblebees feed on nectar and pollen. The most important component for bumblebee larvae is pollen as proteins are needed for growth and development, while the main component for adult bumblebees is sugar which is needed as energy source, and also some nutrients from the pollen (Goulson, 2010).

Nectar is dominated by three sugars: glucose, fructose and sucrose (Percival, 1961; Bernardello et al., 2007). The amount and relative concentration of the main sugars vary among plant species (Percival, 1961). Pollen provides important nutrients such as proteins, minerals, lipids and vitamins (Stanley & Linskens, 1974). Pollen grains are different from

other plant cells, as they have a thick pollen wall that surrounds the inner nutrient-rich protoplasm. The pollen grain is composed of four main layers as presented in Figure 1.2.

The pollen coat is a thin outer skin, which consists of carbohydrates, surface lipids, proteins, (carotenoid) pigments and is also a source of volatiles such as aliphatics, aromatics and terpenoids, which guide pollen-foraging insects to the flowers (Stanley & Linskens, 1974; Klungness & Peng, 1984).

The exine layer is located underneath the pollen coat. It is a complex, richly sculptured and ornamented layer. The exine layer is highly resistant to decay and digestion and even withstands fossilization of pollen grains during millions of years (Jungfermann et al., 1997; Meuter-Gerhards et al., 1999). This layer is composed of the poorly characterized compound sporopollenin, in addition to glycoproteins, carbohydrates, proteins, phenolics, terpenoids, lipids and cellulose. The exine is perforated by germination pores that lead to the under laying intine layer.

The intine layer is rich in proteins, cellulose, hemicellulose and pectic acids (Stanley & Linskens, 1974). Underneath the pollen wall is the nutrient-rich protoplasm, which harbors proteins, lipids, sterols, sugars and starch (Stanley & Linskens, 1974).

It is not completely understood how nutrients are released from the pollen grain as the protoplasm content gradually disappears during digestion, without breaking the structural basis of the pollen wall as empty pollen shells are detected in the feces (Crailsheim et al., 1992). Different mechanisms have been suggested how insects can achieve nutrients from pollen. Specifically for bees the following are possible: osmotic shock, pseudo-germination and/or penetration of the pollen wall by digestive enzymes (Roulston & Cane, 2000). The bacterial members of the gut community can contribute in the production of digestive enzymes, supplementing the endogenous enzymes that are already produced by the host (Suen et al., 2010). It is unknown in which ratio the endogenous and exogenous enzymes are used during digestion. Metagenome studies and metatranscriptome studies on the honeybee have predicted several digestive functionalities of the microbiota in the honeybee (Engel et al., 2012; Lee et al., 2015).

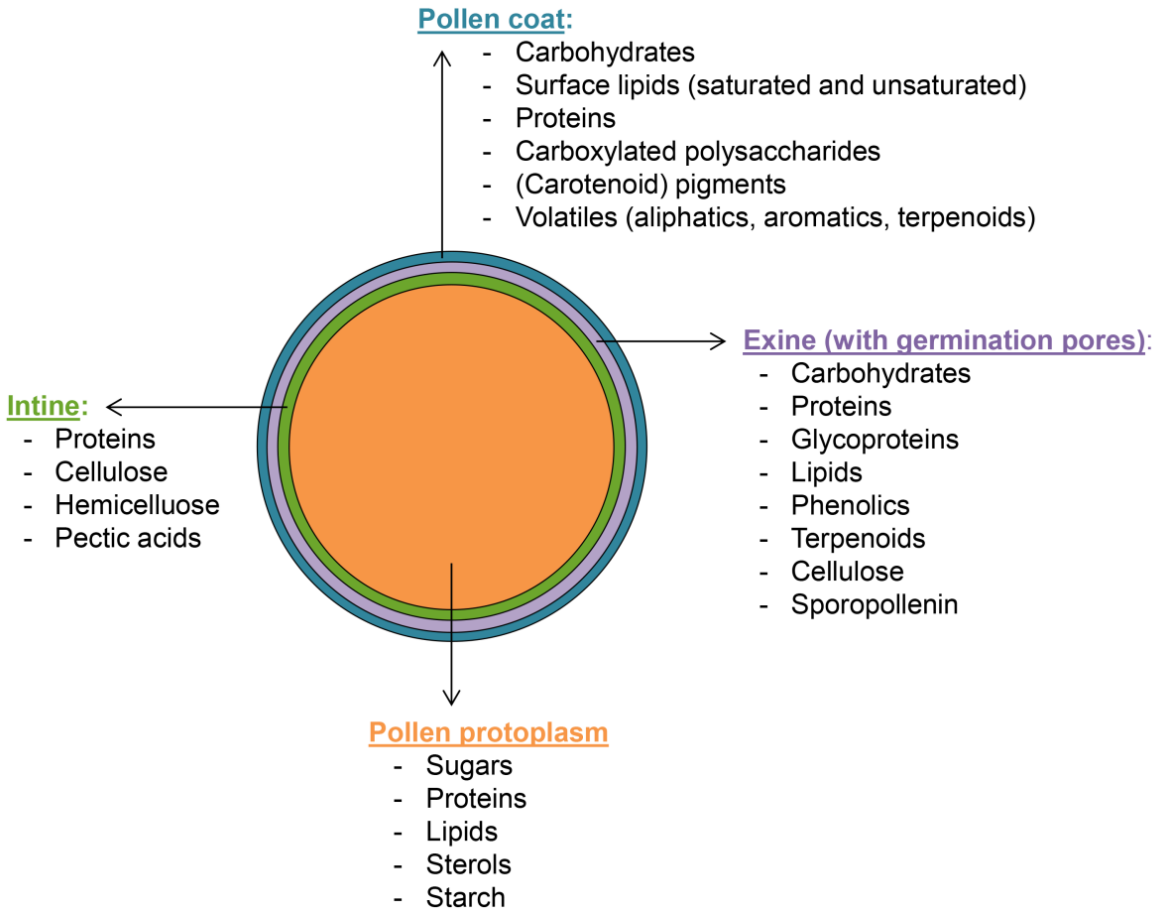

Figure 1.2. Schematic overview of the pollen structure with the different layers, pollen coat, exine, intine and protoplasm with their components.

The digestibility of the components of the pollen and nectar diet of honeybees and bumblebees can be divided into three groups:

(i) A first group consists of carbohydrates, lipids, proteins, glycoproteins and starch which are mainly located in the pollen protoplasm. Usually, carbohydrates and proteins are easily broken down and quickly absorbed in the gut (Klungness & Peng, 1984). The host is able to metabolize these components by use of endogenous enzymes, but the microbiota can assist in their digestion. Bees encounter a broad spectrum of carbohydrate substrates from the nectar or originating from the pollen cell walls. The carbohydrate metabolism and transport are one of the most common functionalities of the honeybee gut microbiota and are found in bacterial classes Gammaproteobacteria, Bacilli

and bifidobacteria (Engel et al., 2012; Lee et al., 2015). Bacilli occurring in the honeybee gut were also predicted to aid in the breakdown of peptides and starch (Lee et al., 2015).

(ii) Components such as sporopollenin, pectin, cellulose and hemicellulose are mainly responsible for the structure in the pollen grain and are present in the intine and the exine layers and are difficult to digest. Some endogenous enzymes can help in the breakdown of these components, but mainly the microbiota could help in the softening of this structure in order to improve the accessibility of nutrients of the protoplasm.

Sporopollenin is a biopolymer which appears to consist of an aliphatic chain with aromatic groups including monomers of para-coumaric acid and ferulic acid with a high degree of cross-linking derived from the polymerization of polyunsaturated fatty acids (Dominguez et al., 1999; Rozema et al., 2001; Smirnova et al., 2012; Mao et al., 2013). Para-coumaric acid has shown to up-regulate all classes of detoxification genes and some antimicrobial peptide genes in *Apis mellifera* (Mao et al., 2013), and could play a role in bee health. However it remains unknown which enzymes are needed to release p-coumaric acid from the sporopollenin.

Histochemical studies revealed that pectin is digested in the midgut of honeybees (Klungness & Peng, 1984). The metagenome study of Engel et al. (2012) showed that Gammaproteobacteria have genes encoding pectin-degrading enzymes, debranching enzymes and pectate lyases, which target the polygalacturonic acid backbone of pectin.

Also the breakdown of cellulose could enhance the release of the nutrients from the protoplasm. However, cellulose is very difficult to hydrolyze and requires the collaboration of several enzymes that have only been detected in wood-eating insects consuming cellulose-rich foods, such as termites (Nation, 2002). Glycoside hydrolases can assist in the hydrolysis of glycosidic bonds in complex sugars such as hemicelluloses and cellulose, by degrading alpha-1,4-glycosidic linkages between simple sugars and the alpha-1,6 linkages in oligosaccharides. According to Lee et al. (2015), the honeybee genome itself encodes a few endogenous glycoside hydrolases, but Bacilli were found to

transcribe enzymes such as glucan 1,6-alpha-glucosidase (EC 3.2.1.70) and neopullulanase (EC 3.2.1.135) highly. Also beta-glucosidase (EC 3.2.1.21) can help in the breakdown of oligosaccharides found in cellulosic material, and was predicted to be transcribed by Actinobacteria, Gammaproteobacteria and Betaproteobacteria from the honeybee gut (Lee et al., 2015). Hemicellulose of the intine is broken down partially as it proceeds through the alimentary tract of honey bees, however it is not clear if this is broken down by the host or its gut microbiota (Peng & Dobson, 1997).

(iii) A last group of components in pollen and nectar is not directly associated with nutrition. These components are mainly located in the exine layer and the pollen coat. Terpenoids have antimicrobial activity and produce volatiles and cannot be related to essential nutrients (Goff & Klee, 2006). However, some terpenoids are reported to be toxic (Ortego et al., 1999) and might cause an antifeeding effect. It is possible that gut bacteria can detoxify some food components. For example, some digestive enzymes of microbial origin of the *Tenebrio molitor* beetle larvae (mealworms) have potential to hydrolyze toxic plant glucosides (Genta et al., 2006). Some sugars like mannose and melibiose from nectar cannot be metabolized by bees and are poisonous to newly emerged workers (Barker & Lehner, 1974; Barker, 1977). Gammaproteobacterial genes were predicted to encode alpha-mannosidase (EC 3.2.1.24). By metabolizing such components, the gut microbiota could be critical for the detoxification of food components.

1.3.2. Production of beneficial nutrients

In certain insect species, typical bacteria prove to have a valuable role in the production of nutrients. This mutualism has mainly been observed in insects feeding on an unbalanced or nutrient-poor diet, for instance blood, plant sap and cellulose. In these cases, host and microbiota have co-evolved, leading towards a beneficial or ultimately to an obligate interaction (Rosenberg & Zilber-Rosenberg, 2011). Gut microbiota can aid in the production of essential components, which are lacking in the host organism or its diet.

Amino acids

The classic example of an obligate symbiont is *Buchnera* sp.. These bacteria are responsible for the biosynthesis of amino acids in pea aphids (*Acyrthosiphon pisum*), which are not included in the aphid's natural diet of plant sap (Sasaki & Ishikawa, 1995; Shigenobu et al., 2000). Another example is *Blochmannia,* which produces essential amino acids for the ant *Camponotus* (Sauer et al., 2000). Pollen can sometimes lack essential components for bee development. For pollinators, tryptophan and phenylalanine are the only two essential amino acids sometimes lacking in pollen (Roulston & Cane, 2000). Actinobacteria and Gammaproteobacteria in honeybee guts are predicted to synthesize all essential amino acids as well as other non-essential amino acids (Engel et al., 2012; Lee et al., 2015). Contrary, Bacilli from the honeybee gut are predicted to carry out relatively few biosynthesis of amino acids, except the alanine anabolism. These amino acids can be useful for the host, or for the bacteria itself as Bacilli probably take up amino acids that were derived from the diet or from biosynthesis by other bacterial members in the honeybee gut (Lee et al., 2015).

Vitamins

Synthesis of vitamins has already been reported for a considerable number of bacteria. Blood feeding insects such as the tsetse fly (*Glossina morsitans morsitans)* and the bedbug (*Cimex lectularius*) have the respective bacterial symbionts *Wigglesworthia* and *Wolbachia*, and these are reported to be necessary for a good reproduction and fitness. Lack of these bacteria can only be substituted by the addition of vitamins B to the diet, indicating that these bacteria play a role in vitamin B production (Hosokawa et al., 2010; Rosenberg & Zilber-Rosenberg, 2011). However it is not always clear if bacteria produce these vitamins for their host. In some cases the bacteria itself needs the vitamins as cofactors for its own metabolism, especially in an environment where these cofactors are scarce (Douglas, 2009). In the case of pollen-feeding insects like bees, Roulston & Cane (2000) reported that pollen is rich in water-soluble vitamins, however poor in fat-soluble vitamins like A, D, E and K.

Pollen is a rich source of vitamin B, but several studies indicated that also certain strains of lactobacilli and bifidobacteria can produce vitamin B (LeBlanc et al., 2011). B vitamins seemed to be essential in the nutrition of honey bee larvae (Haydak & Dietz, 1965).

***Short-chain fatty acids* (SCFAs)**

As reported for humans and rodents, gut bacteria are able to produce short-chain fatty acids such as acetate (C2), propionate (C3) and butyrate (C4) by anaerobic fermentation of carbohydrates and proteins. This is a beneficial process because 60-70 % of the energy requirements for colonic epithelial cells comes from these bacterial fermentation products in mammals (Macfarlane & Macfarlane, 2012). Actinobacteria, Bacilli and Gammaproteobacteria in the honeybee gut were predicted to produce SCFAs lactate, formate and acetate (Lee et al., 2015). Acetate and lactate may then be utilized by the host or by other members of the microbial community (Lee et al., 2015).

1.3.3. Biofilm formation

Fluorescence microscopy showed that lactic acid bacteria (LAB) form a biofilm layer attached to the crop of honeybees (Vasquez et al., 2012) (Figure 1.3). Metagenome studies on the microbiota of the honeybee indicated that also *Snodgrassella* and *Gilliamella* harbor functional capabilities for biofilm formation (Engel et al., 2012), which was confirmed by use of fluorescence microscopy revealing that *Snodgrassella* and *Gilliamella* form biofilm-like layers on the epithelium of the ileum (Martinson et al., 2012; Lim et al., 2015).

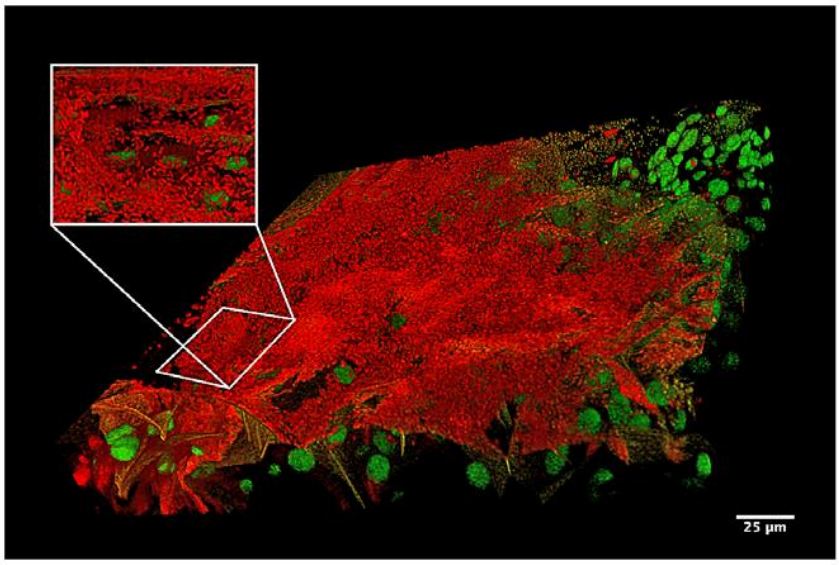

Figure 1.3. *In vivo* visualization of LAB biofilm. The red fluorescence shows live-stained bacteria in a LAB biofilm attached to a honey crop. The green fluorescence shows the nuclei of the honeybee crop cells (Vasquez et al., 2012).

1.3.4. Pathogen protection

Although the gut microbiota of insects is much less complex than in humans, the microbiota has demonstrated to play a role in the protection against parasites and pathogens. For example, the production of toxic phenols by the microbiota of the desert locust *Schistocerca gregaria* showed protection against pathogenic bacteria and ensured the suppression of fungal infections (Dillon et al., 2005). The presence of the facultative symbiont *Hamiltonella defensa* protects *Acyrthosiphon pisum* aphids against larval growth of the parasitic wasp *Aphidius ervi* (Oliver et al., 2003; Moran et al., 2005) and Ryu et al. (2008) showed a mutualistic relationship between the fruit fly *Drosophila* and its endogenous gut microbiota as it was able to suppress the growth of pathogenic bacteria.

Also bee symbionts have proven to play a role in pathogen protection. Genomic analyses have revealed many genes in *Snodgrassella* and *Gilliamella* in honeybees that can produce toxins, potentially affecting bee parasites (Kwong et al., 2014). Bumblebees with a deficient gut microbiota have been shown to be less protected against infection with the protozoan gut parasite *Crithidia bombi* (Koch & Schmid-Hempel, 2011b) and the variation in gut microbiota

was correlated with the variation in resistance to particular strains of *C. bombi* (Koch & Schmid-Hempel, 2012). Cariveau et al. (2014) showed a higher *Crithidia* incidence in bumblebees with a low colonization of the core gut bacteria *Gilliamella* and reported a positive correlation between *C. bombi* infection and the richness of non-core bacteria. Also LAB could play an important role in pathogen protection in bees. Forsgren et al. (2010) and Vasquez et al. (2012) proved that LAB had an antagonistic effect against *Paenibacillus larvae* causing American Foulbrood and *Melissococcus plutonius*, the etiological agent of European Foulbrood. Both are serious honeybee diseases in which bacterial spores germinate in the midgut lumen. Vasquez et al. (2012) discovered 50 novel LAB of the genera *Lactobacillus* and *Bifidobacterium* in the crop of honeybees and stingless bees, which are exchanged between bees via throphallaxis. *Lactobacillus kunkeei*, the most common and dominant lactic acid bacterium in the crop of bees, was able to inhibit 55 bacterial strains and 5 yeasts isolated from flowers. The honeybees' beebread and honey itself also contained these LAB and other antimicrobial substances, which suggests a possible role for LAB to prevent spoiling of beebread and defense against honeybee diseases (Vasquez & Olofsson, 2009).

The mechanism of the beneficial effect of LAB in general lies in competition with pathogens for nutrients and sites of accession, production of antimicrobial metabolites, changes in environmental conditions (e.g. pH), modulation of immune response of the host, producing end-products of anaerobic fermentation of carbohydrates and contribution in digestion (Van den Abbeele et al., 2011; Vyas & Ranganathan, 2012; Saad et al., 2013). Some of the produced organic acids possess broad-spectrum inhibition against bacteria, fungi and yeasts (Corsetti et al., 1998; Mu et al., 2012). Besides that, LAB are able to produce bacteriocins, a group of proteinaceous toxins which have a strong antimicrobial activity against pathogens. LAB strains also have the ability to produce the antimicrobial substance H_2O_2, which is also involved in the maintenance of the vaginal ecosystem as it is produced by LAB in that region (Lahtinen et al., 2011). Furthermore, lactobacilli are involved in biofilm formation (Vasquez et al., 2012) and have been shown to be able to outcompete other pathogens for attachment,

resulting in competitive exclusion and this way, protecting tissues from pathogen colonization (Johnson-Henry et al., 2007).

1.4. The use of probiotics in insects

According to FAO/WHO, probiotics are “live microorganisms which, when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2001). In order to commercialize, a probiotic should be able to survive manufacturing and storage during large-scale industrial preparation. The probiotic should be able to survive the intestinal ecosystem and the host animal should benefit from its presence (Vyas & Ranganathan, 2012). The most widely used microorganisms in probiotics are lactobacilli, bifidobacteria and certain yeasts (*Saccharomyces cerevisiae*). Different strains, even within species, might have diverse health effects. Positive results with probiotic strains have already been applied in mammals and fish hatcheries, where the results show an induced growth and a reduction of diseases without the use of antibiotics (Kawakami et al., 2010; Magnadottir, 2010; Prado et al., 2010; Ringo et al., 2010; Soccol et al., 2010; Delzenne et al., 2011; Bovera et al., 2012; Piccolo et al., 2015). Also in insects, the administration of bacterial (probiotic) strains has proven to be successful. Positive effects were observed on mating, survival, body size, growth rate and fecundity in tests with fruit flies (*Ceratitis capitata* (Wiedemann)), silkworms (*Bombyx mori*), moths (*Hepialus gonggaensis*) and olive flies (*Bactrocera oleae*) (Niyazi et al., 2004; Masthan et al., 2010; Gavriel et al., 2011; Yin et al., 2011; Hamden et al., 2013; Sacchetti et al., 2014). Honeybees have also been the subject of studies for probiotic applications for example in pathogen protection. A mix of *Bifidobacterium* and *Lactobacillus* strains was able to induce the immune response in honeybees (Evans & Lopez, 2004). *In vitro* tests and *in vivo* tests with a mix of LAB on honeybee larvae (*Apis mellifera*) showed inhibitory capacities against *Paenibacillus larvae*, the causal agent of American foulbrood disease (Forsgren et al., 2010). *Melissococcus plutonius*, the causal agent of European foulbrood disease, was also inhibited by application of a *Bacillus* isolate leading to a significant reduction of mortality

of infected larvae of *Apis mellifera japonica* (Wu et al., 2014). Besides the inhibitory effect on pathogens, also other positive effects of probiotics have been investigated on honeybees. Biogen-N and Trilac are two commercial probiotic formulations which were originally produced for humans, containing a mixture of one or more strains of *Bifidobacterium*, *Lactobacillus*, *Enterococcus* and *Pediococcus*. Both probiotics were used in several studies on honeybees (*Apis mellifera carnica*). Their application proved successful as the results showed a decrease of mortality, and showed increases of body mass, fat body, faucal gland, merocrin-type secretion and quantities of peritrophic membranes providing a better digestion (Kaznowski et al., 2005; Kazimierczak-Baryczko & Szymas, 2006; Szymas et al., 2012). Also the combination of commercially available probiotics (Enterobiotic and Enterolactis Plus) and prebiotics (lactic acid or acetic acid) led to an increased colony wax production as a result of an improved wax cell development in honeybees (Patruica & Mot, 2012).

In recent years, several probiotic formulations or the use of particular bacteria have been patented, as they showed potential to enhance the health of honeybees. '*MICRO4BEE*' is a patent (Patent WO2011138310 A2) to protect honeybees against microbial pathologies such as American foulbrood. *Pseudomonas fons MS-1* strain (accession number: FERM P-21673) was patented (Patent TW201036551A) to promote the growth of honeybees and it inhibits infections of bacteria, fungi and viruses in honeybees. Another example is a feed mix supplemented with potential beneficial enterobacteria (Patent JPH02222654A) to enhance the immunology of the honeybees. It contains sugar mixed with useful bacteria like *bifidobacteria*, *Lactobacillus lactis*, *Lactococcus* (*Streptococcus*) *lactis* and *Bacillus subtilis*. A last example of a patent to improve the health of honeybees contains a mix of several strains of *Lactobacillus*, *Lactococcus* and *Bifidobacterium* in a 5 % sucrose solution (Patent WO2014097338 A1). The use of this bacterial mix is described to induce a positive effect on the health conditions of a bee colony as it helps in treatment and prevention of Chronic Bee Paralysis Virus (CBPV) and *Nosema ceranae* infection.

These patent examples reflect an active search and interest in probiotics for bee rearing. The probiotics which have been studied and published in peer-reviewed papers, demonstrate that

these improve either fitness effects, pathogen protection or digestion. However the patented bee probiotics are not yet produced on large scale, but it merely seems a matter of time before commercially produced bee-specific probiotics are applied to promote health or protect bees from infectious diseases.

For most applications, the use of probiotics for honeybees focuses on the protection against pathogens. But the use of beneficial bacterial strains could also prove useful in restoring the gut microbiota. Observations have shown that LAB are highly sensitive to antibiotics such as oxytetracycline and tylosin which are frequently used in apiculture in the USA (Vasquez et al., 2012). The loss of LAB could lead to a higher susceptibility to pathogens. Also indoor-reared bumblebees could benefit from the use of probiotics, as it has been shown that commercially reared bumblebees harbor only a subset of the microbiota of wild bumblebees (Meeus et al., 2015). As the microbiota is predicted to play a role in digestion and pathogen protection, it is important to keep an eye on this microbial community.

Chapter 2.

Assessment of mutualism between *Bombus terrestris* and its microbiota by use of microcolonies

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Redrafted from Meeus, I., Mommaerts, V., Billiet, A., Mosallanejad, H., Van de Wiele, T., Wäckers, F., Smagghe, G. (2013) Assessment of mutualism between *Bombus terrestris* and its microbiota by use of microcolonies. Apidologie. 44 (6), 708-719

2.1. Abstract

Social pollinators, like honeybees and bumblebees, have a specific gut microbiota. The specific association between the insect and its gut microbiota could lead to a functional dependence. This chapter investigates whether an alteration in microbiota could influence the fitness of the generalist pollinator *Bombus terrestris*. We used bumblebee workers in microcolonies to assess effects on colony development and reproduction. Treatment with 200 ppm streptomycin showed an improved reproduction, compared to the control treatment. The gut microbial patterns were investigated using DGGE. In streptomycine-treated samples, the pattern was clearly different from the control treatment. The results are discussed in relation to effects of the gut microbiota on the bumblebee fitness and potential beneficial effects of *Lactobacillus* sp. These data may open new avenues in the mass-rearing of bumblebees used for the biological pollination in agriculture.

2.2. Introduction

Insects are arguably the most successful group of animals on the planet, occupying a diverse range of ecological niches. This ecological success is often facilitated by bacterial symbionts (Brownlie & Johnson, 2009). These acquired microbial genomes, among others, can synthesize required nutrients and cofactors for the insect host, enable the host to utilize unusual or low-nutrient diets, give protection against parasitism and/or influence patterns of host plant use (Dillon et al., 2005; Riegler & O'Neill, 2007; Moran et al., 2008). Within the *Apidae*, a family of bees with an important ecological value in maintaining plant diversity and providing pollination services (Ghazoul, 2005; Steffan-Dewenter et al., 2005), especially honeybees and bumblebees are well studied. This is due to their economic value for the cultivation of pollinator-dependent crops (Velthuis & van Doorn, 2006; Aizen et al., 2008). The role of beneficial host-microbe interactions in social insects is currently under investigation. The honeybee and bumblebee gut microbiota has recently been identified, revealing a highly specific, yet overlapping community composition with low species diversity (Koch & Schmid-Hempel, 2011a; Martinson et al., 2011; Koch et al., 2013). In other insects, the microbiota may be more variable within a single species. Indeed, the gut microbiota of common fruit flies from different locations showed variable species richness (Corby-Harris et al., 2007; Chandler et al., 2011), and diet plays a major role in shaping the gut microbiota of larvae of the gypsy moth (Broderick et al., 2004). The specific relation between the gut microbiota and social pollinators creates the opportunity to evolve a functional dependence.

In this chapter, we investigated this host-microbe interaction to see if alterations of the microbiota in the gut of bumblebee workers can influence their fitness. *Bombus terrestris* from a mass-rearing program were used, as this species offers the unique opportunity to use microcolonies, a standardized setup to study colony development and reproduction following exposure to different treatments (Mommaerts et al., 2006). Although microcolonies have already been used before in insecticide testing, we applied them for the first time to test whether the microbiota patterns in the gut of these workers are representative for a queen-right colony. With these microcolonies, we then determined whether antibiotic treatments like

streptomycin can induce changes in the microbiota of bumblebees compared to untreated microcolonies. Subsequently, we scored the fitness of these microcolonies to evaluate if changes in microbiota affect colony development and reproduction in order to identify certain beneficial bacterial species.

2.3. Materials and methods

2.3.1. Insects

All bumblebees were obtained from a continuous mass rearing program (Biobest, Westerlo, Belgium) and fed on commercial sugar syrup, containing methyl and propyl hydroxybenzoate at 0.6 % (BIOGLUC®, Biobest) and honeybee-collected pollen (Soc. Coop. Apihurdes, Pinofranqueado-Cáceres, Spain) as energy and protein source, respectively. The insects were kept under standardized laboratory conditions with 28-30 °C, 60-65 % relative humidity, and continuous darkness.

2.3.2. Microcolonies and measuring fitness of microcolonies

We used microcolonies to quantify fitness effects of an antibiotic treatment on bumblebees. These microcolonies are created by placing six or seven newly emerged workers (exact numbers of bumblebees are given in each specific experiment) in an artificial nest box (15×15×10 cm). After 1 week, one worker becomes dominant and starts laying unfertilized eggs that develop into drones, while the other workers take care of the brood and forage for food. These microcolonies follow a well-defined development pattern with quantifiable parameters (i.e., time point of egg laying, larval development time, pupation time, reproductive output, and larval mortality). Microcolonies contain unfertilized workers that only produce male offspring. Hence the reproduction output can be determined by counting the numbers of drones and by weighing (Adventurer Pro AV413C) their mass. The microcolonies were kept at the standardized rearing conditions as reported above for the rearing.

Normal distribution of data was confirmed by Kolmogorov–Smirnov test (P=0.05) and the mean ± SD were analyzed by an independent-sample t test (P=0.05).

2.3.3. Microbiota of workers in microcolonies compared to queen-right colonies

Seven newborn workers were selected from one queen-right colony and placed in one microcolony box. This setup was performed for six queen-right colonies as biological replicates (n=6). From each microcolony, we sampled one 4-days-old and one 8-days-old worker to determine the gut microbiota as described below. From the queen-right colony, the newborn workers were labeled and placed back in the colony. Then, three 0-day-old, three 4-days-old, and three 8-days-old workers were sampled per colony and we characterized the gut microbiota as described below.

2.3.4. Fitness and gut microbiota of workers in microcolonies treated with 200 ppm streptomycin

Twenty microcolonies were set up with each six newly emerged workers that were randomly selected from different queen-right colonies. Two different treatment groups were created each consisting of ten microcolonies: control microcolonies (C) were fed normal untreated sugar syrup, while in treated microcolonies the sugar syrup was supplemented with 200 ppm streptomycin (S). Fitness parameters of each microcolony were recorded as described above. To determine the gut microbiota we sampled one 8-days-old worker from each of the 20 microcolonies.

2.3.5. Characterization of the gut microbiota

2.3.5.1. DNA extraction

The mid- and hindgut of the worker bumblebee were dissected and stored individually in 200 µL of acetone for later analysis. Stored samples were washed three times with sterile phosphate-buffered saline (PBS, pH 7.4). For this purpose the gut was opened by gentle crushing in a microtube using a Teflon pestle in 200 µL of PBS and then centrifuged at

10,600×g. The pellets were incubated at 37 °C for 2 h in a 170 µL lysozyme solution (100 mg/mL) followed by treatment with 25 µL of Proteinase K (Sigma-Aldrich) for 1 h at 56 °C. In order not to preferentially extract gram negative bacteria, the samples were transferred to 300 µL of lysis buffer from the EZNA® Insect DNA Kit (Omega Bio-Tek) supplemented with 0.3 g of zirconia beads (diameter 0.1 mm; BioSpec Products) and further homogenized by bead beating in the Fastprep TM FP 120 (Thermo Scientific). Further extraction was performed according to manufacturer's instructions.

2.3.5.2. Fingerprinting the microbial community

We generated a 16S rDNA gene molecular fingerprint of the microbiota of the bumblebee gut in order to analyze its structure and diversity. The bacterial 16S rDNA was amplified without co-amplification of predominant eukaryotic DNA by nested PCR with the primers Eub8F and 984R for external PCR and 338FGC and 518R for internal PCR (Bakke et al., 2011). The reaction mixture contained 2 mM $MgCl_2$, 0.3 mM dNTP, 0.3 µM primers, recombinant Taq DNA polymerase (Invitrogen), and 5 µL of DNA extract for the external PCR and 1 µL (1/50 diluted external PCR product) for the internal PCR. A total of 25 µL of external PCR reaction mix was placed in the Sensoquest Labcycler for 2 min at 94 °C followed by 25 amplification cycles (30 s at 94 °C, 30 s at 53 °C, and 60 s at 72 °C) and then by 3 min at 72 °C. For the internal PCR, the same procedure was followed, except the annealing temperature was 50 °C, 21 amplification cycles were used and the final elongation was 10 min to complete the polymerization.

The 16S rDNA gene molecular fingerprint was generated by denaturing gradient gel electrophoresis (DGGE). 10 µL of the PCR products were loaded on an 8 % (*w/v*; 40 % acrylamide, 2 % bisacrylamide) gel with a denaturing gradient of 45 to 60 %; 100 % denaturation refers to 7 M urea and 40 % deionized formamide. Electrophoretic separation was performed on the INGENYphorU (Ingeny) for 16 h at 120 V in 1× TAE buffer (20 mM Tris, 10 mM acetate, 0.5 mM EDTA pH 7.4) at 60 °C. The gels were stained using SYBR

Green (1:10,000 dilution, FMC BioProducts) in 1× TAE for 20 min and visualized by UV transillumination (Vilbert Lourmat).

2.3.5.3. Diversity analysis

Statistical comparison of the DGGE patterns was done with BioNumerics software (Applied Maths, Kortrijk, Belgium). Similarity matrices were based on the Pearson correlation coefficient using data from matched bands. Dendrograms were calculated using the clustering algorithm of Ward (Van de Wiele et al., 2004). Differences in similarity were calculated by analysis of similarities (ANOSIM) (Clarke & Green, 1988; Clarke, 1993) using Primer version 6.1.10 software. It calculates a global R statistic which lies between −1 and +1, with high values indicating a large degree of discrimination among groups.

The structural diversity of the microbiota is characterized by the Shannon index of general diversity *H* (Vervaeren et al., 2006). The equation for the Shannon index is: $H=-\Sigma\ (n_i/N)\ \log(n_i/N)$, where n_i is the height of the peak and N the sum of all peak heights of the densitometric curve (Vervaeren et al., 2006). Microbiota diversity analysis was based on the method of Lorenz as described (Mertens et al., 2005; Wittebolle et al., 2009). In short, two parameters were derived from the bacterial fingerprint. Firstly, species richness (*R*), which is the number of bands. Secondly, the community organization (*Co*), for which a low number (for example 25 %) represents a community with high evenness or with no specific dominant species (Marzorati et al., 2008).

For all data, normal distribution was confirmed by Kolmogorov-Smirnov test (P=0.05) and then the means ± SD were analyzed by an independent-sample *t* test (P=0.05) or one-way ANOVA and separated by a post hoc Tukey–Kramer test (α=0.05) using SPSS v. 16.0.

2.3.5.4. Identification of bacteria

Bacterial species were identified by cloning the external PCR fragment of one control bumblebee and one streptomycin-treated bumblebee from a microcolony into a pJET1.2/blunt vector with the CloneJET PCR Cloning Kit (Thermo Fisher Scientific) following

manufacturer's instructions. Afterwards, plasmids were transformed in competent *Escherichia coli* XL-1 Blue Cells by heat shock and then plated out on a carbenicillin-containing LB agar plate. After 16 h incubation, only three and two colonies were formed, respectively. From each clone a plasmid was purified using the Plasmid mini prep kit (Omega Bio-Tek) and sent for sequencing to LGC Genomics (Berlin, Germany). The place of the bacterial plasmids on the DGGE profile was determined after nested PCR with external vector-specific primers and the internal primers as described above.

2.4. Results

2.4.1. Microbiota of workers in microcolonies compared to queen-right colonies

The development of the microbiota was followed in untreated queen-right colonies (n=6) and from each colony we sampled nine workers (three newly emerged workers (callow workers) and three workers of 4 and 8 days old, respectively). Newly emerged workers showed no or hardly any microbiota in their gut with a species richness R of 0.17 ± 0.41. By day 4, the gut microbiota had developed and the richness was significantly different from day 0 ($P < 0.05$, ANOVA). Over the subsequent 4 days, the community richness R = 10.14 ± 3.62 (P=0.53), the Shannon index of general diversity H = 0.87 ± 0.18 (P=0.43), and the community organization Co = 35.89 ± 4.77 (P=0.73) remained stable (day 4 compared to day 8; ANOVA; Figure 2.1a). Calculating the same parameters, we could not detect any significant difference between bumblebees from microcolonies (n=6) (days 4 and 8) and those from queen-right colonies (n=6) at days 4 and 8 (ANOVA, R: P=0.94; H: P=0.80; and Co: P=0.49; Figure 2.1a). However, looking at the similarity of the DGGE patterns of queen-right colonies (74.8 ± 12.8) and microcolonies (77.5±14.0), a higher similarity was found within each group, opposed to the similarity between queen-right colonies and microcolonies (62.9±9.4; Figure 2.1b). ANOSIM, comparing each queen-right colony with its corresponding microcolony, revealed some separation (R=0.33±0.26), but none were significantly different. However, some were close to the P=0.05 (i.e., P=0.07; P=0.14; P=0.10; P=0.33; P=0.70; P=0.21).

Although most bands found within microcolonies corresponded with those found in queen-right colonies, we speculate that the early separation of callow workers from the nest has a minor effect on the development of the microbiota.

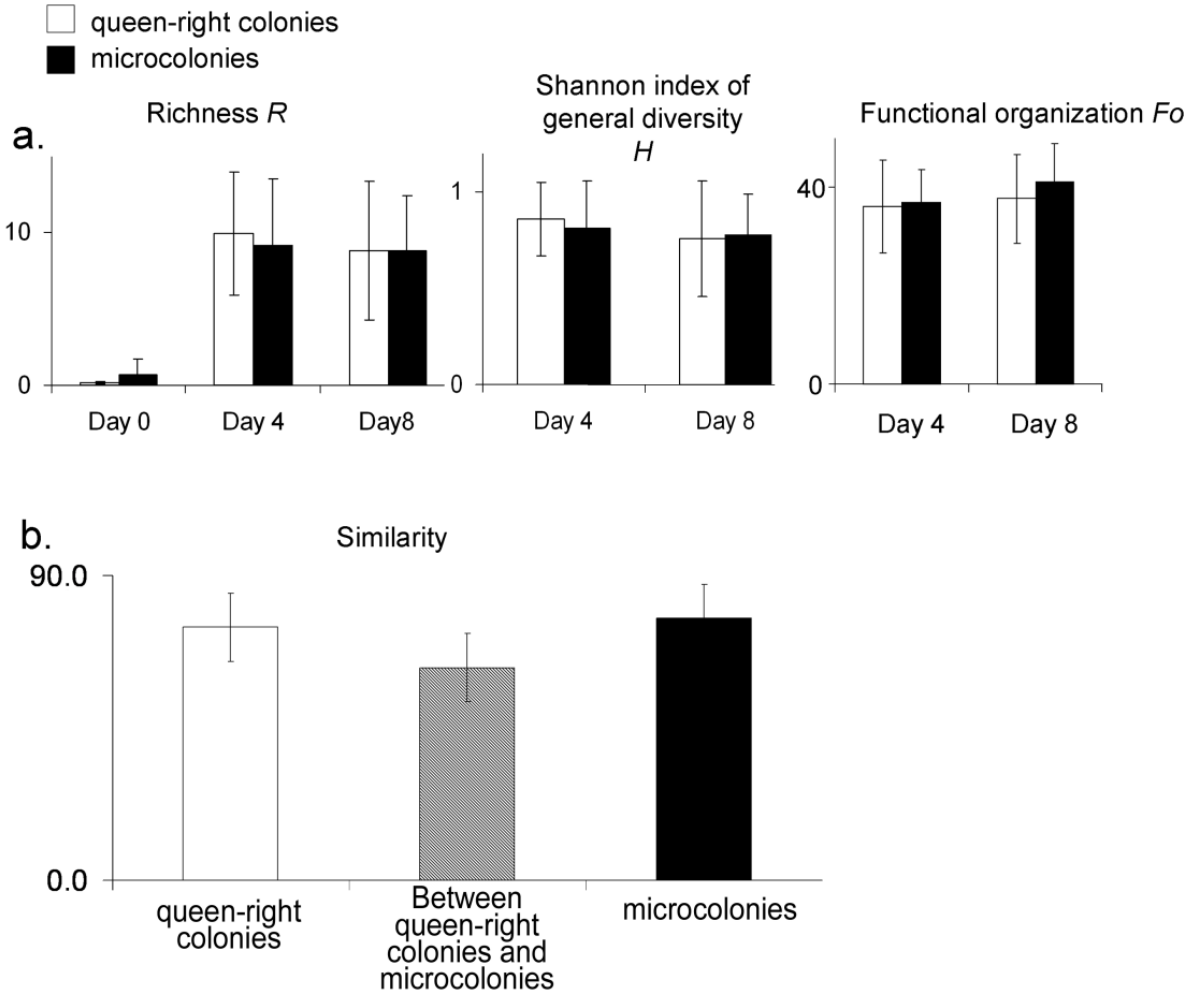

Figure 2.1. (a) The community richness *R*, the Shannon index of general diversity *H*, and the functional organization *Fo* of bumblebee workers from queen-right colonies versus microcolonies. As newly emerged workers (day 0) showed no or hardly any microbiota in their gut (*R*=0.17±0.41), it was not possible to calculate a value for parameters Shannon index and Functional organization for samples of day 0.

(b) Similarity of queen-right colonies and microcolonies and between queen-right colonies and microcolonies, based on the Pearson correlation coefficient using data from matched bands.

2.4.2. Fitness and gut microbiota of workers in microcolonies treated with 200 ppm streptomycin

We recorded a shift in the microbiota of bumblebees receiving 200 ppm streptomycin (S; *n*=10; day 8) compared to control microcolonies (C; *n*=10; day 8). First, the general diversity of the microbiota dropped sharply as illustrated in Figure 2.2b by the Shannon index *H* (independent *t* test with unequal variance $P<0.001$) and species richness *R* (independent *t* test, $P<0.001$). Second, the bacterial identity was also different with control (C) bumblebees clustering separately from streptomycin-treated ones (S; Figure 2.2a). The bands highlighted in red (Figure 2.2a) are unique to the streptomycin-treated colonies. Indeed, their relative position in the DGGE gel is different from the two closest bands present in the untreated control samples (supplemental data Table S1). The red colon punctuations in Figure 2.2a also represent unique bands in the streptomycin treatments, but these have a very weak intensity that was only visible upon contrast and brightness adjustments.

The two red bands unique for the streptomycin-treated samples were identified as the same bacterial species, namely *Lactobacillus* sp. (deposited at GenBank as KC477412) and has been previously isolated from the honeybee (GenBank HM534867). The bands mainly present in untreated bumblebees and here framed in blue are a *Snodgrassella* sp. (deposited at GenBank as KC477411) and a *Bifidobacterium* sp. (deposited at GenBank as KC477410), which both had previously been identified in wild bumblebees (Koch & Schmid-Hempel, 2011a; Koch & Schmid-Hempel, 2011b)

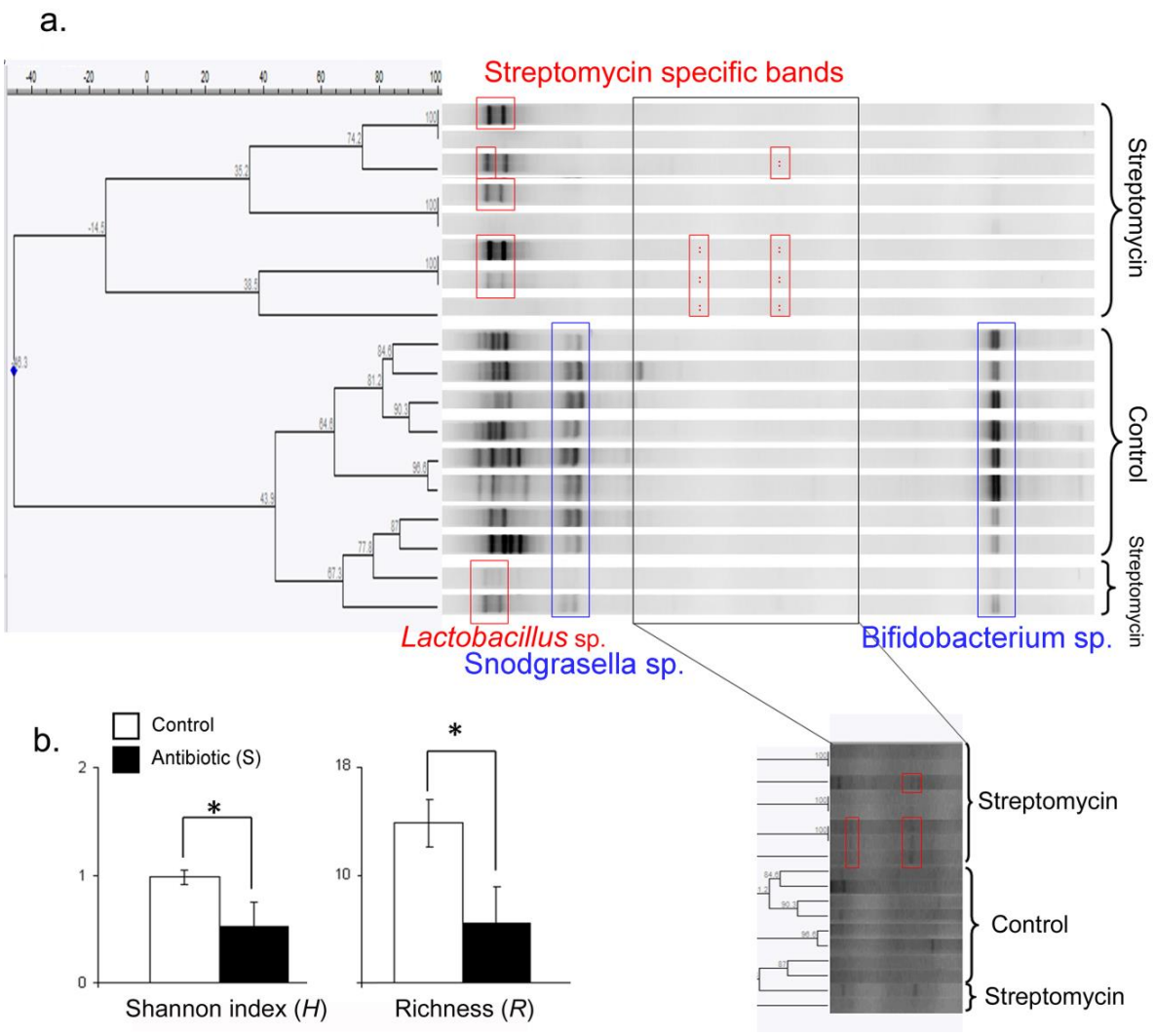

Figure 2.2. (a) The dendrogram shows that major bacterial species mainly disappear after streptomycin treatment. Bands highlighted in black are unique in streptomycin samples. Colon punctuations represent unique low-intensity DNA bands (cropped image below shows the presence of the low-intensity DNA bands after adjusting contrast and brightness). The grey box shows overlapping expression between two streptomycin-treated samples with the control. The bands in the grey box of streptomycin-treated samples show lower expression compared to the band representing *Lactobacillus* sp. (GenBank KC477412). **(b)** Significant drop in mean species richness *R*±SD (*n*=10; *P*<0.001) and the mean Shannon index *H*±SD (*n*=10; *P*<0.001) after streptomycin treatment.

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When evaluating the fitness effects of the 200 ppm streptomycin treatment (S; *n*=10), we observed no lower survival rate of treated workers and also a normal development of the microcolony: the first egg was laid at 8 ± 1 days, the first pupa was formed at day 21 ± 1 and the first drone emerged at day 33 ± 1 (independent sample *t* test; first egg: *P*=0.14, first pupa: *P*=0.50, first drone *P*=1.00). However, the numbers of drones that emerged after 50 days were significantly increased by about 20 % from 32.8 ± 5.9 in the untreated colonies to 39.2 ± 4.3 in streptomycin-treated colonies (S; independent sample *t* test, *P*<0.05). Interestingly, there was also an increase of more than 15 % in the individual drone mass with 345 ± 57 mg in the streptomycin-treated colonies compared to 293 ± 59 mg in the untreated colonies (independent sample *t* test, *P*<0.05; Figure 2.3).

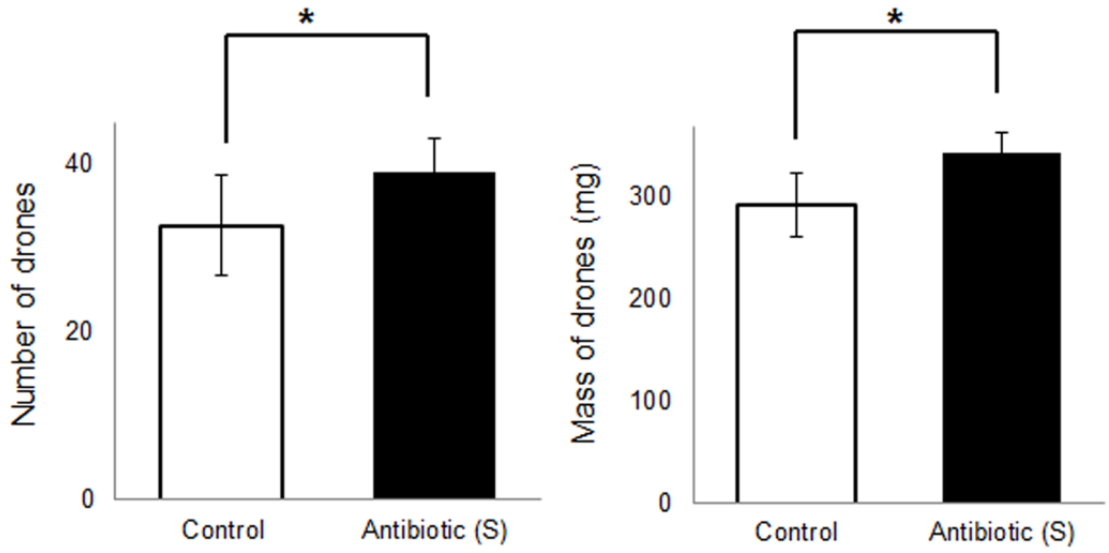

Figure 2.3. Increase in the mean drone production ± SD (*n*=10; *P*<0.05) and mean mass of drones ± SD (*n*=20; *P*<0.01) in streptomycin-treated colonies

2.5. Discussion

In order to create an altered microbiota in microcolonies we used an antibiotic treatment. Microcolonies treated with 200 ppm streptomycin performed better than the controls (Figure 2.3). Streptomycin is bactericidal mainly for gram-negative bacteria but also for some gram-positive ones, and therefore a shift in the bacterial community was to be expected. Since the bumblebee gut microbiota has already been characterized (Koch & Schmid-Hempel, 2011a), we can speculate about the effects of treatment with streptomycin. The gram-negative bacteria (i.e., Gammaproteobacteria, Betaproteobacteria and Bacteroidetes) that are normally present in the bumblebee gut will have difficulties to grow, leaving a selective advantage for the gram-positive bacteria such as Firmicutes (i.e., *Lactobacillus*) and Actinobacteria (i.e. *Bifidobacteriaceae*). Indeed, the microbial fingerprint in streptomycin-treated (S) bumblebees clusters separately from the untreated (C) ones (Figure 2.2a). The DGGE pattern of streptomycin-treated bumblebees mainly consists of *Lactobacillus* sp. However, the fact that the treatment with streptomycin resulted in unique bands, does not mean that the representative bacteria are not present in the control samples. Indeed, some queen-right colonies also harbored this specific *Lactobacillus* sp. (data not shown). We believe that the streptomycin treatment here created an environment for the *Lactobacillus* sp. to further colonize the gut, perhaps by spilling over from biofilms in the insect crop since many *Lactobacillus* species tend to reside in these environments (Vasquez et al., 2012). In order to get more insights into the colonization dynamics of *Lactobacillus* in the bumblebee gut, quantitative PCR of *Lactobacillus* sp. is needed to determine changes in bacterial load. Furthermore by fluorescence in situ hybridization with *Lactobacillus*-specific 16S rRNA probes, one can visualize the microhabitat of the targeted bacteria (Moter & Gobel, 2000).

The identified *Lactobacillus* sp., showed a 100 % max identity with *Lactobacillus* sp. previously identified in honeybees. Based on Babendreier et al. (2007) and Martinson et al. (2011), this bacterium belongs to the Firm-5 phylotype, which is reported as a phylotype specific for corbiculate bees (Martinson et al., 2011). Our specific sequence, KC477412, actually has not been detected in bumblebees before (Koch & Schmid-Hempel, 2011a; Koch

et al., 2013). The closest matches are bumblebee gut bacterial sequences AJ971929 (97 % max identity) (Mohr & Tebbe, 2007), JQ388898, and JQ388899.1 (96 % max identity). On the contrary, in honeybees, the KC477412 is quite common (Martinson et al., 2011). We speculate that *Lactobacillus* sp. (KC477412) only occurs in low numbers in wild bumblebees and it got introduced in commercial bumblebees and further established after streptomycin treatment. When scoring the fitness of the microcolonies treated with streptomycin, it was interesting that these showed an improved fitness with higher drone numbers and higher drone masses. For humans, beneficial properties of LAB have been described as ingestion of LAB has been associated with a range of health benefits, including immune system modulation and increased resistance to malignancy and infectious illness (Soccol et al., 2010). In addition, *Lactobacillus* sp. is known to produce exopolysaccharides for biofilm formation to protect against invading “negative” bacteria (Vasquez et al., 2012). Vasquez et al. (2012) speculated that the potential health benefits of exopolysaccharides in humans could also apply for bees.

In conclusion, in this chapter we altered the gut microbiota of bumblebees (*B. terrestris*) by use of antibiotics, which in turn had effects on the fitness of these bumblebee workers. We mainly explain our results through beneficial effects by *Lactobacillus* sp. but the possibility also remains that the positive fitness effects are a consequence of inhibition or elimination of “negative” bacteria. Further research on these aspects of microbiological communities in the insect gut and insect-microbe interactions may open new innovative avenues in the mass-rearing of bumblebees used for the biological pollination in agriculture.

Chapter 3.

Effect of oral administration of lactic acid bacteria on colony performance and gut microbiota in indoor-reared bumblebees (*Bombus terrestris*)

Redrafted from Billiet, A., Meeus, I., Cnockaert, M., Vandamme, P., Van Oystaeyen, A., Wäckers, F., and Smagghe, G. (2016) Effect of oral administration of lactic acid bacteria on colony performance and gut microbiota in indoor-reared bumblebees (*Bombus terrestris*). Apidologie. DOI: 10.1007/s13592-016-0447-5

3.1. Abstract

In this study we investigated if oral administration of lactic acid bacteria (LAB) could increase the colony performance of reared bumblebees. We found that a continuous administration of *Lactobacillus kunkeei* LMG 18925 and *Lactobacillus crispatus* LMG 9479 could partly compensate the effects of low nutritional pollen, but a permanent colonization in the gut was not detected with Illumina MiSeq 16S rRNA sequencing. Contrary to expectations, a single administration of *L. kunkeei* LMG 18925 in a high nutritional pollen led to a lower total drone mass. The *Bombus*-specific strain *Bifidobacterium actinocoloniiforme* R-53049 showed potential to colonize the gut permanently after three administrations. Our study represents a first screening for the potential use of probiotic strains in bumblebees. We conclude that both diet and host-specificity of bacteria might have an effect on colony performance of indoor-reared bumblebees and play a role in the gut colonization success.

3.2. Introduction

Many endogenous bacteria have a mutualistic relationship with their insect host and play a role in digestion, nutrient production and pathogen protection (Koch & Schmid-Hempel, 2012; Engel & Moran, 2013; Cariveau et al., 2014). The distinct gut microbiota of honeybees and bumblebees (Martinson et al., 2011) probably contributes to the success of the colony, as dysbiosis of the microbiota has been associated with a reduced health in bees (Sabree et al., 2012; Vasquez et al., 2012). Some of the rearing techniques used in the production of honeybees and bumblebees can have a detrimental effect on the bacterial community in the gut. For example, in the US, domesticated honeybees are often treated with antibiotics to control foulbrood infections (Tian et al., 2012), but this is also impairing the gut bacteria. The mass-rearing of bumblebees typically takes place in closed facilities to avoid disease contamination, but at the same time it can also result in a reduction of the bacterial diversity in the bumblebee gut (Meeus et al., 2015). Gaining further insight into the functionality of the bee gut microbiota holds promising opportunities to enhance the fitness of the colony. In humans and several animals and even insects, strains of *Lactobacillus* and *Bifidobacterium* are already widely used in probiotic formulations, as mentioned in chapter 1.

As several positive effects on honeybees have been demonstrated in previous experiments, we wanted to investigate the effect of the administration of *Lactobacillus* and *Bifidobacterium* strains to indoor-reared bumblebees. We used bumblebee microcolonies to assess their performance based on parameters such as colony development and drone production. We also investigated, the colonization of the supplemented bacteria, by use of Illumina MiSeq sequencing, and checked if there was an effect on the general microbial gut community. In a first experiment, we tested if a continuous supplementation of several strains of *Lactobacillus* and *Bifidobacterium* could compensate the effects of low nutritional pollen. In a second experiment we investigated if *Lactobacillus kunkeei* LMG 18925 would also be able to increase the reproduction when supplied only once and high nutritional pollen was provided. In a third experiment, we provided bumblebee microcolonies at three different points in time, with *Bifidobacterium actinocoloniiforme* R-53049, isolated from the gut of a wild bumblebee.

The objective here was to realize a better colonization, as we expected that a bumblebee-specific bacterium is better adapted to the bumblebee gut than a non-host bacterium.

3.3. Materials and methods

3.3.1. Bumblebees and their developmental parameters in microcolonies

In all bioassays, we used microcolonies consisting of 5 newly emerged *Bombus terrestris* workers. The bumblebees were obtained from an indoor mass-production facility (Biobest, Westerlo, Belgium). In each microcolony, one worker becomes dominant and starts laying unfertilized eggs that develop into drones, while the other workers take care of the brood. Several developmental parameters were assessed daily during 50 days, in 10 microcolonies for each treatment: the number of days until first egg (± day 7), first pupa (± day 21), first drone emergence (± day 33), the number of drones, the total drone mass per microcolony and the average mass per drone. The microcolonies were kept under standardized laboratory conditions at 30 °C and continuous darkness during the experiments. All microcolonies were provided with Biogluc® sugar syrup *ad libitum*. The pollen and the bacterial treatments are described below for each experiment. All pollen was 15 kGy radiation-sterilized.

3.3.2. Cultivation of bacterial strains

Lactobacillus acidophilus LMG 11430, *Lactobacillus crispatus* LMG 9479, *Lactobacillus kunkeei* LMG 18925, *Bifidobacterium asteroides* LMG 10735 and LMG 11581, *Bifidobacterium coryneforme* LMG 19811 and *Bifidobacterium actinocoloniiforme* R-53049 were cultured on selective agar plates (supplemental data Table S2). All bacterial strains were obtained from the BCCM/LMG bacterial culture collection (Belgium), except *B. actinocoloniiforme* R-53049 was isolated and identified from a bumblebee gut in the lab. All bacteria were cultured at 37 °C in anaerobic conditions, except *L. kunkeei* LMG 18925 which was aerobically cultured at 28 °C. Bacterial colonies were picked up from their agar plates 2

to 3 days after inoculation and cells were suspended into physiological saline. This bacterial suspension was then added to the pollen provided to the bumblebee microcolonies.

3.3.3. Illumina MiSeq sequencing

The composition in the gut microbiota was assessed by dissection of mid- and hindgut, using disinfected dissection material. The gut was subsequently crushed in a 170 µL lysozyme solution (100 mg/mL) and DNA-extraction was performed as described in Meeus et al. (2013). The hypervariable V4 region (254 bp) of the 16S rRNA was amplified in triplicate, using the 515F and 806R primers designed by Caporaso et al. (2011). Sample preparation and Illumina sequencing were performed as described in Billiet et al. (2015b). Sequences derived from the Illumina MiSeq sequencing were analyzed with the mothur software v. 1.31.1 (Schloss et al., 2009), mainly following the standard operating procedure available on http://www.mothur.org/wiki/MiSeq_SOP, date December 2013. The raw data are publicly available on NCBI's Sequence Read Archive (SRA) under accession number SRP065023. The analysis of the Illumina data was performed as described in Billiet et al. (2015b). The reads of the samples were calculated in percentages, expressing the relative abundance of each OTU. In this analysis we chose to retain the OTUs that were represented by more than 0.05 % of the reads. Community richness was calculated with the Chao1 estimator (alpha diversity) and community diversity with the Shannon index (beta diversity). The bacterial evenness (*e*) was calculated as $e=H/\ln S$, where H is the Shannon-index and S is the number of OTUs.

3.3.4. Experimental set-ups

3.3.4.1. Continuous supplementation of *Lactobacillus* and *Bifidobacterium* strains to low nutritional pollen

Lactobacillus and *Bifidobacterium* are well-known for their beneficial effects and several commercial probiotics containing *Lactobacillus* and *Bifidobacterium* strains had demonstrated their positive effects for instance in honeybees. For this reason we investigated the effects of 6 bacterial strains in this experiment: *L. acidophilus* LMG 11430, *L. crispatus* LMG 9479, *L. kunkeei* LMG 18925, *B. coryneforme* LMG 19811, and *B. asteroides* LMG 10735 and LMG 11581. These bacterial species were identified in the honeybee gut (Forsgren et al., 2010; Audisio et al., 2011), and have not or rarely been detected in the guts of reared bumblebees. This should give the opportunity to distinguish the administered bacteria from microbiota of untreated bumblebees.

The concentration of the bacteria in the physiological saline were measured and standardized at optical density (OD) of 1.5. The corresponding number of colony forming units (CFU) per gram pollen is specific for each treatment and is shown in Table 3.1.The strains were continuously administered in the pollen mixture which was replaced every 2 or 3 days with a freshly prepared pollen mixture. All microcolonies were fed with low nutritional pollen consisting of a honeybee-collected pollen mixture, further referred as pollen A. The pollen mixture consisted of pollen (90.32 % *w/w*), sugar syrup (6.45 % *w/w*) and one bacterial strain suspended in physiological saline (3.23 % *w/w*). The 10 microcolonies in the control group received the same pollen mixture with pollen (90.32 % *w/w*), sugar syrup (6.45 % *w/w*) and physiological saline (3.23 % *w/w*), without bacterial supplementation.

Table 3.1. The identification details, biological origin and the number of colony forming units (CFU) per gram pollen of the six bacterial strains used in the first experiment.

Bacterial species	Strain number	Biological origin	CFU per g pollen
Lactobacillus crispatus	LMG 9479*	Eye	3.23×10^3
Lactobacillus kunkeei	LMG 18925*	Partially fermented grape juice	3.55×10^4
Lactobacillus acidophilus	LMG 11430*	Human	1.00×10^5
Bifidobacterium asteroides	LMG 10735	Honeybee, hindgut	1.19×10^7
Bifidobacterium asteroides	LMG 11581	Honeybee, hindgut	1.29×10^7
Bifidobacterium coryneforme	LMG 18911	Honeybee, hindgut	5.10×10^5

* The 16S DNA sequence of this strain was 100 % identical with the 16S DNA sequence of the isolate from the honeybee gut.

Each bacterial strain was supplied to 10 microcolonies from the start of the experiment (day 0) until day 42. From day 42 to day 50, all microcolonies received pollen without bacterial supplementation. We examined the gut microbial composition of the bumblebee workers of the control treatment, *L. kunkeei* LMG 18925 treatment and *L. crispatus* LMG 9479 treatment by use of Illumina sequencing. We therefore sampled one bumblebee worker of 5 to 6 microcolonies of each of these treatments at day 44. At this point, we expected the bacterial strain to be present in the digestive tract of the bumblebees. At day 50, we sampled again one bumblebee worker of the same microcolonies of these treatments. Detection of the bacterial strain at this stage would point toward a potential colonization of the strain in the bumblebee gut.

Statistical differences in the days until first egg, first pupa and first drone were analyzed using a Kruskal-Wallis test. Statistical differences in the number of drones, the drone mass per microcolony and the mass per drone were analyzed using ANOVA with two-sided Dunnett's post hoc test. This test allows comparison of multiple treatment groups to one control group. The critical value was P=0.050.

3.3.4.2. Fitness effects of a single supplementation of *L. kunkeei* LMG 18925 to pollen types with a different nutritional value

We tested the effect of a single supplementation of *L. kunkeei* LMG 18925 to two types of high nutritional pollen, which will be further referred to as pollen B and pollen C. Hence, we created 4 groups of 10 microcolonies each: a treatment group and a control group for both pollen types.

Pollen mixtures were prepared, as described in the previous bio-assay, with 3.55×10^4 colony forming units (CFU) of *L. kunkeei* LMG 18925 per gram pollen mixture. The control treatment received the same pollen mixture, but without bacterial administration. After the first week, a freshly prepared pollen mixture was provided *ad libitum* without bacterial supplementation, and replaced weekly. Statistical differences in the days until first egg, first pupa and first drone were analyzed using a Mann-Whitney U test. Statistical differences in the number of drones, the drone mass per microcolony and the mass per drone were analyzed using a two sided t-test.

3.3.4.3. Three supplementations of bumblebee isolate *B. actinocoloniiforme* R-53049

In a third experiment, we tested the effect of administration with *B. actinocoloniiforme* R-53049. This strain was isolated from the gut of *Bombus pascuorum* (Belgium) and showed a 100 % (434/434 bp) similarity with the 16S rRNA sequence of *B. actinocoloniiforme* strain DSM 22766^T (data not shown). This bacterium was never detected in the guts of reared bumblebees. This should give the opportunity to distinguish the administered bacteria from microbiota of untreated bumblebees. The bacterium was supplemented to the food by spraying a bacterial suspension on the pollen at three points of time: on day 0, day 14 and a last time at day 28. Each microcolony was provided with 0.5 mL physiological saline (0.86 % NaCl) in which 1.76×10^9 CFU of *B. actinocoloniiforme* R-53049 was suspended. This concentration corresponded to an optical density of 2.5. In the control treatment, 0.5 mL of physiological saline was sprayed on the pollen. To ensure low nutritional conditions, the microcolonies were fed a pollen mixture consisting of 30 % pollen and 70 % of a pollen

substitute. The composition of the pollen substitute was developed by Biobest and is confidential business information.

The days until first egg, first pupa and first drone between the *B. actinocoloniiforme* R-53049 treatment and the control group were analyzed using a Mann-Whitney U test. Differences in the number of drones, drone mass per microcolony and mass per drone were analyzed using a two-sided t-test. The critical value was P=0.050.

At day 50, we sampled 6 bumblebees, originating from 3 microcolonies to investigate the gut microbiota.

3.4. Results

3.4.1. Continuous supplementation of *Lactobacillus* and *Bifidobacterium* strains to low nutritional pollen

3.4.1.1. Assessment of developmental parameters in microcolonies

In the first experiment, we used pollen A for which the control group produced an average of 18.2 ± 2.80 drones per microcolony, a total drone mass per microcolony of 5.27 ± 0.44 g and an average mass per drone of 290.2 ± 2.6 mg.

A Kruskal-Wallis test showed no significant differences between treatments in the number of days until first eggs (P=0.235), days until first pupa (P=0.870), days until first drone emergence (P=0.574). An overall ANOVA test showed no significant effects on the total drone mass per microcolony (P=0.161; F=1.603). However, significant differences could be shown for the number of drones (P=0.020; F=2.734) and drone mass per microcolony (P=0.004; F=3.707). A two-sided Dunnett's post hoc test revealed that the continuous treatment with *L. crispatus* LMG 9479 resulted in a higher number of drones per microcolony (28.1 ± 1.24 drones; P=0.056) than the control treatment, with a similar mass per drone (289.8 ± 6.9 mg; P=1.000). Compared to the control treatment, also the continuous supplementation of *L. kunkeei* LMG 18925 resulted in a higher drone production (27.1 ± 3.05

drones; *P*=0.090), with a similar mass per drone (286.0 ± 5.0 mg; *P*=0.998). The other bacterial treatments showed no significant differences when compared to the control group.

3.4.1.2. Gut microbiota in samples of treatments *L. kunkeei* LMG 18925, *L. crispatus* LMG 9479 and control

As a continuous administration of *L. kunkeei* LMG 18925 and *L. crispatus* LMG 9479 showed some positive effects on reproduction, we investigated the microbial gut composition for these 2 treatments and the control group, using Illumina MiSeq Sequencing.

Taxonomic identification of the OTUs and their closest match in GenBank or EzBioCloud are presented in Table 3.2. The genetic distance of an OTU with its closest bacterial family members is shown for *Lactobacillaceae* (Figure 3.1) and for the *Bifidobacteriaceae* (Figure 3.2), based on the 254 bp sequence.

L. kunkeei was detected in very low relative abundances in some samples of all three treatments (Figure 3.3. A, B). Therefore we cannot conclude whether the administration of *L. kunkeei* LMG 18925 contributed to colonization of the bacterium in the gut. *L. crispatus* was detected in only 1 of the 11 samples; this was in the *L. crispatus* LMG 9479 treatment in a sample taken at day 44. The administration of *L. kunkeei* LMG 18925 or *L. crispatus* LMG 9479 did not impact the overall relative abundance of *Lactobacillaceae* (*L. kunkeei* treatment: *P*=0.754; *Z*=-0.313; *L. crispatus* treatment: *P*=0.465; *Z*=-0.730) and *Bifidobacteriaceae* (*L. kunkeei* treatment: *P*=0.251; *Z*=-1.149 ; *L. crispatus* treatment: *P*=0.200; *Z*=-1.281) (Figure 3.3. C, D) and the treatments did not induce major impacts on the community richness (Chao1 estimator), the community diversity (Shannon index) nor evenness (Figure 3.3. E, F).

Table 3.2. Taxonomic identification of the OTUs and their closest match in GenBank or EzBioCloud.

Identification of OTUs **Phylum** Class *Family* *Genus*	**Matching base pairs to best match in *Bombus* or *Apis***	**Name used here**
Proteobacteria Betaproteobacteria		
Neisseriaceae *Snodgrassella*	253/253 JQ746649 *Snodgrassella alvi* strain wkB29	*Snodgrassella*
Neisseriaceae	251/253 HM215015 Uncultured Betaproteobacterium	*Neisseriaceae* sp.
Proteobacteria Gammaproteobacteria		
Orbaceae *Gilliamella*	253/253 JQ936676 *Gilliamella apicola* strain wkB30	*Gilliamella*
Orbaceae *Schmidhempelia*	253/253 HM215025 *Schmidhempelia* strain D08049A2	*Schmidhempelia*
Firmicutes Bacilli		
Lactobacillaceae *Lactobacillus*	253/253 LK054485 *Lactobacillus bombicola* LMG 28288^T	*L. bombicola*
Lactobacillaceae *Lactobacillus*	253/253 KJ078643 *Lactobacillus bombi* BTLCH M 1/2^T	*L. bombi*
Lactobacillaceae *Lactobacillus*	253/253 Y11374 *Lactobacillus kunkeei* YH-15^T	*L. kunkeei*
Lactobacillaceae *Lactobacillus*	253/253 Y17362 *Lactobacillus crispatus* DSM 20584^T	*L. crispatus*
Lactobacillaceae *Lactobacillus*	250/254 KJ078643 *Lactobacillus bombi* BTLCH M 1/2^T	*Lactobacillus sp.*
Actinobacteria Actinobacteria		
Bifidobacteriaceae	253/253 FJ858733 *Bombiscardovia coagulans* LISPASI-P3	*B. coagulans* LISPASI-P3
Bifidobacteriaceae *Bifidobacterium*	253/253 LK054489 *Bifidobacterium commune* LMG 28292^T	*B. commune*
Bifidobacteriaceae *Bifidobacterium*	252/253 AB437355 *Bifidobacterium asteroides* YIT 11866^T	*Bifidobacterium sp.*
Bifidobacteriaceae *Bifidobacterium*	253/253 JDUR01000035 *Bifidobacterium actinocoloniiforme* DSM 22766^T	*B. actinocoloniiforme*
Bacteroidetes Flavobacteria		
Flavobacteriaceae	253/253 HM215036 Uncultured Bacteroidetes	Bacteroidetes
	252/253 LN713847 *Apibacter mensalis* LMG 28357^T	

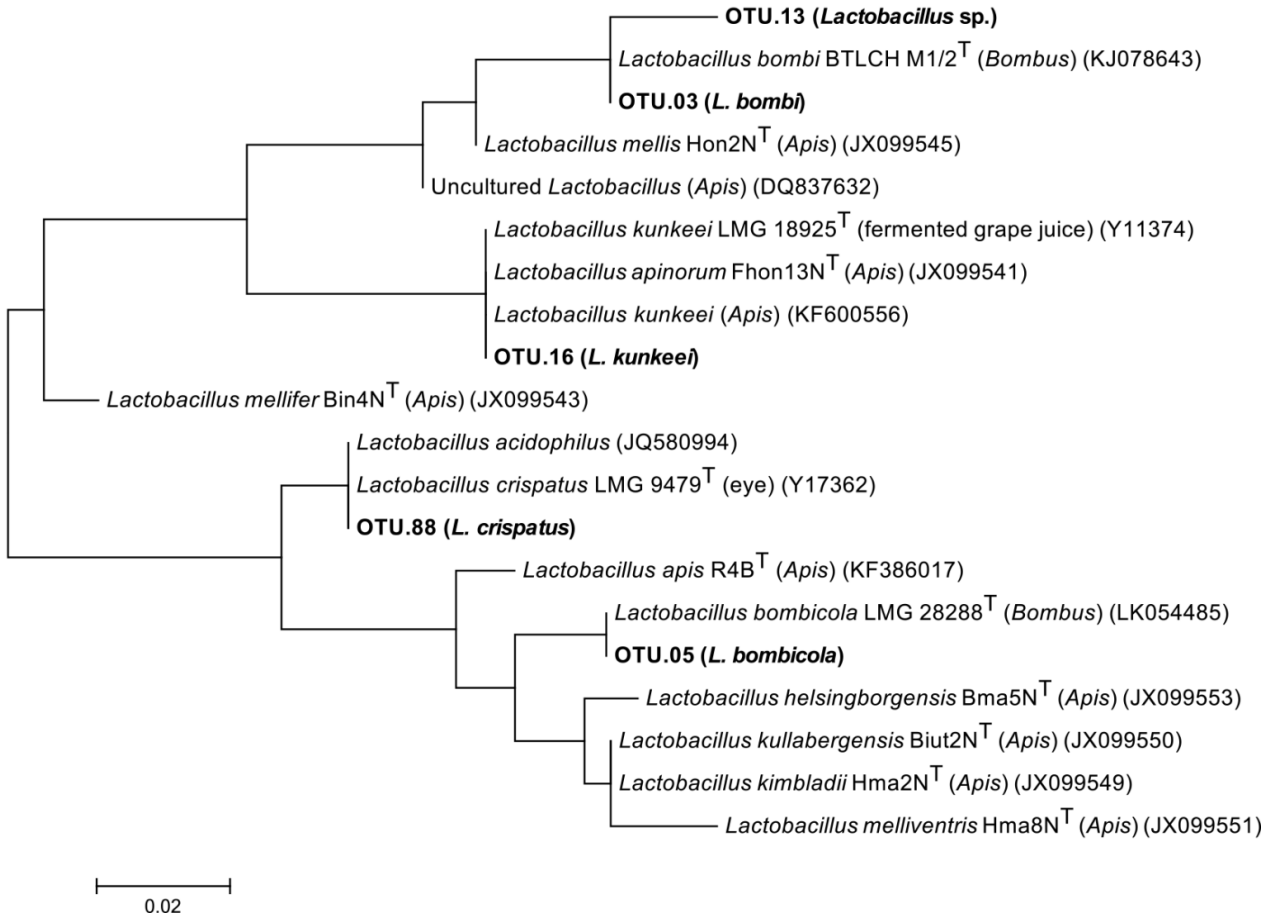

Figure 3.1. The genetic distance of the OTUs with their closest bacterial family members for *Lactobacillaceae*. Species *L. crispatus* and *L. acidophilus* cannot be distinguished, based on the 254 bp sequence. We named OTU.88 *L. crispatus*, because this OTU appeared only once in the *L. crispatus* treatment, while *L. acidophilus* was never before observed in the gut of bumblebees. Also *L. kunkeei* and *L. apinorum* cannot be distinguished. Both were originally found in the honeybee crop (Olofsson & Vasquez, 2008; Olofsson et al., 2014), but only *L. kunkeei* has before been detected in the gut of *Bombus* with culture-dependent techniques (unpublished data). We therefore assigned OTU.16 to *L. kunkeei*.

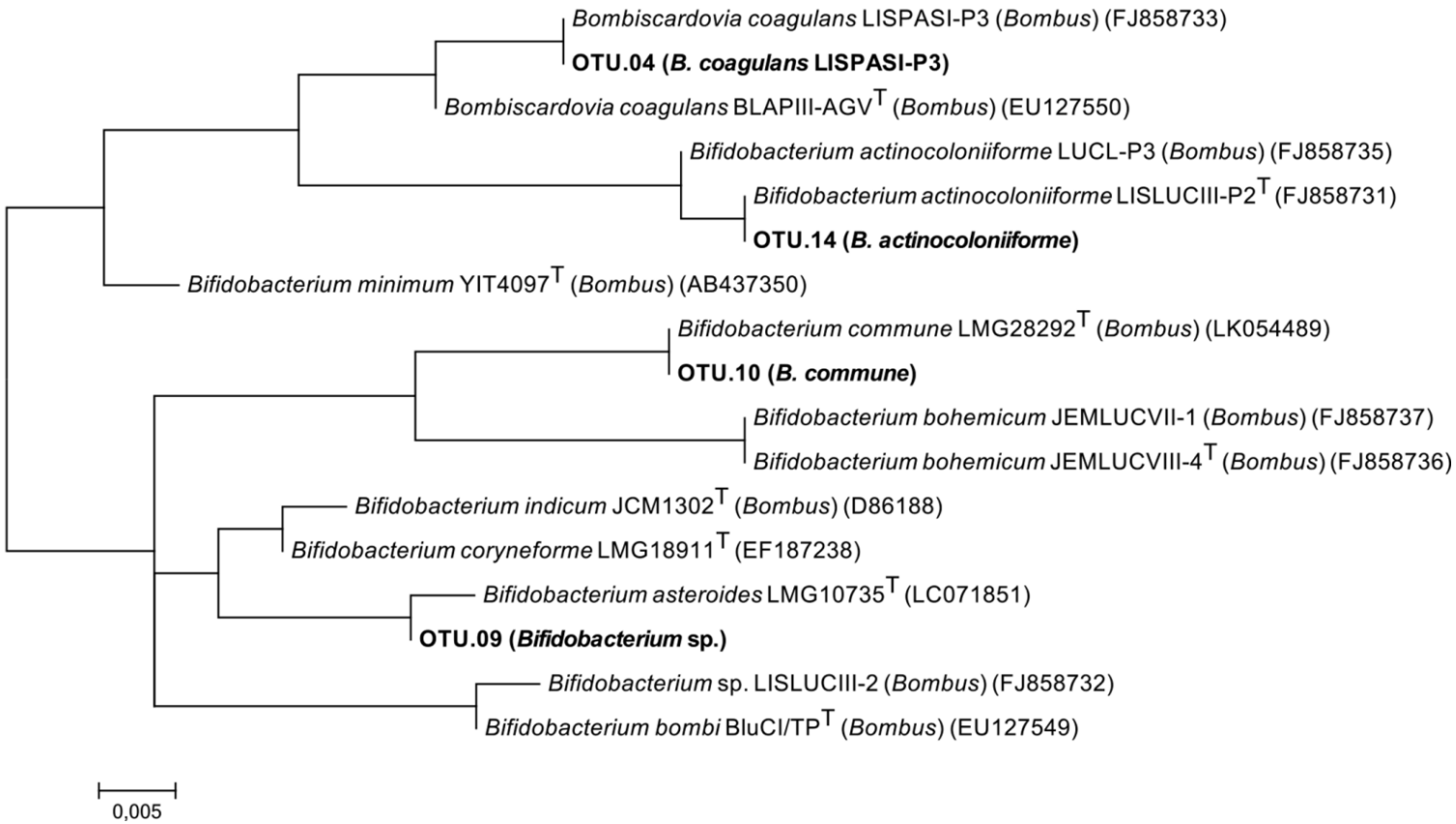

Figure 3.2. The genetic distance of the OTUs with their closest bacterial family members is shown for *Bifidobacteriaceae*, based on the 254 bp sequences.

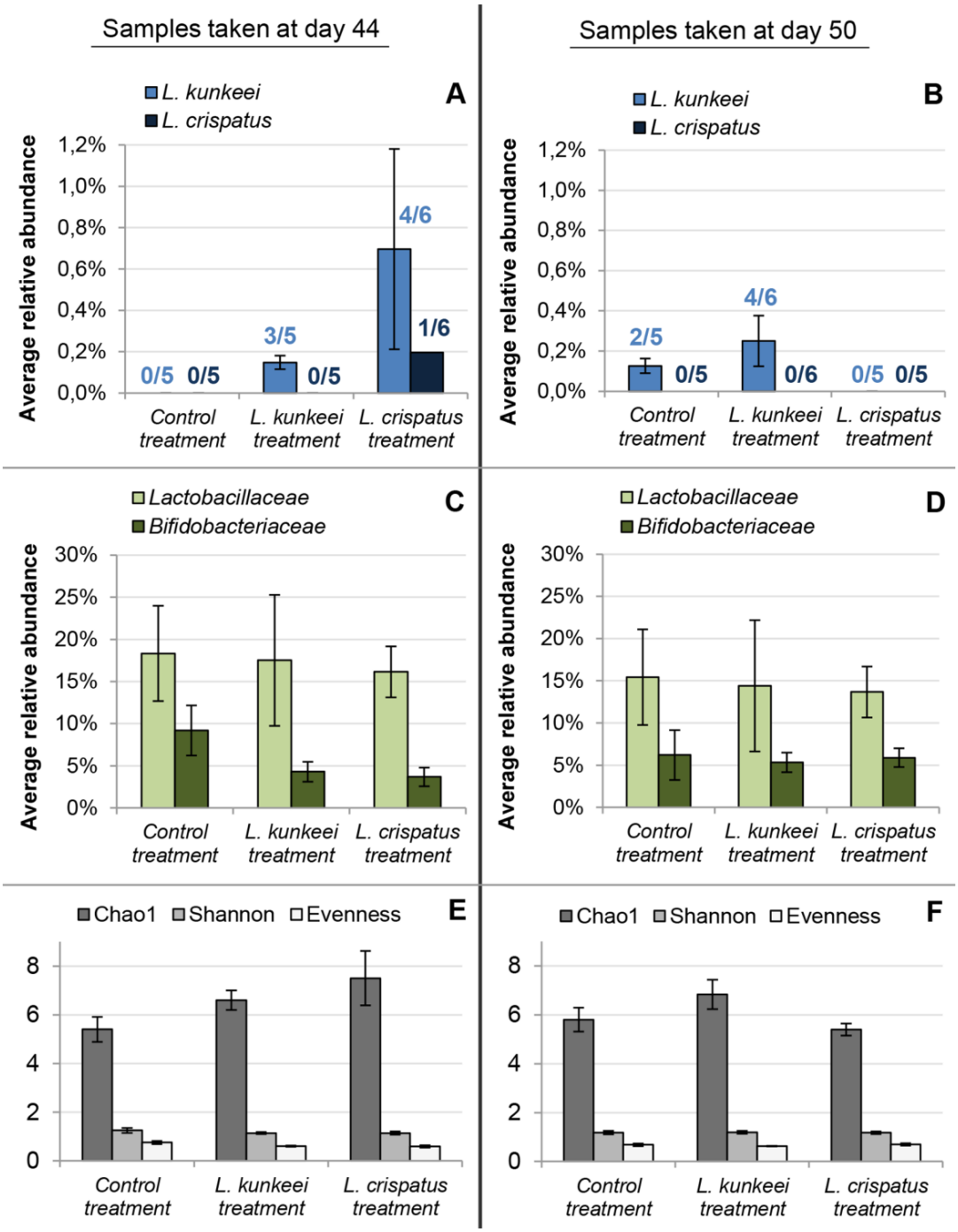

Figure 3.3. Left column (A, C, E) shows the results of the samples taken at day 44, the right column (B, D, F) represents the samples taken at day 50 of the control group, the treatments with *L. kunkeei* LMG 18925 and *L. crispatus* LMG 9479. Error bars represent the standard error. **A and B:** The charts represent the average relative abundance of *L. kunkeei* and *L. crispatus* in the samples in which the bacterium was present. The numbers above the graph represent the prevalence of this sequence in the corresponding treatment. **C and D:** The chart shows the relative abundance of all *Lactobacillaceae* and all *Bifidobacteriaceae*. **E and F:** The average community richness (Chao1 estimator), community diversity (Shannon index) and evenness for each treatment.

3.4.2. Fitness effects of a single supplementation of *L. kunkeei* LMG 18925 to pollen types with a different nutritional value

The control treatment with high nutritional pollen B produced 44.1 ± 4.17 drones per microcolony in 50 days, with a total drone mass per microcolony of 13.13 ± 0.84 g and an average mass per drone of 283.2 ± 14.8 mg. The single supplementation of *L. kunkeei* LMG 18925 to pollen B did not result in a significantly faster colony development, screened by days until first eggs (*P*=0.125; *Z*=-1.534), first pupa (*P*=0.741; *Z*=-0.331) and first drone emergence (*P*=0.600; *Z*=-0.524). Compared to the control treatment, microcolonies that were supplemented with *L. kunkeei* LMG 18925, produced fewer drones per microcolony (33.8 ± 3.04 drones; *P*=0.067) with a similar mass per drone (285.6 ± 6.9 mg; *P*=0.890), resulting in a significantly lower total drone mass per microcolony (9.54 ± 0.75 g; *P*=0.006).

The control group fed pollen C produced 27.9 ± 2.00 drones per microcolony in 50 days, a total drone mass per microcolony of 8.95 ± 0.55 g and a mass per drone of 323.8 ± 8.9 mg. The supplementation of *L. kunkeei* LMG 18925 to pollen C did not induce significant differences in days until first egg (*P*=0.503; *Z*=-0.669), days until first pupa (*P*=0.841; *Z*=-0.200) and days until first drone (*P*=0.412; *Z*=-0.820). The number of drones per microcolony was higher (32.9 ± 3.50) than in the control group, but this effect was not significant (*P*=0.234). The total drone mass was also similar (9.56 ± 1.01 g; *P*=0.603). The slightly higher number of drones, but the similar total drone mass, resulted in a significantly lower mass per drone (290.9 ± 5.3 mg; *P*=0.005), compared to the control group fed with pollen C.

3.4.3. Three supplementations of bumblebee isolate *B. actinocoloniiforme* R-53049

3.4.3.1. Assessment of developmental parameters in microcolonies

The control group produced an average number of drones of 17.7 ± 1.08 with an average drone mass of 323.5 ± 9.7 mg. Supplementation of *B. actinocoloniiforme* R-53049 to the pollen showed a faster development in days until first egg (P=0.029; Z=-2.189) and a slightly faster development in days until first pupa (P=0.067; Z=-1.831), compared to the control treatment. There was, however, no significant effect on time of first drone emergence (P=0.914; Z=-0.108) and it also did not impact the number of drones (18.7 ± 2.28 drones; P=0.696) nor the mass per drone (325.6 ± 4.0 mg; P=0.843).

3.4.3.2. Gut microbial composition

B. actinocoloniiforme was never detected in the samples of the control group (0.00 ± 0.00 %), while it was present in 4 out of 6 sampled bumblebees of the treatment group. The samples that were positive for *B. actinocoloniiforme* had an average relative abundance of 2.09 ± 1.49 %. The overall relative abundances of *Lactobacillaceae* (*L. bombi*, *L. bombicola*, *L. apis*, *L. kunkeei*) or *Bifidobacteriaceae* (*B. commune, B. coagulans*) were not significantly influenced by the *B. actinocoloniiforme* R-53049 treatment. No major changes were observed in the community richness, community diversity and evenness.

3.5. Discussion

In this chapter, it was demonstrated that a continuous administration of *L. kunkeei* LMG 18925 and *L. crispatus* LMG 9479 can positively affect the drone production, when providing low nutritional pollen. It remains unclear which mechanisms are responsible for the increased drone production. Possibly, the supplemented bacterial strains were able to aid in nutrient production. It seems likely that certain bacterial products can benefit bee health, as it was recently demonstrated that metabolites, such as lactic acid, phenyl-lactic acid and acetic acid, produced by *Lactobacillus johnsonii* CRL 1647 can increase the honeybee colony fitness (Maggi et al., 2013). Besides nutrient production, bacteria can also help in the digestion of the pollen grain. A thick pollen wall surrounds the inner nutrient-rich protoplasm (Roulston & Cane, 2000). Bacteria can produce digestive enzymes softening the pollen wall and helping to release the nutrients from the pollen grain (Engel et al., 2012), and this way they are able to release more proteins needed for reproduction and ovary development (Hoover et al., 2006). We could, however, not demonstrate a similar positive effect when *L. kunkeei* LMG 18925 was administered only once to high nutritional pollen B, as it even led to a decrease in drone production. A possible explanation for these findings could be that the effect of microorganisms on their host could be dependent on environmental variables, such as dietary composition. In the case of nutritionally optimal pollen, bumblebees might not need supplementary nutrients provided by the bacteria nor their help in digestion and *L. kunkeei* LMG 18925 might consume more nutrients from the pollen than it produces for the host. Only for the digestion of low nutritional pollen, which could be either difficult to digest or lacking certain nutrients, bumblebees seemed to benefit from the bacterial administration. The principle of context dependent benefits of bacteria has been described in aphids: when a parasitic wasp deposits an egg inside its aphid host, the developing parasitoid wasp larva ultimately kills the host (Oliver et al., 2003; Oliver et al., 2008). The secondary symbiont *Hamiltonella defensa* is able to inhibit the parasitoid development and protects the aphid. Although the aphid does not get killed and thus benefits from the bacterial presence, the protection of *H. defensa* is not free of costs to the aphid, as *H. defensa* consumes many

nutrients from the aphid. Aphids carrying *H. defensa* have reduced fecundity, and are outcompeted by aphids that do not carry the costly secondary symbiont in environments where no parasitoids are present (Vorburger & Gouskov, 2011). Thus, in optimal conditions, the bacterium has a negative effect on the host, while in unfavorable conditions, it is beneficial for the host.

Even though we found positive effects in the continuous administration of *L. kunkeei* LMG 18925 and *L. crispatus* LMG 9479, we could not confirm their presence in the gut. For the microbial analysis, we only sampled the mid- and hindgut of adult workers. It remains possible that these bacteria did colonize other parts of the digestive tract, such as the crop. In honeybees, it has been shown that the crop harbors a rich diversity of LAB which are tightly attached to the crop wall in a biofilm layer (Olofsson & Vasquez, 2008; Olofsson et al., 2014). For future investigations, we propose to investigate the crop, midgut and hindgut separately, as some bacteria might be restricted to a certain region in the digestive tract.

Host-specificity might also play a role in the colonization success of a bacterium. Earlier studies in *Apis* and *Bombus* demonstrated that native strains of *Snodgrassella* showed higher levels of colonization, than non-host *Snodgrassella* strains (Kwong et al., 2014), which points toward a certain degree of host-specificity between microbes and their host. Most of the bacteria administered in our experiments were not *Bombus* specific, with the exception of *B. actinocoloniiforme* R-53049 which was isolated from the gut of a wild *Bombus pascuorum* and which was previously also found in the guts of wild *B. terrestris* (Meeus et al., 2015). Our experiments confirmed that *B. actinocoloniiforme* was not present in the gut of the reared bumblebees of the control treatment, but three administrations of this bacterium seemed sufficient to colonize the gut of the majority of the sampled bumblebees. Regarding administration frequency, possibly a few administrations will be sufficient to colonize the gut when applying a host-specific bacterium.

Although *B. actinocoloniiforme* R-53049 was not able to improve microcolony performance, it is still worth to look further into the effects of the gut bacteria and expand the parameters that we assessed. One interesting parameter would be immunity. In our experiments, all colonies were kept under optimal conditions and did not come into contact with potential pathogens. In previous studies with honeybees, beneficial effects by the presence of certain LAB on immunity could be demonstrated (Koch & Schmid-Hempel, 2011b). When bumblebees are placed outside in the field and encounter bee diseases from wild bees, the reared bumblebees should be as immunocompetent as possible and the introduction of host specific bacteria might be helpful.

We can conclude that our study represents a first screening of the potential of probiotic strains in bumblebees and the possible effects for bumblebee rearing. We identified two bacteria, *L. kunkeei* LMG 18925 and *L. crispatus* LMG 9479, showing potential to improve colony performance, but we could not demonstrate their ability to colonize the gut. Further research is needed to identify the underlying mechanisms of their beneficial effect. We also demonstrated that *B. actinocoloniiforme* R-53049 could effectively colonize the bumblebee gut, but this bacterium did not improve colony performance under laboratory conditions. Further research could expand this study to field conditions to assess possible effects on immunity. At this point, there is still not enough knowledge on the functions of the bacteria in the bumblebee gut. In order to develop probiotics for bumblebees, more research should be done with other bacterial species, the combination of several bacteria or prebiotics, and the application methods like the administration frequency.

Chapter 4.

Impact of sugar syrup and pollen diet on the bacterial diversity in the gut of indoor-reared bumblebees (*Bombus terrestris*)

Redrafted from: Billiet, A., Meeus, I., Van Nieuwerburgh, F., Deforce, D., Wäckers, F., Smagghe, G. (2015) Impact of sugar syrup and pollen diet on the bacterial diversity in the gut of indoor-reared bumblebees (*Bombus terrestris*). Apidologie. DOI: 10.1007/s13592-015-0399-1

4.1. Abstract

In this chapter we showed the influence of diet on the microbial composition in the gut of indoor-reared bumblebees (*Bombus terrestris*), using Illumina MiSeq 16S rRNA sequencing. Three sugar syrups and sterilized pollen from three suppliers were tested. Different effects were observed depending on the developmental stage of the gut microbiota at the start of the dietary treatment. Fructose-rich sugar syrup prevented the colonization of *Bifidobacteriaceae* in the microbiota of newly emerged bumblebees. This effect was correlated with a lower bacterial community richness and diversity. The pollen diet with the best parameters, regarding the protein content and bumblebee offspring, showed the lowest bacterial richness and diversity. The interaction between diet and the microbiota of bumblebees provides new insights for bumblebee breeders. Diet could be used to modulate the bacterial composition in the gut to improve the health of mass-produced bumblebees used for biological pollination.

4.2. Introduction

Diet has proven to play an important role in modulating the gut microbiota of mice (Murphy et al., 2010) and humans, over other possible variables such as ethnicity, sanitation, hygiene, geography or climate (De Filippo et al., 2010). The bacterial colonization dynamics in the gut of insects tend to be different as their guts often present unstable habitats for bacteria (Engel & Moran, 2013). Holometabolous insects molt several times during their life. The radical remodeling of the organs during metamorphosis severely disrupts or eliminates attached bacterial populations (Moll et al., 2001). Opportunities for direct transfer of gut symbionts between conspecifics are also rather limited, as in most insects females abandon their eggs after oviposition. Hence in many insects, gut bacterial communities vary among individuals within a species and are highly influenced by the environment and diet (Engel & Moran, 2013). As discussed in chapter 1, this does not apply to honeybees and bumblebees as they show social behavior, which enables direct or indirect transmission of microbiota between and within generations, resulting in a more stable gut microbiota (Koch & Schmid-Hempel, 2011a; Martinson et al., 2011). Bacterial gut symbionts in bees have been predicted to play a role in digestion (Engel et al., 2012; Lee et al., 2015) and contribute in parasite defense (Koch & Schmid-Hempel, 2012; Cariveau et al., 2014), and thus, knowledge about the bacterial composition and factors that can alter the bacterial composition are important for rearing facilities. The impact of diet on the gut microbiota of bumblebees is not known yet and possibly this could be an interesting means for a commercial breeder to shift the microbial composition of the bumblebee gut.

The diet of bumblebees consists of pollen and nectar. Pollen is the main source of protein naturally exploited by bees. The protein content ranges between 2.5 and 61 % of the dry mass of pollen (Roulston & Cane, 2000). It also provides important nutrients such as lipids, minerals, sterols, and vitamins (Stanley & Linskens, 1974; Roulston & Cane, 2000). In rearing facilities, bumblebee colonies are kept indoors and are usually provided with a mix of honeybee-collected pollen (Velthuis & van Doorn, 2006). In nature, the principal carbohydrate source for bees is nectar collected from flowers (Goulson, 2010). Nectar is

dominated by three sugars: glucose, fructose and sucrose (Percival, 1961; Bernardello et al., 2007). The amount and relative concentration of the main sugars vary among plant species (Percival, 1961). In rearing facilities, the nectar is replaced by a sugar syrup (Velthuis & van Doorn, 2006).

In this chapter, we investigated the impact of diet on the microbial composition of indoor-reared *B. terrestris*, using MiSeq multiplexed 16S ribosomal RNA (rRNA) amplicon sequencing. We studied the effect of diet on the gut microbial composition with different kinds of pollen and sugar diets. We fed different diets to newly emerged bumblebees and bumblebees of 4 days old. This setup allowed us to differentiate between the effect of diet on an undeveloped gut microbiota and an already more established microbiota.

4.3. Materials and methods

4.3.1. Diet

The effect of diet was tested using combinations of different pollen diets and sugar syrups. Three pollen diets from different suppliers (further referred to as pollen A, pollen B and pollen C) were tested. They are all polyfloral, collected by honeybees, and as a standard procedure, sterilized by 15 kGy radiation. The three pollen diets were previously tested for their impact on the number of progeny in bumblebee colonies as shown in Table 4.1. Pollen A had the least favorable properties, while pollen C proved to have the best properties regarding protein content and drone production. The other pollen components such as lipids, vitamins, minerals, … were not determined. We also prepared three kinds of sugar syrup (50 % *w/w*): fructose-rich sugar syrup, sucrose-rich sugar syrup, and Biogluc sugar solution (Biobest), each with its own composition as presented in Table 4.2. The effect of sugar syrup was studied by providing bumblebees with the least favorable pollen diet (pollen A) in combination with Biogluc sugar syrup (pollenA+Biogluc), sucrose-rich sugar syrup (pollenA+sucrose) or fructose-rich sugar syrup (pollenA+fructose). The impact of pollen diet was studied by providing Biogluc sugar syrup, the standard sugar syrup used in rearing

facilities, in combination with pollen A (pollenA+Biogluc), pollen B (pollenB+Biogluc) or pollen C (pollenC+Biogluc).

Table 4.1. Bumblebees were fed three different pollen diets ranging from low pollen quality (pollen A), good pollen quality (pollen B), to very good pollen quality (pollen C), expressed in protein content per dry mass, the average drone production in microcolonies during 50 days and the average drone weight. This data was obtained from Biobest NV.

	Protein content per dry mass (%)	Average drone production in micro-colonies in 50 days	Average drone weight (g)
Pollen A	15.00 ± 0.00	31.50 ± 1.67 *	0.3300 ± 0.0117
Pollen B	15.81 ± 0.23	47.41 ± 1.59 *	0.2608 ± 0.0038 °
Pollen C	23.60 ± 1.19	55.52 ± 1.76 *	0.3179 ± 0.0071

* Drone production during 50 days was significantly different ($p<0.050$) between the three pollen diets.

° Drones weighed significantly less when fed pollen B. There was no significant difference between the drone weight of pollen A and pollen C.

Table 4.2. Bumblebees were fed three kinds of sugar syrup (50 % w/w): fructose-rich sugar syrup, sucrose-rich sugar syrup and commercial Biogluc sugar syrup. The sugar composition of each syrup is presented.

	Sucrose (%)	Fructose (%)	Dextrose (%)	Maltose (%)	Higher sugars (%)	Preservatives (%)
Fructose rich	25.00	56.25	18.75	0.00	0.00	0.00
Biogluc	25.00	37.50	34.50	2.00	1.00	0.05
Sucrose rich	75.00	18.75	6.25	0.00	0.00	0.00

4.3.2. Bumblebees

The experiment was performed with bumblebees of *B. terrestris* from a continuous mass-rearing program (Biobest, Westerlo, Belgium). The bumblebees were kept under standardized laboratory conditions at 30 °C and continuous darkness (Mommaerts et al., 2006). All bumblebees used in the following experiments originated from the same queen-right colony, ensuring little variation in microbial composition between individuals (Meeus et al., 2015). This queen-right colony had been fed *ad libitum* on pollen B and Biogluc sugar syrup.

4.3.3. Experimental set-up

Newly emerged bumblebees, which just completed their metamorphosis, show hardly any microbiota in their gut, and their bacterial gut community develops in the first 4 days after eclosion (Chapter 2). Between day 4 and 35, their bacterial composition and diversity are stable under controlled conditions (Parmentier et al., 2015b). To investigate the effect of diet in both developmental stages of the gut microbiota, we set up two groups.

The first group consisted of newly emerged adult bumblebees of less than 1 day old. They were transferred from the queen-right colony into five microcolony boxes with four bumblebees each. We will further use the term “undeveloped microbiota” to describe this group of bumblebees, referring to the condition of their gut microbiota when they started the dietary treatment.

In the second group we labeled newly emerged adult bumblebees and kept them the first 4 days of their adult life in their queen-right colony fed on pollen B and Biogluc sugar syrup. Spending time in the colony allowed these bumblebees to colonize their gut with a set of bacterial species in accordance with the microbiota of their queen-right colony. At 4-5 days old, the bumblebees were transferred into five microcolonies with four bumblebees each. We will further use the term “established microbiota” to describe this group of bumblebees, referring to the condition of their gut microbiota when they started the dietary treatment.

In both groups, the bumblebees were provided one of the five diets *ad libitum* during the following 16 days, after which they were sampled.

4.3.4. Sample preparation and Illumina sequencing

Mid- and hindgut were dissected from bumblebees using disinfected dissection material and stored at -20 °C. The gut was crushed in a 170 µL lysozyme solution (100 mg/mL) and DNA-extraction was performed as described in Meeus et al. (2013). The hypervariable V4 region (254 bp) of the 16S rRNA was amplified in triplicate, using the 515F and 806R primers designed by Caporaso et al. (2011). The 806R primer was barcoded with a different nucleotide for each sample, and both primers contained Illumina adapter sequences necessary for the bridge amplification on the Illumina MiSeq flow cell (Caporaso et al., 2011). Further sample preparation and Illumina sequencing were performed as described in Meeus et al. (2015).

4.3.5. Data analysis

Sequences derived from Illumina MiSeq were analyzed with the mothur software v. 1.31.1 (Schloss et al., 2009), mainly following the standard operating procedure available on http://www.mothur.org/wiki/MiSeq_SOP, date February 2014. The raw data are publicly available on NCBI's Sequence Read Archive (SRA) under accession number SRP059174. We followed standard operating procedures to optimize the quality of our dataset. At this point the dataset contained a total of 2,994,069 demultiplexed paired-end reads, representing 311,396 unique sequences. We further removed sequences that contained more than 8 homopolymers, sequences that were not complete, chimeras, sequences that only occurred once ("singletons") and sequences that were not correlated with a bacterial taxonomy (e.g., chloroplasts, mitochondria, *Archaea* or *Eukarya*). This resulted in a total of 2,730,002 reads whereof 5,855 unique sequences. The previous steps clearly retained the majority of the reads (91.2 %). Calculating the distance matrix and clustering with a 0.03 cutoff level on this dataset resulted in 230 operational taxonomic units (OTU). The taxonomic

identity of each OTU was revealed by alignment of each sequence with the Bacterial SILVA SEED database, supplemented with host specific sequences (i.e., host *Apis* or *Bombus*) to improve classification (Newton & Roeselers, 2012b). In this analysis, we chose to only retain the OTUs that were represented by more than 0.5 % of the reads per sample as we aimed to focus on the core bacteria in the bumblebee gut. This resulted in seven OTUs covering 99.7 % of the reads of the 230 OTUs. The reads of the samples were calculated in percentages, expressing the relative abundance of each OTU. Community richness was calculated using the Chao1-estimator (alpha diversity) and community diversity was calculated with the Shannon-index (beta diversity).

4.3.6. Statistical analysis

A two-way ANOVA was used to assess the impact of diet (eight samples per diet) and the interaction between diet and the developmental stage of the gut microbiota. The statistical differences in relative abundance of OTUs between diets within each group of bumblebees (four samples per diet) were analyzed using non-parametric overall and pairwise Kruskal-Wallis tests. The values are reported throughout the study as the means ± standard error (SE). Differences were deemed significant at $P<0.050$.

4.4. Results

4.4.1. Identified OTUs

The seven OTUs mainly represented the four core bacterial families in *B. terrestris*: *Neisseriaceae* (*Snodgrassella*), *Orbaceae* (*Gilliamella*), *Lactobacillaceae* (*Lactobacillus bombi* and *Lactobacillus bombicola*) and *Bifidobacteriaceae* (*Bombiscardovia coagulans* LISPASI-P3 and *Bifidobacterium commune*). We also found Firmicutes *Bacillaceae* in nine samples, in very low relative abundances. Taxonomic identification of the OTUs and their closest match in GenBank or EzBioCloud are presented in Table 4.3. The genetic distance of

an OTU with its closest bacterial family members is shown for *Bifidobacteriaceae* (Figure 4.1) and for the *Lactobacillaceae* and *Bacillaceae* (Figure 4.2).

4.4.2. Impact of diet on the gut microbial composition of *B. terrestris*

Diet had a significant impact on both the community richness (P<0.001; F=17.716) and the community diversity (P=0.001; F=6.093) when taking all eight samples per diet in consideration. We also observed a significant interaction between the diet and the developmental stage of the microbial gut community for community richness (P<0.001; F=7.525) and a trend toward this interaction for community diversity (P=0.075; F=2.393). As a result of this interaction, effects of diet will be considered within each group of bumblebees.

A Kruskal-Wallis test on the first group (undeveloped microbiota) revealed significant differences between diets in community richness (P=0.005) and community diversity (P=0.039), a consequence of significant differences in the relative abundances of *L. bombicola* (P=0.014), *L. bombi* (P=0.037), *B. coagulans* LISPASI-P3 (P=0.033), *B. commune* (P=0.005) between diets. As a result of these differences, the total relative abundances of *Lactobacillaceae* (P=0.033) and *Bifidobacteriaceae* (P=0.022) were significantly different between diets.

In the second group (established microbiota), we observed significant effects of diets on the community richness (P=0.008) and community diversity (P=0.016) as a result of differences in the relative abundance of *L. bombi* (P=0.049) and *L. bombicola* (P=0.005) and the overall relative abundance of *Lactobacillaceae* (P=0.044). The relative abundance of *B. coagulans* LISPASI-P3 and *B. commune* was not significantly altered by diet in this group. One bumblebee died in the diet pollenB+Biogluc, leaving three samples in this diet group.

We will further analyze the specific impact of the sugar syrup and also the effect of the pollen diet on the microbial composition in the gut of bumblebees.

Table 4.3. Taxonomic identification of OTUs in reared bumblebees and their closest match in GenBank and EzBioCloud.

Identification of OTUs **Phylum** Class *Family Genus*	**Matching base pairs to best match in *Bombus* or *Apis***	**Name used here**
Proteobacteria Betaproteobacteria *Neisseriaceae Snodgrassella*	253/253 JQ746649 *Snodgrassella alvi* strain wkB29	*Snodgrassella*
Proteobacteria Gammaproteobacteria *Orbaceae Gilliamella*	253/253 JQ936676 *Gilliamella apicola* strain wkB30	*Gilliamella*
Firmicutes Bacilli *Lactobacillaceae Lactobacillus*	253/253 LK054485 *Lactobacillus bombicola* LMG 28288^{T} 253/253 Lacto1-Firm5 (Meeus et al., 2015)	*L. bombicola*
Firmicutes Bacilli *Lactobacillaceae Lactobacillus*	253/253 KJ078643 *Lactobacillus bombi* BTLCH M 1/2^{T} 253/253 Lacto2-Firm4 (Meeus et al., 2015)	*L. bombi*
Firmicutes Bacilli *Bacillaceae*	252/253 AJ971876 Uncultured *Bacillus* sp. Bt27	*Bacillaceae*
Actinobacteria Actinobacteria *Bifidobacteriaceae*	253/253 FJ858733 *Bombiscardovia coagulans* LISPASI-P3 253/253 Bifido3 (Meeus et al., 2015)	*Bombiscardovia coagulans* LISPASI-P3
Actinobacteria Actinobacteria *Bifidobacteriaceae Bifidobacterium*	253/253 LK054489 *Bifidobacterium commune* LMG 28292^{T} 253/253 BifidoX (Meeus et al., 2015)	*B. commune*

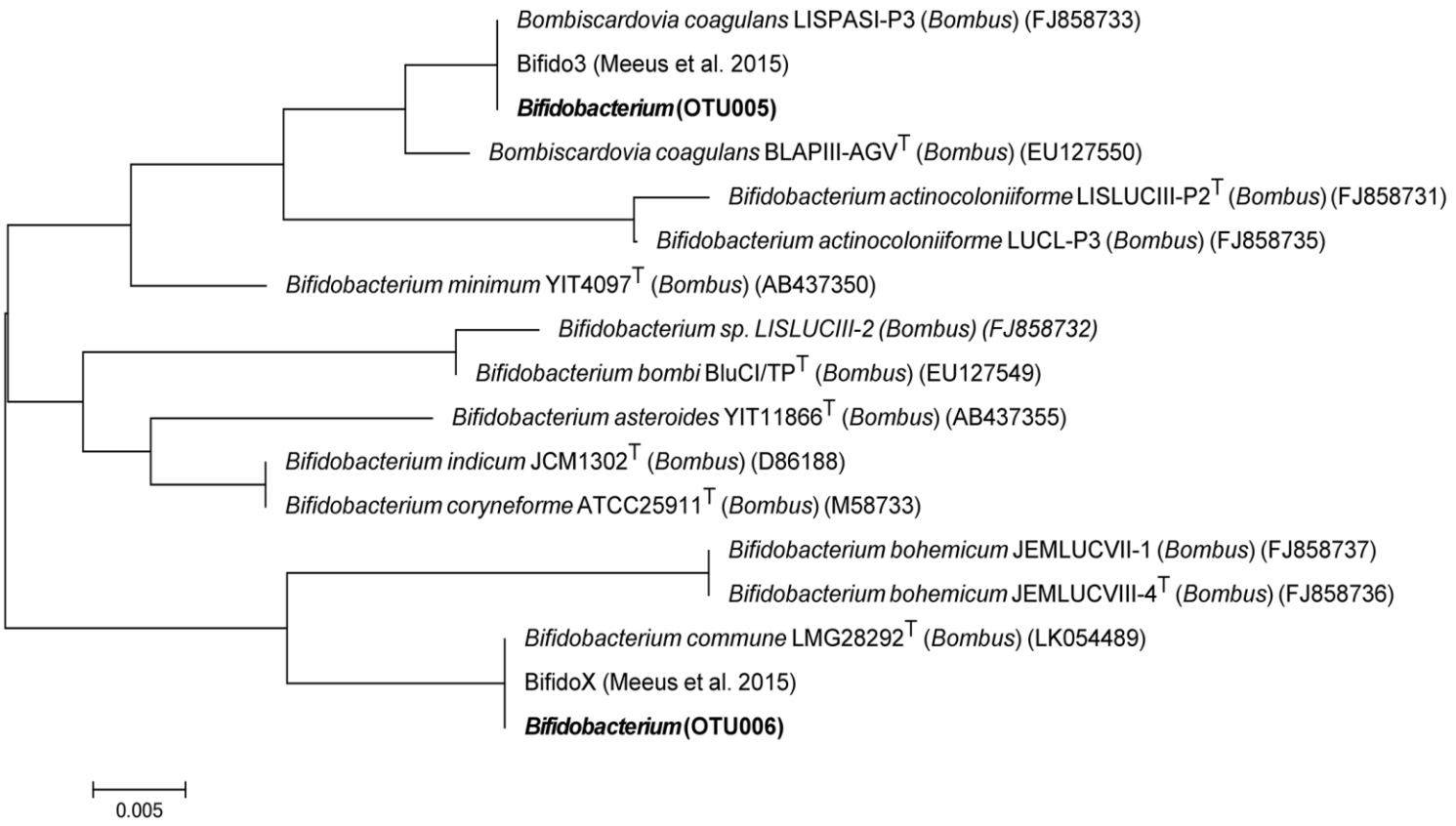

Figure 4.1. Phylogenetic tree derived from the V4 region of the 16S rRNA gene sequence of *Bifidobacteriaceae* occurring in the digestive tract of *Bombus*, showing the position of the *Bifidobacteriaceae* (OTU005 and OTU006) found in our study.

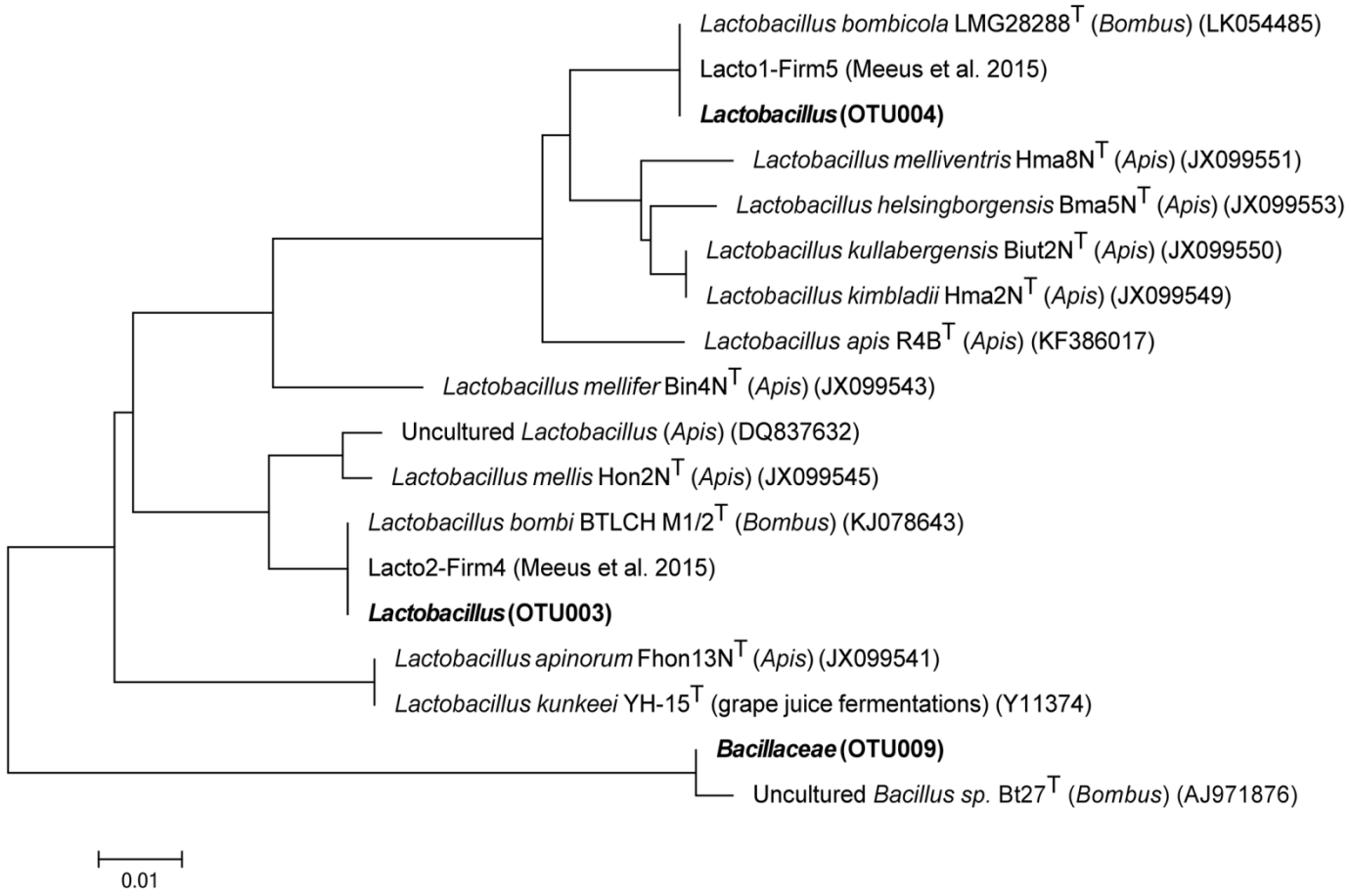

Figure 4.2. Phylogenetic tree derived from the V4 region of the 16S rRNA gene sequence of *Lactobacillaceae* and *Bacillaceae* occurring in the digestive tract of *Bombus* and *Apis*, showing the position of the *Lactobacilli* (OTU003 and OTU004) and *Bacillaceae* (OTU009) found in our study.

4.4.3. Impact of sugar syrup

We studied the effect of the sugar syrup using diets pollenA+Biogluc, pollenA+sucrose and pollenA+fructose. Bumblebees of the group with an undeveloped microbiota (Figure 4.3) showed a significant lower community richness when fed a fructose-rich sugar syrup (Chao1 = 3.75 ± 0.48) compared to the sucrose-rich sugar syrup (Chao1 = 6.00 ± 0.00; *P*=0.013). This is an immediate consequence of the complete lack of *Bifidobacteriaceae* (*B. coagulans* LISPASI-P3 and *B. commune*) in individuals that consumed pollenA+fructose. A pairwise Kruskal-Wallis tests revealed that pollenA+fructose resulted in a significant lower relative abundance of *Bifidobacteriaceae* compared to bumblebees fed with pollenA+Biogluc (*P*=0.048).

The bacterial composition in the gut of bumblebees in the group with an established microbiota (Figure 4.4) showed no significant differences in community richness, diversity or any bacteria between the three sugar syrups.

4.4.4. Impact of pollen diet

The impact of pollen diet on the gut microbiota of *B. terrestris* was studied using pollenA+Biogluc, pollenB+Biogluc, and pollenC+Biogluc as diets. Bumblebees from the group with an undeveloped microbiota showed the lowest community richness and community diversity for pollen C; however, there were no significant differences compared to other pollen diets (Figure 4.5). In bumblebees with an established microbiota, pollen C led to a complete lack of both *L. bombicola* and *L. bombi*. This resulted in a significantly lower Chao1 estimator (3.75 ± 0.25; *P*=0.022) and Shannon index (0.81 ± 0.05; *P*=0.017) compared to the pollenA+Biogluc diet (Chao1 = 6.75 ± 0.25; Shannon = 1.24 ± 0.07). The relative abundance of *L. bombi* was significantly higher (*P*=0.037) with the pollen A diet (12.75 ± 5.98 %) than with pollenC+Biogluc (0.00 ± 0.00 %) (Figure 4.6).

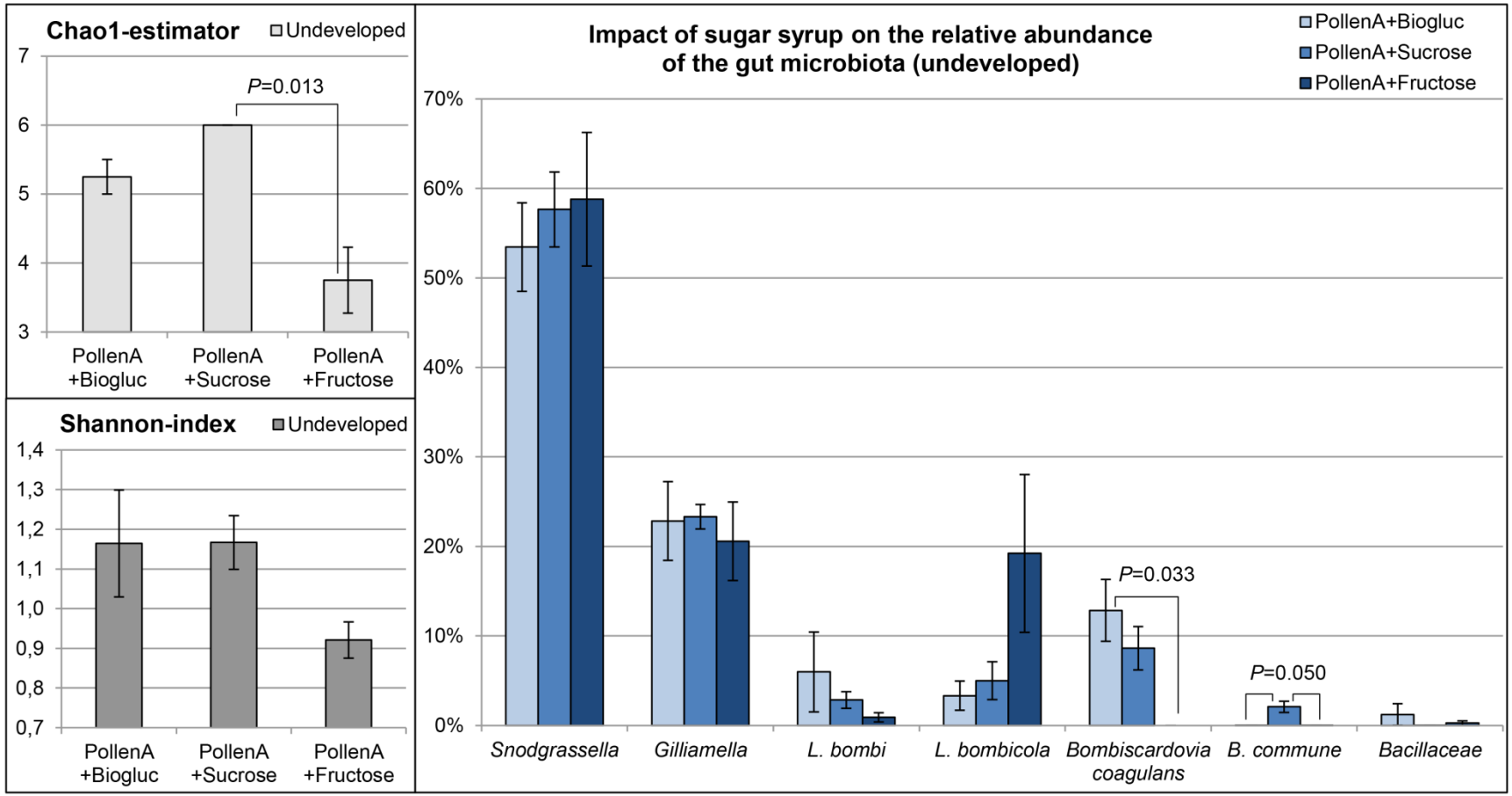

Figure 4.3. The impact of sugar syrup (Biogluc, sucrose-rich or fructose-rich) on the community richness (Chao1-estimator), community diversity (Shannon-index) and the relative abundance of each OTU for bumblebees that received the specific diet when their gut microbiota was still undeveloped.

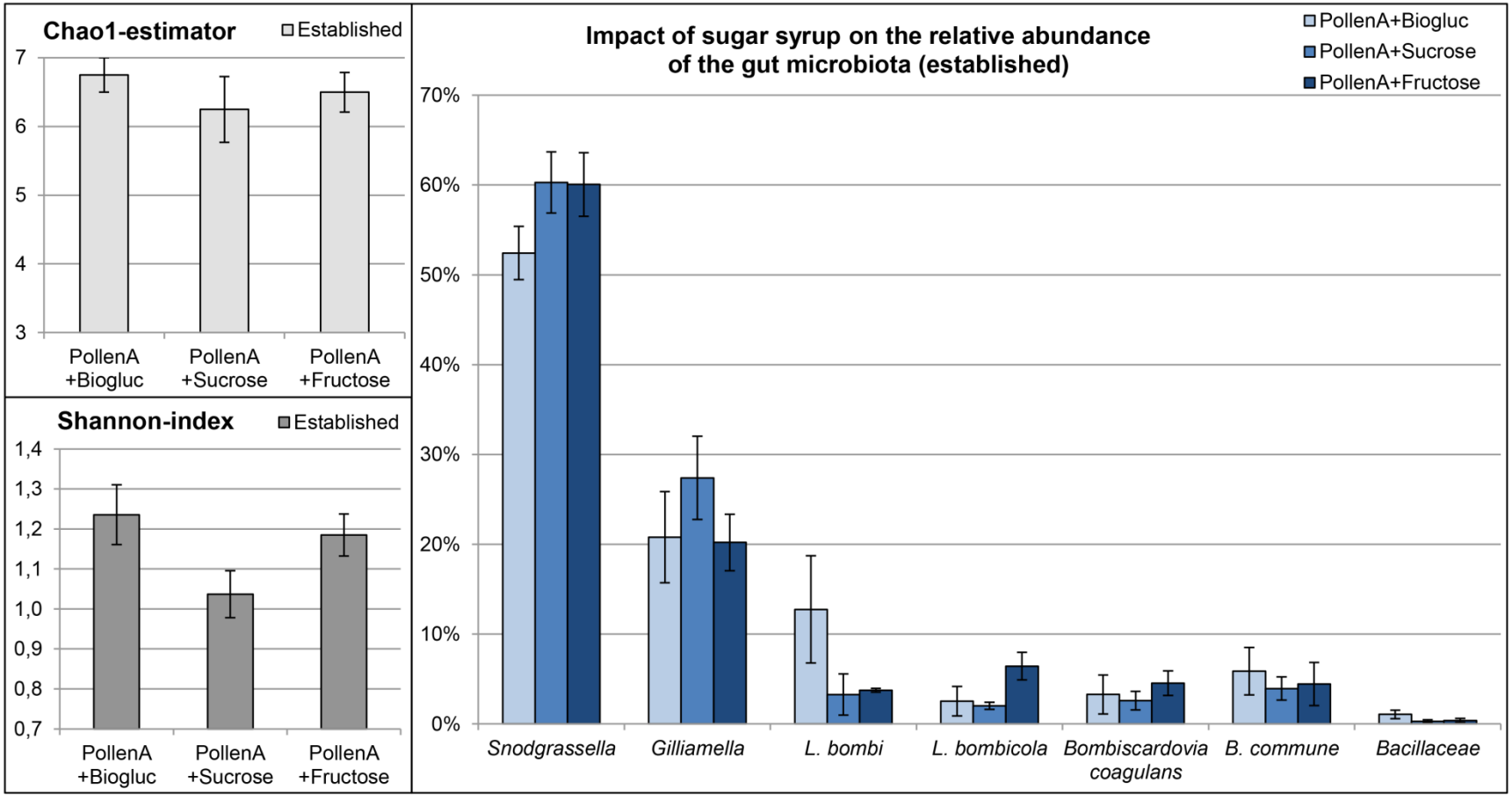

Figure 4.4. The impact of sugar syrup (Biogluc, sucrose-rich or fructose-rich) on the community richness (Chao1-estimator), community diversity (Shannon-index) and the relative abundance of each OTU for bumblebees that received the specific diet when their gut microbiota was already established after 4-5 days.

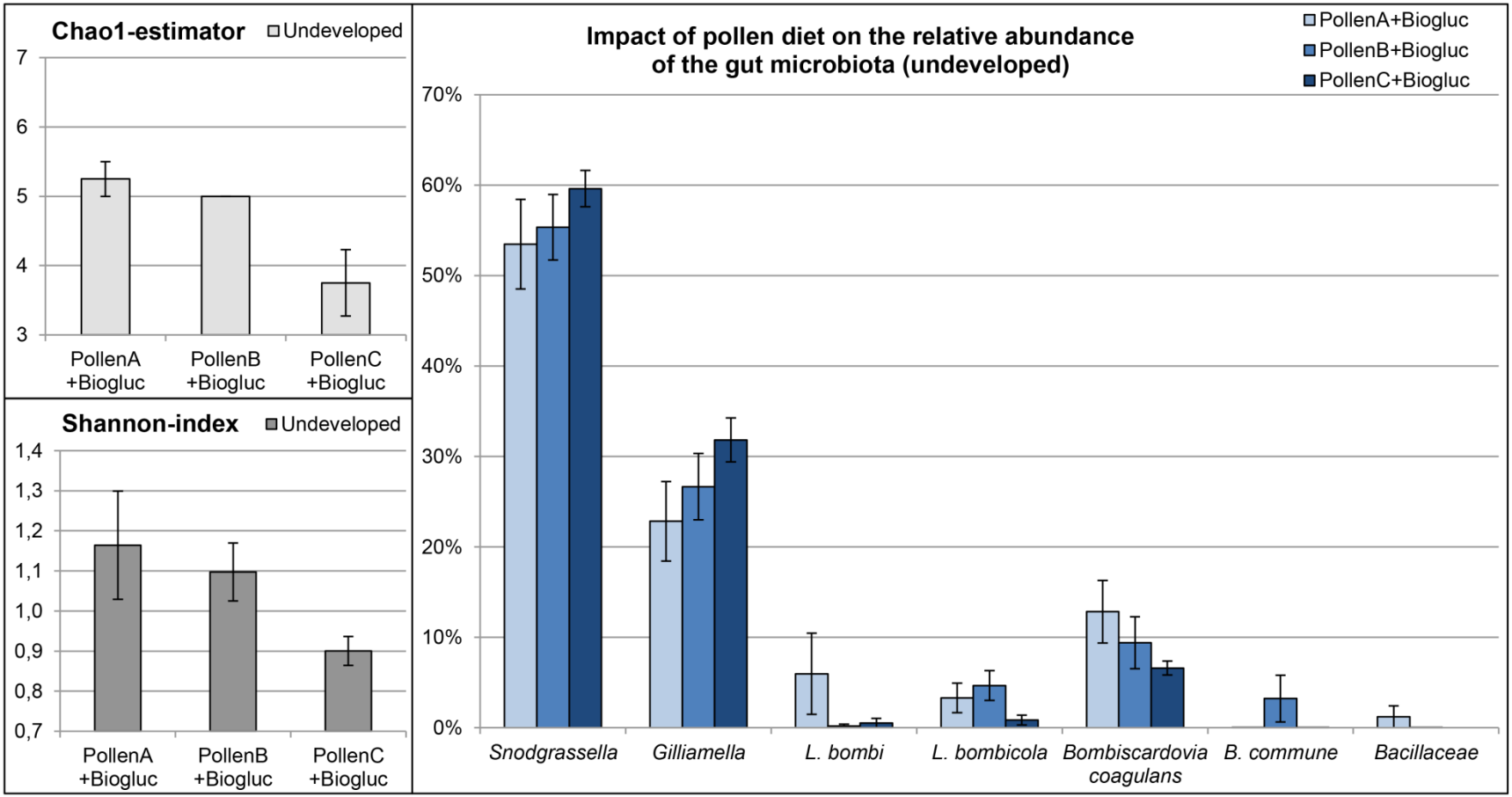

Figure 4.5. The impact of pollen diet (pollen A, pollen B or pollen C) on the community richness (Chao1-estimator), community diversity (Shannon-index) and the relative abundance of each OTU for bumblebees that received the specific diet when their gut microbiota was still undeveloped.

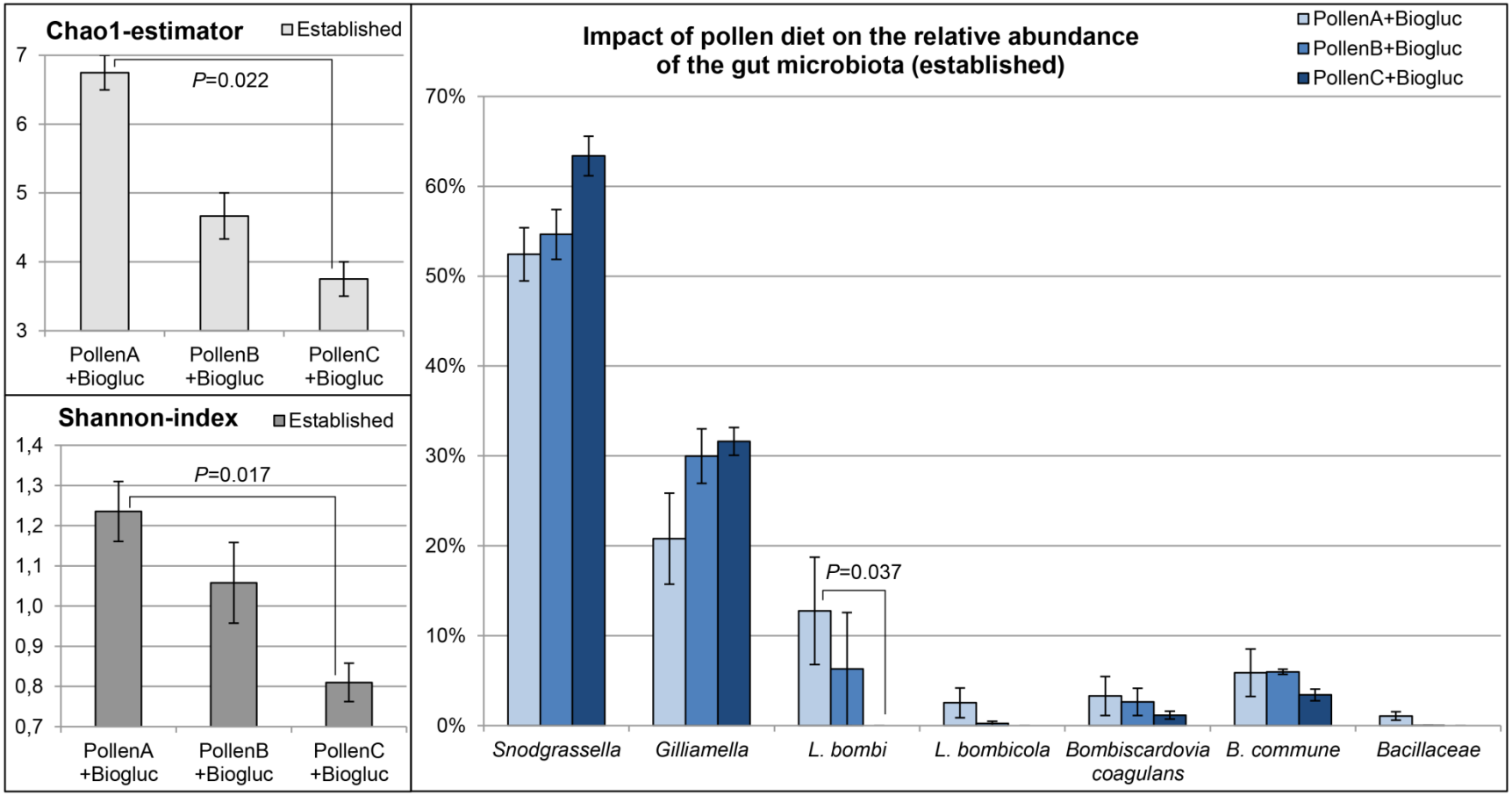

Figure 4.6. The impact of the pollen diet (pollen A, pollen B or pollen C) on the community richness (Chao1-estimator), community diversity (Shannon-index) and the relative abundance of each OTU for bumblebees that received the specific diet when their gut microbiota was already established after 4-5 days.

4.5. Discussion

The gut microbiota of indoor-reared bumblebees has been described as consistent and mainly harboring the core bacteria (Meeus et al., 2015). Here we could confirm their presence, enabling us to study their relative abundance in relation with the diet of the host. More specifically, we determined whether the pollen diet and sugar syrup influenced the gut microbial diversity and whether the developmental stage of the gut microbiota also played a role. The experiments demonstrated that diet was indeed able to induce some shifts in the relative abundance of the microbial gut composition of indoor-reared bumblebees; however, the effects were rather limited. In this study we did not investigate if diet was able to affect the absolute community size, which could also change under different dietary conditions.

4.5.1. Relative abundances of *Snodgrassella* and *Gilliamella* were not altered by diet

Complete genome sequencing of *Gilliamella apicola* and *Snodgrassella alvi* strains, isolated from honeybee guts, suggested that these bacteria contribute in nutrition of the host. Pectate lyase is present and functional in the bacterial genome of some strains of *G. apicola*. This enzyme can digest pectin, which is present in the cell wall of pollen. *G. apicola* also contains a large number of sugar transporters and sugar utilization pathways, whereas *S. alvi* can only use carboxylates, the metabolites of the sugar metabolism. This shows the complementary capacities of *G. apicola* and *S. alvi* (Engel et al., 2014; Kwong et al., 2014; Moran, 2015). In our study, the relative abundances of the most dominant bacteria *Snodgrassella* and *Gilliamella* seemed not significantly susceptible to dietary changes. However, our data do not provide information on strain level. Possibly, diet induced a shift in the strains of *Gilliamella* and *Snodgrassella*. Variation in the presence of hundreds of genes has been observed between strains of both *G. apicola* and *S. alvi* (Engel et al., 2014; Kwong et al., 2014).

4.5.2. Diet affects the relative abundance of *Lactobacillaceae* and *Bifidobacteriaceae*

In contrast to the stability in the relative abundances of *Snodgrassella* and *Gilliamella*, the relative abundances of *Lactobacillaceae* and *Bifidobacteriaceae* were influenced by changes in diet.

4.5.2.1. A high fructose concentration can prevent the growth of *Bifidobacteriaceae* in a developing microbiota

Providing fructose-rich sugar syrup to newly emerged bumblebee workers resulted in an absence of both *Bombiscardovia coagulans* LISPASI-P3 and *B. commune* in their gut microbiota.

Several *Bifidobacteriaceae* and *Lactobacillaceae* were already isolated from the bumblebee gut and taxonomically described, including their carbohydrate fermenting capacities (Killer et al., 2010b; Killer et al., 2014; Praet et al., 2015a; Praet et al., 2015b). We summarized the carbohydrate fermenting capacities of described *Lactobacillaceae* and *Bifidobacteriaceae* in supplemental data Table S3 (A, B, C). These characteristics show that *B. coagulans* LISPASI-P3 and two of the four strains of *B. commune* are not able to ferment fructose. On the other hand, *L. bombi and L. bombicola* are able to ferment fructose, giving them an advantage to colonize the gut.

The microbiota of bumblebees with an established microbiota did harbor *Bifidobacteriaceae*. This suggests that the *Bifidobacteriaceae* were obtained during the first 4 days while living in the original queen-right colony which was fed Biogluc sugar syrup and pollen B. Once the *Bifidobacteriaceae* colonized the gut, they could maintain their prevalence even under high fructose concentrations.

4.5.2.2. Pollen diet impacts the diversity of the gut microbiota

Proteins are important for ovary development (Hoover et al., 2006) and larval development (Tasei & Aupinel, 2008; Quezada-Euán et al., 2011). Protein levels can be reflected in the number of progeny, but also the accessibility of proteins and the pollen species composition are important factors which will greatly influence the biomass production (Vanderplanck et al., 2014). In this study, pollen C had the highest protein content and the highest drone production of the three pollen diets (Table 4.1.). However the microbiota of bumblebees fed on this pollen diet showed the lowest community richness and diversity due to the low abundances of *L. bombi* and *L. bombicola* in both groups of bumblebees. This observation does not necessarily point toward a correlation between protein levels, high reproduction, and low bacterial diversity. However, our results indicate that high quality in pollen, in terms of biomass production, does not necessarily correlate with a diverse microbiota and can influence the prevalence of *Lactobacillaceae*.

Earlier studies in honeybees showed that LAB might improve the resistance against *Paenibacillus larvae* (Forsgren et al., 2010; Vasquez et al., 2012). We speculate that a low abundance of *Bifidobacteriaceae* and *Lactobacillaceae* might have negative health consequences when pathogens appear. It is known that the gut microbiota of bees plays a role in the protection against pathogen infection, as bumblebees with a heavily impaired microbiota have been proven to be more susceptible to pathogen intrusion than bumblebees with the typical core gut microbiota (Koch & Schmid-Hempel, 2011b). In rearing facilities, the quality of pollen is assessed by the number of drones produced in microcolonies, but this is not a measure for immune competence. Our results show that diet can alter microbial diversity, which might impact the resistance to diseases. Reared bumblebees are used for biological pollination in agriculture in open field and thus also forage on the same flowers as wild bumblebees. Immune competence of reared bumblebees is important as it could affect their susceptibility to get infected by wild bees and this in turn may affect the spread of pathogens (Meeus et al., 2011; Murray et al., 2013; Graystock et al., 2014). However it remains speculative whether the microbial composition can be actively modified to make

bees less prone to disease infections, but we believe that efforts should be made into this direction.

4.5.2.3. The choice of sugar syrup and pollen diet as a tool to alter the gut microbiota of reared bumblebees

The gut microbiota of indoor-reared bumblebees is a subset of the gut microbiota of wild bumblebees (Meeus et al., 2015). At this point, the effect of a lower community richness or diversity and what an optimal gut microbiota looks like, is not known. As functionalities of the bumblebee microbiota have not yet been completely elucidated, we would encourage to choose diets that either maintain the core gut microbiota or diets that enhance the relative abundance of *Bifidobacteriaceae* and *Lactobacillaceae*. Within this context we would not recommend pollen C or fructose-rich sugar syrup, which had a negative impact on the relative abundance of *Lactobacillaceae* and *Bifidobacteriaceae*. To date, application of probiotics is the most conventional method to manipulate or optimize the gut microbiota in order to ensure higher reproduction (Drillet et al., 2011), better survival (Kaznowski et al., 2005), increased larval growth (Lauzon et al., 2010), or decreased pathogen infection (Evans & Lopez, 2004; Forsgren et al., 2010; Kawakami et al., 2010). Our results imply that diet is an important parameter to consider when applying a probiotic to enhance the colonization of typical host-specific bacteria. We speculate that also other carbohydrates could potentially enhance the colonization potential of *Bifidobacteriaceae* and *Lactobacillaceae*. Examples are amygdalin, L-arabinose, cellobiose, gentiobiose, (D-)melibiose, D-mannose and salicin, as the *Lactobacillaceae* and/or *Bifidobacteriaceae* that occur in the indoor-reared bumblebees show positive fermenting capacities for these carbohydrates (supplemental data Table S3.). However their effect on the gut microbial composition and their nutritional values remain to be studied, as these carbohydrates were not included in the tested diets in this chapter.

In conclusion, the composition of the sugar syrup and pollen diet contributed to differences in community richness, community diversity, and the relative abundance of the gut bacteria of

indoor-reared bumblebees. Rearing facilities should not only monitor the reproduction numbers in bumblebee colonies when choosing their diets, but the impact on the composition of the gut microbiota should also be taken into consideration. In addition, further studies are needed to understand the role of the gut microbial composition in protecting bees against pathogens. Our findings provide new insights that the gut microbiota can be modulated through diet types and offers opportunities to shift the gut microbiota of mass-reared bumblebees improving bee health as well as productivity.

Chapter 5.

Colony contact contributes to the diversity of gut bacteria in bumblebees (*Bombus terrestris*)

Redrafted from: Billiet, A., Meeus, I., Van Nieuwerburgh, F., Deforce, D., Wäckers, F. and Smagghe, G. (2015) Colony contact contributes to the diversity of gut bacteria in bumblebees (*Bombus terrestris*). Insect Science. DOI: 10.1111/1744-7917.12284

5.1. Abstract

Social bees, like honeybees and bumblebees, have a close contact with nest mates of different developmental stages and generations. This could enhance bacterial transfer between nest mates and offers opportunities for direct transfer of symbionts from one generation to the next, resulting in a stable host specific gut microbiota. Gut symbionts of honeybees and bumblebees have been suggested to contribute in digestion and protection against parasites and pathogens. Here we studied the impact of contact with the bumblebee colony on the colonization potential of the bacterial families (i.e. *Neisseriaceae*, *Orbaceae*, *Lactobacillaceae* and *Bifidobacteriaceae*) occurring in the gut of adult bumblebees (*Bombus terrestris*). Bacterial profiles of the gut microbiota of *B. terrestris* were determined based on the hypervariable V4 region of the 16S rRNA using paired-end Illumina sequencing. In our experiments, we created different groups in which we gradually reduced the contact with nest mates and hive material. We made 3 observations: (i) reducing the contact between the colony and the bumblebee during adult life resulted in a significant drop in the relative abundance of *Lactobacillus bombicola* and *Lactobacillus bombi*; (ii) *Bifidobacteriaceae* required contact with nest mates to colonize the gut of *B. terrestris* and a significant lower bacterial diversity was observed in bumblebees that were completely excluded from colony contact during the adult life; (iii) *Snodgrassella* and *Gilliamella* were able to colonize the gut of the adult bumblebee without any direct contact with nest mates in the adult life stage. These results indicate the impact of the colony life on the diversity of the characteristic bumblebee gut bacteria.

5.2. Introduction

In many insects, gut bacterial communities vary among individuals within a species and are highly influenced by bacteria present in the insect's environment. Opportunities for direct transfer of symbionts from one generation to the next are rather limited, as most insects have no brood care or contact with their offspring (Engel & Moran, 2013). In contrast to most insects, social insects live together in one colony with many individuals in different developmental stages going from eggs, larvae, pupae to adults and harbor a much more stable microbiota (Engel & Moran, 2013). The close contact in the colony between nest mates and offspring could create a specific and interesting opportunity to enhance a close relationship between the host and its microbiota (Koch et al., 2013). An example of this social behavior in insects is seen in social bees like honeybees (*Apis*) and bumblebees (*Bombus*). Because of the ecologic and economic importance of bumblebees and honeybees, their bacterial symbionts have already been the subject of many studies. Their characteristic gut bacteria harbor genes that may contribute to defense against parasites and pathogens (Evans & Armstrong, 2006; Koch & Schmid-Hempel, 2011b; Cariveau et al., 2014), and digestion of carbohydrates and pollen walls (Engel et al., 2012; Lee et al., 2015). However the true impact of living together in one colony on the transmission of the gut microbiota was not yet studied in bumblebees.

The social behavior of honeybees and bumblebees is different at some points, which might impact the transmission routes of gut bacteria. Honeybee nurses feed beebread to the larvae by oral trophallaxis, a transfer of food through mouth-to-mouth contact, which is also a transmission route for some bacterial symbionts (Powell et al., 2014). Bumblebees feed their larvae a mixture of pollen and nectar, however not through direct mouth-to-mouth contact as seen in honeybees. Whereas honeybee colonies are perennial, bumblebee colonies are annual, meaning only the daughter-queen hibernates, and thus she is the only one able to transmit her microbiota to her offspring after winter. While some differences are observed in the social behavior between social honeybees and bumblebees, both harbor a set of gut bacteria that are similar on genus level, as previously mentioned in chapter 1.

Holometabolous insects molt several times during their life and undergo radical remodeling of the gut and other organs at metamorphosis (Hakim et al., 2010) and this severely disrupts or eliminates attached bacterial populations (Moll et al., 2001). During the pupation stadium, a cocktail of antimicrobial peptides is produced and therefore it was suggested that newly emerged adults have a sterile gut (Russell & Dunn, 1996). However, Hroncova et al. (2015) reported a significant decrease of bacterial counts in pupae of *A. mellifera* but not a complete sterile gut. Microbial counts quickly recovered 4-6 days after adult emergence (Meeus et al., 2013; Hroncova et al., 2015).

The social lifestyle of honeybees and bumblebees is probably an important advantage in the transmission of gut bacteria with functionalities in digestion and pathogen protection. Living together in a colony provides opportunities for vertical transmission (from queen to daughter) and horizontal transmission (between workers), enabling them to evolve towards a symbiotic relationship with a specialized and specific gut microbiota. Our aim is to investigate which of the core gut bacteria in the adult bumblebee are obtained as a result of their social behavior.

Here we studied the effect of reduced contact of emerging bumblebees with the colony, nest mates or hive material by creating four groups of bumblebees, divided in two experiments. In the first experiment we reduced the time of contact with the colony. In the second experiment we excluded all contact with nest mates and the colony. The gut microbial composition of each group was analyzed by use of MiSeq multiplexed 16S rRNA amplicon sequencing.

5.3. Materials and methods

5.3.1. Bumblebees

Bumblebees were used from indoor mass-reared queen-right colonies of *Bombus terrestris* (Biobest, Belgium). All individuals were fed on Biogluc sugar syrup and 15 kGy radiation-sterilized honeybee-collected pollen (Soc. Coop. Apihurdes, Pinofranqueado-Cáceres, Spain). The bumblebees were kept under standardized laboratory conditions at 30 °C and continuous darkness.

5.3.2. Experimental conditions

5.3.2.1. Interaction with nest mates and hive material

It is known that the typical gut microbiota of bumblebees is accomplished within the first four days after adult eclosion (Chapter 2). This experiment was designed to determine the effect on the gut microbiota of adult bumblebees when removed from the colony before their gut microbiota was completely established. Hereto we defined two groups of bumblebees: first, a control group consisted of newly emerged bumblebee workers (n=9) that were labeled with a marker and immediately placed back in their colony. These bumblebees were able to interact with hive material and nest mates during the next 4 days. We will further refer to this group of bumblebees as ‘group A’.

Second, newly emerged bumblebee workers of less than 1 day old were transferred from their queen-right colony (n=8) into microcolony boxes (5 sister bees/microcolony). During the following 4 days these bumblebees were only able to interact with the other newly emerged bumblebees in the microcolony. The microcolony did not contain hive material. After 4 days in the microcolonies, the composition of the gut microbiota of one of the workers of each microcolony was analyzed. We will further refer to this group of bumblebees as ‘group B’.

5.3.2.2. Exclusion of all physical contact with nest mates

The aim of this experiment was to study which gut bacteria were still able to colonize the gut of bumblebees, when interaction with the colony was decreased even more than in group B. We created two groups of bumblebees, excluding them from any social interaction with nest material and nest mates. First, we transferred 6 bumblebees of less than 1 day old from their queen-right colony into individual boxes, without hive material. These bumblebees had interaction with their queen-right colony during the first hours after emergence, followed by a solitary life during the next 12 days. We will further refer to this group of bumblebees as 'group C'.

Second, we transferred 5 pupal cocoons from the colony into individual boxes. All pupae were white and had black eyes at the time of transfer. They completed their pupation isolated from the colony and emerged in the individual box, without hive material with the exception of their own cocoon. During the 12 following days, this group of bumblebees had a solitary life, excluded from all possible interaction with the colony and other nest mates. We will further refer to this group of bumblebees as 'group D'.

In normal conditions the gut microbiota is accomplished within 4 days after eclosion and stays stable for the following days (Meeus et al., 2013; Parmentier et al., 2015a). However by isolating bumblebees from social contact, an impaired gut microbiota is expected in these bumblebees. We chose to investigate their gut microbiota after 12 days, as we expected this to be enough time for gut bacteria to colonize.

5.3.3. Sample preparation and Illumina sequencing

Mid- and hindgut were dissected from bumblebees using disinfected dissection material and stored at -20 °C. The gut was crushed in a 170 µL lysozyme solution (100 mg/mL) and DNA-extraction was performed as described in Meeus et al. (2013). The hypervariable V4 region (254 bp) of the 16S rRNA was amplified in triplicate, using the 515F and 806R primers designed by Caporaso et al. (2011). The 806R primer was barcoded with a different nucleotide for each sample and both primers contained Illumina adapter sequences

necessary for the bridge amplification on the Illumina MiSeq flow cell (Caporaso et al., 2011). Further sample preparation and Illumina sequencing were performed as described in Meeus et al. (2015).

5.3.4. Sequence data analysis

Sequences derived from the Illumina MiSeq were analyzed with the mothur software v. 1.31.1 (Schloss et al., 2009), mainly following the standard operating procedure available on http://www.mothur.org/wiki/MiSeq_SOP, date December 2013. The raw data are publicly available on NCBI's Sequence Read Archive (SRA) under accession number SRP049766. We followed standard operating procedures to optimize the quality of our dataset. At this point the dataset contained a total of 2,335,395 demultiplexed paired-end reads, representing 303,738 unique sequences. We further removed sequences that contained more than 8 homopolymers, sequences that were not complete and we also removed chimeras. After these steps, our dataset still contained 2,258,041 reads representing 154,095 unique sequences. In a following step we removed sequences that only occurred once ('singletons'), which brought the number of unique sequences down to 6,471. In a last step we removed sequences from our dataset that were not correlated with a bacterial taxonomy (e.g. chloroplasts, mitochondria, *Archaea* or *Eukarya*), resulting in a total of 2,025,941 reads whereof 6,406 unique sequences, with an average of 49,727 ± 3,322 reads per specimen. The previous steps clearly retained the majority of the reads (86.5 %).

Calculating the distance matrix and clustering with a 0.03 cutoff level resulted in 546 OTUs (Operational Taxonomic Unit). The taxonomic identity of each OTU was revealed by alignment of each sequence with the Bacterial SILVA SEED database. This database (training set) was supplemented with host specific sequences (i.e. host *Apis* or *Bombus*) to improve classification (Newton & Roeselers, 2012a). In this analysis we chose to only retain the OTUs that were represented by more than 0.5 % of the reads in a sample, as we aimed to focus on the core bacteria in the bumblebee gut. This resulted in 7 OTUs covering 99.7 % of the reads of the 546 OTUs. The reads of the samples were calculated in percentages,

expressing the relative abundance of each OTU. Community richness was calculated using the Chao1-estimator (alpha diversity) and community diversity was calculated with the Shannon-index (beta diversity). The bacterial evenness (e) was calculated as e = H/ lnS, where H is the Shannon-index and S is the number of OTUs.

The statistical differences between the relative abundance, Chao1-estimator or Shannon index between two groups were analyzed using the non-parametric Mann-Whitney U test.

5.4. Results

5.4.1. Identified OTUs

The 7 OTUs mainly represented the four core bacterial families i.e. *Neisseriaceae (Snodgrassella)*, *Orbaceae (Gilliamella)*, *Lactobacillaceae* (*Lactobacillus bombi* and *Lactobacillus bombicola*), *Bifidobacteriaceae* (*Bombiscardovia coagulans* LISPASI-P3 and *Bifidobacterium commune*). Only one sample (in group B) contained Bacteroidetes, a bacterium that already has been identified in *B. terrestris* in earlier studies, but does not belong to the core bacteria (Meeus et al., 2015). Taxonomic identification of the OTUs and their closest match in GenBank or EzBioCloud are presented in Table 5.1. The genetic distance of an OTU with its closest bacterial family members is shown for *Bifidobacteriaceae* in Figure 5.1 and for the *Lactobacillaceae* in Figure 5.2.

Table 5.1. Taxonomic identification of the OTUs and their closest match in GenBank or EzBioCloud.

Identification of OTUs **Phylum** Class *Family Genus*	**Matching base pairs to best match in *Bombus* or *Apis***	**Name used here**
Proteobacteria Betaproteobacteria *Neisseriaceae Snodgrassella*	253/253 JQ746649 *Snodgrassella alvi* strain wkB29	*Snodgrassella*
Proteobacteria Gammaproteobacteria *Orbaceae Gilliamella*	253/253 JQ936676 *Gilliamella apicola* strain wkB30	*Gilliamella*
Firmicutes Bacilli *Lactobacillaceae Lactobacillus*	253/253 LK054485 *Lactobacillus bombicola* LMG 28288^T 253/253 'Lacto1-Firm5' (Meeus et al., 2015)	*L. bombicola*
Firmicutes Bacilli *Lactobacillaceae Lactobacillus*	253/253 KJ078643 *Lactobacillus bombi* BTLCH M 1/2^T 253/253 'Lacto2-Firm4' (Meeus et al., 2015)	*L. bombi*
Actinobacteria Actinobacteria *Bifidobacteriaceae*	253/253 FJ858733 *Bombiscardovia coagulans* LISPASI-P3 253/253 'Bifido3' (Meeus et al., 2015)	*Bombiscardovia coagulans* LISPASI-P3
Actinobacteria Actinobacteria *Bifidobacteriaceae Bifidobacterium*	253/253 LK054489 *Bifidobacterium commune* LMG 28292^T 253/253 'BifidoX' (Meeus et al., 2015)	*B. commune*
Bacteroidetes Flavobacteria *Flavobacteriaceae*	253/253 HM215036 Uncultured Bacteroidetes 252/253 LN713847 *Apibacter mensalis* LMG 28357^T	Bacteroidetes

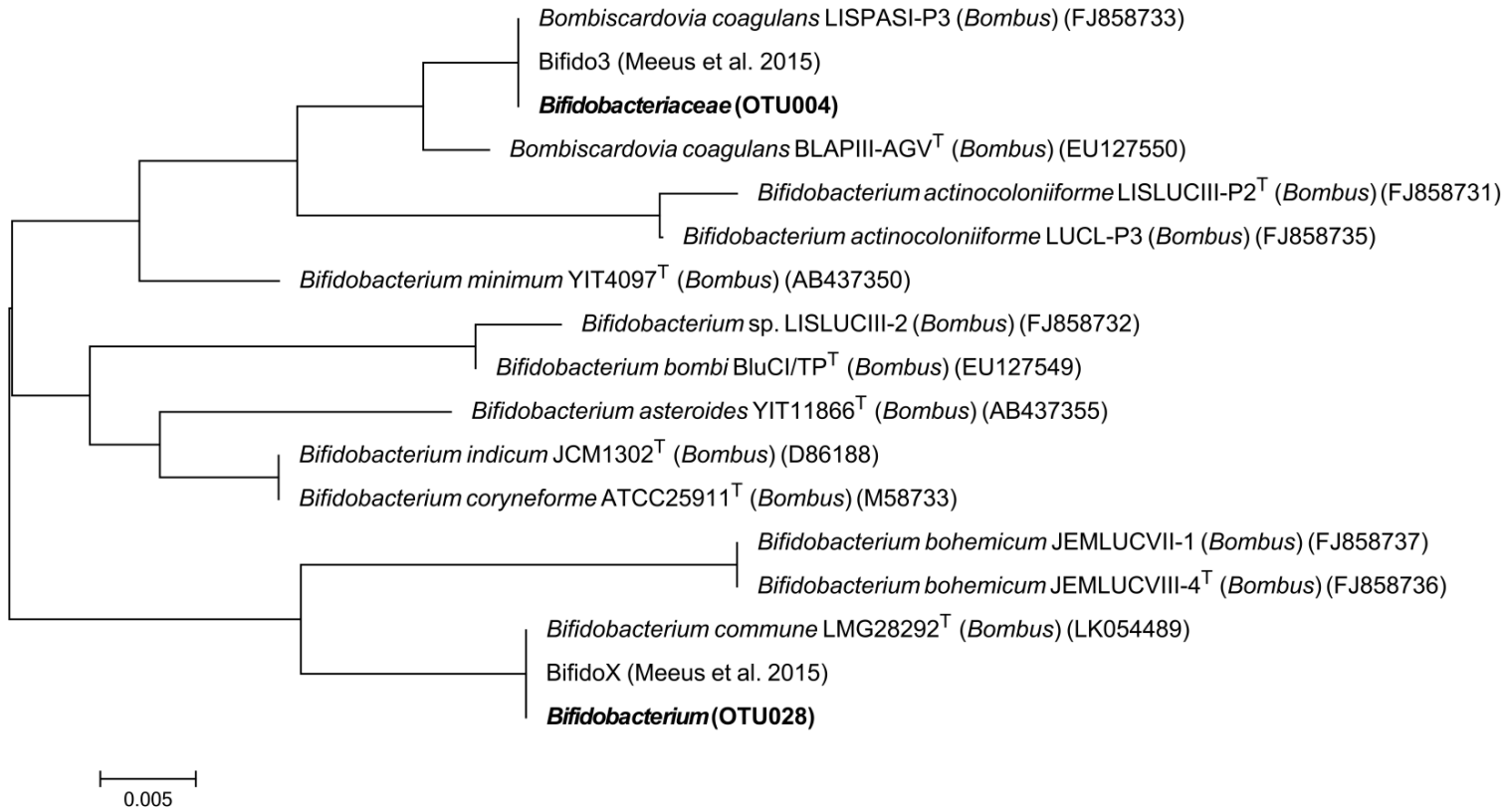

Figure 5.1. Phylogenetic tree derived from the V4 region of the 16S rRNA gene sequence of *Bifidobacteriaceae* occurring in the digestive tract of *Bombus*, showing the position of the *Bifidobacteria* (OTU004 and OTU028) found in our study

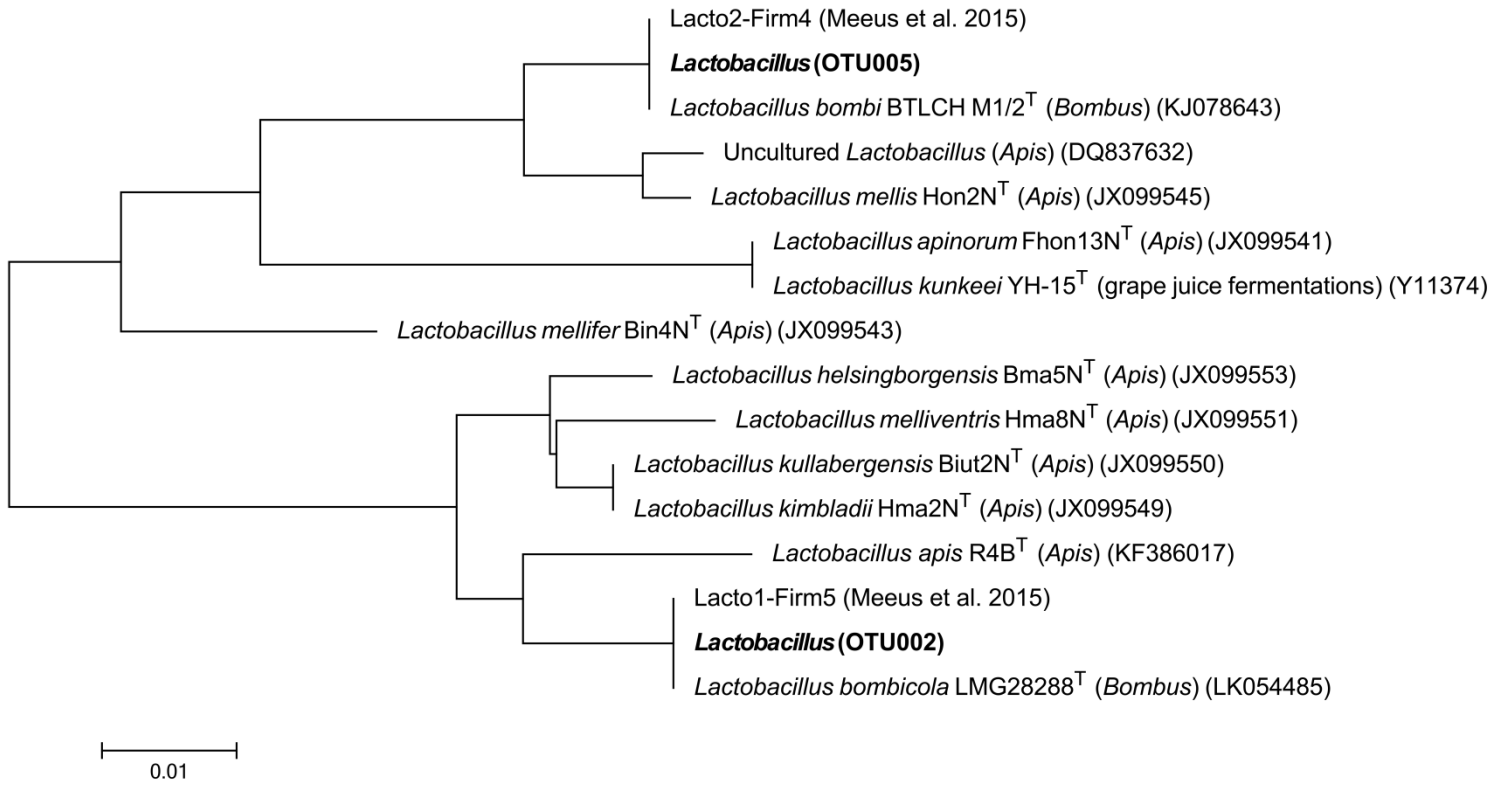

Figure 5.2. Phylogenetic tree derived from the V4 region of the 16S rRNA gene sequence of *Lactobacillaceae* occurring in the digestive tract of *Bombus* and *Apis*, showing the position of the *Lactobacilli* (OTU002 and OTU005) found in our study.

5.4.2. Interaction with nest mates and hive material

Snodgrassella, *L. bombicola* and *Gilliamella* dominated the bacterial composition in bumblebee guts of group A (n=9). The short time of contact in group B (n=8) between newly emerged bees and the colony, seemed enough to have the gut colonized with the core set of bacterial families *Neisseriaceae*, *Orbaceae*, *Lactobacillaceae* and *Bifidobacteriaceae*. Yet, reducing the time of contact with the colony resulted in a significant reduction of the relative abundance for *L. bombi* (P=0.01; Z=-2.709) and *L. bombicola* (P=0.01; Z=-2.598) (Figure 5.3). While noticing a significant drop in relative abundance for *L. bombicola* and *L. bombi*, there was no significant effect on the community richness (P=0.19; Z=-1.314) (Figure 5.4a), community diversity (P=0.29; Z=-1.060) (Figure 5.4b), or the bacterial evenness (P=0.56; Z=-0.578) (Figure 5.4c).

5.4.3. Exclusion of all physical contact with nest mates

Comparison of groups C (n=6) and D (n=5) revealed that complete exclusion of physical contact with nest mates had no significant effect on the community richness (P=0.37; Z=-0.898), however, there was a significant drop in community diversity (P=0.02; Z=-2.379) and bacterial evenness (P=0.04; Z=-2.008) (Figures 5.4 a, b, c). Workers that hatched in a sterile environment without colony contact (group D) had a significantly lower relative abundance of *Bombiscardovia coagulans* LISPASI-P3 (P=0.02; Z=-2.298) and a lower, not significant, relative abundance of *L. bombi* (P=0.08; Z=-1.742) (Figure 5.3).

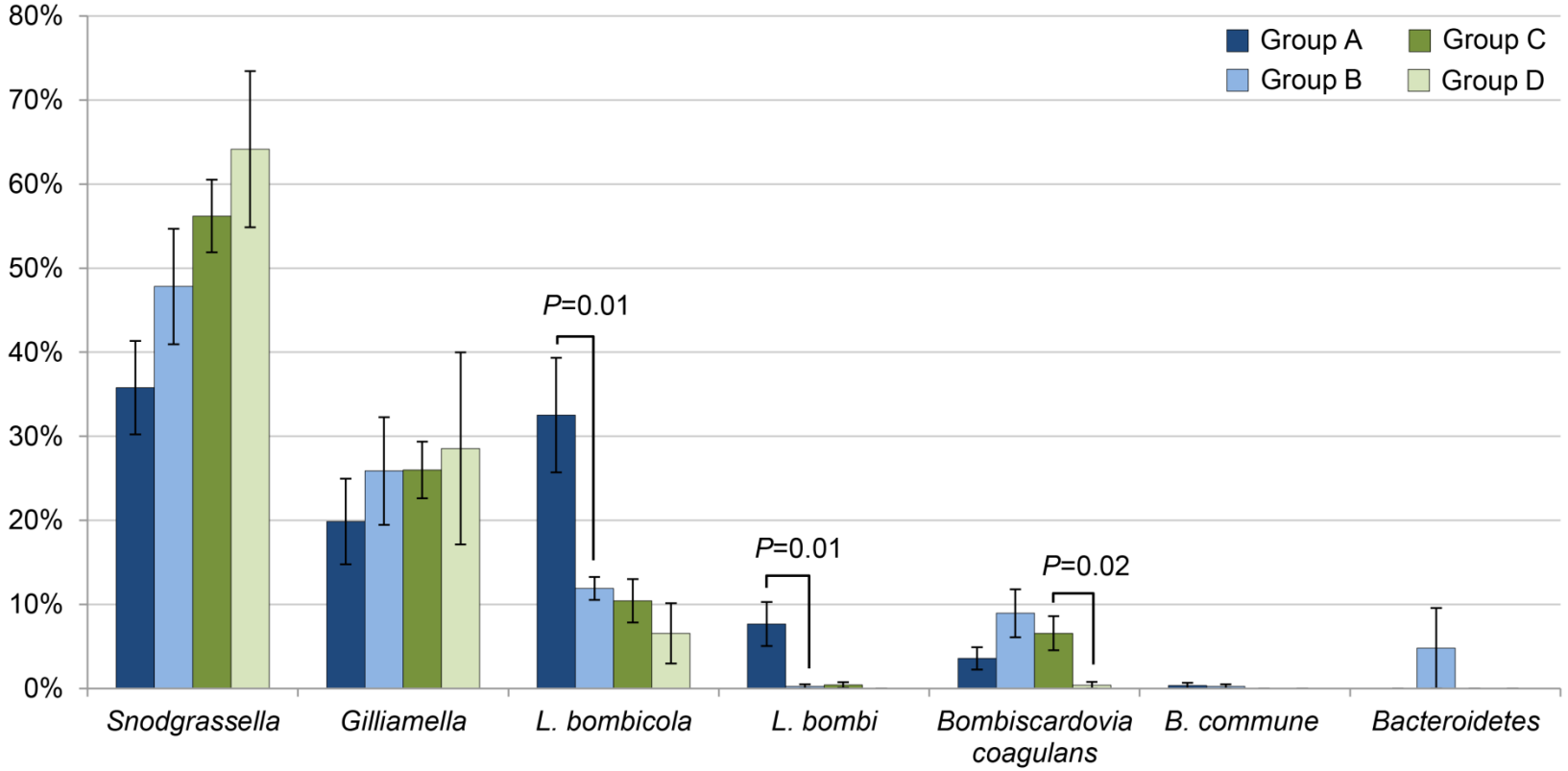

Figure 5.3. The average relative abundance of gut bacteria in adult bumblebee workers, exposed to different degrees of contact with their colony. Significant differences in relative abundances are indicated with their p-values for comparisons between groups A-B and groups C-D. Between group A (full interaction with the colony) and group B (newly emerged bumblebees transferred from their colony into a microcolony box) there is a significant reduction of the relative abundance for *L. bombi* and *L. bombicola*. Between group C (newly emerged bumblebees transferred from their colony into a sterile individual box) and group D (transfer to a sterile individual box took place during pupation stadium) there is a significant reduction of the relative abundance for *Bombiscardovia coagulans* LISPASI-P3.

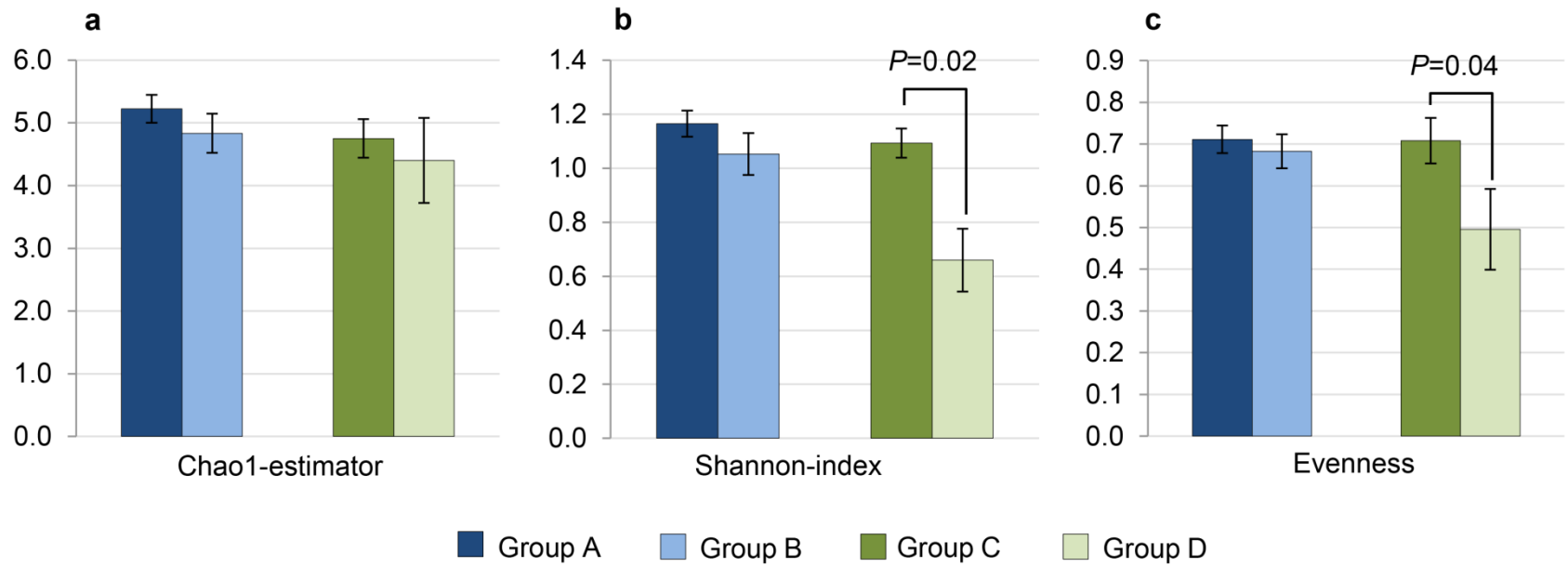

Figure 5.4. (a) The average Chao1-estimator for groups A to D with the standard error. No significant differences were detected in community richness. **(b)** The average Shannon-index for groups A to D with the standard error. In the second experiment, a significantly lower community diversity was observed in group D compared to group C. **(c)** The average bacterial evenness of the gut microbiota of bumblebees from groups A to D with the standard error. There was a significant lower evenness in group D, when compared to group C.

5.5. Discussion

Bacterial taxa that are repeatedly found exclusively or primarily in bee guts are described as the core gut bacteria. *Apis* and *Bombus* share many of the same bacterial groups, including species within Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Lactobacillales (Cariveau et al., 2014). The core gut bacterial genera of *B. terrestris* consist of *Snodgrassella* (Betaproteobacteria), *Gilliamella* (Gammaproteobacteria), *Lactobacillus* (Firmicutes) and *Bifidobacteriaceae* (Actinobacteria) (Meeus et al., 2015). The gut of indoor-reared bumblebees mainly harbors these core bacteria and very few non-core bacteria (Meeus et al., 2015), which is in agreement with our findings in this study in bumblebees of the control treatment (group A).

Comparison of the bacterial gut composition of bumblebees in the colony (group A) and microcolonies with newly emerged bumblebees (group B) showed a significant reduction of *L. bombi* and *L. bombicola* in group B. Lactobacilli have been suggested to be beneficial for bees, as *in vitro* tests with bee specific lactobacilli demonstrated a certain degree of pathogen protection towards *Paenibacillus larvae*, causing American foulbrood in honeybees and bumblebees (Forsgren et al., 2010; Vasquez et al., 2012; Killer et al., 2013). The gut microbiota of bees plays a role in protection against pathogen infection (Koch & Schmid-Hempel, 2011b). The natural behavior of bees in which the newly emerged workers first have tasks in the hive before they start to forage (Goulson, 2010), allows them to establish their gut microbiota before encountering environmental bacteria while foraging.

Bombiscardovia coagulans was able to colonize the gut after short contact with nest mates (groups B and C), but was mostly missing when deprived from all contact with nest mates (group D), resulting in a significant lower bacterial diversity and evenness. Bumblebees from group D only had contact with the cocoon and although not having any direct contact with the colony or other nest mates during their adult live, *Snodgrassella*, *Gilliamella* and *L. bombicola* were still able to colonize their gut.

Different options are possible to explain the colonization of these bacteria. First of all, the cocoon possibly contained fecal material with bacterial remnants from the larval stage or from the adults before the cocoons were removed from the colony. When emerging, the callow bumblebee worker bites through the cocoon and this is possibly a first inoculation source. These results seem to fit the assumptions of Powell et al. (2014) where *Snodgrassella* and *Gilliamella* could be transferred via a fecal inoculation route in *A. mellifera*, while lactobacilli and bifidobacteria were mainly transferred by oral contact and hive material such as beebread, honey and comb (Powell et al., 2014).

Another inoculation source could be bacterial remnants which may have survived the gut metamorphosis by use of biofilm protection. Both *Gilliamella* and *Snodgrassella* are able of biofilm formation (Martinson et al., 2012). It remains the question if they could survive the pupation stadium in very low abundances. This hypothesis could explain the observation of occasional bacterial prevalence in honeybee pupae (Hroncova et al., 2015) but is in contrast to previous assumptions of a sterile gut after pupation (Russell & Dunn, 1996).

Or the inoculation originated from other bumblebee tissues, for example the hemolymph which might store certain symbionts able to recolonize the gut of adult bumblebees. However, further investigation is needed to indicate the exact inoculation source of these bacteria.

Gilliamella and *Snodgrassella* are two recently discovered bacterial taxa, exclusively present in honeybees and bumblebees (Kwong & Moran, 2013). Some strains of *Gilliamella apicola*, isolated from the honeybee gut, have functional pectate lyase enzyme in their genome, which can digest pectin from the cell walls of pollen. Both *Gilliamella apicola* and *Snodgrassella alvi* play a role in the carbohydrate metabolism, complementing each other's functionality (Engel et al., 2014; Kwong et al., 2014; Moran, 2015). It remains undetermined if these functionalities are the same in the strains of *Gilliamella* and *Snodgrassella* occurring in the bumblebee. According to Koch et al. (2013), both *Snodgrassella* and *Gilliamella* have a close association with their host. This was confirmed in our results as both bacteria were able to colonize even without any contact with other nest mates.

On the other hand, lactobacilli and bifidobacteria were obtained as a result of direct contact with other nest mates during the first hours and days in the colony after emerging. These bacteria have functionalities in pathogen protection and seem to be maintained in the colony as a result of direct social contact.

Chapter 6.

Bacteria in the ovaries of indoor-reared bumblebee queens (*Bombus terrestris*)

6.1. Abstract

Bumblebees are nowadays mass-reared by specialized companies. Although parameters important to bumblebee rearing are well controlled in the facilities, some queens do not start laying eggs after hibernation, while others stop laying eggs after a few weeks. Our aim was to investigate if the bacterial community in the gut and the ovaries of reared bumblebee queens (*Bombus terrestris*) could be correlated with the colony success. Queens were classified in four groups, ranging from no reproduction at all, to a very good reproduction. The ovary length and mass were clearly correlated with the colony success. The bacterial fingerprint of the gut and ovaries of the four groups was determined by use of denaturing gradient gel electrophoresis (DGGE). The bacterial profiles on the DGGE were not able to elucidate the differences in colony success. In a last step we identified the bacterial community in well-developed ovaries using Illumina MiSeq Sequencing. The typical core gut bacteria *Snodgrassella*, *Gilliamella*, *Lactobacillus* and *Bifidobacterium*, were also detected in the ovaries, and covered two third of the relative abundance. Other bacteria that were not yet described in the bumblebee microbiota were also detected, but in very low abundances. This study provides a new insight in the microbial communities present in tissues of bumblebee queens.

6.2. Introduction

Bumblebees (*Bombus terrestris*) are nowadays mass-reared and sold worldwide for the pollination of crops in the open field or in green houses (Velthuis & van Doorn, 2006). Mass-rearing companies use quality-controlled rearing techniques to secure a successful production of bumblebee colonies. An important step for rearing in synchrony with commercial demand is the artificial management of the hibernation of the queens. In contrast to honeybees which are perennial, bumblebees are annual and only the bumblebee queen survives winter while she is hibernating (Velthuis & van Doorn, 2006; Goulson, 2010). In the early spring, the bumblebee queen awakes from hibernation and starts a new colony. Mass-rearing companies are capable to simulate the winter period and the awaking spring period afterwards, and in this way, they can start up bumblebee colonies within the indoor facilities (Velthuis & van Doorn, 2006). The right temperature is crucial for ovary development after hibernation, together with the presence of pollen and nectar (Vogt et al., 2011). In rearing facilities, these parameters are controlled, however some queens do not start laying eggs, while others stop laying eggs after a few weeks. Here, we investigated if this incidence could be correlated with the bacterial community. So far, all studies on the bumblebee microbiome have focused on the bacteria in the gut, while ovaries are a biologically important tissue in which the inhabiting microbes have not yet been described. The aim of the present study is to fill the gap on the bacterial content in the reproductive system of bumblebee queens and investigate if the ovary microbiota could play a role in the colony success. Several bacteria in ovaries have already been described in the red palm weevil (*Dryophthoridae*) (Montagna et al., 2015) and the mosquito (*Anopheles*) (Tchioffo et al., 2016). Other studies showed the presence of symbionts enclosed within specialized cells in different organs in insects. For example the endosymbiont *Buchnera* is maternally passed to the insect offspring through the ovary and eggs (Buchner, 1965). The ovaries, the eggs, the fat body and the salivary glands of adult females and nymphs of the leafhopper (*Scaphoideus titanus*) harbor *Cardinium* symbionts (Sacchi et al., 2008), and in the ovaries of the tsetse fly (*Glossina morsitans*), *Wolbachia*-like Rickettsia was detected (O'Neill et al., 1993). The genus *Wolbachia*

(Rickettsiales, Alphaproteobacteria) encompasses intracellular bacteria widespread in arthropods and in filarial nematodes (O'Neill et al., 1993; Zchori-Fein et al., 1998; Kozek & Rao, 2007; Hosokawa et al., 2010). This bacterium has been linked with various reproductive disorders such as cytoplasmatic incompatibility (the inability of *Wolbachia*-infected males to successfully reproduce with uninfected females or females infected with another *Wolbachia* strain) (Louis & Nigro, 1989; Breeuwer & Werren, 1990), parthenogenesis (infected females reproduce without males, with the help of *Wolbachia*) (Stouthamer et al., 1993), male killing (infected males die during larval development, which increases the rate of *Wolbachia* infected females) (Hurst et al., 1999) and feminization of genetic males (Hurst, 1993).

In this chapter, the bacterial community in the gut and ovaries of reared bumblebee queens (*Bombus terrestris*) was studied. Queens were given 7-8 weeks to start a colony after hibernation. Based on their colony success after this period, four groups of queens were formed, ranging from no reproduction, to very good reproduction. The queens were sacrificed to determine the ovary length and mass. A correlation between the colony success and their microbial diversity in the gut and ovaries was determined, comparing the 16S rDNA gene molecular fingerprint by use of denaturing gradient gel electrophoresis (DGGE). The identity of the bacteria in the ovaries was identified by Illumina MiSeq Sequencing. Finally, we discussed the correlation between the ovary development, microbial characteristics and the colony success. This study gives a first insight in the microbiota in the ovaries of bumblebees.

6.3. Materials and methods

6.3.1. Bumblebee queens

We selected bumblebee queens with a different reproduction capacity from an indoor bumblebee mass-production facility (Biobest, Westerlo, Belgium). Queens followed the standard start-up procedure and were reared at 30 °C and 60 % relative humidity. After 7-8 weeks, 4 groups of bumblebees were formed, based on their colony start-up success and the reproduction capacities. Bumblebee queens that did not lay eggs during these 7-8 weeks will further be referred to as group A. Bumblebee queens that initially started laying eggs but quickly stopped reproducing were classified into group B. Bumblebee queens with a normal or good reproduction were assigned to group C (± 50 workers/colony after 7-8 weeks). The last group D consisted of queens that produced even more workers (> 70 workers/colony after 7-8 weeks) than group C. Each group consisted of 15 bumblebee queens.

6.3.2. Dissection and assessing the ovary development

Both the ovaries and the gut were dissected from the bumblebee queens, using disinfected dissection material. Dissection material was disinfected between each dissection of different tissues. We made sure to dissect the ovaries first, without touching the gut in order to avoid bacterial contamination from the gut. Subsequently the gut (mid- and hindgut) was dissected. The ovaries were washed in bleach (1:20) for 30 s, and then in 1x PBS for 30 s and stored in sterile Eppendorf tubes. To extract the bacterial DNA, each tissue was individually crushed in a 170 µL lysozyme solution (100 mg/mL) and DNA was extracted as described in Meeus et al. (2013). The length and mass of the ovaries were determined. Statistical differences between the four groups were determined using ANOVA and Tukey post-hoc test.

6.3.3. PCR and bacterial fingerprint by use of denaturing gradient gel electrophoresis (DGGE)

We generated a 16S rDNA gene molecular fingerprint of the microbiota of 12 ovaries of each group and 4 guts of each group. The bacterial 16S rDNA was amplified by nested PCR.

Primers Eub8F and 984R were used in the external PCR and primers 338F-GC and 518R for the internal PCR (Bakke et al., 2011), targeting the V3 region. The PCR was performed as described in Meeus et al. (2013). 10 µL of the internal PCR product was loaded onto an 8 % (*w/v*) polyacrylamide gel (*w/v*; 40 % acrylamide, 2 % bisacrylamide) in 1× TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA pH 7.4), with 100 % denaturant containing 7M urea and 40 % *v/v* formamide. We made a denaturing gradient, ranging from 45 % to 60 %. An internal marker was used to align different DGGE gels. Electrophoretic separation was performed on the INGENYphorU (Ingeny) for 16 h at 120 V in 1× TAE buffer at 60 °C. The gels were stained using SYBR Green (1:10,000 dilution, FMC BioProducts) in 1× TAE for 20 min and visualized by UV transillumination (Vilbert Lourmat).

The patterns of DGGE bands were further analyzed using BioNumerics software version 6.0 (Applied Math, Belgium). Clustering for the banding patterns was based on Dice correlation coefficient, in the BioNumerics software. The community richness (R) was calculated based on the total number of bands. The community organization (Co) was calculated by means of the Gini coefficient, calculating the normalized area between the Lorenz distribution and the perfect evenness line. In other words, a low Co number represents a community with a high evenness or no specific dominant species (Marzorati et al., 2008). Statistical differences between the groups of bumblebee queens in richness and community organization were analyzed using ANOVA and Tukey post-hoc test.

6.3.4. Identification of the bacteria by use of MiSeq Illumina sequencing

In order to identify the bacteria occurring in the ovaries of the queens, we analyzed 9 ovaries from bumblebees in groups C and D, as these were fully developed, while in groups A and B the ovaries were not or less developed.

The hypervariable V4 region (254 bp) of the 16S rDNA was amplified in triplicate, using the 515F and 806R primers designed by Caporaso et al. (2011). Sample preparation and Illumina sequencing were performed as described in chapter 4. Sequences derived from the Illumina MiSeq sequencing were analyzed with the mothur software v. 1.31.1 (Schloss et al., 2009), mainly following the standard operating procedure available on http://www.mothur.org/wiki/MiSeq_SOP, date December 2013. The raw data are publicly available on NCBI's Sequence Read Archive (SRA) under accession number SRP068799. The analysis of the Illumina data was performed as described in chapter 4. The reads of the samples were calculated in percentages, expressing the relative abundance of each operational taxonomic unit (OTU). The taxonomic identity of each OTU was revealed by alignment of each sequence with the Bacterial SILVA SEED database. In this analysis we chose to retain the OTUs that were represented by more than 0.5 % of the reads. Community richness was calculated with the Chao1 estimator (alpha diversity) and community diversity with the Shannon index (beta diversity). The bacterial evenness (*e*) was calculated as $e = H/\ln S$, where H is the Shannon-index and S is the number of OTUs.

6.3.5. Pathogen detection

In order to investigate if failed colony success could be correlated with the presence of pathogens, we screened the DNA-extractions of the gut on the presence of *Apicystis*, *Nosema* and *Crithidia* by use of PCR (Table 6.1). The presence of *Apicystis bombi* was checked using primers NeoF and NeoR and for the pathogen *Crithidia bombi* primers SEF and SER were used. The PCR mix and the protocol were followed as described in Meeus et al. (2010). To screen for the presence of the *Nosema* species, a multiplex PCR was used

with forward primer UF reverse primers Ra, Rc and Rb for respectively *N. apis*, *N. ceranae, N. bombi*. (Table 6.1). The PCR mix was prepared with 15 µL nuclease free water, 2.5 µL of 10×PCR buffer, 0.75 µL of 50 mM $MgCl_2$, 0.5 µL of 10 mM dNTPs, 0.25 µL of 5 U/µL Taq DNA Polymerase (Life Technologies, Belgium), 1.25 µL of each primer (10 µM), and 1µL template DNA. The PCR protocol was as follows: 2 min at 95 °C, 35 amplification cycles of 30 s at 95 °C, 30 s at 60 °C, 1 min at 72 °C and a final step of 2 min at 72 °C. During the PCR, a negative control (water sample) and a positive control was used for each pathogen. The samples used as positive controls were present in the bee disease diagnostic unit of the lab Agrozooloy (Ghent University).

Table 6.1. The bumblebee queens were screened for pathogens. The primers are shown with their sequences.

Pathogen	**Primer name**	**Sequence**
Apicystis bombi	Forward primer NeoF:	CCAGCATGGAATAACATGTAAGG
	Reverse primer NeoR:	GACAGCTTCCAATCTCTAGTCG
Crithidia	Forward primer SEF:	CTTTTGGTCGGTGGAGTGAT
	Reverse primer SER:	GGACGTAATCGGCACAGTTT
Nosema	Forward primer UF:	GGATTGTGCGGCTTAATTTGACTC
Nosema apis	Reverse primer Ra:	CCTCAGATCATATCCTCGCAGAAC
Nosema bombi	Reverse primer Rb:	ATTCTCGAATCAGGATTCTCTCAGAA
Nosema ceranae	Reverse primer Rc:	ACCACTATTATCATTCTCAAACAAAAAACC

6.4. Results

6.4.1. Ovary development

The mean values for each group regarding the ovary length and ovary mass are shown in Figure 6.1. An ANOVA revealed significant differences between groups in both ovary length (P<0.001; F=55.411) and ovary mass (P<0.001; F=50.171). A pairwise post-hoc Tukey test showed that only groups C (good colony development) and D (very good colony development) were similar to each other in ovary length (P=0.998) and ovary mass (P=0.997). All other pairwise comparisons between groups were significantly different (P<0.002) in both ovary length and ovary mass.

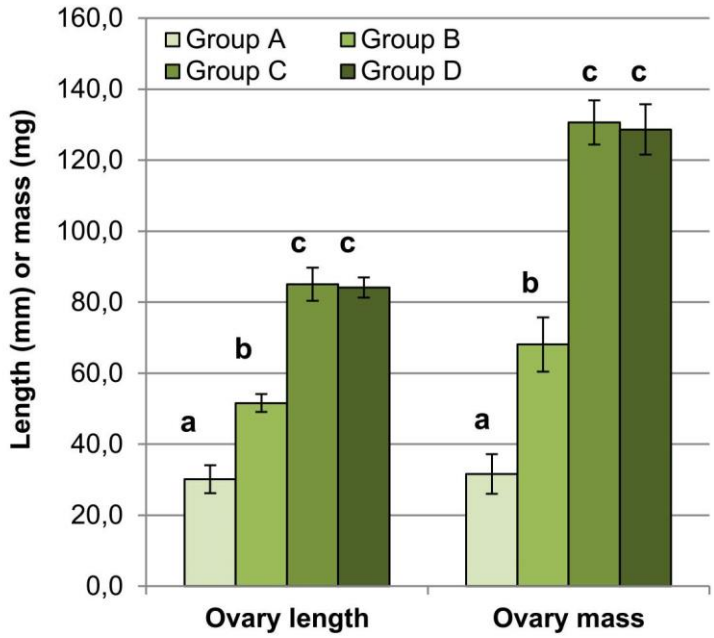

Figure 6.1. Ovary development, expressed by the length (mm) and mass (mg) of the bumblebee queen ovaries for each group (n=15/group). The error bars represent the standard error. Significant differences are indicated by different letters above the charts.

6.4.2. Bacterial fingerprinting by use of DGGE

The bacterial profiles of the bumblebee gut showed no clear clustering between groups (Figure 6.2), and no significant differences were observed between groups in richness (*P*=0.859; *F*=0.322) or community organization (*P*=0.158; *F*=1.950) (Figure 6.3 A, B).

For the bumblebee queen ovaries, an ANOVA revealed no significant differences in the community organization (*P*=0.163; *F*=1.795) (Figure 6.3 D), but there was a significant difference between groups in richness (*P*=0.002; *F*=5.997). A Tukey post-hoc test showed a significant lower richness in group C, compared to group A (*P*=0.012) and group B (*P*=0.003) (Figure 6.3 C). This correlates to the clustering of the DGGE profiles, as one of the clusters did not contain any samples of groups A or B but only contained samples of groups C and D with a lower richness (Figure 6.4). Apart from this observation, the DGGE lanes of group A did not show any bands that did not occur in the other groups and also groups C and D did not have bands that seemed specific for good colony success. Overall, the ovaries harbored a higher richness and community organization, compared to the gut.

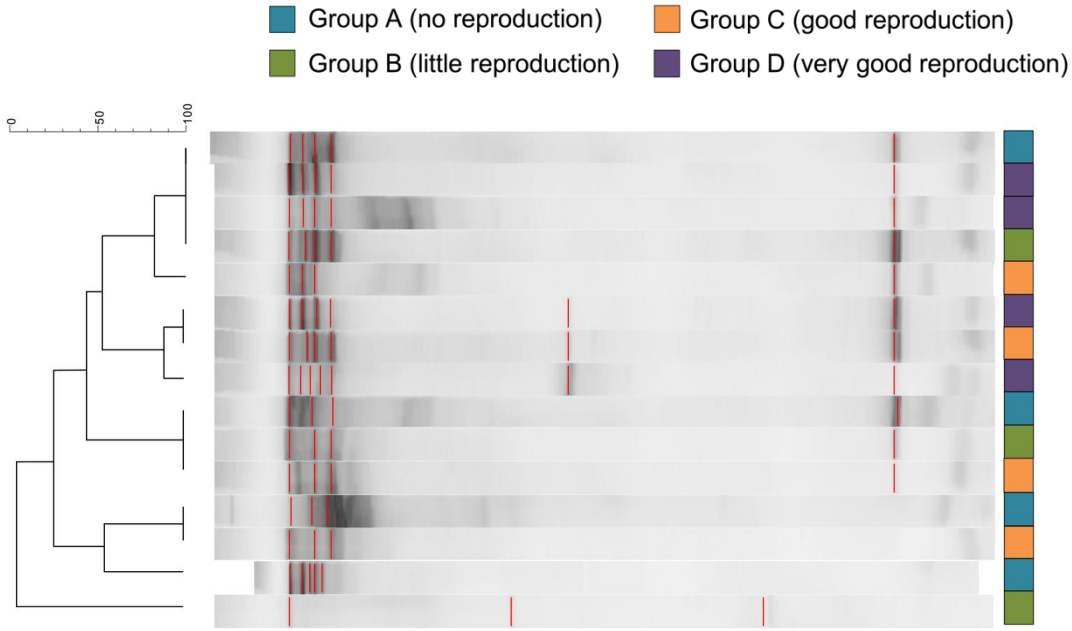

Figure 6.2. DGGE clustering of the bacterial patterns from the gut of the bumblebee queen (n=4/group)

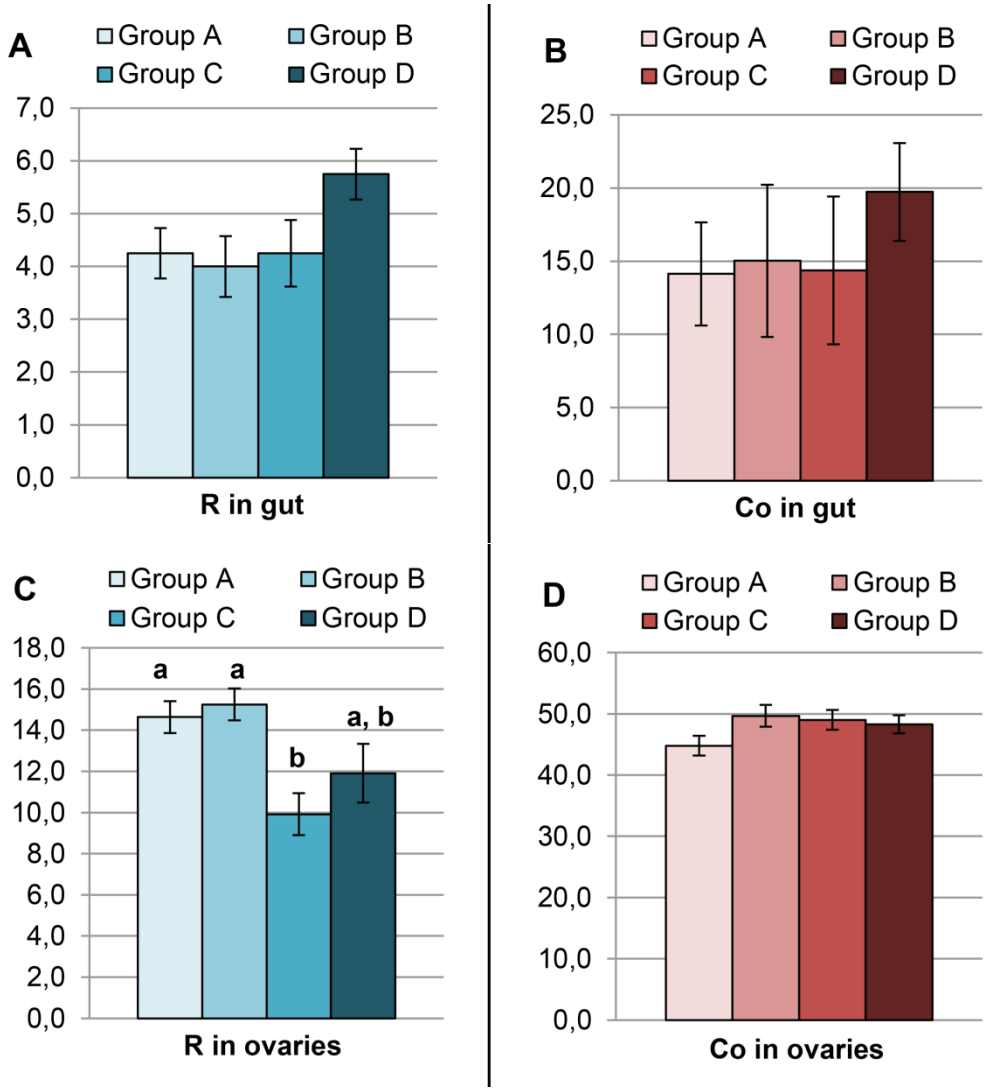

Figure 6.3. **(A)** Mean of richness R of each group for the gut (n=4/group). No significant differences were observed in this parameter. **(B)** Mean of community organization Co of each group for the gut (n=4/group). No significant differences were observed in this parameter. **(C)** Mean of richness R of each group for the bumblebee queen ovaries (n=12/group) observed on the DGGE. The different letters above the charts indicate significant differences between groups. **(D)** Mean of community organization Co of each group for the ovaries (n=12/group) on the DGGE. No significant differences were observed in this parameter.

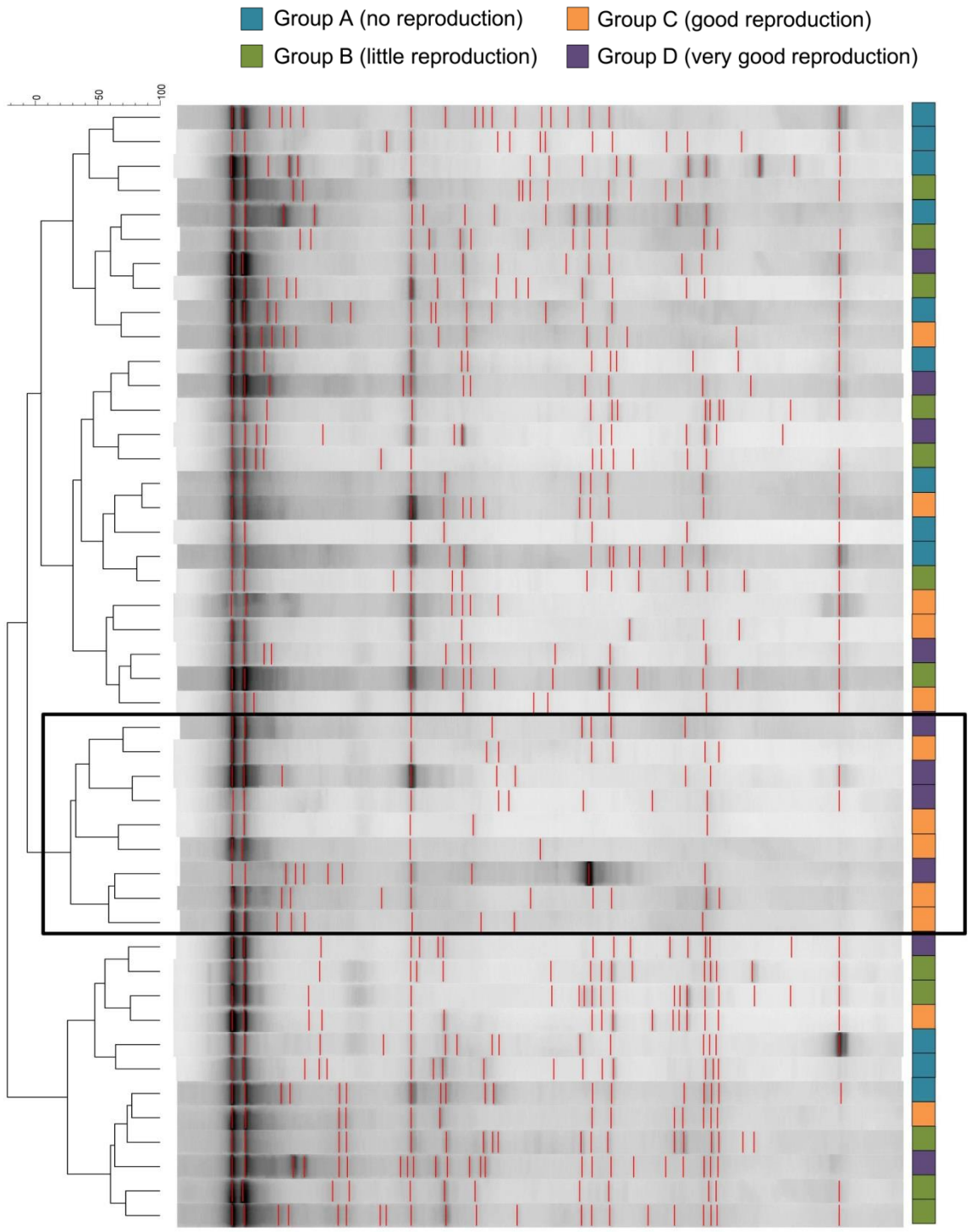

Figure 6.4. DGGE clustering of the bacterial patterns from the ovaries of the bumblebee queen (n=12/group)

6.4.3. Identified OTUs

We chose to identify the bacterial composition of ovaries from groups C and D, as groups A and B did not show clear differences with the successful groups C and D, based on the DGGE gels. Besides this, groups C and D showed similar ovary development, and thus possible differences in bacterial composition would not be due to differences in the amount of tissue. The taxonomic identification of the bacterial species occurring in the ovaries of the bumblebee queens from groups C and D is presented in Table 6.2. As no significant differences (GLM, Poisson distribution, P=0.522) between the bacterial composition of samples from group C or group D were observed, the average relative abundances were calculated on the samples from groups C and D together. The main bacteria detected in the ovaries were *Bradyrhizobiaceae*, *Snodgrassella*, *Gilliamella*, *Enterobacteriaceae*, *Lactobacillus*, *Staphylococcaceae*, *Bifidobacterium* and *Corynebacteriaceae*.

6.4.4. Pathogen detection

None of the indoor-reared bumblebee queens showed positive signals for any of the screened pathogens *Nosema*, *Crithidia* and *Apicystis*.

Table 6.2. Taxonomic identification of OTUs and their closest match in GenBank and EzBioCloud with the relative abundance in the ovaries (n=9) of reared bumblebee queens of groups C and D. The names indicated with gray, are also found in the gut of reared bumblebees in earlier studies.

Phylum Class Order *Family*	*Genus species*	**Otu**	**Relative abundance (%)**
Proteobacteria Alphaproteobacteria			
Rhizobiales *Bradyrhizobiaceae*		Otu012	1.03 ± 0.28
Hyphomicrobiaceae		Otu024	0.34 ± 0.18
Methylobacteriaceae		Otu158	0.08 ± 0.08
Rhodobacterales *Rhodobacteraceae*		Otu065	0.11 ± 0.11
Sphingomonadales *Sphingomonadaceae*		Otu018	0.09 ± 0.09
Proteobacteria Betaproteobacteria			
Neisseriales *Neisseriaceae*	*Snodgrassella alvi*	Otu001	34.53 ± 3.09
Burkholderiales *Burkholderiaceae*		Otu058	0.06 ± 0.06
Comamonadaceae 1		Otu021	0.07 ± 0.07
Comamonadaceae 2		Otu022	0.62 ± 0.24
Rhodocyclales *Rhodocyclaceae*		Otu144	0.16 ± 0.10
Proteobacteria Gammaproteobacteria			
Orbales *Orbaceae*	*Gilliamella apicola*	Otu003	18.26 ± 2.46
Enterobacteriales *Enterobacteriaceae* 1		Otu008	3.85 ± 0.56
Enterobacteriaceae 2		Otu010	4.42 ± 3.69
Pseudomonadales *Moraxellaceae*		Otu016	0.19 ± 0.14
Firmicutes Bacilli			
Lactobacillales *Lactobacillaceae*	*Lactobacillus bombi*	Otu005	5.56 ± 0.69
Lactobacillaceae	*Lactobacillus bombicola*	Otu004	2.85 ± 0.48
Lactobacillaceae	*Lactobacillus kunkeei*	Otu189	0.14 ± 0.09
Streptococcaceae		Otu020	0.55 ± 0.19
Bacillales *Staphylococcaceae*		Otu009	9.65 ± 4.83
Paenibacillaceae		Otu541	0.11 ± 0.08
Actinobacteria Actinobacteria			
Bifidobacteriales *Bifidobacteriaceae*	*Bifidobacterium commune*	Otu002	4.58 ± 0.67
Bifidobacteriaceae	*Bombiscardovia coagulans*	Otu006	0.57 ± 0.16
Actinomycetales *Corynebacteriaceae* 1		Otu015	1.17 ± 0.37
Corynebacteriaceae 2		Otu076	0.06 ± 0.06
Micrococcaceae 1		Otu040	0.25 ± 0.17
Micrococcaceae 2		Otu029	0.11 ± 0.07
Intrasporangiaceae		Otu025	0.11 ± 0.11
Bacteroidetes Flavobacteria			
Flavobacteriales *Flavobacteriaceae*		Otu152	0.07 ± 0.07
Sphingobacteriales *Chitinophagaceae*		Otu033	0.78 ± 0.25
Cytophagaceae		Otu129	0.09 ± 0.09

6.5. Discussion

Bumblebee queens with a good reproduction (group C) and a very good reproduction (group D) showed a lower bacterial community richness in the ovaries than bumblebees with less successful reproduction (groups A and B). One explanation could be that certain bacteria were less abundant, creating opportunities for other bacteria, resulting in the higher bacterial diversity, but responsible for the lower colony success. However, no specific bands could be detected in the analysis. We rather speculate that the detected difference in microbial community richness is a consequence of the different physiological status of the ovaria, as the successful queens had a fully developed microbiota and the ovaries of unsuccessful queens were not or very little developed. Although the ovary mass and length were clearly correlated with the colony success of the bumblebee queens, we could not clearly elucidate the differences in colony success based on the clustering and the bacterial profiles of the DGGE. The lack of reproduction can however still have many other reasons, such as problems with yolk synthesis, the juvenile hormone, the social environment, age and the pollen quality (Bonhag, 1958; Bloch et al., 2000; Human et al., 2007), although the last three options are expected to be controlled in a rearing facility. Besides these, it is also known that pathogens can have an impact on the reproduction (Hurd, 2001), and therefore we screened for the presence of the most common bumblebee pathogens. None of our samples were infected with *Nosema*, *Crithidia* or *Apicystis*, which was expected, knowing that the bumblebee queens came from a rearing facility. Another pathogen, the nematode *Sphaerularia bombi*, is known to infect several species of *Bombus* queens while hibernating underground (Poinar & Van Der Laan, 1972; Sayama et al., 2007; Plischuk & Lange, 2012; Kadoya & Ishii, 2015). This parasite infects and sterilizes the overwintering queen (Poinar & Van Der Laan, 1972). We did not screen on its presence in our samples, given that this parasite is not present in rearing facilities, due to the fact that it requires to molt to the adult stage in the soil in order to infect new hibernating queens (Poinar & Van Der Laan, 1972). Moreover, it would be noticed in standard quality control tests of the rearing company, should it occur.

Our analysis could not correlate any specific bacterial species with the ovary development, however about one third of the relative abundance of the bacteria identified in the ovaries was not previously detected in the bumblebee gut microbiota. Although the bacterial genus *Wolbachia* is often found in the reproductive system of arthropods (O'Neill et al., 1993; Zchori-Fein et al., 1998; Kozek & Rao, 2007; Hosokawa et al., 2010), we did not detect the presence of any typical endosymbionts, such as *Wolbachia* in the ovaries of reared bumblebee queens. The other two third of the relative abundance in the ovaries, was covered by bacteria that were previously described as the core gut microbiota of *Bombus terrestris* (Meeus et al., 2015), namely *Snodgrassella*, *Gilliamella*, *Lactobacillus* and *Bifidobacterium*. The detection of the same core bacteria in different tissues of the insect species is not uncommon. For example in the red palm weevil, several bacteria were isolated from the hemolymph, testis, gut and the ovaries using both culture-independent as culture-dependent methods. Although there were differences in the abundance of the bacteria between the different tissues, all of them mainly harbored the same set of bacteria: Actinobacteria (Actinomycetales), Alphaproteobacteria (Rhodospirillales), Betaproteobacteria (Burkholderiales), Firmicutes (Bacillales and Lactobacillales), Gammaproteobacteria (Enterobacteriales, Pseudomonadales and Xanthomonadales) and Bacteroidetes (Cytophagales) (Montagna et al., 2015). Also the Malaria mosquito showed the presence of a core microbiota in the midgut, the salivary glands as well as the ovaries, which is represented by Gammaproteobacteria (*Acinetobacter*, *Pseudomonas*, and members of the *Enterobacteriaceae* family), Betaproteobacteria (*Comamonas*, *Burkholderia*) and Alphaproteobacteria (*Rhizobium*) (Tchioffo et al., 2016).

The presence of bacteria in tissues is usually linked with certain functionalities. Gut bacteria of social bees have been described to contribute in pollen digestion, the fermentation of sugars, detoxification of food components and pathogen protection (Engel et al., 2012; Engel & Moran, 2013). It remains difficult to determine the functionality of these bacteria in the

ovaries at this point. The large fraction of the typical core bacteria could suggest a transovarial transmission route ensuring transmission of bacteria between the queen and her offspring. However the mechanism behind it remains undetermined and examples of transovarial transmission are usually described when discussing endosymbionts in insects (Mira & Moran; Hosokawa et al., 2007). Besides transovarial transmission, the microbiota in the ovaries could also play a role in the pathogen protection as the endogenous bacteria compete with possible incoming pathogens. For example, fruit flies that were injected with pathogenic bacteria displayed degenerated ovaries and more importantly these pathogenic bacterial species were able to colonize the ovaries (Brandt & Schneider, 2007). Some bacteria have been reported to aid in pathogen protection, such as LAB which can synthesize bacteriocins, H_2O_2 and organic acids creating an environment with low pH (Lahtinen et al., 2011). Also *Snodgrassella* and *Gilliamella* were correlated with pathogen protection in earlier studies (Martinson et al., 2012; Kwong et al., 2014).

We can conclude that this study contributes in the research of microbial communities in tissues of insects, more specifically bumblebee queens. The ovaries of less successful queens were clearly less developed than successful queens, but this could not be linked with the bacterial profiles occurring in the ovaries. The core bacteria previously described in the bumblebee gut were also detected in the ovaries, along with small fractions of other bacteria that were not yet reported in bumblebees before.

Chapter 7.

Bacterial community in the fat body and the gut of wild *Bombus terrestris* and the correlation with the prevalence of *Apicystis bombi*

Redrafted from: Billiet, A.°, Parmentier, A.°, Smagghe, G., Vandamme, P., Deforce, D., Van Nieuwerburgh, F., Meeus, I. (*in preparation*) Bacterial community in the fat body and the gut of wild *Bombus terrestris* and the correlation with the prevalence of *Apicystis bombi*

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7.1. Abstract

Symbiosis between the insect host and its microbiota is mostly studied in relation with the digestive tract, while there are multiple habitats for microorganisms on and within a single insect. Here we studied the microbial communities residing in the gut and fat body of wild bumblebees (*Bombus terrestris*) using V4 16S rRNA sequencing with the Illumina MiSeq technology. Foraging bumblebees were caught at two locations (Sterre and Bramier) in Belgium and their infection status with the neogregarine *Apicystis bombi*, which resides in the fat body, was determined. Although common OTUs (Operational Taxonomic Unit) were identified in both the gut and fat body microbiota, the fat body microbiota also harbors multiple unique OTUs. For instance a fat body specific OTU, having a closest match with the genus *Phyllobacterium*, has never been described before in insects. The fat body microbiota was correlated with its sampling location and with *Apicystis bombi* infection and *A. bombi*-infected bumblebees had positive correlations between the OTUs residing in the gut and the fat body. The OTU identified as *Arsenophonus* had a location-dependent interaction with *A. bombi* infection.

7.2. Introduction

Animals are characterized by ubiquitous bacterial communities residing across multiple host niches. Depending on the chemical, biological and physical conditions of the environment they inhabit, bacterial strains or species can survive and modify the habitat to create their own micro-environment. The intimate association between host and symbiont, defined as symbiosis, is marked by a series of complex interactions (Moran, 2006). This range of symbiotic relationships can take place at particular sites inside and outside the insect's body cavity. Outside the insect's body cavity, for example in the gut, microbial communities appear with associated functionalities (Engel & Moran, 2013), while inside the insect's body cavity it is not a community, but rather individual bacteria. These bacteria can be obligate and facultative symbionts, colonizing the cytosol of host cells, called bacteriocytes. Although their interactions with the host can range from mutualistic to parasitic, a context-dependent interaction may still exist. For example, *Hamiltonella defensa* inhibits the parasitic wasp egg development in aphids, but also has trade-offs toward reduced fecundity of its host (Vorburger & Gouskov, 2011). Therefore, to understand insect-microbe interactions, the dependency on the environmental conditions needs to be considered. Especially the presence of potential parasitic organisms is an important factor determining the host-microbe interactions.

Here we study the host-microbe interactions in a social insect, the bumblebee. It has been argued that the social life style of bumblebees enables a specific "core" microbiota and predisposes them to coevolution (Kaltenpoth, 2011). We expand the current focus on the gut microbiota when studying host-parasite interactions in bees. We hypothesize that a bacterial community is present within the insect's body cavity and that context-dependent conditions determine the composition of this symbiotic interaction. Recently, the presence of bacterial communities in different body tissues has been described in other invertebrates, for example in the haemolymph and body tissues of aquatic invertebrates (Lokmer et al., 2015; Lokmer & Wegner, 2015; Wang & Wang, 2015), in the red palm weevil (Montagna et al., 2015) and the Malaria mosquito (Tchioffo et al., 2016).

We analysed both gut and fat body microbial communities in two landscape contexts and explored the relation with the neogregarine parasite *Apicystis bombi*, which preferentially resides in fat body tissues (Lipa & Triggiani, 1996). The sporozoites of *Apicystis* emerge in the intestine, penetrate through the midgut wall into the body cavity and infect the fat body cells, in which they grow, develop and multiply (Lipa & Triggiani, 1996). Associations between the fat body microbiota and its host infection status and/or environment will broaden the view on bacterial niches and functionalities within insects.

7.3. Materials and methods

7.3.1. Bumblebees

Foraging wild bumblebees (*Bombus terrestris*) were caught at two locations in Belgium. A first site, called Sterre, is located in the city of Ghent (51.05 N, 3.71 E). At this location 17 bumblebees were sampled in an urban environment. The second location, called Bramier, is a small natural domain in a more suburban environment of Menen (50.79°N, 3.12°E), where 18 bumblebees were caught. The sampled bumblebees were put individually in falcon tubes and frozen (-20 °C) within the 2h after collection. Bumblebees were caught at both locations on a regular basis during June and July 2014.

7.3.2. Dissection and DNA extraction

The surface of the bumblebees was washed with bleach (1:20) and 1x PBS before dissection, to avoid external contamination of the inner tissues. Both the fat body and the gut (mid- and hindgut) were dissected, using disinfected material. Bumblebees from both locations were dissected in no particular order, and the gut was dissected very carefully to avoid contamination of the fat body. To extract bacterial DNA, each tissue was individually crushed in a 170 µL lysozyme solution (100 mg/mL) and DNA was extracted as described in Meeus et al. (2013).

7.3.3. Prevalence of *Apicystis bombi*

A PCR was performed on the DNA extractions of the fat body to screen for infection with *A. bombi*. Universal primers NeoF and NeoR were used and the PCR mix and program were followed according to Meeus et al. (2010). PCR products were analyzed by electrophoresis in 1.5 % (*w*/*v*) agarose gel, stained with ethidium bromide. The correlation between the prevalence of *A. bombi* and the location was statistically analyzed using a Pearson Chi-Square test.

7.3.4. Identification of the bacteria by use of MiSeq Illumina sequencing

To identify the bacteria occurring in both the gut and the fat body of the bumblebees, the hypervariable V4 region (254 bp) of the 16S rRNA was amplified in triplicate, using the 515F and 806R primers designed by Caporaso et al. (2011). Sample preparation and Illumina sequencing were performed as described in chapter 4. Sequences derived from the Illumina MiSeq sequencing were analyzed with the mothur software v. 1.31.1 (Schloss et al., 2009), mainly following the standard operating procedure available on http://www.mothur.org/wiki/MiSeq_SOP, date December 2013. The raw data are publicly available on NCBI's Sequence Read Archive (SRA) under accession number SRP069342. We followed the standard operating procedures to optimize the quality of our dataset: at this point the dataset of the gut microbiota contained 3,521,553 demultiplexed paired-end reads, representing 448,669 unique sequences. The dataset of the fat body microbiota contained 1,844,860 demultiplexed paired-end reads, representing 342,182 unique sequences. Further analysis of the Illumina data was performed as described in chapter 4. Calculating the distance matrix and clustering with a 0.03 cut-off level, the dataset of the gut microbiota retained a total of 2,967,992 reads whereof 7,206 unique sequences and resulted in 330 OTUs. For the dataset of the fat body microbiota, this retained a total of 1,303,156 sequences whereof 6,140 unique sequences and resulted in 1,176 OTUs. In this analysis we chose to retain the OTUs that were represented by more than 0.5 % of the reads. For the gut microbiota, this step resulted in 30 OTUs covering 99.1 % of the reads of the 330 OTUs and

for the fat body microbiota this resulted in 57 OTUs covering 94.4 % of the reads of the 1,176 OTUs. For further analysis, the reads of the samples were calculated in percentages, and thus expressing the relative abundance of each OTU. Community richness was calculated with the Chao1 estimator and community diversity with the Shannon index. The effect of location, *A. bombi* infection and the interaction between the location and *A. bombi* on the Chao 1 estimator and Shannon index were studied with a two-way ANOVA in R. The effect of the parameters location and prevalence of *A. bombi* on the bacterial abundance data was analyzed using GLM, Poisson distribution in the mvabund package, as described in Wang et al. (2012). Heatmaps were created in R studio, using R packages gplots and RColorBrewer. Correlations between OTUs were inferred by Sparse Correlations for Compositional data (SparCC) (Friedman & Alm, 2012), implemented in mothur software v.1.31.1. The microbial association networks were drawn with the network analysis and visualization R package igraph. The percentage of positive edges (PEP) was calculated, with positive edges representing $|r|>0.3$ and $P<0.01$ over all possible edges (Faust et al., 2015).

Sequences were identified using EzTaxon (EzBioCloud), searching the database of both cultured and uncultured bacteria. Bacteria were identified to the level upon which they could be identified with 100 % similarity. This was usually genus or species level. When there was no 100 % match available in the database, the closest match was given and indicated with the number of matching base pairs (xxx/253), as shown in Table S4.

7.4. Results

7.4.1. *Apicystis bombi* prevalence in two locations

At location Sterre, 12 of the 17 bumblebees were infected with *A. bombi*, and 9 of the 18 bumblebees were infected at location Bramier. A Pearson Chi-Square test revealed no significant correlation ($P=0.214$) between location and the prevalence of *A. bombi*.

7.4.2. Interaction of microbiota, location and *Apicystis bombi* infection

The correlation between the sampling location, *A. bombi* infection status, and the global bacterial community composition of both gut and fat body, was examined by a multivariate analysis (GLM, Poisson distribution). To explain changes in the microbial community, a two-way ANOVA was performed on the community richness and community diversity and a univariate analysis (GLM, Poisson distribution) was performed on the bacterial OTUs. Heatmaps were used to visualize the different microbial profiles occurring in the gut and the fat body.

7.4.2.1. Microbiota in the gut

30 bacterial OTUs were detected in the gut of the wild bumblebees, which are listed with their average relative abundances and standard errors in the supplemental data Table S4.

A multivariate GLM demonstrated that differences in the overall composition of the gut microbiota were significantly correlated with the location ($P_{location}$=0.001; *Dev*=670.0), while there was no significant correlation with *A. bombi* infection ($P_{infection}$=0.114; *Dev*=334.8). Here it is important to mention the significant interaction between location and infection status on the bacterial composition in the gut ($P_{location \times infection}$=0.001; *Dev*=487.9). The overall location effect was also visible in the univariate GLM analysis, as there was a significant correlation between location and the relative abundance of *Arsenophonus* (otu19) ($P_{location}$=0.001; *Dev*=221.284) in the gut, which was much more abundant at location Bramier, compared to location Sterre. The overall interaction effect was detectable when the diversity parameters were calculated in the gut, with the community richness (Chao1 estimator) ($P_{location \times infection}$=0.045; *F*=4.353) and community diversity (Shannon index) ($P_{location \times infection}$=0.060; *F*=3.826). At the location Sterre, the microbiota of non-infected bumblebee guts had a higher community richness compared to infected samples (*P*=0.006), while no differences were observed at the location Bramier (*P*=0.635) (Figure 7.1 A). The community diversity had a similar tendency, as only in location Sterre there was a

significantly higher community diversity (*P*=0.006) in samples that were not infected with *A. bombi* (Figure 7.1 B).

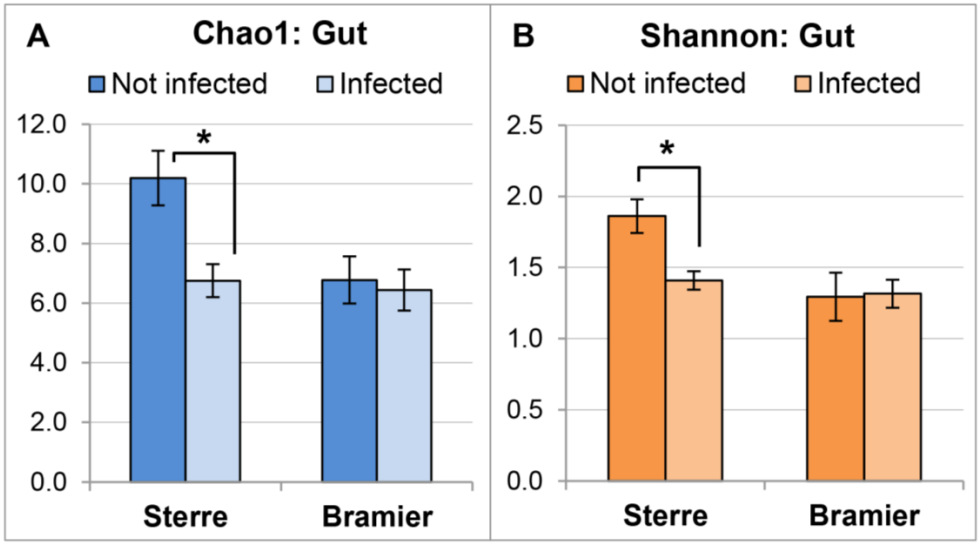

Figure 7.1. (A) Chao1 estimator (community richness) and (B) Shannon index (community diversity) in the gut of bumblebees for both locations. Statistical differences are indicated with *****.

A heatmap of the 13 most abundant gut bacterial OTUs displays a visual representation of two microbial community profiles. A first profile was defined with samples in which the microbiota was dominated by *Snodgrassella* and *Gilliamella*. A second profile gathered different branches which were not dominated by *Snodgrassella* and *Gilliamella*, but either *Arsenophonus* (otu19), *Pseudomonas* (otu29), *Lactococcus* (otu45), *Fructobacillus* (otu54) or *Saccharibacter* (otu05) had a higher relative abundance instead (Figure 7.2). At location Sterre, 13 out of 17 samples belonged to the *Snodgrassella* and *Gilliamella* dominant profile, while at Bramier 10 of the 18 samples were dominated by *Snodgrassella* and *Gilliamella*. However, no significant correlation between these two microbial profiles and the location was retrieved (*P*=0.193). Also no significant correlation (*P*=0.884) was found between the prevalence of *A. bombi* and the two profiles.

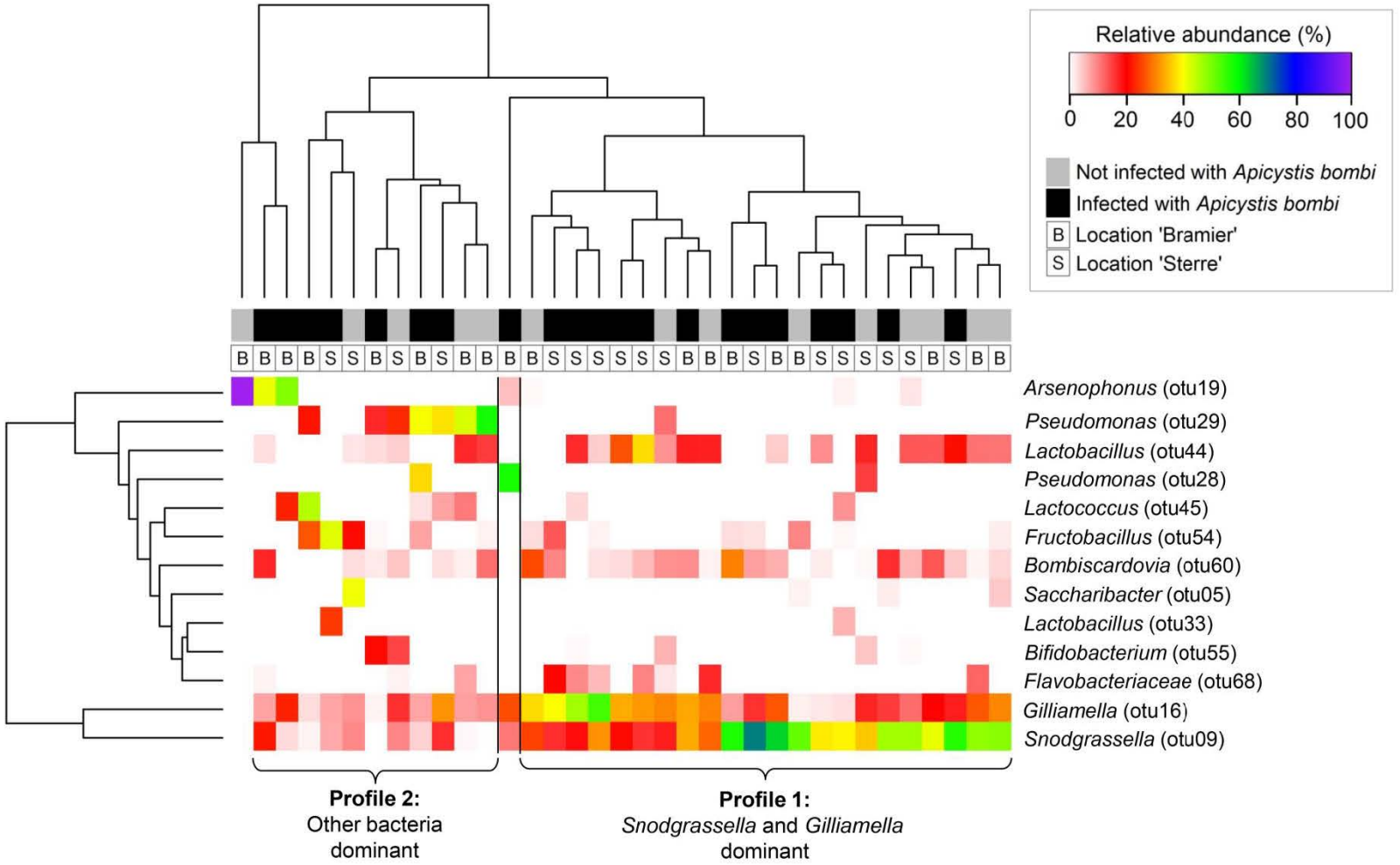

Figure 7.2. The heatmap shows the three bacterial profiles occurring in the gut of bumblebees from the sampling locations Bramier and Sterre. The heatmap was created with the most abundant bacteria in the gut.

7.4.2.2. Microbiota in the fat body

In the fat body, a total of 57 bacterial OTUs were detected, which are listed with their mean relative abundances and standard errors in the supplemental data Table S5.

A multivariate GLM (Poisson distribution) on the overall microbial composition showed a significant correlation with the location ($P_{location}$=0.001; *Dev*=1616.4), a significant correlation with the *A. bombi* infection status ($P_{infection}$=0.001; *Dev*=597.2) and a trend toward interaction of location and infection status on the microbiota in the fat body ($P_{location \times infection}$=0.087; *Dev*=146.5). The main effect of location in the multivariate analysis was also observed in the diversity parameters: location was correlated with the community richness (Chao1 estimator) ($P_{location}$=0.005; *F*=9.342) (Figure 7.3 A) and the community diversity (Shannon index) ($P_{location}$<0.001; *F*=16.719) (Figure 7.3 B).

Figure 7.3. (A) Chao1 estimator (community richness) and (B) Shannon index (community diversity) in the fat body of bumblebees for both locations. Statistical differences between locations are indicated with * for both the community richness and the community diversity.

The main effect of *A. bombi* infection status and the interaction of location and infection were not significant for these parameters. A univariate GLM (Poisson distribution) on the relative abundance data of the fat body microbiota revealed a significant correlation between the

location and the relative abundances of *Phyllobacterium* (otu02) ($P_{location}$=0.002; *Dev*=345.432), *Snodgrassella* (otu09) ($P_{location}$=0.004; *Dev*=87.276), *Gilliamella* (otu16) ($P_{location}$=0.002; *Dev*=123.193), *Arsenophonus* (otu19) ($P_{location}$=0.001; *Dev*=568.89), *Lactobacillus ozensis* (otu33) ($P_{location}$=0.036; *Dev*=39.173), *Lactobacillus* sp. (otu36) ($P_{location}$=0.014; *Dev*=47.007), *Lactococcus* (otu45) ($P_{location}$=0.004; *Dev*=74.733), *Convivina intestini* (otu53) ($P_{location}$=0.004; *Dev*=61.269) and *Bifidobacterium bombi* (otu55) ($P_{location}$=0.023; *Dev*=42.919).

The heatmap (Figure 7.4) with the most abundant bacteria revealed several profiles based on the differences in relative abundances of these bacteria. The first profile contained samples in which *Snodgrassella* and *Gilliamella* were dominant. The second profile was not dominated by *Snodgrassella* and *Gilliamella* and was a collection of branches in which other bacteria were dominant, such as *Arsenophonus* (otu19), *Phyllobacterium* (otu02), *Lactococcus* (otu45), *Bifidobacterium bombi* (otu55), *Lactobacillus ozensis* (otu33), *Lactobacillus* sp. (otu36) or *Convivina intestini* (otu53). Three samples were an intersection of the first and second profile as both *Snodgrassella* (otu09) and *Gilliamella* (otu16) had comparable relative abundances with *Arsenophonus* (otu19) and *Phyllobacterium* (otu02). The above reported location effect was confirmed as 14 out of the 17 samples from Sterre belonged to the profile dominated by *Snodgrassella* and *Gilliamella*, while at location Bramier, 14 out of the 18 samples belonged to the second profile. A Pearson Chi-Square test demonstrated a significant correlation of the occurrence of a profile type with the location (P<0.001), but no significant correlation with the infection status with *A. bombi* (P=0.909). *Arsenophonus* had a location-dependent interaction with *A. bombi*, as only in the location Bramier a higher relative abundance of *Arsenophonus* was recorded in function of *A. bombi* infection (Mann-Whitney U; P=0.005; Z=-2.78) as shown in Figure 7.5.

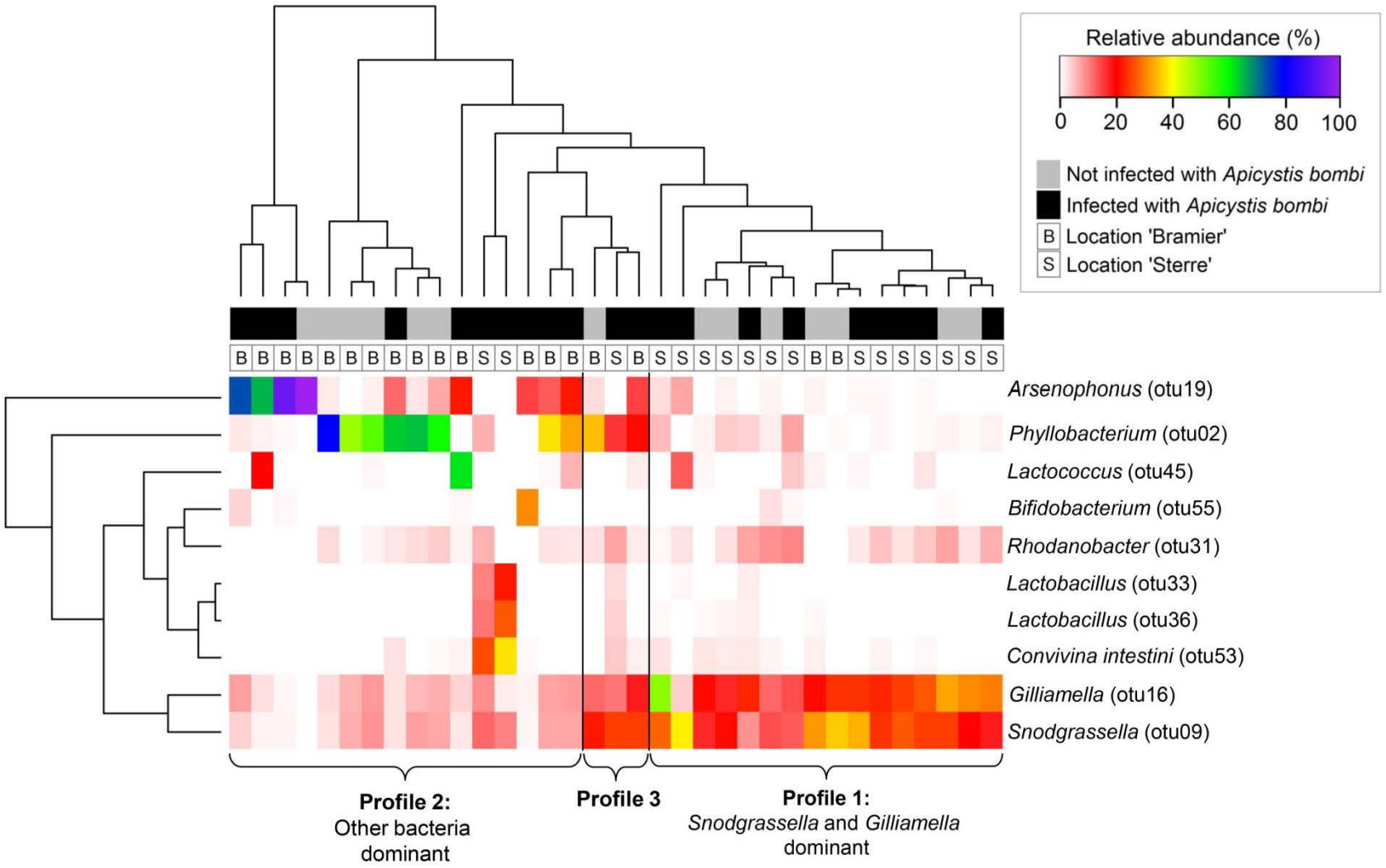

Figure 7.4. The heatmap shows the three bacterial profiles occurring in the fat body of bumblebees from the sampling locations Bramier and Sterre. The heatmap was created with the most abundant bacteria in the fat body.

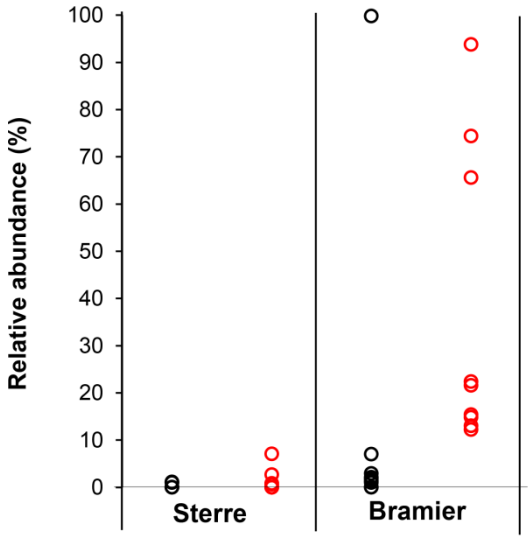

Figure 7.5. The relative abundance of *Arsenophonus* (otu19) was higher when infected with *A. bombi*. Black circles represent the relative abundance in samples that were not infected, while the red circles display the relative abundance in infected samples.

7.4.2.3. Microbial associations between bacteria in the gut and the fat body

The microbial association network in non-infected samples showed few significant associations between bacteria residing in the gut and the fat body (P<0.01 and |r|>0.3). The percentage of positive edges (PEP) was 0.8 % looking at all OTUs, of which 23 % represents interactions between OTUs in the gut and the fat body (Figure 7.6 A). In infected bumblebees, the PEP increased to 1.6 %, of which 43 % of the correlation were between OTUs from fat body and gut (Figure 7.6 C). When we restricted our analysis to those bacteria present in both tissues, we can infer that the most significant correlations were found between the same bacterial OTUs and again mainly present in *A. bombi*-infected bumblebees (Figure 7.6 B and 7.6 D). This demonstrates an association of the gut bacteria with fat body bacteria for *A. bombi*-infected bumblebees, in contrast to non-infected bumblebees.

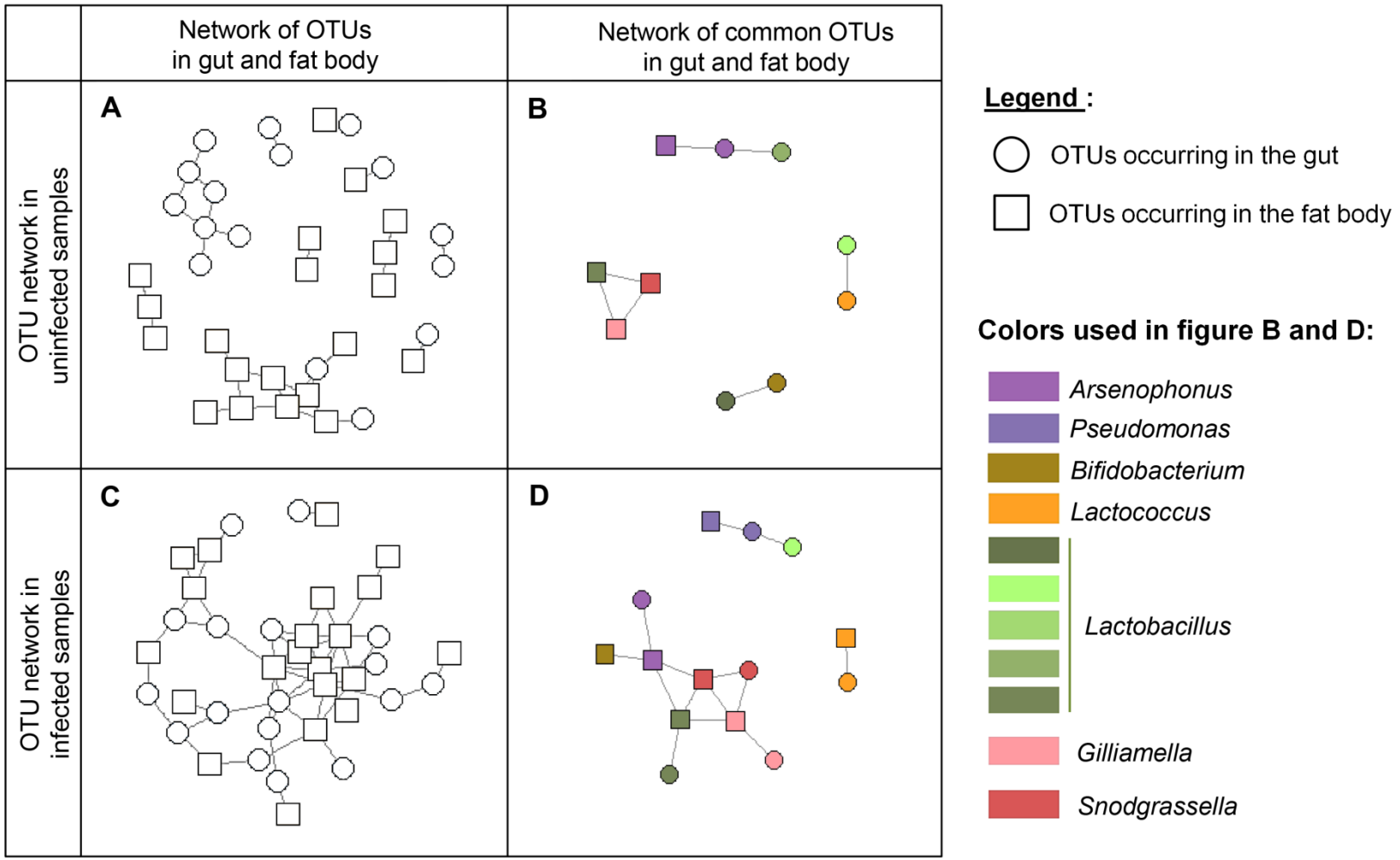

Figure 7.6. Microbial association network of OTUs in uninfected samples (**A** and **B**) and in samples infected with *A. bombi* (**C** and **D**). (**A** and **C**) OTU network for all OTUs ; (**B** and **D**) OTU network for the OTUs that occurred in both the gut and the fat body, the same color represents the same OTU. Circles represent OTUs occurring in the fat body, squares represent OTUs occurring in the gut.

7.5. Discussion

7.5.1. The fat body of bumblebees, a niche for a specific bacterial community

Metazoan organisms are not autonomous entities, but rather holobionts, biomolecular networks composed of the host and its symbiotic microbes (Bordenstein & Theis, 2015). In analogy with vertebrates, the main studied symbiotic bacterial niches in insects are outside the hemocoel, which protects the sterile body tissues. In this study, we showed that the bumblebee carries a microbiota associated with the fat body, an adipose tissue located within the open circulatory system. Besides some overlap between the microbiota of the gut and fat body, the latter also harbored some unique bacteria. For instance, the OTU identified as *Phyllobacterium* was restricted to the fat body in both sampling locations and is probably adjusted to the specific micro-environment of the fat body. *Phyllobacterium* isolates are associated with plant rhizosphere or plant nodes (Mergaert et al., 2002; Valverde et al., 2005; Mantelin et al., 2006; Flores-Felix et al., 2013; Sanchez et al., 2014) and have never been observed within bees. The short 16S rRNA sequence reads (253 base pairs) showed a 100 % match with different *phyllobacteria*. Longer sequence reads or cultivation will be required to fully characterize this bacterium.

The role of bacterial symbionts in eukaryotic ecology and evolution was until now mainly focused on functionalities associated with the gut, with specific functions in nutrition, detoxification, protection against pathogens and the corresponding modulation of the immune response (Engel & Moran, 2013). The existence of a fat body microbiota opens new avenues, as the fat body is essential in the dynamic process of energy storage and release, important for example to survive non-feeding periods (Arrese & Soulages, 2010), like bumblebee queens face in hibernation periods.

7.5.2. The fat body microbiota has a closer association with the environment than the gut microbiota

Li et al. (2015a) defined two enterotypes in the bumblebee gut microbiota, based on their community richness, community evenness and microbial composition, after screening 142

workers of 28 species of Chinese bumblebees. Similar to the first enterotype of Li et al. (2015a), our study also showed a gut bacterial profile dominated by *Gilliamella* and *Snodgrassella*. The second enterotype defined by Li et al. (2015a) in the Chinese bumblebees was dominated by *Lactobacillus*, *Serratia* and *Hafnia*, while in our study in *Bombus terrestris* a dominance of *Arsenophonus*, *Pseudomonas*, *Lactococcus* or *Fructobacillus* was observed. As only the relative abundance of *Arsenophonus* was correlated with the sampling location, we can confirm the strong association between the gut microbiota and its host, a prerequisite to evolve a functional dependence.

A similar observation can be made for the fat body microbiota. The fat body was dominated by *Snodgrassella* and *Gilliamella* in the majority of the samples from location Sterre, while *Arsenophonus*, *Phyllobacterium* or *Lactococcus* showed the highest relative abundance in most samples from location Bramier. The fat body microbial composition is more associated with the sampling location and thus less associated with the host. This indicates a lower potential toward functional dependence of the host, compared to the gut microbiota.

7.5.3. The interaction between the microbiota and *Apicystis bombi*

In this chapter, the fat body microbiota differed not only between sampling locations, but also in function of *A. bombi* infection. The causality of this microbiota-pathogen interaction remains unknown at this point, but several scenarios are plausible.

A first hypothesis is that the composition of the fat body microbiota is driven by the *A. bombi* infection. *A. bombi* primarily infects the fat body tissue (Lipa & Triggiani, 1996). Infection could induce changes in the fat body microenvironment, which influences the viability of bacteria. Changes in pH, oxygen concentration, and nutrient resources influence the competition between bacteria and thus affect the bacterial composition. Also the fact that the *A. bombi* sporozoite migrates through the gut wall, could create opportunities for an exchange of bacteria between tissues. The microbiota in the fat body and the gut displayed more associations when bumblebees were infected, compared to non-infected samples. This probably indicates that *A. bombi* is facilitating gut bacteria to enter the fat body, or at least

creating an environment in which typical gut bacteria can survive. Here *A. bombi* could be a facilitator for opportunistic bacteria to infect and become pathogenic. The general concept of opportunistic infection is well described, where an infection can take place because of an impaired host immunity or microbiota. It remains to be determined which bacteria could inflict damage to the host. Besides this, also the fact that *A. bombi* can alter the fat body microbiota could enhance opportunistic pathogenic bacteria. Under stressful conditions, such as infection with *A. bombi*, opportunistic bacteria can transform into true pathogens.

A second hypothesis is that the *A. bombi* infection is driven by the microbial composition. Certain bacteria could play a role in the protection against *A. bombi* or enhance infection success of *A. bombi*. As the fat body microbiota proved to be location-dependent, a different environment could result in a different microbial protection, leading to a different pathogenicity of *A. bombi*.

Aside from being the cause or effect, in all cases, a microbial-related pathogenicity of *A. bombi* is probable. The detected association with the microbial community could also be important when describing the multi-host character of the parasite. Indeed *A. bombi* has been detected in several bumblebee species, honeybees and even solitary bees (Plischuk et al., 2011; Ravoet et al., 2014; Gamboa et al., 2015). The latter harbor a variable gut microbiota that is not related with the gut microbiota of corbiculate bees (Martinson et al., 2011) and thus an even wider variety of bacteria could potentially colonize the gut after *A. bombi* infection. Until now pathogenicity studies of *A. bombi* have been restricted to reared bumblebees (Graystock et al., 2015), but here we can conclude that the microbial community is an important factor to consider for pathogenicity effect of *A. bombi* in different hosts.

7.5.4. *Arsenophonus*: a candidate for a context dependent pathogenicity

In this chapter, the bacterium *Arsenophonus* is an important candidate that fits the concept of this context-dependent pathogenicity. *Arsenophonus* was mainly present at the location Bramier and its presence was associated with *A. bombi* infection. The genus *Arsenophonus* represents one of the richest and most widespread clusters of insect symbiotic bacteria. It

groups intracellular symbionts in insects, which vary from parasitic male-killers, to host-specific primary beneficial endosymbionts, to non-specific putative mutualists and plant pathogens (Thao & Baumann, 2004; Nováková et al., 2009). *Arsenophonus* has already been detected in the hemolymph of the parasitic *Varroa destructor* mites (Hubert et al., 2015) which could be vectors and/or reservoirs of (pathogenic) bacteria. *Arsenophonus* has been detected in honeybees as well, and Cornman et al. (2012) suggested that an increased presence of *Arsenophonus* was associated with honeybee hives that were affected by colony collapse disorder (CCD). Therefore, this bacterium could thus be associated with reduced colony performance. However, it remains speculative who, *Arsenophonus* or *A. bombi*, enables who and which of both is perhaps most pathogenic, if pathogenic at all, it is still clear that this prokaryotic/eukaryotic interaction could determine the bee's health.

Chapter 8.

Discussion and future perspectives

In this dissertation we searched a balance between applying current knowledge on microbial communities in bees and gaining new insight into its importance for the host. While the first chapters describe potential beneficial functions of LAB and how to influence the gut microbiota in reared bumblebees, the final chapters expand this viewpoint exploring the microbiota in other bee organs and their potential associated functionalities. In the discussion, we will follow a similar structure, first gathering all generated knowledge to evaluate the potential of optimizing the microbial community for bumblebee rearing, in order to finish with some new insights on bee-microbe interactions.

8.1. An optimized microbiota results in a fitter bee?

8.1.1 What does an optimal microbiota look like ?

In the recent years, whole genome sequencing has given more insight in the functionalities of bacteria, but at this point the interaction between microbe-host-environment is not understood well enough, in order to define the “optimal microbiota”.

Although there is evidence that severe dysbiosis results in a lower pathogen protection in bumblebees (Koch & Schmid-Hempel, 2011b), it is not yet proven that a slight lower or higher bacterial diversity is good or bad for bee hosts. Several concepts are possible when studying an ecosystem and this may also apply to a bacterial community:

(i) Several species have a similar function in the system and thus are primarily redundant. Addition of new species does not add anything new to the system, but species with a similar function can compensate the loss of other species. This means that a higher diversity might be more stable under fluctuating environmental conditions, and shows the context-dependent ‘redundancy’ (Barthlott et al., 2009). An example of a functionality covered by several bacteria in bumblebees is the carbohydrate metabolism, as *Gilliamella*, *Lactobacillus* and *Bifidobacterium* are all able to ferment sugars (Engel et al., 2012; Lee et al., 2015).

(ii) Each species makes a unique contribution to the ecosystem and the loss or addition of species causes detectable changes in the ecosystem (Barthlott et al., 2009). It is also possible that some bacteria might not have a direct impact on the host, but provide essential

nutrients or metabolites for other important bacteria (e.g. SFCA). This ecosystem without redundancies probably works well as long as the environmental conditions are stable.
(iii) The impact of the loss or addition of species depends on the environmental conditions (Barthlott et al., 2009). In a bumblebee context, this might apply for pathogen protection or detoxification of specific components in some kinds of pollen, nectar or pesticides.

Functional groups which are beneficial for the host are the most important and within these beneficial functional groups, redundant bacteria could be valuable. Possibly, the limited gut microbiota of reared bumblebees represents the minimal set of functionalities that functions well in a stable indoor environment. However it is not clear whether this limited set of bacteria is still sufficient in outdoor conditions. As these interactions are still poorly understood, we suggest to be careful not to lose any of the core gut bacteria in reared bumblebees, and some redundancy makes the microbial system more stable.

8.1.2 The microbiota and reproductive output of the reared bumblebee

The use of the antibiotic streptomycin (chapter 2) resulted in a shift in the composition of the gut microbiota towards *Lactobacillus* sp. and showed an increased reproduction and drone mass in the bumblebee microcolonies. However, it must be mentioned that the use of antibiotics should not be encouraged in rearing programs. It is rather a tool to alter bacterial communities or to eliminate endosymbionts in hosts (Pfarr & Hoerauf, 2006). The use of antibiotics is commonly known to result in undesirable long-term effects such as antibiotic resistance of pathogens (WHO; Smith, 2008). Antibiotics only result in a loss of bacterial species. In contrast, probiotics are able to enrich the diversity in the gut and are thus a safer option. In chapter 3, different lactobacilli and bifidobacteria were administered to improve the reproductive success of reared bumblebees. Here the results were less straight forward, because probiotics do not eliminate bacteria and the newly introduced bacteria have to compete with the endogenous bacteria for colonization spots in the gut. Chapter 3 represents a very first screening of the potential of *Lactobacillus* and *Bifidobacterium* strains in a context

of indoor-reared bumblebees. All used strains were previously detected in honeybees and bumblebees. Supplementation of *Lactobacillus kunkeei* and *Lactobacillus crispatus* to the pollen, resulted in a higher drone production, but colonization of these bacteria was not detected in the mid- and hindgut. Positive effects were either linked with the continuous supplementation or the nutritional quality of the pollen. Indeed, a trend to a lower reproductive output was noticed when bumblebees received high nutritional pollen supplemented with *L. kunkeei*, while increased colony performance was noticed when low nutritional pollen was provided. In a setting with optimal conditions as organized in rearing facilities, where good nutritional food is present *ad libitum* and bumblebees are not exposed to pathogens, it is probably hard to demonstrate positive effects on the bumblebee fitness by use of probiotics. The added value of the microbiota is rather to upgrade effects of less nutritional diets. As breeders often supplement pollen diets with other cheaper protein sources, the use of probiotics is a potential measure to support reproduction in a cost effective way.

This dissertation already revealed some potential effects of the use of *Lactobacillus* and *Bifidobacterium* strains, however there are still a lot of other aspects which have not yet been investigated: What is the colonization potential of strains in each of the different gut regions? What is the most efficient application method? Is the use of a mix with several strains better? What are the underlying mechanisms to clarify the effects of probiotics on a higher reproduction?

8.1.3 A broader perspective on the higher reproduction as a result of the streptomycin treatment

The streptomycin treatment in chapter 2 resulted in a higher number of drones and higher drone mass in the bumblebee colonies. Streptomycin is bactericidal and mainly inhibits growth of gram-negative bacteria and some gram-positive ones. The antibiotic showed to have a selective advantage toward *Lactobacillus* sp., as only this bacterium was detected in the guts of treated bumblebees. However, the antibiotic alters probably more than only the

gut microbiota of the adult bee. The positive effect on the reproduction could work on different levels. First, the higher reproduction in streptomycin-treated bees could be a result of a higher number of eggs produced by the dominant worker. Chapter 6 demonstrates that the ovaries also harbor a microbiota mainly consisting of the core bacteria. It is likely that also in this organ *Lactobacillus* sp. was the remaining bacterium after antibiotic treatment. An antibiotic such as streptomycin might eliminate the “negative” bacteria and retain the beneficial bacteria, such as *Lactobacillus*, in the gut as well as in the ovaries.

Second, the antibiotic added in the diet most probably also targets the larvae. The observed positive effect can be a result of a higher survival rate of the larvae during growth and pupation. LAB have already been associated with a range of health benefits, aiding in resistance to pathogens (Forsgren et al., 2010; Vasquez et al., 2012). Also the highest biomass growth is realized during the larval stage, hence an optimal microbiota in terms of nutrient provisioning will positively affect the reproductive output in microcolonies.

8.1.4 Effects of the microbiota on pathogen protection

The importance of the microbiota might prove to be more essential when bumblebees are exposed to unfavorable outdoor conditions. It is known that bumblebees with a heavily impaired microbiota are more susceptible to pathogen intrusion than bumblebees with the typical core gut microbiota (Koch & Schmid-Hempel, 2011b). The beneficial effects of LAB on the immunity of honeybees have already been demonstrated as they can improve the resistance against *Paenibacillus larvae* and *Melissococcus plutonius* (Forsgren et al., 2010; Vasquez et al., 2012). In this dissertation, the impact of the probiotic bacteria on the immune capacity of the reared bumblebees was not monitored. Besides the use of probiotics for a better pathogen protection, also other metabolites could prove useful as the organic acids produced by *Lactobacillus johnsonii* CRL1647 were able to reduce *Nosema* infection (Maggi et al., 2013; Audisio et al., 2015). Also some pollen are known to have antibacterial substances, antioxidant properties and are able to inhibit certain (human) pathogens (Graikou et al., 2011; Khider et al., 2013). These pollen have been proposed to use as a

supplement for humans. It is not known yet what the impact is on the gut microbiota and the immune capacity of bumblebees.

We believe that immune competence should be considered as a valuable endpoint to define the health of bumblebees. This is an important topic in regard with pathogen spillover, as commercial bumblebees have been suspected to be sources of pathogens spilling over toward native bees (Meeus et al., 2011; Arbetman et al., 2012; Graystock et al., 2013; Murray et al., 2013), which encourages breeders to produce pathogen-free bees.

8.1.5 Future perspectives

In order to provide better tools for bumblebee breeders to optimize the bumblebee microbiota, more insight is still needed in the functionalities of the bacteria in the different bumblebee organs. The focus could be on bacteria occurring in wild bumblebees, as our first results showed a higher colonization success when working with *Bombus* specific bacteria. Furthermore wild bumblebees harbor a more diverse collection of bacteria compared to reared bumblebees (Meeus et al., 2015). The aim should be to create a richer microbiota in reared bumblebees which remains stable indoors and outdoors and functions better in fitness and pathogen protection.

Three main axes need to be investigated: (1) bacterial assistance in nutrient provisioning, be it the release of proteins from the pollen, metabolizing different sugars, or the production of essential nutrients; (2) the detoxification potential of plant metabolites; (3) protection against pathogenic bacteria, protozoa or fungi. These topics can be studied on several levels: whole-genome sequencing can be implemented to provide insight in the metabolic pathways present in bacterial symbionts of the bumblebee and this can form a steady basis to develop a hypothesis on mutualistic interactions with the host. As efforts are made to culture the bumblebee bacteria, functional *in vitro* assays can be developed to specifically test this

metabolism. The creation of gnotobiotic[1] bees could provide the opportunity to perform functional tests *in vivo*.

8.2. What is the required nutrient balance for the host and its gut microbiota ?

Chapter 4 shows that different diets result in different gut bacterial compositions and in the case of pollen, diet also plays a role in the number of progeny. This opens the question which nutrients are primarily needed by the host and which nutrients are mainly needed by the gut microbiota.

The dietary needs change during the different developmental stages of the bumblebee. Adult bumblebees mainly consume nectar and a smaller amount of pollen, as the adult relies more on sugars as an energy source. Larvae primarily consume pollen, as they need proteins and other nutrients to grow and to pupate (Goulson, 2010). This will certainly have an impact on the gut microbiota in both the adult and the larva. In this dissertation, the focus went to the gut microbiota in adult bumblebees, but also the gut microbiota of larvae could reveal new functionalities or contribute in the understanding of the interaction between host-microbiota-diet and its impact on larval development.

Both the host as well as its microbiota rely on proteins, lipids, carbohydrates, … but the exact ratio between the nutrients necessary for the host or for the microbiota is unknown. Regarding the carbohydrates, we can speculate that the low molecular weight sugars are easily digested and thus are mainly digested by the host in the fore- and midgut. The more complex high molecular weight sugars, such as pectin and cellulose, are mainly found in the pollen wall. Most of the pollen wall remains visible in feces (Crailsheim et al., 1992). As these high molecular weight sugars are more difficult to digest, we can speculate that the highly abundant bacteria in the hindgut could prove useful for further digestion of these components and are possibly more important to the microbiota. The amount of nutrients consumed by the bacteria should not all be considered as a loss of nutrients for the host, as fermentation

[1] A gnotobiotic animal is an animal in which only certain known strains of bacteria and other microorganisms are present.

products, such as SCFA produced by bacteria, are also nutritional or beneficial for the host or other bacterial members. Besides this, more insight is needed if the transition time through the gut is long enough to digest these complex sugars.

8.3. Detection and description of organ-specific bacterial communities in bumblebees

Previous studies have focused on the bacteria in the gut of bumblebees and honeybees and the use of traditional culture-dependent techniques has allowed to describe new bacterial genera and species in the bee gut (Olofsson & Vasquez, 2008; Killer et al., 2009; Killer et al., 2010a; Killer et al., 2010b; Killer et al., 2011; Engel et al., 2013; Killer et al., 2013; Kwong & Moran, 2013; Killer et al., 2014; Olofsson et al., 2014; Praet et al., 2015a; Praet et al., 2015b; Kwong & Moran, 2016; Praet et al., 2016). In this research, we did not only focus on the gut microbiota (chapters 2-5), but also the bacterial communities in the ovaries (chapter 6) and the fat body (chapter 7) of *Bombus terrestris* were studied. The bacterial species in the different organs showed some overlap, while other bacteria were restricted to one habitat in the bumblebee. This observation is intriguing and forces us to rethink some ideas:

First, bacterial functionalities were until now often restricted to functionalities associated with the gut. A special interest should go out to identify potential opportunistic pathogens, bacteria residing in low numbers in the gut, but start to proliferate in a specific context and thereby have a negative impact on the health of the host. It is possible that bacterium *Arsenophonus* (chapter 7) is associated with *A. bombi* infected bees. For now, not many bacterial pathogens have been described and are mainly restricted to true pathogens like *Melissococcus plutonius* and *Paenibacillus larvae*, causing respectively European foulbrood disease and American foulbrood disease in honeybees.

Second, further research is needed to determine how these bacteria adjust themselves to colonize different habits and how they are associated with the host tissue. Are the bacterial cells located between the fat body cells, or are they able to form a thin biofilm layer on top of

tissue? This has not been investigated yet, although several examples of microbiota in organs and hemolymph have been reported in other invertebrates as well, but in none of these papers the bacterial colonization of the organs has been investigated (Lokmer et al., 2015; Lokmer & Wegner, 2015; Montagna et al., 2015; Wang & Wang, 2015; Tchioffo et al., 2016). Besides this, we question if different strains of bacteria occurring in several organs, such as *Snodgrassella alvi* and *Gilliamella apicola*, have different colonization potential in different tissues or if they can adjust their protein expression profile depending on the bacterial habitat in the host.

Third, the term 'core bacteria' was used to describe the bacteria found exclusively or primarily in the guts of honey bees and bumblebees. The term 'core' is host specific: for honeybees (*Apis*), this core set of bacteria consists of species within Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Lactobacillales (Cariveau et al., 2014), while for bumblebees (*Bombus terrestris*) bacterial families *Neisseriaceae* (*Snodgrassella*), *Orbaceae* (*Gilliamella*), *Lactobacillaceae* (*Lactobacillus*), and *Bifidobacteriaceae* (*Bifidobacterium*) were assigned as the core set of gut bacteria (Meeus et al., 2015). It becomes clear that the 'core' set of bacteria should be defined for each host specifically. This research shows that the 'core' bacteria in bumblebees were found in the gut as well as in the fat body and the ovaries. However the term 'core' does not define in which ratios this set of bacteria is present. Both the observations in Li et al. (2015a) and the observations in chapter 7 have demonstrated that different profiles of bacterial communities can be detected in organs of wild bumblebees. The first profile was dominated by the core bacteria, while other samples were dominated by non-core bacteria. It is currently unknown what the cause or the consequence is of a microbiota dominated by non-core bacteria and the impact it has on the host. A lot of questions remain unanswered: Should we consider a microbiota dominated by non-core bacteria as dysbiosis? Is this bacterial composition less effective in pathogen protection? Are there non-core bacteria with protective functionalities? What is the impact of certain non-core bacteria that were detected in high relative abundances such as

Phyllobacterium and *Arsenophonus* in the fat body? What is the cause or consequence of strongly different bacterial profiles between organs within one bumblebee?

8.3.1 Future perspectives

As most of our identifications were based on 254 bp of the V4 region by use of the 16S rRNA Illumina MiSeq sequencing technique, the exact identification of the bacteria on strain level remains unknown. In analogy with the gut microbiota of bees, also the bacteria occurring in other tissues should be cultured in order to describe new bacterial strains which are restricted to one or more body organs, such as the ovaries, fat body, hemolymph, salivary glands, ... This would open new insights to further explore the complexity of host-microbe interactions.

8.4. Bacterial communities in different organs of the bumblebee and the implications for bacterial transmission

With the knowledge that bumblebees are social insects and their organs harbor a microbiota, there are several ways how bacteria can be transmitted between and within hosts and also other external factors can have an impact on the host and its microbiota (Figure 8.1).

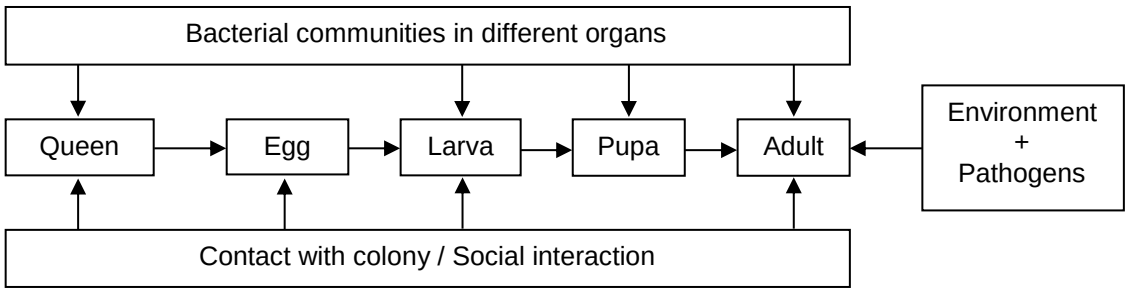

Figure 8.1. Schematic overview of the different inoculation sources and transmission routes for microbiota in bumblebees. The arrows show the directions of the potential bacterial transfers. The upperpart shows the internal transmission routes of bacteria in different organs within a single host. The bottom part shows the transmission routes between different bumblebees in the same nest.

Chapter 6 demonstrated the presence of a bacterial community in the ovaries of bumblebee queens. Transovarial transmission is usually described when discussing endosymbionts in insects (Mira & Moran; Hosokawa et al., 2007), but it has not yet been proven if the bacterial community that was detected in the ovaries of bumblebee queens also plays a role in the vertical transmission of bacteria. It would be interesting to investigate if for example a bacterial community could be detected on or in the eggs.

Besides the vertical transmission route, there is also a horizontal transmission route where contact with the colony and social interactions between several developmental stages play an important role in bacterial transmission. The impact of colony contact on the gut microbiota of larvae has not yet been investigated, but chapter 5 focused on the impact of colony contact on the gut microbiota of adult bumblebees. Lactobacilli and bifidobacteria were mainly obtained as a result of direct contact with other nest mates shortly after emerging and showed the added value of a social life in a colony. In contrast, *Snodgrassella*, *Gilliamella* and a small fraction of *Lactobacillus bombicola* were still able to colonize the gut, even when bumblebees emerged from their cocoon outside the nest in complete isolation. One of the given hypotheses mentioned that certain bacteria could be able to survive the metamorphosis in the gut or in other body organs. Recolonization of the gut does not only occur after eclosion, but also occurs in hibernating queens. An exploratory analysis during my PhD demonstrated that the gut of hibernating bumblebee queens was devoid of bacteria, based on PCR bands and DGGE profiles. But already 7 days after the end of hibernation, the typical gut microbial community was again fully established within a lab environment. In the lab, the bumblebee was not even exposed to other external inoculation sources. It remains unknown whether there is a very small undetectable fraction of the microbiota that survives in the gut, or whether the bacteria are surviving in other body organs that serve as a bacterial stock to recolonize the gut. It is remarkable that the core bacteria *Snodgrassella*, *Gilliamella*, *Lactobacillus* and *Bifidobacterium* were also detected in several organs: the gut, the fat body (chapter 7) as well as the ovaries (chapter 6) and this suggests an internal transmission route within the host. Possibly the fat body not only stores energy for the bumblebee queen

to survive winter, but is also a bacterial storage place (chapter 7). As the bumblebee queen is the only one surviving the winter period and founds a new nest after hibernation, she is the only one that transmits the typical bumblebee microbiota to her off-spring. Theoretically, this means that, creating bumblebee queens with the desired gut microbiota is the most efficient way to improve the fitness of the whole future colony. Studying this principle would not only interest microbial ecologists, but could be a key in unlocking the issues on the optimization of the microbiota for bumblebee rearing.

In nature, also the impact of the environment plays a role in the colonization of bacteria in the bumblebee. This became clear as the gut microbiota of reared bumblebees can shift after colonies were placed outside and new bacteria were picked up from the environment (Parmentier et al., 2015b). One of the earlier studies by Koch & Schmid-Hempel (2011a), demonstrated that the sampling location had an impact on the core gut bacteria of bumblebees, but there was still a closer association with the bumblebee species than with the environment. Also the data of chapter 7 showed that the gut microbiota was correlated with the location. The fat body microbiota was also correlated with the sampling location, but here also an extra factor played a role, i.e. the infection status with *A. bombi*. As there were more associations between the microbiota of the gut and the fat body in infected bumblebees, compared to non-infected bumblebees, it is possible that pathogens might interact in the internal transmission routes as well. Also the causality and interaction between fat body microbiota and the infection status remains undetermined and shows that the combination of all these factors is not yet elucidated.

All these observations and remaining questions are very interesting and demonstrate that the complexity of the transmission routes of bacteria between hosts, within a host and other factors such as environment and pathogens, are poorly understood at this point and there is without any doubt a lot more to discover.

Summary

Many endogenous bacteria have a mutualistic relationship with the insect host and play a role in digestion, nutrient production and pathogen protection. Knowledge about the interaction between the host and its microbiota and the associated functionalities holds promising applications, for instance to improve the production and the health of mass-reared bumblebees (*Bombus*). Their buzz pollination properties turned bumblebee breeding from a Belgian innovation into a worldwide product, mainly to pollinate tomatoes in greenhouses. Aside from the general introduction on bumblebees, chapter 1 also presents the beneficial properties of bacteria for insects. There is a focus on the gut microbiota of indoor-reared bumblebees (*Bombus terrestris*), mainly consisting of core gut bacteria Betaproteobacteria (*Snodgrassella*), Gammaproteobacteria (*Gilliamella*), Firmicutes (*Lactobacillus*) and Actinobacteria (*Bifidobacterium*), their predicted functionalities, and the potentials of probiotics.

In the first research chapters, indoor-reared bumblebees were used as a model with minimal biological variance, allowing to study the interaction between the host and its core gut bacteria. As a proof of concept, the microbial communities were altered by a treatment with the antibiotic streptomycin (chapter 2), while the bumblebee colony development and reproduction were assessed. The treatment improved reproduction and the gut microbiota was mainly dominated by *Lactobacillus* sp.. Therefore, oral administration of *Lactobacillus* and *Bifidobacterium* strains were tested in chapter 3 and effects on colony performance and the gut microbial composition were evaluated. A continuous administration of *Lactobacillus kunkeei* LMG 18925 and *Lactobacillus crispatus* LMG 9479 were able to partly compensate the effects of low nutritional pollen, but permanent colonization of these strains in the mid- and hindgut was not detected. A *Bombus*-specific strain, *Bifidobacterium actinocoloniiforme* R-53049, showed potential to colonize the gut permanently after three administrations, however no beneficial effect was detected in microcolonies. This study represents a first screening for the potential use of probiotic strains in bumblebees and it shows that host-specificity of bacteria might play a role. Investigations in chapter 4 showed that besides the administration of bacterial strains, also the composition of sugar syrup and the pollen type

have an impact on the gut bacterial composition. Fructose-rich sugar syrup prevented the colonization of *Bifidobacteriaceae* in the gut of newly emerged bumblebees and was correlated with a lower bacterial community richness and diversity. The pollen diet with the highest protein content resulting in the highest bumblebee offspring, showed the lowest bacterial richness and diversity in the gut. We conclude that both probiotic strains and diet can be used to modulate the bacterial composition in the gut in order to improve the health of mass-produced bumblebees used for biological pollination.

The last three chapters focus more on some general biological questions in relation with the bumblebee and its microbiota. Chapter 5 studies the transmission dynamics between nest mates and gut colonization potential of the core bacterial families (*Neisseriaceae*, *Orbaceae*, *Lactobacillaceae* and *Bifidobacteriaceae*). The fact that *Bombus terrestris* has a close contact with nest mates of different developmental stages in the colony, could play an important role to establish their gut microbiota. Three observations were made: (i) the relative abundance of *Lactobacillus bombicola* and *Lactobacillus bombi* dropped significantly when reducing the contact between the colony and the bumblebee during its adult life; (ii) *Bifidobacteriaceae* required contact with nest mates to colonize the gut of *B. terrestris* and bumblebees that were completely excluded from colony contact during the adult life showed a significantly lower bacterial diversity; (iii) *Snodgrassella* and *Gilliamella* were able to colonize the gut of the adult bumblebee without any direct contact with nest mates in the adult life stage. These results indicate the impact of sociality on the diversity of the bumblebee gut microbiota.

The gut microbiota of insects has been a topic of interest for many researchers, however also other habitats in the insect host are associated with microbiota. Chapter 6 focusses on the ovaries of indoor-reared bumblebee queens and investigates if there is a correlation between their microbial pattern and the colony development performance of these queens. The ovary length and mass were clearly correlated with the number of offspring, but the bacterial profiles on the Denaturing Gradient Gel Electrophoresis were not able to elucidate

the correlation with colony success. The ovaries of successful queens harbored the typical core gut bacteria *Snodgrassella*, *Gilliamella*, *Lactobacillus* and *Bifidobacterium*, as well as several other bacteria detected in low relative abundances such as *Enterobacteriaceae* and *Staphylococcaceae* among others. In chapter 7, we widened the view of the bumblebee as a holobiontic insect harboring multiple bacterial habitats. The microbiota in the fat body of wild bumblebees was studied with the aim to investigate the correlation between bacteria in the fat body, sampling location of the bumblebees and the infection status with the neogregarine *Apicystis bombi*, which resides in the fat body. Common bacteria were identified in both the gut and fat body microbiota. However, the fat body microbiota also harbors unique bacteria underlining a specific functionality. The fat body microbiota was correlated with its sampling location and with *Apicystis bombi* infection. The OTU identified as *Arsenophonus* was correlated with *A. bombi* infection in bumblebees of one location. Moreover, in samples infected with *A. bombi*, there was a higher correlation between bacteria residing in the gut and in the fat body, suggesting that *A. bombi* is facilitating an exchange of bacteria between gut and fat body.

Finally, all data are discussed in chapter 8 and some perspectives for future research and optimization of bumblebee rearing are formulated. Throughout this PhD research, it has become clear that microbiota plays a role in the bumblebee colony performance. New insight was achieved, not only about the influences on the gut microbiota, but also on microbiota in other organs of the bumblebee which most likely also play a role in the fitness of the bumblebee host.

Samenvatting

Vele endogene bacteriën hebben een mutualistische relatie met hun insect gastheer en dragen bij in vertering, nutriënt productie en pathogeen protectie. Kennis over de interacties tussen de microbiota en de gastheer en de functionaliteiten die daaraan gekoppeld zijn, kunnen leiden tot veelbelovende toepassingen bijvoorbeeld voor de productie en gezondheid van commercieel gekweekte hommels (*Bombus*). Hommels zijn zeer efficiënte pollinatoren als gevolg van hun 'buzz'-bestuivingstechniek. Dit heeft er toe geleid dat deze Belgische innovatie is uitgegroeid tot een wereldwijd export product, hoofdzakelijk voor de bestuiving van tomaten in serres. In hoofdstuk 1 wordt naast de algemene introductie over hommels, ook dieper ingegaan op de interessante eigenschappen van bacteriën in insecten. De focus ligt op de darmmicrobiota van gekweekte hommels (*Bombus terrestris*) waarvan de kernset van bacteriën bestaat uit Betaproteobacteria (*Snodgrassella*), Gammaproteobacteria (*Gilliamella*), Firmicutes (*Lactobacillus*) en Actinobacteria (*Bifidobacterium*), hun voorspelde functies en het potentieel van probiotica.

In de eerste onderzoekshoofdstukken werden commercieel gekweekte hommels gebruikt als een model met minimale biologische variatie, wat toelaat om de interactie van de gastheer en zijn darmmicrobiota te bestuderen. Als 'proof-of-concept' werd in hoofdstuk 2 de microbiële gemeenschap van hommels veranderd door middel van een behandeling met het antibioticum streptomycine. Hierbij werd de kolonieontwikkeling en de reproductie opgevolgd in microkolonies met hommels. De behandeling leidde tot een verhoogde reproductie en de darmmicrobiota werd gedomineerd door *Lactobacillus* sp. Daarom werd in hoofdstuk 3 gekozen om verschillende bacteriële stammen van *Lactobacillus* en *Bifidobacterium* via het voedsel toe te dienen aan hommels. De effecten van deze behandeling werden geëvalueerd op kolonieontwikkeling en de impact op de darmmicrobiota. Een continue toediening van *Lactobacillus kunkeei* LMG 18925 en *Lactobacillus crispatus* LMG 9479 konden deels de effecten van laag nutritioneel pollen compenseren, maar een permanente kolonisatie van deze stammen kon niet worden gedetecteerd in de midden- en einddarm. Een *Bombus*-specifieke bacteriële stam, *Bifidobacterium actinocoloniiforme* R-53049, toonde potentieel om de darm permanent te koloniseren na drie orale toedieningen, maar had geen effect op

vlak van kolonieontwikkeling. Deze studie is een eerste screening van het potentieel gebruik van probiotische stammen in hommels en toont aan dat gastheer-specificiteit van de bacteriën mogelijk een rol kan spelen in het kolonisatie succes in de darm. Onderzoek in hoofdstuk 4 toonde aan dat naast de toediening van bacteriële stammen, ook de samenstelling van het suikerwater en het pollen type een impact kan hebben op de bacteriële darmgemeenschap. Fructose-rijk suikerwater verhinderde de kolonisatie van *Bifidobacteriaceae* in de darm van pas ontpopte hommels en dit was gecorreleerd met een lagere bacteriële rijkheid en diversiteit van de darmmicrobiota. Het pollen type met het hoogste eiwitgehalte zorgde voor het hoogst aantal nakomelingen, maar toonde anderzijds ook de laagste bacteriële rijkheid en diversiteit in de darm. Hieruit kan geconcludeerd worden dat zowel het gebruik van probiotische stammen als de samenstelling van het dieet kan gebruikt worden om de darmmicrobiota van gekweekte hommels aan te passen met het oog op een verhoogde gezondheid van commercieel gekweekte hommels.

De laatste drie onderzoekshoofdstukken focussen meer op algemene biologische vraagstellingen over de interactie tussen de microbiota en de hommel. Hoofdstuk 5 bestudeerde de transmissie van de ‘kernset’ darmbacteriën *Neisseriaceae*, *Orbaceae*, *Lactobacillaceae* en *Bifidobacteriaceae.* Het feit dat *Bombus terrestris* in het nest nauw contact heeft met de andere nestgenoten in diverse ontwikkelingsstadia, kan een rol spelen in de manier waarop hun darmmicrobiota tot stand komt. Hierbij werden drie vaststellingen gedaan: (i) de relatieve abundantie van *Lactobacillus bombicola* en *Lactobacillus bombi* was significant lager wanneer het contact tussen de kolonie en de hommel werd gereduceerd tijdens het adulte levensstadium van de hommel; (ii) kolonisatie van *Bifidobacteriaceae* in de darm vereist contact tussen nestgenoten, en hommels die volledig afgezonderd werden van de kolonie tijdens het adulte levensstadium vertoonden een significant lagere bacteriële diversiteit; (iii) *Snodgrassella* en *Gilliamella* konden de darm van de adulte hommel koloniseren zonder enig direct contact met andere hommels. Deze resultaten wijzen op de impact van het sociaal gedrag op de bacteriële diversiteit in de darm van de hommel.

De darmmicrobiota van insecten werd reeds door vele onderzoekers bestudeerd, maar ook andere organen in insecten kunnen een habitat vormen voor microbiota. Hoofdstuk 6 nam de ovaria van commercieel gekweekte hommels onder de loep. Hierbij werd onderzocht of er een correlatie is tussen de bacteriële profielen in de ovaria en het koloniesucces van gekweekte hommelkoninginnen. De lengte en het gewicht van de ovaria waren duidelijk gerelateerd met het aantal nakomelingen en het opstartsucces van de kolonies, maar de bacteriële profielen op de Denaturerende Gradiënt Gel Elektroforese konden niet worden gerelateerd aan het opstart succes van de koninginnen. In de ovaria van koninginnen met een goede nestopstart werden hoofdzakelijk bacteriën *Snodgrassella*, *Gilliamella*, *Lactobacillus* en *Bifidobacterium* geïdentificeerd. Daarnaast werden ook nog andere bacteriën gedetecteerd waaronder bacteriën uit de families *Enterobacteriaceae* en *Staphylococcaceae*, maar deze waren meestal in zeer kleine fracties aanwezig. In hoofdstuk 7 werd de hommel beschouwd als een holobiont die meerdere bacteriële habitats huisvest. Er werd bestudeerd of er een correlatie was tussen de bacteriën in het vetlichaam van wilde hommels, de locatie waar de hommels gevangen werden en de infectie met het neogregarine *Apicystis bombi*, die zich in het vetlichaam vestigt. In het vetlichaam werden bacteriën geïdentificeerd die zowel voorkomen in de darm als in het vetlichaam. Daarnaast werden er ook meerdere bacteriën geïdentificeerd die enkel voorkwamen in het vetlichaam, wat wijst op een specifieke functionaliteit. De microbiota in het vetlichaam was gecorreleerd met de locatie waar de hommels werden gevangen en ook met *Apicystis bombi* infectie. Een OTU die geïdentificeerd werd als *Arsenophonus,* was gecorreleerd met hommels die geïnfecteerd waren met *Apicystis bombi*. Bovendien was er een sterkere correlatie tussen bacteriën in de darm en het vetlichaam in hommels die geïnfecteerd waren met *A. bombi*, wat er op kan wijzen dat *A. bombi* bijdraagt aan een uitwisseling van bacteriën tussen darm en vetlichaam.

Tenslotte werden alle bevindingen in hoofdstuk 8 besproken en werden perspectieven voor toekomstig onderzoek voor de optimalisatie voor de hommelkweek geformuleerd. Doorheen dit doctoraatsonderzoek is het duidelijk geworden dat de microbiota invloed heeft op de

kolonieontwikkeling van de hommel. Nieuw inzicht werd bekomen, niet alleen over de impact van de darmmicrobiota, maar ook de microbiota in andere organen van de hommel die een rol spelen in de gezondheid en fitheid van de hommel.

Supplemental data

Table S1. *Lactobacillus* sp. (deposited at GenBank as KC477412) (represented by two bands in streptomycin treated bumblebees) does not appear in the DGGE pattern of untreated microcolonies. Its normalized position (%) in the DDGE pattern is compared with the two closest bands in untreated colonies. Each band present in untreated bumblebees has another normalized position. Values are reported as mean ± SD (n=10).

Streptomycin	**Closest match in controls**	
Normalized position (%) of *Lactobacillus* sp.	Lower normalized position (%)	Higher normalized position (%)
9.64 ± 0.12	9.20 ± 0.06 ($P < 0.001$)	10.26 ± 0.16 ($P < 0.001$)
11.55 ± 0.07	10.93 ± 0.06 ($P < 0.001$)	11.97 ± 0.01 ($P < 0.001$)

Table S2. Composition of the agar used for the cultivation of lactobacilli and bifidobacteria.

Lactobacilli: MRS agar	
10 g	Peptone
8 g	Lab-Lemco Powder
4 g	Yeast extract
20 g	Glucose
1 mL	Tween 80
2 g	Tri-ammonium citrate
5g	Sodium acetate x $3H_2O$
0.2 g	$MgSO_4$ x $7H_2O$
0.05 g	$MnSO_4$ x $4H_2O$
2 g	K_2HPO_4
15 g	Agar
Up to 1 L	Distilled water

Bifidobacteria	
23 g	'special peptone'
1 g	Soluble starch
5 g	NaCl
0.3 g	Cysteine hydrochloride
5 g	Glucose
15 g	Agar
1 L	Distilled water

Table S3A. Described strains of *Bifidobacterium* and *Lactobacillus*, isolated from the gut of bumblebees and honeybees along with the numbers and letters further used in Tables S3B and S3C.

Table S3A	*Genus species* strain	ncbi accession number	Reference	
1	*Bifidobacterium actinocoloniiforme* sp. nov. LISLUCIII–P2^T	FJ858731	Killer et al. (2010b)	a
			Killer et al. (2011)	c
2	*Bifidobacterium asteroides* YIT 11866 = ATCC 25910 = DSM 20089	AB437355	Killer et al. (2010b)	a
			Killer et al. (2011)	c
3	*Bifidobacterium indicum* JCM 1302 = DSM 20214	D86188	Killer et al. (2010b)	a
			Killer et al. (2011)	c
4	*Bifidobacterium coryneforme* ATCC 25911 = DSM 20216	M58733	Killer et al. (2010b)	a
			Killer et al. (2011)	c
5	*Bifidobacterium* sp. LISLUCIII–2	FJ858732	Killer et al. (2010b)	a
6	*Bombiscardovia coagulans* LISPASI–P3	FJ858733	Killer et al. (2010b)	a
7	*Bifidobacterium minimum* ATCC 27538 = YIT 4097	AB437350	Killer et al. (2011)	c
8	*Bifidobacterium bohemicum* sp. nov. JEMLUCVII–1^T	FJ858737	Killer et al. (2010b)	a
			Praet et al. (2015)	
9	*Bifidobacterium bohemicum* sp. nov. JEMLUCVIII–4^T = LMG 27797^T	FJ858736	Killer et al. (2011)	c
10	*Bifidobacterium bombi* BluCI–TP^T = DSM 19703 = ATCC BAA–1567	EU127549	Killer et al. (2009)	g
11	*Bifidobacterium commune* LMG 28292^T	LK054489	Praet et al. (2015a)	f
12	*Bombiscardovia coagulans* BLAPIII–AGV^T = DSM 22924^T = ATCC BAA–1568^T	EU127550	Killer et al. (2010a)	b
13	*Lactobacillus kunkeei* YH–15^T	Y11374	Olofsson et al. (2014)	e
14	*Lactobacillus apinorum* Fhon13N^T = DSM 26257^T = CCUG 63287^T	JX099541	Olofsson et al. (2014)	e
15	*Lactobacillus mellis* Hon2N^T = DSM 26255^T = CCUG 63289^T	JX099545	Olofsson et al. (2014)	e
16	*Lactobacillus mellifer* Bin4N^T = DSM 26254^T = CCUG 63291^T	JX099543	Olofsson et al. (2014)	e
17	*Lactobacillus melliventris* Hma8N^T = DSM 26256^T = CCUG 63629^T	JX099551	Olofsson et al. (2014)	e
18	*Lactobacillus helsingborgensis* Bma5N^T = DSM 26265^T = CCUG 63301^T	JX099553	Olofsson et al. (2014)	e
19	*Lactobacillus kimbladii* Hma2N^T = DSM 26263^T = CCUG 63633^T	JX099549	Olofsson et al. (2014)	e
20	*Lactobacillus kullabergensis* Biut2N^T = DSM 26262^T = CCUG 63631^T	JX099550	Olofsson et al. (2014)	e
21	*Lactobacillus apis* R4B^T	KF386017	Olofsson et al. (2014)	e
			Killer et al. (2014)	d
22	*Lactobacillus bombi* BTLCH M1/2^T	KJ078643	Killer et al. (2014)	d
23	*Convivina intestini* LMG 28291	LK054488	Praet et al. (2015b)	
24	*Lactobacillus bombicola* LMG 28288	LK054485	Praet et al. (2015b)	
25	*Weissella bombi* LMG 28290 = LK054487	LK054487	Praet et al. (2015b)	

Table S3B. The carbohydrate fermenting capacities and enzyme activity of different strains of *Bifidobacterium*, isolated from the bumblebee gut. '+' positive, '(+)' weakly positive, '–' negative, 'ND' no data, '*' number of isolates with a reaction identical to that of the type strain. Numbers in the title refer to bacteria and letters represent the reference, which are both explained in **Table S3A**.

Table S3B: Part 1	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Carbohydrate fermenting capacities												
adonitol	– c	– c	– c	– c	ND	ND	–	ND	–	–	ND	–
D–adonitol	– a	– a	– a	– a	–	–	ND	–	(+) f	ND	–	ND
aesculin	+a	ND	ND	ND	+	+	ND	+a	ND	ND	ND	ND
amygdalin	+	+	+	(+) c	+	+	–	–	– c + f	+	+ (3/4)*	+
L–arabinose	+	– a (+) c	+	+	–	+	–	+	+	–	–	+
D–arabinose	– b	– a	– a	– a	–	–	ND	–	– f	ND or –	–	ND
arabitol	–	–	–	–	–	–	–	–	–	–	ND	ND
D–arabitol	–	–	–	–	ND	ND	– c	ND	–	– c	–	–
L–arabitol	–	–	–	–	ND	ND	– c	ND	–	– c	–	–
arbutin	+	+ c	+ c	+ c	+	+	+	+a	+	+	+	ND
cellobiose	+ a (+) c	–	– a + c	– a + c	–	–	+ a – c	–	–	– g + c	ND	ND
D–cellobiose	ND	– a	– a	– a	ND	ND	ND	ND	(+) f	ND	–	+
dulcitol	–	–	–	–	–	–	–	–	– c (+) f	–	–	–
erythritol	–	–	–	–	–	–	–	–	–	–	–	–
esculin ferric citrate	ND	ND	ND	ND	ND	ND	ND	ND	+ f	ND	+	ND
D–fructose	+ a – c	+	– a + c	– a + c	(+)	–	– a + c	(+)	(+) c + f	+	+ (2/4)*	ND
fucose	–	–	–	–	–	–	–	–	–	–	ND	ND
D–fucose	–	–	–	–	ND	ND	– c	ND	–	– c	–	–
L–fucose	–	–	–	–	ND	ND	– c	ND	–	– c	–	–
D–galactose	– a + c	(+)	– a + c	– a + c	(+)	–	+ a – c	+	+	–	+	–
gentiobiose	+	+ c	+ c	+ c	+	+	+	+a	+	+	–	+
(D–)glucose	+	+ c	+ c	+ c	+	+	+	+a	+	+	+	ND
glycerol	–	–	–	–	–	–	–	–	–	–	–	–
glycogen	– a	– a	– a	– a	–	–	ND	–	– f	ND	–	–
inulin	–	–	–	–	–	–	–	–	–	–	–	–
inositol	–	–	–	–	–	–	–	–	– c (+) f	–	–	–
D–lactose	– a	– a	– a	– a	–	–	ND	–	– f	ND	–	–
D–lyxose	–	–	–	–	–	–	–	–	–	–	–	–
maltose	–	–	+	+	–	–	– b + c	–	–	–	ND	ND
D–maltose	ND	–	+	+	ND	ND	ND	ND	–	ND	–	–
mannitol	– c	– c	– c	– c	ND	ND	–	ND	–	–	ND	–
D–mannitol	– a	– a	– a	– a	–	–	ND	–	– f	ND	–	ND
D–mannose	–	+	–	–	–	–	– c	+	+	+	–	–
melezitose	– c	+ c	– c	– c	ND	ND	–	ND	–	–	ND	ND
D–melezitose	– b	+ b	– b	– b	–	–	ND	–	– f	ND	–	–
melibiose	– a (+) c	–	(+)	+	+	+	+ a – c	+	+ c	+	ND	ND
D–melibiose	ND	–	(+)	+	ND	ND	ND	ND	+ f	ND	+	–

Table S3B: Part 2	1	2	3	4	5	6	7	8	9	10	11	12
Carbohydrate fermenting capacities												
methyl β–D–xylopyranoside	–	–	–	–	–	–	–	–	– c (+) f	–	–	–
methyl α–D–mannopyranoside	–	–	–	–	–	–	–	–	–	–	–	–
methyl α–D–glucopyranoside	+ a – c	–	+ a (+) c	+ a – c	–	+	ND	+	– c (+) f	(+)	+ (2/4)*	+
N–acetylgluosamine	–	–	–	–	–	–	–	–	– c + f	–	–	–
raffinose	+ a – c	–	–	+	+	–	– c	+	+	+	+	+
D–raffinose	ND	–	–	+	ND	ND	ND	ND	ND	ND	+	ND
rhamnose	– c	– c	– c	– c	ND	ND	–	ND	–	–	ND	–
L–rhamnose	– a	– a	– a	– a	–	–	ND	–	– f	ND	–	ND
ribose	+ c	+ c	+ c	+ c	ND	ND	–	ND	+ c	+	ND	ND
D–ribose	ND	ND	ND	ND	ND	ND	ND	ND	+ f	ND	+	ND
salicin	+	+ c	+ c	+ c	+	+	–	+a	+	+	+	ND
sorbitol	– c	– c	– c	– c	ND	ND	–	ND	–	–	ND	–
D–sorbitol	– a	– a	– a	– a	–	–	ND	–	– f	ND	–	ND
L–sorbose	–	–	–	–	–	–	–	–	– c (+) f	–	–	–
starch	–	–	–	–	–	–	+	–	– c + f	–	–	–
sucrose / D–saccharose	+	+	(+)	(+)	–	+	– a + c	–	– c (+) f	–	+ (2/4)*	+
D–tagatose	–	–	–	–	–	–	–	–	– c (+) f	–	–	–
trehalose	+	–	–	–	–	–	–	–	–	–	ND	+
D–trehalose	ND	– a	– a	– a	ND	ND	ND	ND	ND	ND	–	ND
D–turanose	– a	– a	– a	– a	–	–	ND	–	– f	ND	–	–
xylitol	–	–	–	–	–	–	–	–	–	–	–	–
D–xylose	– a + c	+	+ a – c	+ a – c	–	+	–	+	+	–	–	–
L–xylose	– a	– a	– a	– a	–	–	ND	–	(+) f	ND	–	ND
2–ketoglucanate	– a	– a	– a	– a	–	–	ND	–	ND	ND	ND	–
potassium 2–ketogluconate	ND	ND	ND	ND	ND	ND	ND	ND	– f	ND	–	ND
5–ketogluconate	–	–	–	–	–	–	–	–	–	–	ND	–
potassium 5–ketogluconate	ND	ND	ND	ND	ND	ND	ND	ND	(+) f	ND	(+)	ND
(Potassium) gluconate	–	–	–	–	–	–	–	–	– c (+) f	–	–	–

Table S3B: Part 3	1	2	3	4	5	6	7	8	9	10	11	12
Activity of enzymes												
acid phosphatase	ND	ND	ND	ND	ND	ND	ND	ND	+	ND	+	ND
α–arabinosidase	+	+	+	+	+	+	+	+	+	+	ND	+
alkaline phosphatase	–	–	–	–	–	–	–	–	–	–	–	–
arginine dihydrolase	–	–	–	–	–	–	–	–	–	–	ND	ND
catalase	– c	– c	– c	– c	ND	ND	–	ND	–	–	ND	–
α–chymotrypsin	ND	ND	ND	ND	ND	ND	ND	ND	–	ND	–	ND
esterase (C4)	ND	ND	ND	ND	ND	ND	ND	ND	–	ND	–	ND
esterase lipase (C8)	ND	ND	ND	ND	ND	ND	ND	ND	–	ND	–	ND
α–fucosidase	–	–	–	–	–	–	–	–	–	–	–	ND
α–galactosidase	+	+	+	+	+	+	+	+	+	+	+	+
β–galactosidase	+	+	+	+	+	+	+	+	+	+	+	+
β–galactosidase–6–phosphate	–	–	–	–	–	–	–	–	–	–	ND	ND
gelatinase / gelatin hydrolysis	ND	ND	ND	ND	ND	ND	ND	ND	–	ND	–	ND
α–glucosidase	+	+	+	+	+	+	+	+	+	+	+	+
β–glucosidase	+	+	+	+	+	+	+	+	+	+	+	+
β–glucuronidase	–	–	–	–	–	–	–	–	–	–	–	ND
glutamic acid decarboxylase	–	–	–	–	–	–	–	–	–	–	ND	ND
indole production (from L–tryptophan)	–	– c	– c	– c	ND	ND	–	ND	–	–	ND	ND
lipase (C14)	ND	ND	ND	ND	ND	ND	ND	ND	–	ND	–	ND
α–mannosidase	ND	ND	ND	ND	ND	ND	ND	ND	–	ND	–	ND
naphtol–AS–BI–phosphohydrolase	ND	ND	ND	ND	ND	ND	ND	ND	+	ND	+	ND
nitrate reduction	–	– c	– c	– c	ND	ND	–	ND	–	–	ND	ND
N–acetyl–β–glucosaminidase	–	–	–	–	–	–	–	–	–	–	–	ND
pyroglutamic acid arylamidase	–	–	–	–	–	–	–	–	–	–	ND	–
oxidase	– c	– c	– c	– c	ND	ND	–	ND	–	–	ND	–
trypsin	ND	ND	ND	ND	ND	ND	ND	ND	–	ND	–	ND
urease	–	–	–	–	–	–	–	–	–	–	ND	–
alanine arylamidase	– a + c	–	+	(+) a + c	–	–	ND	–	ND	–	ND	–
arginine arylamidase	+	+	+	+	+	+	+	+	+	+	ND	+
cysteine arylamidase	ND	ND	ND	ND	ND	ND	ND	ND	–	ND	–	ND
glutamyl glutamic acid arylamidase	–	–	–	–	–	–	–	–	–	–	ND	–
glycine arylamidase	+	+	+	+	+	–	+	+	+	+	ND	+
histidine arylamidase	+	+	+	+	+	+	+	+	+	+	ND	+
leucine arylamidase	+	+	+	+	–	+	+	+	+	–	+	+
phenylalanine arylamidase	+	+	+	+	–	–	+	+	+	–	ND	+
proline arylamidase	+	+	+	+	+	+	+	+	+	+	ND	+
serine arylamidase	– a	+ a	+ a	+ a	+	(+)	ND	+	ND	ND	ND	–
tyrosine arylamidase	+	+	+	+	+	+	+	+	+	+	ND	+
valine arylamidase	ND	ND	ND	ND	ND	ND	ND	ND	–	ND	–	ND

Table S3C. The carbohydrate fermenting capacities and enzyme activity of different strains of *Lactobacillus*, '+' positive, '(+)' weakly positive, '–' negative, 'ND' no data, '*' number of isolates with a reaction identical to that of the type strain. Numbers in the title refer to bacteria and letters represent the reference, which are both explained in **Table S3A**.

Table S3C: Part 1	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**
Carbohydrate fermenting capacities													
adonitol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
D–adonitol	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	–	–	–
aesculin	– e	–	+	+	+	+	+	–	+ d, e	+	ND	ND	ND
amygdalin	– e	–	–	–	–	(+)	–	–	(+) d,e	+	–	+	–
L–arabinose	– e	–	–	–	–	–	–	–	– d,e	+	–	+	+
D–arabinose	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	–	–	–
arabitol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
D–arabitol	– e	–	–	+	–	–	–	–	– d,e	–	–	–	–
L–arabitol	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	–	–	–
arbutin	– e	–	+	+	–	(+)	+	–	+ d, e	+	–	+	+
cellobiose	– e	–	–	–	–	–	–	–	– d, e	+	ND	ND	ND
D–cellobiose	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	+	+
dulcitol	– e	–	–	–	–	(+)	–	–	– d,e	–	–	–	–
erythritol	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	–	–	–
esculin ferric citrate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	+
D–fructose	+ e	+	+	+	(+)	(+)	+	+	+ d,e	+	+	+	+
fucose	ND	ND	ND	ND	ND	ND	ND	ND	– d	ND	ND	ND	ND
D–fucose	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	–	–	–
L–fucose	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	–	–	–
D–galactose	+	–	–	–	–	(+)	–	–	– d,e	–	–	–	+
gentiobiose	–	–	–	–	+	–	–	+	+ d – e	+	–	+	+
(D–)glucose	ND	+	+	+	+	+	+	+	+ d	+	+	+	+
glycerol	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	–	–	–
glycogen	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	–	–	–
inulin	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	–	–	–
inositol	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	–	–	–
lactose	– e	–	–	–	–	–	–	–	– de	–	ND	ND	ND
D–lactose	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	–	+
D–lyxose	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	–	(+)	–
maltose	– e	–	–	–	–	–	(+)	–	– d,e	–	ND	ND	ND
D–maltose	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	–	+
D–mannitol	+ e	–	–	–	–	–	–	–	– d,e	–	+	–	–
D–mannose	– e	–	–	–	+	+	+	–	+ d,e	+	–	+	+
melezitose	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	ND	ND	ND
D–melezitose	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	–	–
melibiose	– e	–	–	–	–	–	–	–	– d,e	+	ND	ND	ND
D–melibiose	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	–	+
methyl β–D–xylopyranoside	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	–	–	–
methyl α–D–mannopyranoside	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	–	–	–
methyl α–D–glucopyranoside	– e	–	–	–	–	(+)	–	–	– d,e	–	–	–	+

Table S3C: Part 2	13	14	15	16	17	18	19	20	21	22	23	24	25
Carbohydrate fermenting capacities													
N–acetylgluosamine	– e	–	–	–	–	+	+	–	– e	(+)	–	+	+
raffinose	+ e	–	–	–	–	(+)	–	–	– d,e	+	ND	ND	ND
D–raffinose	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	–	+
L–rhamnose	ND	ND	ND	ND	ND	ND	ND	ND	– d	(+)	–	–	–
ribose	– e	–	–	–	–	–	–	–	– e	ND	ND	ND	ND
D–ribose	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	+	+	+
salicin	– e	–	+	(+)	–	+	+	+	+ d,e	+	–	+	+
D–sorbitol	– e	–	–	–	–	+	–	–	– d,e	–	–	–	–
L–sorbose	– e	–	–	–	–	+	–	–	– d,e	–	–	–	–
starch	– e	–	–	–	–	–	–	–	– d,e	–	–	–	–
sucrose / D–saccharose	+ e	–	–	–	–	+	–	–	– d,e	+	+	–	+
D–tagatose	– e	–	–	–	(+)	–	+	–	– d,e	–	–	(+)	–
trehalose	– e	–	–	–	–	–	–	+	(+) d,e	(+)	ND	ND	ND
D–trehalose	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	+
turanose	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	ND	ND	ND
D–turanose	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	–	+
xylitol	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	–	–	–
D–xylose	– e	–	–	–	–	–	–	–	– d,e	+	–	–	–
L–xylose	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	–	–	–
2–ketoglucanate	ND	ND	ND	ND	ND	ND	ND	ND	– d	ND	ND	ND	ND
potassium 2–ketogluconate	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	–	–	+
5–ketogluconate	ND	ND	ND	ND	ND	ND	ND	ND	– d	ND	ND	ND	ND
potassium 5–ketogluconate	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	–	(+)	+
(Potassium) gluconate	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	+	–	+
Activity of enzymes													
acid phosphatase	– e	+	+	+	+	+	+	+	+ d, e	+	+	+	+
α–arabinosidase	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	ND	ND	ND
alkaline phosphatase	+ e	–	ND	ND	–	–	–	–	– d,e	–	+	–	–
arginine dihydrolase	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	ND	ND	ND
catalase	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	ND	ND	ND
α–chymotrypsin	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	–	–	–
esterase (C4)	– e	–	+	+	+	+	–	(+)	(+) d,e	ND	–	–	–
esterase lipase (C8)	– e	–	+	+	–	–	–	–	– d, e	–	–	–	–
α–fucosidase	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	–	–	–
α–galactosidase	ND	ND	ND	ND	ND	ND	ND	ND	– d	+	–	–	–
β–galactosidase	– e	–	–	–	–	+	–	–	(+) d,e	+	–	–	+
β–galactosidase–6–phosphate	ND	ND	ND	ND	ND	ND	ND	ND	+ d	–	ND	ND	ND
gelatinase / gelatin hydrolysis	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	ND	ND	ND

Table S3C: Part 3	13	14	15	16	17	18	19	20	21	22	23	24	25
Activity of enzymes													
α–glucosidase	– e	–	–	–	+	–	–	–	– d,e	–	–	–	+
β–glucosidase	– e	–	+	+	+	+	–	+	+ d,e	ND	–	+	–
β–glucuronidase	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	–	–	–
glutamic acid decarboxylase	ND	ND	ND	ND	ND	ND	ND	ND	+ d	+	ND	ND	ND
indole production (from L–tryptophan)	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	ND	ND	ND
lipase (C14)	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	–	–	–
α–mannosidase	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	–	–	–
naphtol–AS–BI–phosphohydrolase	+ e	+	+	+	+	+	+	+	+ d,e	+	+	+	+
nitrate reduction	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	ND	ND	ND
N–acetyl–β–glucosaminidase	– e	–	–	–	(+)	–	–	(+)	+ d,e	+	–	+	–
pyroglutamic acid arylamidase	ND	ND	ND	ND	ND	ND	ND	ND	– d	ND	ND	ND	ND
oxidase	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	ND	ND	ND
trypsin	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	–	–	–
urease	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	ND	ND	ND
alanine arylamidase	ND	ND	ND	ND	ND	ND	ND	ND	+ d	(+)	ND	ND	ND
arginine arylamidase	ND	ND	ND	ND	ND	ND	ND	ND	+ d	+	ND	ND	ND
cysteine/cystine arylamidase	– e	(+)	+	+	+	–	(+)	–	– d,e	–	–	+	–
glutamyl glutamic acid arylamidase	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	ND	ND	ND
glycine arylamidase	ND	ND	ND	ND	ND	ND	ND	ND	+ d	–	ND	ND	ND
histidine arylamidase	ND	ND	ND	ND	ND	ND	ND	ND	+ d	+	ND	ND	ND
leucine arylamidase	+ e	+	+	+	+	+	+	+	+ d,e	+	+	+	–
leucyl–glycine arylamidase	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	ND	ND	ND
phenylalanine arylamidase	ND	ND	ND	ND	ND	ND	ND	ND	+ d	+	ND	ND	ND
proline arylamidase	ND	ND	ND	ND	ND	ND	ND	ND	– d	+	ND	ND	ND
pyroglutamic acid arylamidase	ND	ND	ND	ND	ND	ND	ND	ND	– d	–	ND	ND	ND
serine arylamidase	ND	ND	ND	ND	ND	ND	ND	ND	+ d	+	ND	ND	ND
tyrosine arylamidase	ND	ND	ND	ND	ND	ND	ND	ND	+ d	(+)	ND	ND	ND
valine arylamidase	+ e	+	+	+	+	+	+	+	+ e – d	+	–	+	–

Table S4. 30 bacterial OTUs were detected in the gut of the wild bumblebees, which are listed with their average relative abundances and standard errors. Sequences were identified in EzTaxon (EzBioCloud), searching the database of both cultured and uncultured bacteria. Bacteria were identified to the level upon which they could be identified with 100 % similarity (usually genus or species level). When there was no 100 % match available in the database, the closest match is given and indicated with the number of matching base pairs (xxx/253). Significant differences (P<0.05) between sampling locations or *A. bombi* infection status of certain OTUs are indicated with a dark background.

Table S4: Part 1			**GUT:** Mean relative abundance ± st. error (%)					
Family	**Genus species**	**otu**	**Sterre**	**Bramier**	**Sterre**		**Bramier**	
			All samples	**All samples**	**No infection**	***A. bombi***	**No infection**	***A. bombi***
Phylum: Proteobacteria - Class: Alphaproteobacteria								
Acetobacteraceae	*Saccharibacter floricola*	otu05	2.48 ± 2.39	0.30 ± 0.24	8.15 ± 8.15	0.12 ± 0.12	0.59 ± 0.47	
Acetobacteraceae	*Asaia astilbis*	otu06	0.38 ± 0.38			0.54 ± 0.54		
Acetobacteraceae	uncultured *Commensalibacter* (ATSX01000009)	otu07		0.91 ± 0.91			1.82 ± 1.82	
Bartonellaceae or *Rhizobiaceae*	--	otu08		0.07 ± 0.07			0.14 ± 0.14	
Phylum: Proteobacteria - Class: Betaproteobacteria								
Neisseriaceae	*Snodgrassella alvi*	otu09	29.10 ± 4.42	24.21 ± 5.37	23.72 ± 7.50	31.35 ± 5.50	27.01 ± 7.35	21.41 ± 8.16
Phylum: Proteobacteria - Class: Gammaproteobacteria								
Orbaceae	*Schmidhempelia bombi*	otu14	2.62 ± 1.42	0.41 ± 0.30	0.59 ± 0.59	3.47 ± 1.97	0.55 ± 0.55	0.26 ± 0.26
Orbaceae	*Gilliamella apicola*	otu16	22.39 ± 3.74	16.31 ± 3.00	16.68 ± 3.75	24.77 ± 5.00	17.88 ± 4.63	14.75 ± 4.01
Enterobacteriaceae	*Arsenophonus* sp.	otu19	**0.18 ± 0.13**	**10.82 ± 6.22**	0.42 ± 0.42	0.09 ± 0.09	11.08 ± 11.01	10.56 ± 6.57
Enterobacteriaceae	*Erwinia* sp. or *Pantoea* sp.	otu20	1.22 ± 0.73	0.31 ± 0.21	1.31 ± 0.83	1.18 ± 1.00		0.62 ± 0.41
Moraxellaceae	*Acinetobacter* sp.	otu23	2.04 ± 1.11	1.09 ± 0.76	0.12 ± 0.12	2.84 ± 1.53	1.96 ± 1.48	0.22 ± 0.22
Pseudomonadaceae	uncultured *Pseudomonas* (FJ904261) (245/253)	otu28	0.88 ± 0.88	5.25 ± 3.70	3.00 ± 3.00		0.00 ± 0.00	10.50 ± 7.16
Pseudomonadaceae	uncultured *Pseudomonas* (HM215023)	otu29	4.24 ± 2.55	9.93 ± 4.33	6.89 ± 4.62	3.13 ± 3.13	11.17 ± 7.49	8.70 ± 4.81

Table S4: Part 2			**GUT:** Mean relative abundance ± st. error (%)					
Family	**Genus species**	**otu**	**Sterre**	**Bramier**	**Sterre**		**Bramier**	
			All samples	**All samples**	**No infection**	***A. bombi***	**No infection**	***A. bombi***
Phylum: Firmicutes - Class: Bacilli								
Lactobacillaceae	*Lactobacillus ozensis*	otu33	1.78 ± 1.45			2.52 ± 2.05		
Lactobacillaceae	*Lactobacillus* sp.	otu34	1.10 ± 0.71	1.00 ± 1.00	3.63 ± 2.13	0.05 ± 0.05		2.00 ± 2.00
Lactobacillaceae	*Lactobacillus kunkeei / apinorum* (253/254)	otu35	2.32 ± 1.41	0.21 ± 0.15	3.78 ± 2.90	1.71 ± 1.64	0.30 ± 0.30	0.13 ± 0.08
Lactobacillaceae	*Lactobacillus bombi*	otu38	0.94 ± 0.53	1.14 ± 1.07	2.84 ± 1.56	0.14 ± 0.14		2.27 ± 2.14
Lactobacillaceae	*Lactobacillus bombicola* (249/253)	otu42	1.20 ± 0.59	1.56 ± 0.92	2.83 ± 1.80	0.52 ± 0.29	1.24 ± 0.46	1.89 ± 1.82
Lactobacillaceae	*Lactobacillus apis*	otu43	0.14 ± 0.10	0.08 ± 0.08	0.47 ± 0.29		0.16 ± 0.16	
Lactobacillaceae	*Lactobacillus* HM215046	otu44	9.30 ± 2.67	6.18 ± 1.69	8.86 ± 2.79	9.48 ± 3.68	9.34 ± 2.45	3.02 ± 1.90
Streptococcaceae	*Lactococcus lactis* (252/253)	otu45	1.11 ± 0.64	4.46 ± 2.75		1.57 ± 0.89	1.15 ± 1.15	7.77 ± 5.29
Enterococcaceae	*Enterococcus* sp.	otu47		0.07 ± 0.07			0.14 ± 0.14	
Leuconostocaceae	*Weissella bombi*	otu51	1.12 ± 1.12			1.59 ± 1.59		
Leuconostocaceae	*Leuconostoc* sp.	otu52		0.03 ± 0.03			0.06 ± 0.06	
Leuconostocaceae	*Fructobacillus* sp.	otu54	4.69 ± 2.71	2.87 ± 1.53	4.15 ± 4.15	4.92 ± 3.53	1.61 ± 1.02	4.12 ± 2.92
Phylum: Actinobacteria - Class: Actinobacteria								
Bifidobacteriaceae	*Bifidobacterium bombi*	otu55	1.53 ± 0.91	1.14 ± 1.14	5.11 ± 2.61	0.05 ± 0.05		2.28 ± 2.28
Bifidobacteriaceae	*Bifidobacterium actinocoloniiforme*	otu59		0.12 ± 0.12				0.23 ± 0.23
Bifidobacteriaceae	*Bombiscardovia coagulans* (252/254)	otu60	4.34 ± 1.03	6.66 ± 2.19	4.39 ± 1.35	4.31 ± 1.39	6.24 ± 2.92	7.07 ± 3.43
Bifidobacteriaceae	*Bifidobacterium commune* (253/254)	otu61						
Bifidobacteriaceae	*Bifidobacterium commune*	otu62	1.18 ± 0.72	2.00 ± 0.90	1.48 ± 0.80	1.06 ± 0.98	2.84 ± 1.47	1.16 ± 1.04
Phylum: Bacteroidetes and Acidobacteria								
Flavobacteriaceae	uncultured HM215036_s	otu68	2.80 ± 1.34	2.02 ± 1.14	0.63 ± 0.42	3.71 ± 1.85	3.95 ± 2.14	0.09 ± 0.09

Table S5:. In the fat body, a total of 57 bacterial OTUs were detected, which are listed with their mean relative abundances and standard errors. Sequences were identified in EzTaxon (EzBioCloud), searching the database of both cultured and uncultured bacteria. Bacteria were identified to the level upon which they could be identified with 100 % similarity (usually genus or species level). When there was no 100 % match available in the database, the closest match is given and indicated with the number of matching base pairs (xxx/253). Significant differences (P<0.05) between sampling locations or *A. bombi* infection status of certain OTUs are indicated with a dark background.

Table S5: Part 1			**FATBODY:** Mean relative abundance ± st. error (%)					
Family	**Genus species**	**otu**	**Sterre**	**Bramier**	**Sterre**		**Bramier**	
			All samples	**All samples**	**No infection**	***A. bombi***	**No infection**	***A. bombi***
Phylum: Proteobacteria - Class: Alphaproteobacteria								
Bartonellaceae	uncultured *Bartonella* (AY370187)	otu01		0.08 ± 0.08			0.16 ± 0.16	
Phyllobacteriaceae	*Phyllobacterium* sp.	otu02	**2.89 ± 0.98**	**27.69 ± 6.66**	1.68 ± 0.65	3.40 ± 1.36	37.79 ± 10.23	17.59 ± 7.63
Rhodobacteraceae	*Paracoccus* sp.	otu03		0.03 ± 0.03		3.40 ± 1.37	0.07 ± 0.07	
Anaplasmataceae	uncultured *Wolbachia* (AE017196) (248/253)	otu04	0.04 ± 0.04			0.05 ± 0.05		
Acetobacteraceae	*Saccharibacter floricola*	otu05	0.20 ± 0.10	0.07 ± 0.07	0.14 ± 0.14	0.23 ± 0.13	0.14 ± 0.14	
Acetobacteraceae	*Asaia astilbis*	otu06	0.06 ± 0.06			0.08 ± 0.08		
Phylum: Proteobacteria - Class: Betaproteobacteria								
Neisseriaceae	*Snodgrassella alvi*	otu09	**21.14 ± 2.01**	**9.48 ± 2.62**	19.28 ± 1.78	21.91 ± 2.76	13.43 ± 4.35	5.53 ± 2.51
Neisseriaceae	*Neisseria* sp.	otu10		0.07 ± 0.07			0.14 ± 0.14	
Sterolibacterium_f	*Methyloversatilis universalis*	otu12	0.27 ± 0.12	0.09 ± 0.09	0.38 ± 0.38	0.23 ± 0.10		0.18 ± 0.18
Phylum: Proteobacteria - Class: Gammaproteobacteria								
Orbaceae	*Schmidhempelia* HM215025	otu13	0.49 ± 0.36	0.19 ± 0.15	0.12 ± 0.12	0.64 ± 0.51	0.38 ± 0.30	
Orbaceae	*Frischella perrara*	otu15	0.04 ± 0.04		0.14 ± 0.14			
Orbaceae	*Gilliamella apicola*	otu16	**20.73 ± 2.85**	**7.58 ± 1.62**	22.74 ± 4.04	19.89 ± 3.75	9.48 ± 2.63	5.68 ± 1.82
Pasteurellaceae	*uncultured Haemophilus* sp.	otu17	0.14 ± 0.14	0.07 ± 0.07	0.46 ± 0.46		0.14 ± 0.14	
Vibrionaceae	*Vibrio* sp.	otu18	0.54 ± 0.54			0.77 ± 0.77		
Enterobacteriaceae	*Arsenophonus* sp.	otu19	**0.82 ± 0.43**	**24.96 ± 7.92**	0.42 ± 0.26	0.99 ± 0.60	**12.89 ± 10.89**	**37.02 ± 10.57**
Enterobacteriaceae	*Erwinia* sp. or *Pantoea* sp.	otu20	1.14 ± 0.39	0.22 ± 0.12	1.58 ± 0.72	0.96 ± 0.47	0.11 ± 0.11	0.33 ± 0.22
Pseudoalteromonadaceae	*Pseudoalteromonas* sp.	otu21	0.19 ± 0.19			0.28 ± 0.28		

Table S5: Part 2			**FATBODY:** Mean relative abundance ± st. error (%)					
Family	**Genus species**	**otu**	**Sterre**	**Bramier**	**Sterre**		**Bramier**	
			All samples	**All samples**	**No infection**	***A. bombi***	**No infection**	***A. bombi***
Phylum: Proteobacteria - Class: Gammaproteobacteria								
Moraxellaceae	*Acinetobacter boissieri*	otu22	2.15 ± 1.18	0.30 ± 0.18	0.68 ± 0.56	2.77 ± 1.64	0.17 ± 0.11	0.43 ± 0.35
Moraxellaceae	*Acinetobacter johnsonii*	otu24	0.39 ± 0.18		0.66 ± 0.41	0.28 ± 0.19		
Moraxellaceae	*Enhydrobacter* sp.	otu25	0.51 ± 0.27	0.04 ± 0.04	0.62 ± 0.27	0.46 ± 0.37	0.08 ± 0.08	
Moraxellaceae	*Enhydrobacter* sp.	otu25	0.51 ± 0.27	0.04 ± 0.04	0.62 ± 0.27	0.46 ± 0.37	0.08 ± 0.08	
Pseudomonadaceae	*Pseudomonas* sp.	otu26	0.04 ± 0.04	0.04 ± 0.04	0.12 ± 0.12		0.08 ± 0.08	
Pseudomonadaceae	uncultured *Pseudomonas* (FJ904261)	otu27	1.03 ± 0.55	1.03 ± 0.42	2.40 ± 1.78	0.46 ± 0.22	0.88 ± 0.70	1.19 ± 0.52
Pseudomonadaceae	uncultured *Pseudomonas* (HM215023)	otu29	1.71 ± 0.95	0.97 ± 0.28	3.40 ± 2.86	1.01 ± 0.69	0.83 ± 0.33	1.11 ± 0.47
Oceanospirillaceae	*Marinomonas* sp.	otu30	0.23 ± 0.23			0.33 ± 0.33		
Xanthomonadaceae	*Rhodanobacter glycinis*	otu31	4.39 ± 0.75	1.28 ± 0.32	4.20 ± 1.67	4.47 ± 0.86	1.52 ± 0.55	1.04 ± 0.35
Xanthomonadaceae	*Stenotrophomonas rhizophila*	otu32		0.04 ± 0.04			0.09 ± 0.09	
Phylum: Firmicutes - Class: Bacilli								
Lactobacillaceae	*Lactobacillus ozensis*	otu33	**2.21 ± 1.39**	**0.00 ± 0.00**		3.13 ± 1.92		
Lactobacillaceae	*Lactobacillus* sp.	otu34	0.04 ± 0.04	0.30 ± 0.27	0.14 ± 0.14		0.07 ± 0.07	0.53 ± 0.53
Lactobacillaceae	*Lactobacillus kunkeei / apinorum*	otu36	**2.69 ± 1.68**	**0.04 ± 0.04**	0.30 ± 0.19	3.68 ± 2.34	0.08 ± 0.08	
Lactobacillaceae	*Lactobacillus mellis* (253/254)	otu37	2.48 ± 0.67	1.31 ± 0.37	5.08 ± 1.50	1.39 ± 0.48	1.42 ± 0.40	1.20 ± 0.66
Lactobacillaceae	*Lactobacillus bombi*	otu38	0.74 ± 0.39	1.22 ± 0.99	0.36 ± 0.25	0.89 ± 0.54	0.13 ± 0.13	2.31 ± 1.97
Lactobacillaceae	*Lactobacillus mellifer*	otu39	0.08 ± 0.06		0.28 ± 0.17			
Lactobacillaceae	*Lactobacillus kitasatonis*	otu40		0.19 ± 0.19			0.38 ± 0.38	
Lactobacillaceae	*Lactobacillus bombicola*	otu41	3.69 ± 0.62	2.93 ± 0.90	5.60 ± 1.18	2.90 ± 0.61	2.59 ± 0.49	3.28 ± 1.79
Lactobacillaceae	*Lactobacillus apis*	otu43	1.00 ± 0.23	0.58 ± 0.17	1.92 ± 0.48	0.62 ± 0.18	0.92 ± 0.26	0.23 ± 0.16
Lactobacillaceae	*Lactobacillus* HM215046	otu44	4.12 ± 1.17	1.91 ± 0.66	5.24 ± 2.87	3.66 ± 1.23	3.03 ± 1.19	0.79 ± 0.39
Streptococcaceae	*Lactococcus lactis* (252/253)	otu45	**1.22 ± 0.78**	**5.16 ± 3.56**	0.14 ± 0.14	1.67 ± 1.08	0.21 ± 0.15	10.10 ± 6.90
Streptococcaceae	*Streptococcus* sp.	otu46	0.48 ± 0.28	0.16 ± 0.11	1.10 ± 0.91	0.23 ± 0.10	0.32 ± 0.22	
Bacillaceae	*Anoxybacillus* sp.	otu48	0.04 ± 0.04			0.05 ± 0.05		
Staphylococcaceae	*Staphylococcus* sp.	otu49	5.12 ± 1.01	2.28 ± 1.00	5.52 ± 1.10	4.95 ± 1.39	3.17 ± 1.81	1.40 ± 0.87
Gemella_f	*Gemella_s*	otu50		0.05 ± 0.05			0.10 ± 0.10	
Leuconostocaceae	*Convivina intestini*	otu53	**4.63 ± 2.54**	**0.38 ± 0.16**	0.94 ± 0.43	6.17 ± 3.54	0.20 ± 0.15	0.57 ± 0.27

Table S5: Part 3			**FATBODY:** Mean relative abundance ± st. error (%)					
Family	**Genus species**	**otu**	**Sterre**	**Bramier**	**Sterre**		**Bramier**	
			All samples	**All samples**	**No infection**	***A. bombi***	**No infection**	***A. bombi***
Phylum: Actinobacteria - Class: Actinobacteria								
Bifidobacteriaceae	*Bifidobacterium bombi*	otu55	**0.23 ± 0.15**	**2.01 ± 1.73**	0.62 ± 0.48	0.07 ± 0.07	0.00 ± 0.00	4.02 ± 3.42
Bifidobacteriaceae	*Bifidobacterium asteroides* (253/254)	otu56	1.59 ± 0.59	0.66 ± 0.26	3.62 ± 1.61	0.74 ± 0.31	0.70 ± 0.28	0.62 ± 0.46
Bifidobacteriaceae	*Bifidobacterium actinocoloniiforme* (253/254)	otu57	0.06 ± 0.06	0.22 ± 0.22		0.09 ± 0.09		0.43 ± 0.43
Bifidobacteriaceae	*Bifidobacterium actinocoloniiforme* (253/254)	otu58	0.24 ± 0.17			0.34 ± 0.24		
Bifidobacteriaceae	*Bombiscardovia coagulans* (252/254)	otu60	2.32 ± 0.36	1.49 ± 0.62	2.24 ± 0.47	2.35 ± 0.48	2.26 ± 1.18	0.73 ± 0.34
Bifidobacteriaceae	*Bifidobacterium commune* (253/254)	otu61	0.58 ± 0.15	0.78 ± 0.66	0.86 ± 0.29	0.46 ± 0.18	1.47 ± 1.31	0.10 ± 0.10
Micrococcaceae	*Kocuria* sp.	otu63	0.15 ± 0.15			0.22 ± 0.22		
Brevibacteriaceae	*Brevibacterium aurantiacum*	otu64	0.09 ± 0.09			0.13 ± 0.13		
Corynebacteriaceae	*Corynebacterium lipophiloflavum*	otu65	0.10 ± 0.07	0.03 ± 0.03	0.20 ± 0.20	0.06 ± 0.06	0.07 ± 0.07	
Corynebacteriaceae	*Corynebacterium* sp.	otu66	0.04 ± 0.04	0.06 ± 0.06	0.12 ± 0.12		0.11 ± 0.11	
uncultured EF188441_f	uncultured EF188441_s	otu67	0.18 ± 0.09	0.10 ± 0.07	0.52 ± 0.23	0.04 ± 0.04	0.20 ± 0.13	
Phylum: Bacteroidetes and Acidobacteria								
Flavobacteriaceae	uncultured HM215036_s	otu68	0.79 ± 0.31	0.20 ± 0.20	0.36 ± 0.22	0.97 ± 0.43	0.40 ± 0.40	
Prevotellaceae	*Prevotella* sp.	otu69	0.05 ± 0.05		0.16 ± 0.16			
Blastocatella	uncultured *Blastocatella* (252/253)	otu70	0.04 ± 0.04			0.05 ± 0.05		
Flavobacteriaceae	*Cloacibacterium* sp.	otu11	0.09 ± 0.07		0.12 ± 0.12	0.08 ± 0.08		

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Curriculum vitae

Annelies Billiet was born on the 10th of February 1989 in Ghent, Belgium. She obtained her high school degree in Science-Mathematics at Sint-Laurensinstituut, Zelzate in 2007. Then she studied Applied Bioscience Engineering, option Food Industry, at University College Ghent in the years 2007-2011. During her master thesis she investigated bioactive peptides with CCK receptor activity.

In January 2012 she obtained her IWT PhD grant for strategic basic research and started her PhD in the Lab of Agrozoology within the Department of Crop Protection at the faculty of Bioscience Engineering under the supervision of Prof. dr. ir. Guy Smagghe and dr. Ivan Meeus. During her PhD research, she investigated parameters that can alter the gut microbiota of mass-reared bumblebees (*Bombus terrestris*) as well as the microbiota in several organs of the bumblebee. Her results were and will be published in international journals.

Publication list

Billiet, A., Meeus, I., Cnockaert, M., Vandamme, P., Van Oystaeyen, A., Wäckers, F. and Smagghe, G. (2016). Effect of oral administration of lactic acid bacteria on colony performance and gut microbiota in indoor-reared bumblebees (*Bombus terrestris*). Apidologie. DOI: 10.1007/s13592-016-0447-5

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Tutorships of undergraduate students

Van Mele, M., 2013-2014. Functionaliteit van de microbiota in de hommel (*Bombus terrestris*). Bachelor thesis: Bachelor in de Biomedische Laboratoriumtechnologie

De Clercq, E., 2012-2013. Invloed van orale toediening van *Lactobacillus* en *Bifidobacterium* op kolonieontwikkeling en microbiota van de hommel (*Bombus terrestris*). Bachelor thesis: Bachelor in de Biomedische Laboratoriumtechnologie

Martens, M., 2012-2013. Invloed op de microbiële darmflora van hommels na blootstelling aan biologische insecticiden gebruikt binnen entomovectoring. Masterthesis: Master na Master in de Milieusanering en het Milieubeheer

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