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Beneficial microorganisms for honey bees health

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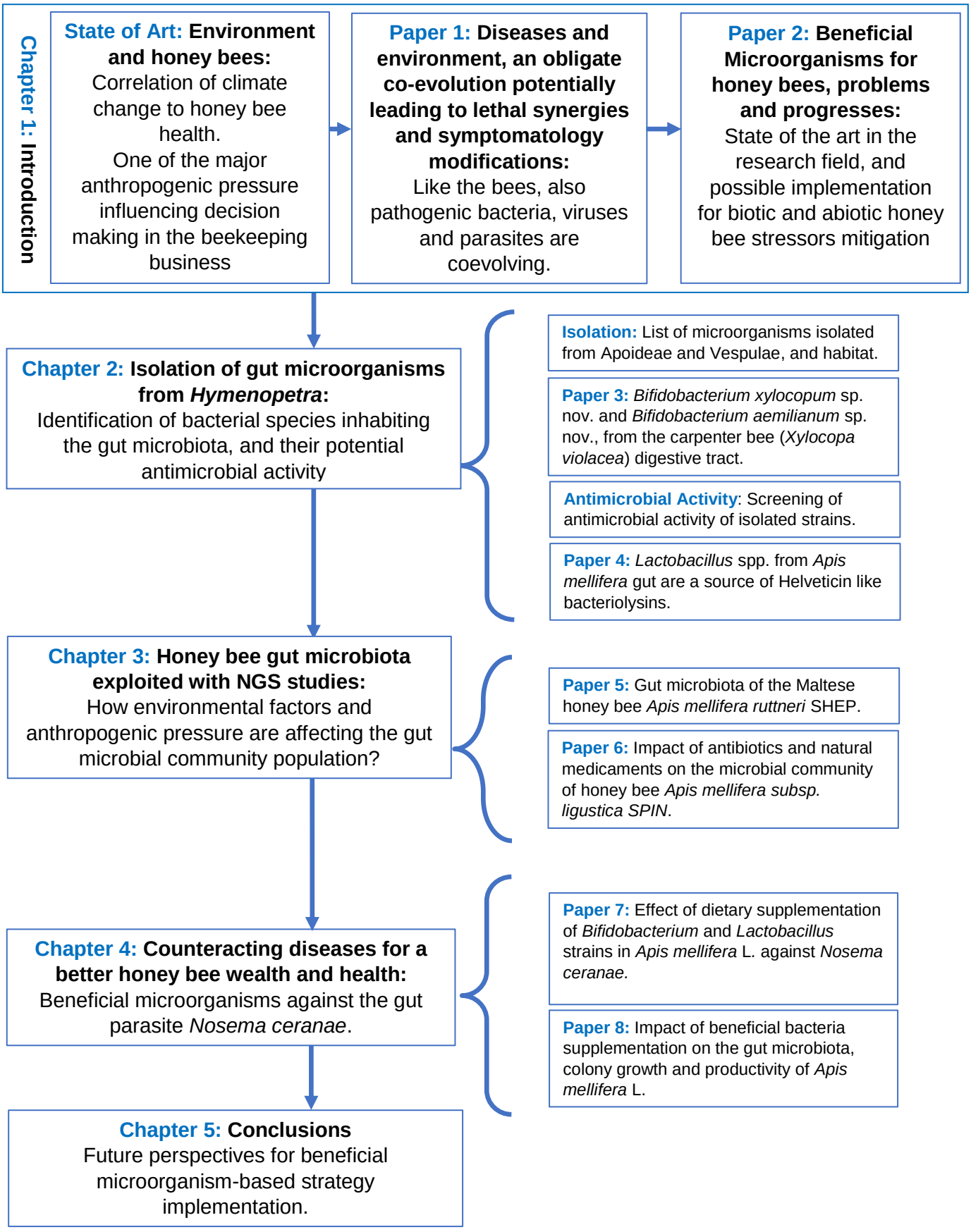
Thesis Abstract

Honeybees (*Apis mellifera* and other species) are considered as the most economically important insect species for humans and the ecosystems, not only as honey producers but also and especially as pollinators of agricultural, horticultural crops and wild plants (approximately 90 different farm-grown foods, including many fruits and nuts, depend on honeybees), contributing at the pollination of 35% of the global food production. Unfortunately, honeybee decline started about 30 years ago, with the arrival from Asia of the bee mite *Varroa destructor.* Since then, honeybees have been damaged by different kinds of biotic and abiotic stressor factors, cumulating any kind of damages, and posing a serious threat to the agricultural field. Many scientists agree that bee decline is a multifactorial process in which a mechanism seems to be more important in a given period of the year than in another, and different mechanisms may predominate in another period or in other environments. Of those multifactorial processes, leading factors are the new emergent pathogens, such as *Nosema ceranae* a gut pathogen causing serious threat to bees and the consequent death of the colony; Viruses such as “deformed wing virus”, “Black queen cell virus”, “chronic paralysis virus” and many others that are often over transmitted by the mite parasite *Varroa destructor*. Pesticides and other environmental stress factors are furthering enhancing the high pathogenicity on bees, weakening more and more the delicate beehive superorganism balance. The major science concern about the bees usually regards the study of the bee pathogens and their interaction with an increasingly anthropized environment (*e.g.*: pollution and sub lethal poisonings). Only few research projects (of high scientific importance) have been carried out using an approach aimed to fix the problems linked with it. Even less are the researches investigating probiotic microorganisms as growth promoter, in order to obtain a better wealth and wellbeing of the bees. In the light of these possibilities the aim of my research is the development of -environmental friendly- microbial technologies aimed to increase the health of the bees.

Keywords: Hymenoptera, Apoidea, *Apis mellifera*, gut microbiota, beneficial bacteri
Nosema ceranae, EFB, *Lactobacillus*, *Bifidobacterium*, Bacteriocins, antimicrobial a
DGGE, next generation sequencing (NGS), climate change.

PhD Thesis Conceptual Flow:



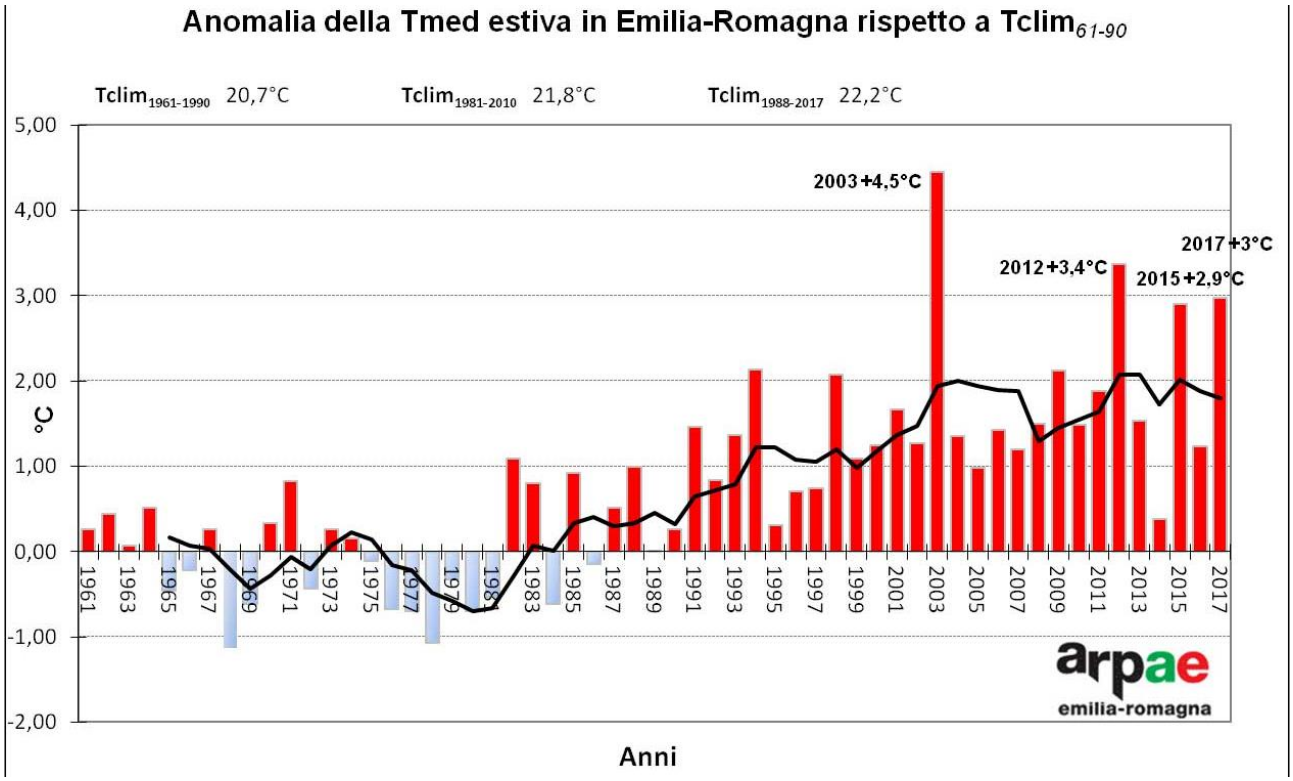
THESIS INDEX

CHAPTER 1

INTRODUCTION

Climate change, a look to a case study: the year 2017.

Extreme and unusual hot temperatures and draught were the ingredients of the past summer 2017. An endless summer that represents a further step towards the overall climate warming of the Emilia-Romagna region, i.e. the Italian region where University of Bologna is located. A problem joined by most of regions facing the Mediterranean Sea. Having a look at the registered records of temperatures, Summer 2017 is the 3rd hottest summer since 1961, preceded only by Summer 2003 and 2012. Taking into account then average temperature from the year 1961 to the year 1991, temperatures have been of +4.5°C degrees above average in year 2003, +3.4°C in year 2012, whereas in 2017 temperatures were "only" +3°C above average (See Bar Chart 1 and Figure 1). Summer 2017 had the prerogative to celebrate its own first place in terms of absolute temperature: +42.2°C represents the highest temperature registered in the region in the last 150 years. This peak was registered the first week of August 2017, and, with the humidity typical of the region (continental climate), the perception by the human population (and consequently by animal and plants) was + 54°C.

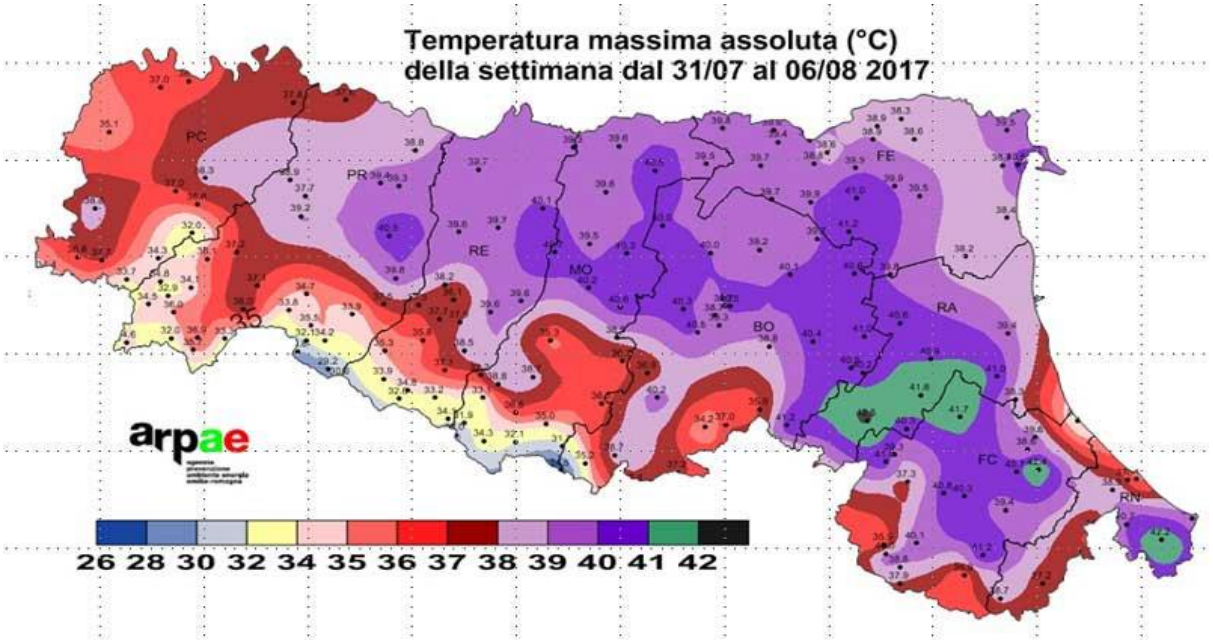


Bar Chart 1: Average temperature anomaly in the Emilia Romagna region in comparison with the average climate temperature calculated from 1961 to 1990. Source ARPA Emilia-Romagna.

Extreme temperatures were not the only deleterious factor. Rainfalls were missing as well, recording a -80% in the period January-October 2017. Moreover, scarce rainfalls and snow accumulation on the Apennine during Autumn 2016 didn't help as well. In 2017, the agricultural sector was brought to its knees already in the spring. The Emilia Romagna regional government was forced to officially claim the natural calamity status at the

beginning of the summer (16 June 2017). Following the claim, the Italian government authorized emergency measures to counteract agricultural production losses on June 22, 2017. Emilia Romagna (E.R.) region was so authorized to increase beyond the traditional limit the water collection amount in the Po river, a measure that, however, was able to satisfy only few areas of the region.

a)



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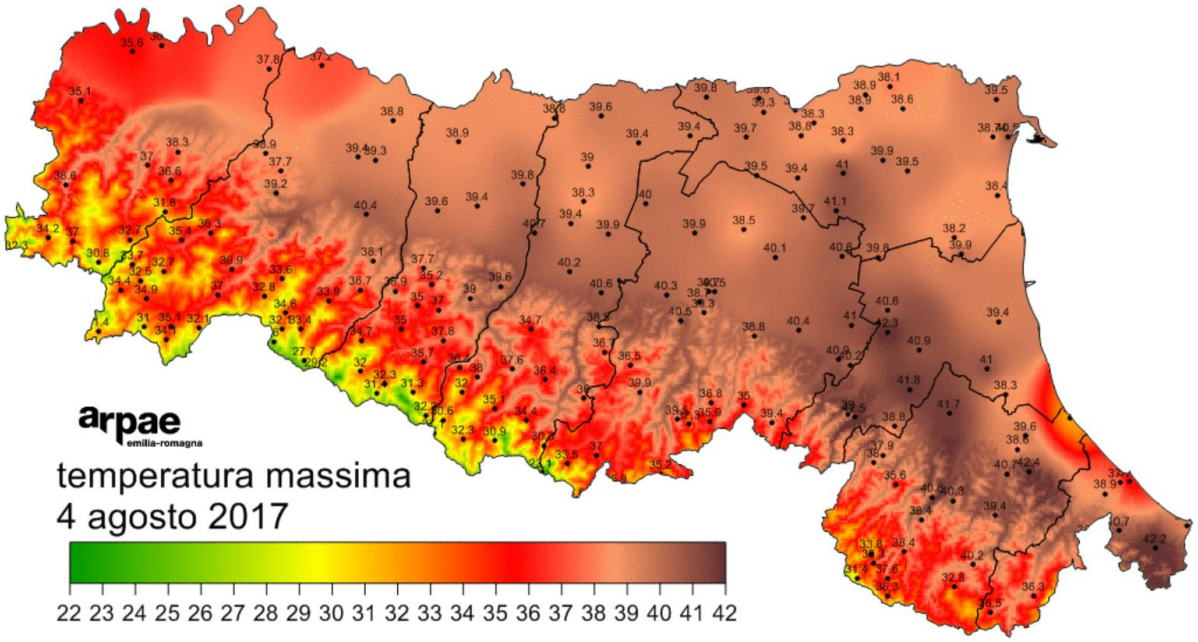


Figure 1: a) Maximum temperature anomaly in the Emilia Romagna region from 31 July to 6 August 2017. b) Maximum temperature anomaly in the Emilia Romagna region on the 4th of August. Source ARPA Emilia-Romagna.

The most significant climatic output of the year 2017 is that high temperatures combined with low rainfalls (Figure 2) determined the worst water deficit (ground water, rivers, basins) of the regional history. This fact determined the first recorded extensive forest death of the modern history. Defoliation, desiccation from the roots of extensive forest areas, especially in the low-medium altitude hills. Forest fruits losses (chestnuts, small fruits, etc…) were impressive, with premature total drop in certain areas. This fact together with an extensive wild animals' death was detected all over the region with different gravity degrees.

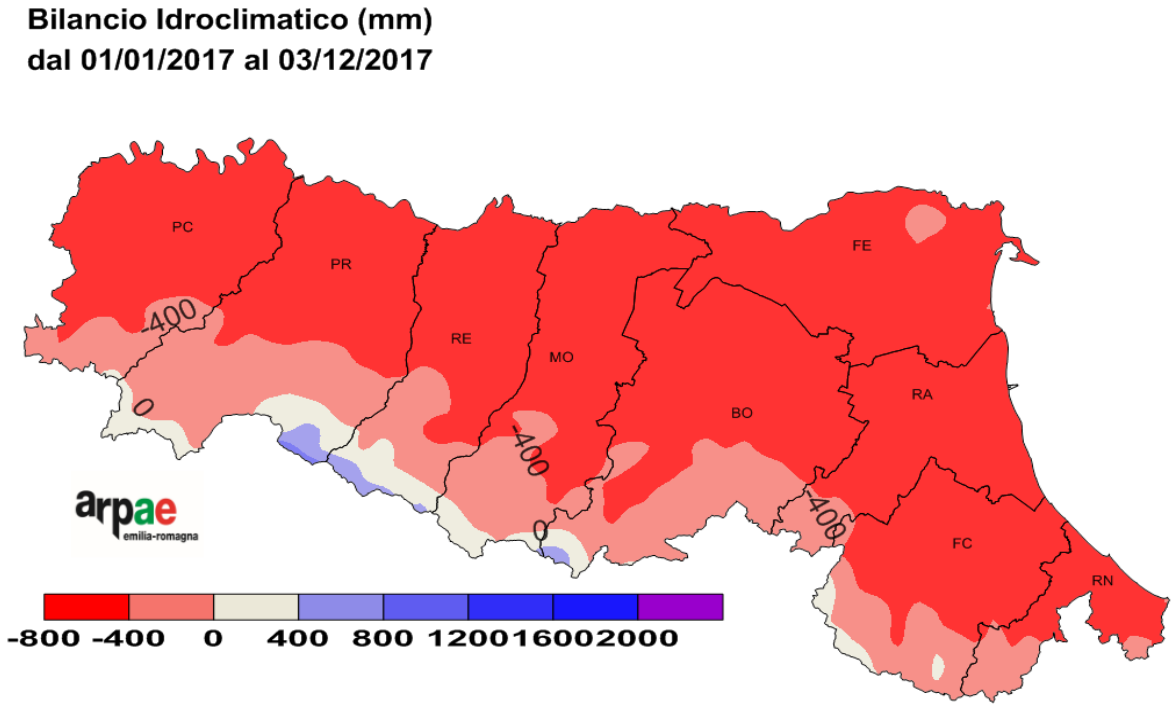


Figure 2: The missing rain; Rainfalls deficit from 1 January to 3 December 2017 expressed in millimetres (mm). Source ARPA Emilia-Romagna.

Unfortunately, 2017 is neither the sole case of emergency in the region, nor the sole region of the Mediterranean basin suffering. In the last decades, climatic instability has primed a fast reshaping of the ecosystem and landscape of the region. Entire plant genus like *Carpinus betulus* have been eradicated from the majority of their natural habitat (Po river valley). Other genus like *Quercus robur* and *Quercus pubescence*, well known for their resistance to extreme climatic condition, were highly stressed (figure 3a; 3b, 3c and 3d). Draught enhances fungal diseases proliferation on *Castanea sativa* (Waldboth and Oberhuber 2009) contributing to the reduction of the chestnut Apennine vegetation belt. Herbaceous vegetation also suffers from this unnatural condition and, for instance, meadow of *Taraxacum officinale* are rarer and rarer to see in spring.

Figure 3: a) *Quercus robur* and *Q. pubescences* mixed forest under drought stress in the high Apennine (Castel D'Aiano, Bologna district, about 800m a.s.l. – 17 August 2017). b) *Q. pubescences* dried out. c) Contrast of irrigated garden with the background forest in draught stress (Castello di Serravalle, Bologna district, about 149m a.s.l. – 8 August 2017). D) young plants in a 10years old reforestation project, dead.

This is the contest in which I wish to introduce the decline of honeybees (*Apis mellifera* subsp. *ligustica*) in the Emilia-Romagna region.

Honeybees suffering climate change

In Italy there are countless honeybees' ecotypes that are closely associated with their environment. They all belong to *Apis mellifera* subsp. *ligustica* that coevolved with the environment in close niches protected by natural barriers. These barriers prevented for thousands of years any cross contact with other neighbour ecotypes, making a unique and diversified genetic patrimony. This genetic diversity is endangered by a fast climate change. In fact, climate change seems to be faster than evolution in remodulating bee ecotypes behaviour and genetic potential to allow survival, making bees weaker and totally dependent on mankind support.

In the 80s, the worldwide spread of *Varroa destructor* declared a stated dependency of honeybees to beekeepers; in other words, bees cannot survive without the help of beekeepers. It's been more than 30 years ago, and since then beekeepers (but not bees) have learnt to live with *Varroa* very well. It is not paradoxical to compare climate change seriousness to a new *Varroa destructor* –like advent. Honeybees require once again human protection, because of their importance not only in agricultural production as pollinators but also for the economical sector. Beekeepers are going to spend again efforts to save them as they did once with Varroa.

Climate change estimations predict, in few decades starting from now, an increased desertification and retreating icecaps or snowmelt. Processes that will somehow change

rainfall patterns leading to a greater frequency of extreme climate events will be generated (Le Conte and Navajas 2008).

The floral distribution is already changing, often reducing colony harvesting capacity due to scarce production or out of time blooming. Honeybees have lost their rhythm and cannot adapt to changing conditions so easily. For instance near Bologna, *Robinia pseudoacacia*, that constitutes the main honey harvest of the region, has bloomed in 1st of May in 2015 and on the 6th of April in 2017. Tree weeks of difference is a big-time shift for the honeybees' biological clock that may lead to the missed harvest of natural resources. In the Emilia-Romagna region, considering the two main food components, i.e. nectar and pollen, it is a fact that a scarcity of pollen and impoverishment of its nutritional quality have been observed since the beginning of summer. Nectar flow is more and more replaced by honeydew. The positive influence of a diet rich in pollen for bees is well known: It stimulates a higher brood deposition, but it can also help fighting diseases, for instance significantly prolonging the lifespan of bees infected with pathogens such as *Nosema ceranae* (Di Pasquale et al., 2013).

How will the pathogen/bee interaction evolve?

Studies related to colony collapse disorder (CCD) in the U.S.A. or colony death in Europe have shown that honeybee colonies are infested by numerous pathogens. Moreover, there is feeling that symptomatology does not frequently reflect the effective causative agent of disease. In fact, it has been shown that biotic and abiotic stressor synergy can modify symptomatology to the extent of sudden colony collapse as happened with CCD in the U.S.A.

Although with a lower extent, also Italy had to face a change of symptomatology for certain diseases. The case of European Foulbrood (EFB), that is going to be fully described in paper 1 (page 7), is a very explicative and clamorous example. Italian beekeepers had to face for years a symptomatology hybrid of both EFB and American Foulbrood (AFB), that has treaked both the veterinary institutions and beekeepers. The major question was "Should I set this beehive on fire or not?". Then beekeepers realized that this peculiar foulbrood was disappearing with a simple queen bee supersedurre. Consequently, it could not be the "true" AFB, known to be incurable. Italian beekeepers developed a new name for the disease that can be translated in "Fake - AFB". A deep investigation on honeybee infected larvae brought to the discovery that the symptomatology change was caused by a substitution of the secondary invader of the main EFB disease agent *Melisococcu plutonius. Paenibacillus alvei* was substituted by *Paenibacillus dendritiformis*, a less virulent secondary invader that brought to death the larvae a couple of days later than normal. The secondary invader allows the complete or partial capping of the diseased brood cells leading to a symptomatology closer to AFB than EFB. This is the first example of symptomatology change. The reason is still unknown, but it might be therefore hypothesized that climate change enhanced also a microbial niche evolution.

A second case is the disappearing of *Nosema apis*. In the last 10 years, no beekeepers had fortunately the chance to face this disease, causing strong dysenteries to honeybees. It seems that *Nosema ceranae* has completely replaced *Nosema apis*, for unknown reasons,

but most probably to be linked once again with climate change. The synergy of climate change and *N. ceranae* has been well experienced by Italian beekeepers in the last years. *N. ceranae* seem to be easily controllable when strong pollen importation into the beehive is possible. On the contrary, pollen deficit has often lead to rapid (3 weeks) honeybee colony death. This rapid death has often been confused by beekeepers as a failure in the *V. destructor* control. Differently, *N. ceranae* is a silent killer, easily taking advantage, in summer, of environmental disorders.

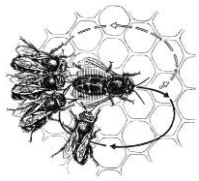
Economic Factors

From the data picked by the “Italian Honey Production Observatory” overall national territory, from January until August 2017 the recorder production of honey is of -80% if compared with the average of past years. The harvest of the main honey kinds (Orange, Acacia, Eucalyptus, Linden) has been disastrous due to excessive draught as main reason followed by out of range temperatures. Beekeepers in the most of Italy were forced to nourish honeybees to winter hives in acceptable conditions (report from Italian National Honey Production Observatory, September 2017).

Mitigation measure is the answer of this thesis to honeybee stressors

Several approaches are feasible to mitigate stressors deriving from climate change. In this thesis we propose a microbial mediated control method. Following laboratory and *In field* test, it was clear how mixtures of beneficial bacteria can:

1. Fight *N. ceranae*, when administered in an early disease proliferation stage
2. Stimulate honeybees’ population development, allowing a better harvest of honey and especially pollen as storage for extreme events.



Diseases and environment, an obligate co-evolution potentially leading to lethal synergies and symptomatology modifications. The case of a **microbial investigation on honey bee larvae showing atypical symptoms of European Foulbrood**

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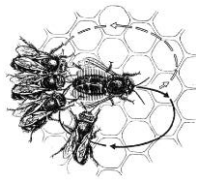


Abstract

Culture-dependent and culture-independent techniques were applied to investigate the bacterial communities occurring in 5-6 days-old honey bee larvae (*Apis mellifera* L.), with evident symptoms of disease and healthy-looking ones. Samples were collected by the Bee Emergency Service Team (BeeNet Project) from two apiaries located in different geographical sites (North and South Italy). Observed symptoms were atypical, but very close to those attributed to the European Foulbrood (EFB) and to the American Foulbrood (AFB), which are severe diseases affecting honey bee larvae worldwide. Isolates from diseased larvae were identified as *Enterococcus faecalis* Schleifer et Kilpper-Balz and *Paenibacillus dendritiformis* Tcherniakov. *Melissococcus plutonius* Bailey et Collins, the causative agent of EFB, was detected by polymerase chain reaction in both diseased and healthy-looking larval samples, whereas *Paenibacillus larvae* White, the causative agent of the AFB, failed to be detected. Microbial profiles obtained by denaturing gradient gel electrophoresis did not show relevant differences among samples, thus evidencing that the healthy-looking samples were partially affected. Besides confirming the presence of *E. faecalis* and *P. dendritiformis,* as found by plate count, the technique confirmed the presence of *M. plutonius* in all samples. The study has evidenced that honey bee larvae were affected by the EFB, with the presence of an atypical *Paenibacillus* species as second invader, which presumably confers a different symptomatology to the diseased brood.

Keywords: European foulbrood, American Foulbrood, honey bee larvae, atypical symptoms, *Paenibacillus dentritiformis*, denaturing gradient gel electrophoresis (DGGE)

Introduction

Honey bees (*Apis mellifera* L.) are one of the insect species more relevant for humans as pollinator for the production of many fruits, vegetables and stimulant crops (Abrol, 2011). In recent years, larvae and adult honey bees are subjected to different kind of biotic and abiotic stresses, leading to a severe colony loss and decrease in hive products with large economic damages. The parasites and pathogens, affecting larvae and bee health, include mites (*Varroa destructor* Anderson et Truemann), microsporidia (*Nosema* spp.), fungi (*Ascosphaera apis* (Maassen ex Claussen) Olive et Spiltoir), bacteria (*Paenibacillus larvae* White, *Melissococcus plutonius* Bailey et Collins) and viruses (Genersch, 2010).

The most known diseases affecting the bee larval stage are the European Foulbrood (EFB) and the American Foulbrood (AFB). EFB is a severe bacterial brood disease, caused by the Gram-positive bacterium *Melissococcus plutonius*. The disease is widely distributed, leading to brood losses and the consequent colony collapse (Bailey and Ball, 1991). The ingestion of contaminated food induces the proliferation of the pathogen in the larval midgut and its consumption of the larval food. Larvae are susceptible at any stage before cell capping and usually death occurs during the 4^{th}-5^{th} day of life. Infected larvae die from starvation (Bailey, 1983), twisted around the cell wall or stretched out, and are then decomposed by secondary invaders like *Paenibacillus alvei* Cheshire et Cheyne and *Enterococcus faecalis* Schleifer et Kilpper-Balz, two saprophytic bacteria frequently associated with EFB (Forsgren, 2010). Diseased larvae are easily identifiable, since larvae move in the brood cell leaving off the

normal coiled position. Moreover, their colour changes from pearly white to yellow, then brown and finally, when they decompose, greyish black (Bailey, 1960). Affected brood may have a very stale or sour odour, sometimes acidic, like vinegar, but often there is no smell (OIE, 2008).

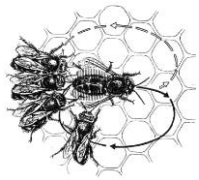
The causative agent of AFB is the Gram positive, spore-forming bacterium *Paenibacillus larvae* (Genersch *et al*., 2006), which contaminates the first instar larvae leading them to death after cell capping. *P. larvae* passes from the midgut throughout the epithelium and invades the hemocoel (Davidson, 1973; Bailey and Ball, 1991). It is reported that during the vegetative growth and infection it secretes highly active extracellular proteases (Hrabak and Martinek, 2007), which probably cause the degradation of the epithelial barrier, thereby allowing the hemocoel invasion. In a second stage the larvae become a brownish, semi-fluid, glue-like colloid (ropy stage) releasing a putrid smell. The ropy aspect (dead larvae adhere and form a thread span when touched with a wooden stick) confirmed the presence of AFB. Finally, the larval remains dry out to a hard scale (foulbrood scale), which tightly adheres to the lower cell wall. The scales contain millions of spores, which could distribute the infection for many years within and between colonies (Bailey and Ball, 1991).

The current work was performed in spring 2012, following the warning of beekeepers from all Italy, denunciating, to the Bee Emergency Service Team (BeeNet Project), misleading symptoms very close to both EFB and AFB, affecting honey bee larvae. Suspected diseased brood looked like a mosaic with open cells (typical symptoms of EFB) and pierced cells (tipycal symptoms of AFB). It was possible to observe simultaneously in the same frame, dead larvae before and after the cell capping. Compared with the classic EFB symptoms, the larvae often died 2-3 days later, closer to AFB death timing). Uncapped diseased larvae quickly lost their shape and then liquefied in the bottom of the cells. Larvae had white-ivory colour without any characteristic smell. Partially capped cells showed diseased larvae (6^{th}-7^{th} day) softly adhering to the lower cell wall; if touched with a wooden stick the larvae explode realising a non-colloid, not smelling, white-brown liquid. Dried scales (similar to those observed in the AFB) were easily removable. To understand deeply the observed symptomatology, available material of diseased and healthy-looking larvae was sampled from two apiaries, one in North Italy and one in South Italy. Culture dependent (plate-count, 16S rDNA sequencing) and independent techniques (denaturing gradient gel electrophoresis, PCR-DGGE) were applied to allow the most accurate microbiological investigation.

Materials and Methods

Larvae sampling

5-10 honey bee larvae (5^{th}-6^{th} day of life) were collected from an infected hive in two apiaries located one at North and one at South Italy. Based on their aspect and position, the larval material was pooled, and samples were classified as follows: NHHL (healthy-looking honey bee larvae from North Italy) NDHL (diseased honey bee larvae from North Italy), SHHL (healthy-looking honey bee larvae from South Italy), SDHL (diseased honey bee larvae from South Italy).



Bacteria isolation and enumeration from diseased honey bee larvae

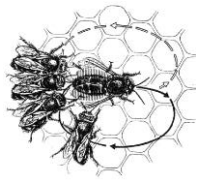
1 gram of pooled diseased honey bee larvae, previously washed in sterile water and homogenised with a plastic pestle, was dissolved in 9 ml of phosphate-buffered saline (PBS) and tenfold serial dilution prepared. For the presumable detection of *M. plutonius*, the resulting suspension was streaked out onto SYPG agar (Bailey and Ball, 1991) and anaerobically incubated at 35±1 °C for 3-5 days. Isolation and enumeration of *P. larvae* was attempted on MYPGP agar (Dingman and Stahly, 1983) after a pasteurization treatment on NDHL and SDHL samples (NDHL-P and SDHL-P) at 80 °C for 10 min (aerobic incubation at 35±1 °C, 24h). Experiments were performed in triplicate and results were expressed as mean $\log_{10}$ of colony-forming units (cfu) per gram of triplicates ± standard deviation (sd). Between 20 and 40 colonies from each sample and from both media (SYPG and MYPGP agar), randomly selected, were re-streaked and purified. For long-term storage, purified isolates were stored at -80 °C.

DNA extraction

DNA was extracted from isolated bacterial colonies, from *M. plutonius* ATCC 35311, *P. larvae* ATCC 9545 and from *P. alvei* ATCC 6344 using the InstaGene Matrix DNA extraction kit following the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Total genomic DNA from pooled samples of diseased and healthy-looking larvae was extracted in duplicate with the QIAamp DNA stool kit (Qiagen, West Sussex, UK). Following extraction, the purity and concentration of DNA were determined by measuring the ratio of the absorbance at 260 and 280 nm (Infinite® 200 PRO NanoQuant, Tecan, Manne-dorf, Switzerland). The DNA was stored at -20 °C.

BOX-PCR analysis

Bacterial isolates were fingerprinted by BOX-PCR. The reaction was carried out in a 30 µl volume containing 1 U Taq DNA polymerase (AmpliTaq Gold, Applied Biosystems, Foster City, CA, USA), 3 µl 10X PCR Gold Buffer (Applied Biosystems), 200 nM of each dNTPs (Fermentas International Inc., Thermo Fisher Scientific Inc., Waltham, MA, USA), 2 mM $MgCl_2$ (Applied Biosystems), 0.4 µM of primer BOXA1R (5'-CTACGGCAAGGCGACGCTGACG-3') (Eurofins Genomics, Ebersberg, Germany), 0.1% (wt/vol) Bovine Serum Albumin (BSA, Fermentas), 2 µl of DNA template, and sterile MilliQ water. The PCR reaction was performed on a TGradient Biometra thermocycler (Biotron, Göttingen, Germany) under the following thermocycling program: 7 min of initial denaturation at 95 °C, 30 cycles of 94 °C for 1 min, 53 °C for 1 min, 72 °C for 3 min followed by a final elongation step of 72 °C for 10 min. After electrophoresis (2% w/v agarose gel at 75 V for four hours), gels were stained with ethidium bromide and visualized with the gel documentation system Gel DocTM X^+R (Bio-Rad). A dendrogram was constructed using the Dice similarity coefficient and the UPGMA (unweighted pair group method with arithmetic mean) algorithm with GelCompar II software, version 6.6 (Applied Maths, Sint-Martens-Latem, Belgium).



16S rDNA amplification

Representative isolates (SDHL-5 and NDHL-P1) were selected after BOX-PCR analysis and the 16S rDNA amplification was performed with universal primers 27f and 1492r, according to Gaggìa *et al*. (2013). The 50 µl reaction mixture contained 1X PCR Gold Buffer (Applied Biosystems), 200 nM of each dNTPs (Fermentas) 0.2 µM of each primer (Eurofins Genomics), 1.5 mM $MgCl_2$ (Fermentas), 1 U Taq polymerase (AmpliTaq Gold, Applied Biosystems), 4 µl of DNA template (20-50 ng/µl) and sterile MilliQ water. The PCR reaction was performed on a TGradient Biometra thermocycler (Biotron, Göttingen, Germany) under the following thermocycling program: 7 min of initial denaturation at 95 °C, 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min and a final extension step at 72 °C for 7 min. After electrophoresis (1.5% w/v agarose gel at 75 V), gels were stained with ethidium bromide and visualized with the gel documentation system Gel DocTM XR (Bio-Rad).

Detection of *Melissococcus plutonius* and *Paenibacillus larvae* by species-specific PCR

A PCR method was used to selectively amplify the 16S rDNA of *M. plutonius* from a pure culture (ATCC 35311) and from DNA samples (NDHL, SDHL, NHHL, SHHL), according to Govan and Brözel (1998). Detection of *P. larvae* from the same samples was performed according to Bakonyi *et al*. (2003) with a modified annealing temperature, which was set at 55 °C. *P. larvae* ATCC 9545 was used as positive control. The molecular weights of the PCR products were determined by electrophoresis in a 1.5 % agarose, stained with ethidium bromide and visualized with the gel documentation system Gel DocTM XR (Bio-Rad).

PCR-DGGE analysis

The V2-V3 region of the 16S rRNA gene (rDNA) of the four samples in duplicate (NDHL, SDHL, NHHL, SHHL,) was amplified by PCR with the universal primer set HDA1-GC (5′-**CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G**AC TCC TAC GGG AGG CAG CAG T-3′) and HDA2 (5′-GTA TTA CCG CGG CTG CTG GCA-3′) (Walter *et al.*, 2000). An identification ladder was also prepared with the DNA of the strains *M. plutonius* ATCC 35311, *Enterococcus faecalis* SDHL-5, and *Paenibacillus dentritiformis* NDHL-P1. The reaction was carried out in a 50 µl volume containing 1.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems), 5 µl of 10X PCR Gold Buffer (Applied Biosystem), 200 µM of each deoxynucleotide triphosphate (Fermentas), 1.50 mM $MgCl_2$ (Fermentas), 0.45 µM of each primer (Eurofins Operon), 2.5% (w/v) bovine serum albumin (BSA; Fermentas), 4 µl DNA template, and sterile MilliQ water for adjustment of the volume to 50 µl. The PCR reaction was performed on a TGradient Biometra thermocycler (Biotron, Göttingen, Germany) under the following thermocycling program: 7 min initial denaturation at 95 °C; 35 cycles of 95 °C for 30 s, 54 °C for 60 s, 72 °C for 40 s; followed by a final elongation step of 72 °C for 7 min. The size and amount of the PCR products were estimated by analysing 5 µl of samples by agarose gel (1.5% w/v) electrophoresis and ethidium bromide staining.

The DGGE analysis was performed as first described by Muyzer *et al*. (1993), using a DCode System apparatus (Bio-Rad). Polyacrylamide gels [8% (w/v) acrylamide:

bisacrylamide (37.5 : 1) (Bio-Rad)] in 1X Tris-Acetate-EDTA (TAE) buffer were prepared using a Bio-Rad Gradient Delivery System (Model 475, Bio-Rad), using solutions containing 40–60% denaturant [100% denaturant corresponds to 7 M urea (Sigma-Aldrich, Milan, Italy) and 40% (v/v) formamide (Sigma-Aldrich)]. The electrophoresis was run at 55 V for 16 h at 60 °C. Gels were stained in a solution of 1X SYBR-Green (Sigma-Aldrich) in 1X TAE for 20 min and their images captured in UV transillumination with Gel Doc™ XR apparatus (Bio-Rad). Cluster analysis was performed with the software GelCompar II version 6.6 (Applied Maths), by the UPGMA algorithm based on the Pearson correlation coefficient with an optimization coefficient of 1%.
Selected bands, particularly those migrated at the same distance of the reference species, were cut from the gel with a sterile scalpel and DNA was eluted by incubating overnight the gel fragments in 50 µl of sterile deionised water at 4 °C. 2 µl of the solution were then used as template to re-amplify the band fragments with the same PCR condition describe above. After amplification and repeated DGGE, purity and co-mobility with amplified DNA obtained directly from larvae samples were assured. Bands were excised again and after overnight elution in sterile deionized water, an amplification without GC-clamp was performed.

Sequence analysis of 16S rDNA of bacterial isolates and DGGE bands
The amplified 16S rDNA from the isolated strains and the obtained amplicons from DGGE bands were then purified (PCR clean-up; Macherey-Nagel GmbH & Co. KG, Germany) and sequenced (Eurofins Operon) with primers 27f and 1492r and HDA-2 respectively. Sequence chromatograms were edited and analysed by using Finch TV software version 1.4.0 (Geospiza Inc., Seattle, WA, USA) and percentage of similarity was determined searching against the NCBI GenBank database using megablast algorithm (http://www.ncbi.nlm.nih.gov/BLAST/).

Results

Plate count, BOX-PCR and sequence analysis of 16S rDNA
The results of viable counts obtained in SYPG and MYPGP agar from diseased larvae samples are reported below, indicating the average of triplicate expressed as $\log_{10}$ (cfu/g of sample) ± standard deviation. High colony counts were obtained from SYPG agar in SDHL (8.75±0.05), whereas less colonies were obtained in plates referred to NDHL samples (3.37±0.04). Enumeration of sporogenic bacteria (NDHL-P and SDHL-P samples) was comparable in both geographical areas (4.57±0.08 vs 5.71±0.02). The cluster analysis of BOX-PCR of all isolates (NDHL, NDHL-P, SDHL and SDHL-P) evidenced two clusters (similarity of 30%). All of the isolates on SYPG agar, belonging to NDHL and SDHL samples, displayed a unique fingerprinting (100% similarity), as well as isolates from MYPGP agar NDHL-P and SDHL-P, whose profiles were 100% similar. The strains SDHL-5 and NDHL-P1 (one from each cluster) were identified by sequence analysis of the 16S rRNA gene, based on the closest match on GenBank database. SDHL-5 sequence was ascribed to *Enterococcus faecalis* (100% similarity; Accession number KR073926) while the strain NHDL-P1 was identified as *Paenibacillus dendritiformis* Tcherniakov (100 % similarity; Accession number KR073927).

***Melissococcus plutonius* and *Paenibacillus larvae* detection by species-specific PCR**

Results of the amplification reaction for the detection of *M. plutonius* are shown inFigure 1a. All the analysed samples (in duplicate), including the positive control *M*. *plutonius* ATCC 35311, produced a band of 812 bp. On the other hand, the amplified product was absent in the negative control (*E*. *faecalis* SDHL-5). With regard to *P. larvae*, all samples were negative except for the positive control *P. larvae* ATCC 9545 (Figure 1b).

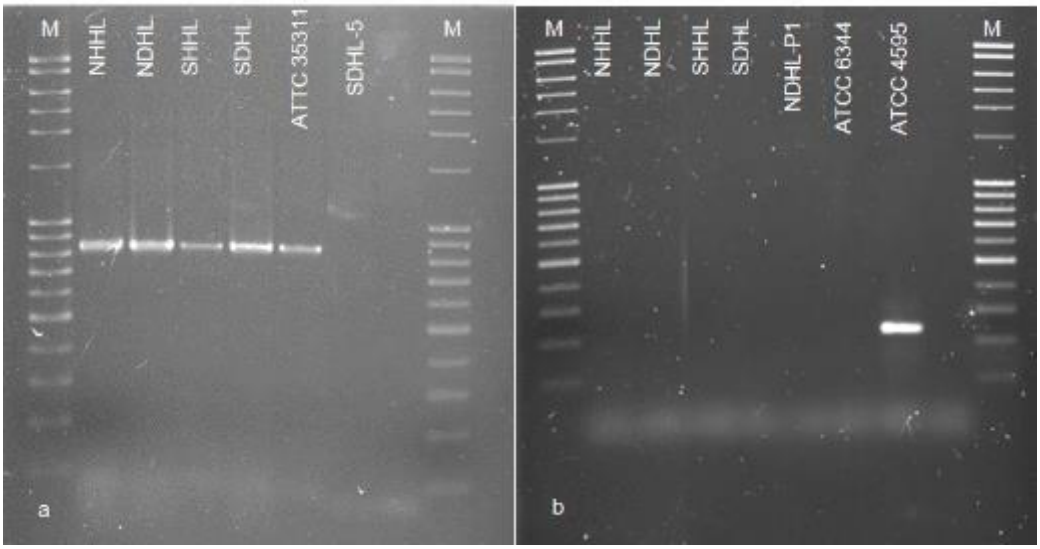


Figure 1. Species-specific PCR for the detection of *M. plutonius* (a) and *P. larvae* (b) from one replicate of DNA samples. M: 100 bp DNA Ladder: NHHL: healthy-looking honey bee larvae from North Italy; NDHL: diseased honey bee larvae from North Italy; SHHL: healthy-looking honey bee larvae from South Italy; SDHL: diseased honey bee larvae from South Italy; ATCC 35311: *M. plutonius*; SDHL-5: *E. faecalis;* NDHL-5: *P. dendritiformis*; ATCC 6344: *P. alvei*; ATCC 9545: *P. larvae*

PCR-DGGE and band sequencing

The PCR-DGGE analysis on diseased and healthy larval duplicate samples showed profiles with a few bands. UPGMA dendrogram and bacterial communities fingerprinting are shown in Figure 3a and 3b, respectively. Overall, the analysis revealed high similarity of the DGGE patterns obtained from each of the two replicates. The cluster analysis showed a distinct division between samples of South Italy and North Italy. The diseased samples in both geographical area clustered separately from healthy samples (similarity less than 60% and 85% respectively).

Sequencing results of excised bands (Figure 3b) are shown in Table 1. Bands 1, 3, 4 and 5 belonged to different species of *Lactobacillus*, detectable in all samples, mainly in SHHL and SDHL. Interestingly, bands 6, 8 and 10 showed a migration distance comparable to the reference strains in the ladder profile (*E. faecalis* SDHL-5, *M. plutonius* ATCC 35311 and *P. dendritiformis* NDHL-P1). Band 6, common to all profiles, was identified as *M. plutonius*. Band 8, excised from sample SDHL was identified as *E. faecalis*. Bands migrating at the same level, although showing a decreased intensity, were also detected in SHHL. Finally,

band 10 excised from SDHL, was identified as *Paenibacillus* spp.. A weak band migrating at the same distance is also present in NDHL. Band 7 (NDHL) was identified as *Gilliamella apicola* Kwong et Moran.

Table 1 Eubacteria sequence alignment with the megablast algorithm in the GenBank database

Band	Closest match	% similarity*	GenBank accession number
1	*Lactobacillus helsingborgensis*	99	KR073912
2	Nd	-	-
3	*Lactobacillus kunkeei*	99	KR073913
4	*Lactobacillus kimbladii*	98	KR073914
5	*Lactobacillus kunkeei*	100	KR073919
6	*Melissococcus plutonius*	100	KR073920
7	*Gilliamella apicola*	97	KR073925
8	*Enterococcus faecalis*	100	KR073918
9	Nd	-	-
10	*Paenibacillus* spp.	100	KR073924

* similarity represents the % similarity shared with the sequences in the GenBank database. nd: not determined (scarce quality of the obtained sequencing)

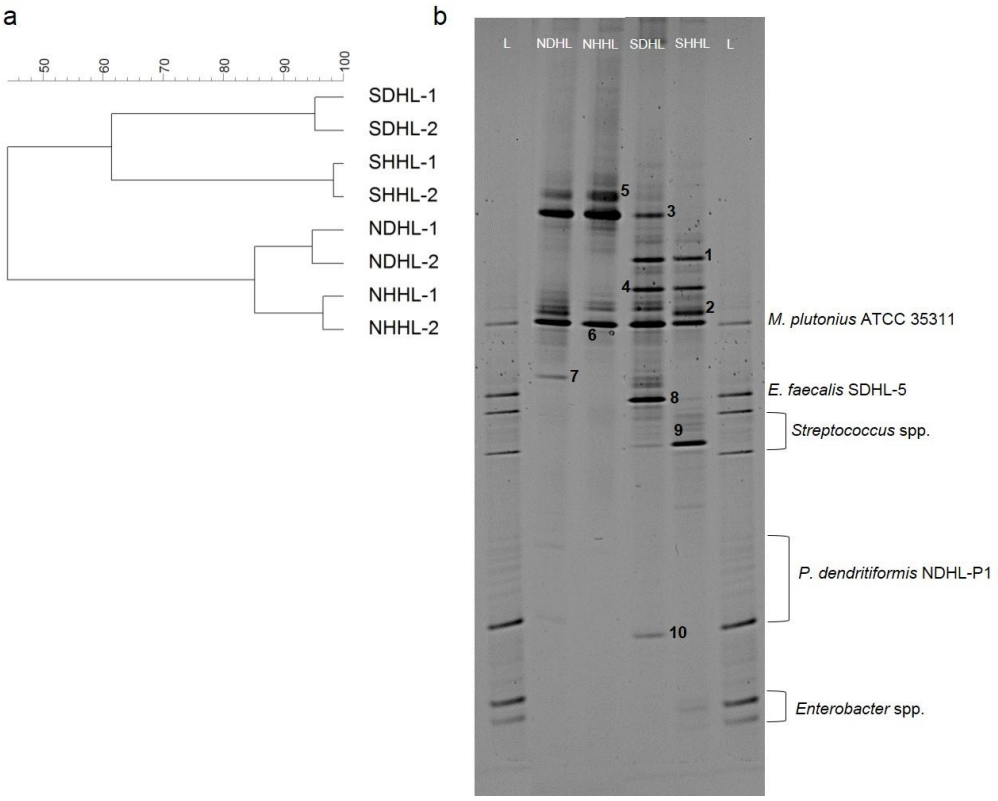


Figure 2 UPGMA dendrogram (a) and DGGE profiles of eubacteria (b) from the amplified V2-V3 region of 16S rRNA obtained from one replicate of DNA samples. The bands indicated by the numbers were excised, re-amplified and subjected to sequencing. L: ladder with reference strains; NDHL: diseased honey bee larvae from North Italy; NHHL: healthy-looking honey bee larvae from North Italy; SDHL: diseased honey bee larvae from South Italy; SHHL: healthy-looking honey bee larvae from South Italy

Discussion

The microbial investigation by plate count on diseased honey bee larvae, exhibiting atypical clinical symptoms closely related to both EFB and AFB, allowed the massive isolation of two microorganisms further identified as *E. faecalis* and *P. dendritiformis* respectively. Plates did not evidence neither *M. plutonius*, nor *P. larvae*. *E. faecalis* is one of the typical second invaders often described in the EFB (Forsgren, 2010). It does not multiply in bee larvae in the absence of *M. plutonius*, and its presence is a presumptive evidence of EFB (OIE, 2008). *E. faecalis* is morphologically similar to *M. plutonius* and it has frequently been confused as being the causative agent of EFB (Bailey and Gibbs, 1962; Hornitzky and Wilson, 1989). Interestingly, pasteurized samples allowed the isolation of *P. dendritiformis* and the unsuccessful recovery of *P. larvae* on MYPGP plates. *P. dendritiformis* is a soil-borne bacterium, which can be found in various plant-related niches (Campos-Herrera *et al*., 2011); nowadays, no data on its presence in honey bee larvae are available. Its genome encodes various genes for the production of offensive compounds (toxins, lytic enzymes, antibiotics) to regulate its population size and compete with other bacteria (Sirota-Madi *et al.*, 2012). It could be hypothesized that, in low nutrient availability as in larval remains, this antimicrobial arsenal could provide a competitive advantage, replacing neighbouring species as the EFB second invader *P. alvei*, conferring atypical symptoms to the disease. Indeed, *P. alvei* usually confers a characteristic stale or sour odour to the EFB (OIE, 2008), which was not perceived in the current study. Moreover, Australian researchers suggested that *in vitro* reared larvae only developed EFB symptoms when both *M. plutonius* and *P. alvei* were present (Hornitzky and Giersch, 2008).

These findings let us suppose that analysed honey bee larvae in both geographical areas exhibited an advanced disease status, presumably ascribed to the EFB. The confirmation came out from molecular techniques (both qualitative PCR and PCR-DGGE), which evidenced the presence of *M. plutonius* in all samples, including larvae without symptoms.

Obtained molecular data are in contrast with plate counts. However, it is always reported that *M. plutonius* is a fastidious microorganism and culture methods could be very insensitive, detecting less than 0.2% of microscopically counted cells (Djordjevic et al., 1998 and Hornitzky and Smith, 1998). Moreover, *E. faecalis*, if present in high quantity as resulted in this study, could overgrow SYPG plates, thus avoiding *M. plutonius* detection (Bailey and Ball, 1991; OIE, 2008). Detection of *M. plutonius* in healthy colonies has been already reported (Pinnock and Featherstone, 1984, Mckee et al., 2003 and Forsgren et al., 2005). It could be supposed that healthy-looking larvae (not affected in the aspect and in the morphological position) were in an early stage of infection and their microbiota was changing; the DGGE profile revealed, indeed, a high similarity to the diseased samples. However, in some cases, infected larvae without apparent symptoms, could survive and successfully pupate and emerge as adults, thus withstanding the infection (OIE, 2008). Contrarily to *M. plutonius*, *P. larvae* was not detected, neither by species-specific PCR nor by DGGE, thus excluding any implication of the AFB.

Overall, the combination of traditional and molecular techniques was successful in evidencing the disease origin. Besides confirming plate count results and species-specific PCR, PCR-DGGE analysis and band sequencing provided a more complete picture of the

bacterial communities shift on the analysed samples. Interestingly, DGGE bands ascribed to *Lactobacillus* spp. were detected in all samples; as reported by Vojvodic *et al*. (2013), *Lactobacillus* spp. is the most representative genus of honey bee larvae gut microbiota.
The absence of the band ascribed to *E*. *faecalis* in NDHL profile probably reflects the low colony detection observed by plate count. It should be emphasized that DGGE shares with the other PCR-based molecular biology techniques some limitations in terms of detection limits and of quantitative comparison of detected populations (Marzorati *et al*., 2008). Sequencing of band 10, identified as *Paenibacillus* spp. in DNHL and SDHL, was not able to discriminate at species level, since the 100% similarity was referred to *P. dendritiformis* but also to *Paenibacillus popiliae* Dutky and *Paenibacillus thiaminolyticus* Nakamura. However, considering that from plate count the only strain isolated and identified by 16S rDNA sequencing was *P. dentritiformis*, band 10 presumably refers to this bacterial species. A further confirmation derives from the presence in the ladder of the isolated strain *P. dendritiformis* NDHL-P1, whose migration distance is comparable to the excised band.

The current work represents a preliminary investigation on the microbiology of the honey bee larvae and put in evidence the importance to combine culture dependent and independent techniques. With the above-described approach, a different bacterial species has been detected in honey bee larvae affected by the EFB. Further research is envisaged (*e.g.* infection trial in healthy larvae) to better understand the role of *P. dentritiformis* in the development of the disease.

Acknowledgment
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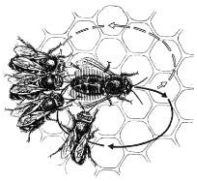

PAPER 2

Beneficial Microorganisms for Honey bees, problems and progresses

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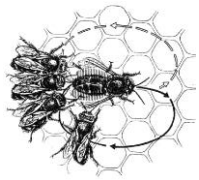
Abstract

Nowadays, honey bees are stressed by a number of biotic and abiotic factors which may compromise to some extent the pollination service and the hive productivity. The EU ban of antibiotics as therapeutic agents against bee pathogens has stimulated the search for natural alternatives. The increasing knowledge on the composition and functions of the bee gut microbiota and the link between a balanced gut microbiota and health status have encouraged the research on the use of gut microorganisms to improve bee health. Somehow, we are assisting to the transfer of the “probiotic concept” into the bee science. In this review, we examine the role of the honey bee gut microbiota in bee health and critically describe the available applications of beneficial microorganisms as pest-control agents and health support. Most of the strains, mainly belonging to the genera *Lactobacillus*, *Bifidobacterium* and *Bacillus,* are isolated from honey bee crop or gut but some applications involve environmental strains or formulation for animal and human consumption. Overall, the obtained results show the favorable effect of applied microbial strains on bee health and productivity, in particular if strains of bee origin are used. However, it is actually not yet possible to conclude whether this strategy will ever work. In particular, many aspects regarding the overall set up of the experiments, the dose, the timing and the duration of the treatment need to be optimized, also considering the microbiological safety of the hive products (*i.e.* pollen, honey). In addition, a deep investigation about the effect on host immunity and physiology is envisaged. Lastly, the final users of the formulations, *i.e.* beekeepers, should be taken into account for the achievement of high quality, cost-effective and easy-to-use products.



Introduction

Pollination is one of the most important service provided by insects, with a strong ecological, economic and cultural impact. The European honey bee *Apis mellifera* is regarded as the most relevant pollinating agent, even if a significant contribution comes also from less known *Apoidea* species, such as bumble bees (*Bombus* spp.) and wild bees (*Macropis* spp., *Osmia* spp. and *Xilocopa* spp.). The maintenance of genetic diversity in plant population, the productivity of crops and orchards for human nutrition and the floral variety in the environment are unequivocally assured and satisfied by this "free" ecosystem service, whose preservation is also dependent on human actions (Gill et al. 2016). Nowadays, bees are stressed by a number of biotic and abiotic factors which affect honey bee health and productivity. In addition to pathogens, pesticides and lack of flowers, whose implications in insect health have been deeply studied (Goulson et al. 2015; Porrini et al. 2016), climate change, habitat loss and invasive species are becoming equally crucial for beehive integrity (Potts et al. 2010; USDA 2012; Nieto et al. 2014). The parasite *Varroa destructor* and the microsporidium *Nosema ceranae* moved, in the last decades, from their natural Asiatic host (*Apis cerana*) to the European one, finding fertile ground for their development (Higes et al. 2010 and Rosenkraz et al. 2010). Moreover, the presence of *V. destructor* in every colony seems to exert an important pressure on bee health since the mite found in *Apis mellifera* a less resistant host (Le Conte et al. 2010). The use of veterinary medicines in the beekeeping sector has a strong limitation due to the big concern about antibiotic resistance acquisition/transmission, antibiotic residues in beehive products and to a lesser extent the risk of unbalancing the bee gut microbiota. Consequently, antibiotics were banned in EU countries, whereas some acaricides are still permitted (Regulation (EU) No 37/2010). Natural substances, such as oxalic acid and thymol, are highly efficient in controlling mite populations if they are correctly applied. The proper handling is important to avoid bee intoxication and, most importantly, to achieve efficacy. Honey bee management needs a deep knowledge of bee behaviour and seasonal cycles and appropriate skills to recognize problems and threats at a given time, in order to successfully employ the colonies for crop pollination or for the hive products. What is often underestimated is that a compromised health status, due to different stressors, can negatively affect the activities of a balanced and healthy gut microbiota both in humans and in animals (Gaggia et al. 2010). The honey bee gut microbiota displays high affinity with that of mammals (Kwong and Moran 2016); the huge number of bacterial symbionts, inhabiting selected niches in the gut (from honey crop to the rectum), are represented by host-adapted species contributing to host defence, nutrition and physiology (Hamdi et al. 2011). Recent advances on metagenomics have brought new insights in the knowledge of honey bee gut microbiota and its genes (Moran 2015). The host-microbe interaction derives from a long co-evolution process strictly associated with insect labour division, developmental stage and social transmission (Hughes et al. 2008). It is quite surprisingly to observe that most members of this gut microbiota are maintained by horizontal social transmission (with the exception of the queen) and interaction with the hive environment (Tarpy et al. 2015), providing unique functions related to food storage and transformation. Moreover, the finding that the honey bee genome has significantly fewer immune genes than expected allowed to speculate a contribution of the gut endosymbiont genes in supporting honey bee immunity (Evans et al. 2006) in

association with the social immune response described in eusocial insects (Wilson-Rich et al., 2009). Recent works on *Drosophila melanogaster* have given a picture of the molecular dialog between the microbiota and the insect gut. Many authors described the role of gut microorganisms in supporting the immune system, influencing the epithelial homeostasis, promoting lifespan, larval growth in food shortage and driving the host mating preference (Brummel et al. 2004; Ryu et al. 2008; Buchon et al. 2009; Sharon et al. 2010; Storelli et al. 2011). For these reasons, as in vertebrates, the prosperous gut symbionts community should be considered pivotal for insect life and should be preserved. Beneficial microorganisms have been widely exploited in humans and animals both as food/feed supplements and as pharmaceutical formulations, representing a valid tool to support gut health and alleviate several disorders (Gaggia et al. 2010; Di Gioia et al. 2014). The use of commensal gut microorganisms and their related secondary metabolites are more and more taken into account to re-establish a disbiotic insect gut community and control disease spread (Crotti et al. 2012; Berasategui et al. 2016). Insects are probably a simpler system to investigate, but such applications, in social bees, could result more difficult to monitor since many variables should be considered (environment, genetic diversity, high complexity at hive level). Researchers are focusing on honey bee microbial gut inhabitants to better understand the host-microbiota interaction and transfer the acquired knowledge from human and animal to bees.

In this review, we discuss the role of the honey bee gut microbiota, focusing on its main activities and we give an overview of the available applications of beneficial microorganism on bee larvae and adults, looking at their potential as pest-control agents and health support.

A look inside the honey bee gut microbiota

In the last decade, the new available techniques led scientists to investigate the microbial gut symbionts with a particular focus on the functional aspect of host-symbiont interaction. Next Generation sequencing (NGS) has allowed the identification of a distinctive gut bacterial community, which consists of eight dominant groups, comprising over 95% of the whole community, as described in Moran (2015) and Kwong and Moran (2016). The Gram-negative *Gilliamella apicola* and *Frischella perrara,* belonging to the *Gammaproteobacteria* class, and the *Betaproteobacterium Snodgrassella alvi* are predominant in the midgut. The rectum is preferentially colonized by the clades Firm-4 and Firm-5, including different *Lactobacillus* species (*e.g. L. mellis*, *L. mellifer L. helsingborgensis*, *L. kullabergensis*, *L. melliventris*, and *L. kimbladii*) and two species belonging to the genus *Bifidobacterium* (*B. asteroides* and *B. coryneforme*). *Alphaproteobacteria* (related to the genera *Bartonella*/*Brucella* and the *Acetobacteraceae* family) have been described but they are less abundant (Moran 2015; Kwong and Moran 2016). The microbial gut community, evolving in the days following pupae hatching, reaches its definition in 3-5 days (Anderson et al. 2016). The same authors hypothesized that many strains of *Lactobacillus* Firm-5 are pioneer species, being particularly abundant within the hive, and that cell cleaning and other early behaviours are pivotal in newly emerging bees for promoting the composition of the adult gut microbial community. However, further behavioural mechanisms, such as the grooming, the oral trophallaxis and the oral-faecal route are reported as well (Martinson et al. 2012;

Powell et al. 2014). As in humans and animals, this bacterial core group is composed of facultative anaerobic and microaerophilic bacteria (Kwong and Moran 2016), which are strictly associated with the gut epithelial cells and are involved in several host functions. It is interesting to point out that several species have been only recently isolated and identified (Engel et al. 2013; Kwong and Moran 2013; Olofsson et al. 2014) and studies on their role and interaction with the host are still at the beginning. Besides this core microbiota, some caste-related differences may be found in relation to the social function that honey bees cover during their life (Kapheim et al. 2015). Moreover, a recent study (Rokop et al. 2015) has suggested the presence of a "non core" microbial group associated with the hive environment, including the food prepared by the bees, which may trigger the development of the gut core microbiota.

The role of gut microorganisms in honey bees

1. Nutritional support

Social insects create a partnership with the microbial gut symbionts as they possess genes encoding for enzymatic activities (*i.e.* cellulases, hemicellulases and lignase) essential for the energy uptake from a plant-based diet (Newton et al. 2013). Moreover, the microbial consortium produces fatty acids, amino acids and other necessary nutrients and metabolites (Gündüz and Douglas 2009). Honey bees also require vitamins, including the vitamin B complex and gut bacteria could represent a relevant source (Brodschneider and Crailsheim 2010). A summary, indicating the main activities of gut symbionts, is reported in Table 1. *Fructobacillus* species, isolated from bee bread, brood cells and larval gut, were found to utilize the plant complex molecule lignin, which is a component of pollen, thus beginning the breakdown of this important high-protein plant-derived food (Rokop et al. 2015). In a recent metagenomic study, involving 150 pooled guts of *A. mellifera* worker bees, Engel and Moran (2013) evidenced the presence of different sugar uptake systems in *Gammaproteobacteria*, *Firmicutes*, and *Bifidobacteriaceae* (phosphotransferase system families and the arabinose efflux permease family). This is in agreement with Lee et al. (2015) who identified, through metatranscriptome sequencing, the aforementioned bacterial groups as the major contributors (91%) of the protein-coding transcripts, participating in the breakdown of plant-derived macromolecules and in the fermentation of the monomeric subunits. Interestingly, the energy uptake of the *betaproteobacterium S. alvi* exclusively relies on the aerobic oxidation of the products of the fermentation process (citrate, malate, acetate and lactic acid), thus avoiding any competition for nutrients with neighbouring species (Kwong et al. 2014). This represents a simple example of co-evolution within the same niche. A further interesting finding (Engel and Moran 2013) is the pectin degradation activity of *G. apicola* that is strain-specific and leads to pollen cell wall degradation, thus leaving the protein content available for the host. It is clear from these studies that a high degree of genetic diversity can be found within the microbial symbionts, thus suggesting a high adaptability of microorganisms to host metabolic requirements within the same niche (Engel and Moran 2013). The catabolic pathways in lactobacilli (commonly defined Lactic Acid Bacteria; LAB)

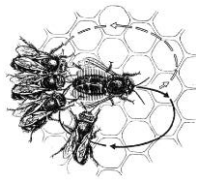
and bifidobacteria are well known since these two microbial groups are involved in numerous fermentation processes and have a long history of safe use as probiotic and protective microorganisms (Gaggia et al. 2011). Lee et al. (2015) described a wide range of glycoside hydrolase (GH) activities in the bee gut, such as GH13 and GH16 families, acting on plant cell wall components and highly transcribed within the lactobacilli group. Other GH families were described for their activities on the soluble disaccharides maltose, cellobiose and sucrose. The importance of LAB is also emphasized by their ecological distribution, which is not limited to adult bee gut. They have been isolated from larval guts (Gaggìa et al. 2015) and from the honey stomach of adult bees (Olofsson and Vasquez 2008), which is a further relevant microbial niche associated with food storage and liquid transfer (water, nectar and royal jelly), adjacent to the midgut. Moreover, LAB are also dominant in the hive environment (bee bread, honey, wax and comb) (Anderson et al. 2013). Among bifidobacteria, some isolates from social insects are known to possess a complete trehalose degradation IV pathway, which is absent in the majority of the other bifidobacterial taxa. Trehalose is indeed used as carbohydrate storage and hemolymph-sugar by many insects including honey bee (Milani et al. 2015). Moreover, Milani et al. (2015) confirmed the significant differences in the glycobiome composition of bifidobacterial taxa isolated from social insects compared with human and animal taxa, highlighting a discrete set of GH43 (for the breakdown of complex plant glycans, xylan and arabinoxylans) and GH3 family members. Bottacini et al. (2012) showed that *Bifidobacterium asteroides* was able to metabolize a range of simple carbohydrates broader than any other tested bifidobacterial species (72 carbohydrate-active proteins). This is consistent with Lee et al. (2015), who detected a class of β-glucosidases within the *Actinobacteriaceae* family, whose activity is addressed towards oligosaccharides with diverse sizes and compositions and it has been associated with pollen cell wall degradation. The genome sequencing of *B. asteroides* also confirmed the presence of a complete biosynthetic pathway for folate (vitamin B9), but not for other B vitamins (Bottaccini et al. 2012). Overall, the above studies showed again that species isolated from different hosts possess specific gene sets, suggesting host-specific adaptation. Bifidobacteria are recognized as strictly anaerobic microorganisms, but *B. asteroides*, inhabiting the honey bee hind gut, possesses genes associated with a respiratory metabolism that help the bacterium to adapt to the oxygen-rich bee gut environment (Bottaccini et al. 2012; Sun et al. 2015).

2. Immunity support

Host protection is another important aspect that is frequently associated with a balanced gut microbiota. It is a fact that different stress factors, such as parasites/pathogens, deficient nutrition and pesticides, can cause immunosuppression (Antúnez et al. 2009; Alaux et al. 2010b; Anbutsu and Fukatsu 2010; Fang et al. 2010; Di Prisco et al. 2013). As already mentioned, honey bee has a simpler immune system compared to other model insects (Evans et al. 2006 ; Barribeau et al. 2015), in favour of more convenient and less expensive social defence strategies which combine prophylactic and activated responses as well as behavioural, physiological and spatial mechanisms (Cremer et al. 2007). However, a

significant contribution to host protection is provided by the antagonistic activity of the gut microbiota and its interaction with the humoral and systemic immunity (Dillon et al. 2005; Hedges et al. 2008; Jaenike et al. 2010). In three species of wild bumble bees, a low presence of *S. alvi* and *G. apicola* strains was associated with a higher incidence of the pathogen *Crithidia* spp. (Cariveau et al. 2014). Dillon and Charnley (2002) reported in the desert locust *Schistocerca gregaria* a real contribution of the gut microbiota to host defence against pathogens by producing antimicrobial phenolic compounds and synthesizing key components of the locust cohesion pheromone. Alterations of this microbiota could consequently compromise honey bee defence mechanisms. In particular, this paragraph will focus on how microorganisms could play a role in host protection, *i*) by directly stimulating the bee's immune system; *ii*) by directly inhibiting pathogens through antimicrobial compound production (Table 1).

Given that individual and social defence mechanisms are diverse and complex, one of the main effectors of the innate immunity in honey bee, and more in general in insects, is represented by antimicrobial peptides (AMPs), whose synthesis is under the control of the Toll and Imd signalling pathways (Lemaitre and Hoffmann 2007). Honey bees possess six AMPs, mainly activated at epithelial surfaces, following the exposure to the major cell wall component of Gram-positive bacteria, the Lys-type Peptidoglycan (PG): Abaecin, Hymenoptaecin, Apidaecin, Defensin-1, Defensin-2, and Apisimin (Casteels et al. 1989; Casteels et al. 1990; Casteels et al. 1993; Bilikova et al. 2002; Klaudiny et al. 2005). Antimicrobial activity is mainly achieved through alteration of the microbial membrane properties (Imler and Bulet 2005) and intracellular metabolic processes (Brogden 2005). A selective AMP synthesis is induced following exposure to various honey bee larvae/adult pathogens with variable responses (Evans and Lopez 2004; Jefferson et al. 2013; Yoshiyama et al. 2013). Evans and Pettis (2005) showed a higher abaecin expression in colonies with a lower incidence of *Paenibacillus larvae* (the ethiological agent of the American Foulbrood, AFB). However, some studies also evidenced an increased level of AMPs in response to non-pathogenic bacteria. Higher RNA levels for the abaecin gene have been reported in bee larvae fed with probiotic bacteria of human origin and fermented foods (Evans and Lopez 2004; Yoshiyama et al. 2013). Janashia and Alaux (2016) fed larvae with five different LAB species previously isolated from worker honey bee guts and bee bread, and among them, two strains (*Bifidobacterium asteroides* 26p and *Fructobacillus pseudoficulneus* 57) significantly upregulated the expression of apidaecin, while no effect was observed on abaecin, hymenoptaecin and defensin-1 levels. These results, taken together, showed that the honey bee immune response through AMP synthesis is fairly non-specific and the increase of the transcription levels of the different AMPs genes is strain specific and is not related to either the species or the source of the strains. Jefferson et al. (2013) also found a strong positive correlation between the amount of total honey bee gut bacteria and transcript levels of two AMPs, *defensin-1* and *apidaecin*. The hypothesis that the resident gut microorganisms may determine a basal immune response to control its proliferation and consequently harmful microorganisms through AMPs synthesis has not yet be investigated in honey bee; however, studies on *D. melanogaster* and Anopheles mosquitoes go in that direction. An interesting observation in *D. melanogaster* has revealed



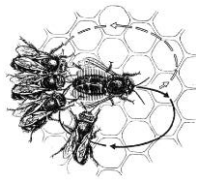
that appropriate AMP levels could guarantee the preservation of a balanced gut microbial community structure, with the species *Commensalibacter intestini* dominant within the *Acetobacteraceae* family. An induced-up regulation of AMP gene expression led to a drastic change in the microbial composition, exerting the growth promotion of the pathogenic

Table 1 Summary of the main activities correlated with honey bee gut symbionts

Generic function of the honey bee gut microbiota	Specific function	Target microorganisms (where available)	References
Nutritional support	Source of vitamins, fatty acids, amino acids	-	Gündüz and Douglas 2009; Brodschneider and Crailsheim 2010
	Lignin degradation	*Fructobacillus* spp.	Rokop et al. 2015
	Sugar uptake systems	*Gammaproteobacteria*, *Firmicutes*, *Bifidobacteriaceae*	Engel and Moran 2013
	Breakdown of plant-derived macromolecules	*Gammaproteobacteria*, *Firmicutes*, *Bifidobacteriaceae*	Lee et al. 2015
	Pectin degradation activity (strain specific)	*G. apicola*	Engel and Moran 2013
	Aerobic oxidation of the end-products of the fermentation process	*S. alvi*	Kwong et al. 2014
	Glycoside hydrolase activities	Lactic acid bacteria	Lee et al. 2015
	Trehalose degradation IV pathway	*Bifidobacterium* spp.	Milani et al. 2015
Direct stimulation of the bee's immune system	Increased expression level of antimicrobial peptides (AMPs) under pathogen exposure in bee larvae	-	Evans and Lopez 2004; Evan and Pettis 2005; Jefferson et al. 2013; Yoshiyama et al. 2013
	Increased expression level of selected AMPs in bee larvae upon feeding with probiotic bacteria	-	Evans and Lopez 2004; Yoshiyama et al. 2013; Janashia and Alaux 2016
	Strong positive correlation between the amount of total honey bee gut bacteria and transcript levels AMPs	-	Jefferson et al. 2013
Host protection: other strategies	Antimicrobial activity against *Paenibacillus larvae*, *Melissococcus plutonius* and *Ascosphaera apis*	*Bacillus* spp., *Lactobacillus* spp., *Bifidobacterium* spp.	Sabaté et al. 2009; Yoshiyama and Kimura 2009; Forsgren et al. 2010; Audisio et al. 2011; Vásquez et al. 2012; Butler et al. 2013; Wu et al. 2013; Killer et al. 2014
	Biofilm formation and structures resembling extracellular polymeric substances	LAB symbionts from honey crop	Vásquez et al. 2012
	Biosynthesis of cell wall exopolysaccharides	"Firm4" and "Bifido" groups	Ellegaard et al. 2015
	Genes encoding a relevant number of functions related to biofilm formation and host interaction (Type IV pili, outer membrane proteins, and secretion)	*G. apicola* and *S. alvii*	Martinson et al. 2012

commensal *Gluconobacter morbifer* (Ryu et al. 2008). *Acetobacteraceae* is indeed a relevant symbiont group of insect gut (adult and larvae) and crop and has significant implications related to both host nutrition and protection (as reviewed by Crotti et al. 2010). Dong et al. (2009) showed that microbe-free aseptic *Anopheles* mosquitoes displayed an increased susceptibility to *Plasmodium* infection with a reduced expression of the anti-*Plasmodium* factors FBNs 6, 9, and 36.

Concerning the production of antimicrobial compounds for host protection, Saraiva et al. (2015) found a relative high presence of genes involved in the biosynthesis of streptomycin and secondary metabolites in the gut microbiota of honey bee, which could play a role in shaping the microbiome. A considerable amount of information also derives from the LAB community and bifidobacteria, which are well-known antimicrobial compound producers. The finding that an important component of the honey bee gut microbiota was represented by lactobacilli and bifidobacteria have increased the interest of scientists in looking for similarity and analogy with the probiotic bacteria widely investigated in humans and animals. Once lactobacilli and bifidobacteria started to be isolated (from honey bee stomach, gut and hive products), numerous *in vitro* trials confirmed their ability to inhibit honey bee pathogens; in particular *Paenibacillus larvae*, *Melissococcus plutonius* and *Ascosphaera apis,* the agents of the American and European foulbrood (AFB and EFB) and Chalkbrood disease



respectively (Sabaté et al. 2009; Yoshiyama and Kimura 2009; Audisio et al. 2011; Vásquez et al. 2012; Wu et al. 2013; Killer et al. 2014). Although *in vitro* activity does not necessarily correspond to action in *in vivo* systems, these assays could provide useful information on the antimicrobial equipment possessed by each strain. Organic acids, strain-specific metabolites and/or bacteriocin production have been described as powerful antimicrobial molecules (Servin 2004; Kleerebezem et al. 2010) and are widely exploited in human and animal food/feed additives, in the food industry to preserve food and in bio-control strategy against phyto-pathogens (Gaggìa et al. 2011; Tontou et al. 2015). Nevertheless, the interactions between microorganisms in the gut of larvae and adult bees are very complex and pathogens are not at all defenceless exposed to the weapons of the gut microbial symbionts. As example, *P. larvae* with its secreted non-ribosomal peptides (NRP) and NRP/polyketide hybrids (Müller et al., 2015) is able to eliminate all microbial competitors, despite their antimicrobials, resulting in a pure *P. larvae* culture in the degraded larval cadavers (Holst 1945).

A recent genomic analysis of 13 LAB strains, isolated from the honey crop, put in evidence that most of them produced extracellular proteins of known/unknown function related with antimicrobial action, host interaction, or biofilm formation. In particular, a putative novel bacteriolysin with 51% homology with Helveticin J was detected in *L. helsinborgensis* Bma5N (Butler et al. 2013). At the same time, some strains did not evidence any "antimicrobial function", thus confirming the high variability among the gut microorganisms inhabiting the same niches. Vásquez et al. (2012) analysed the interaction of some LAB symbionts with the honey crop by SEM and fluorescence microscopy. The resulting images evidenced biofilm formation and structures resembling extracellular polymeric substances (EPS), which are known to be involved in host protection/ colonization and cellular recognition (Flemming and Wingender 2010). A further support comes from the work of Ellegaard et al. (2015), which evidenced at genome level the presence of gene clusters associated with the biosynthesis of cell wall exopolysaccharides in both "Firm4" and "Bifido" groups. Martinson et al. (2012) reported, in honey bee workers, the presence of genes in *G. apicola* and *S. alvii* encoding a relevant number of functions related to biofilm formation and host interaction (Type IV pili, outer membrane proteins, and secretion), whose expression could be relevant for the establishment of a micro-niche insensitive to pathogens colonization. Finally, the *Bacillaceae* family includes several spore forming bacteria, isolated from the bee gut and from the hive environment, showing *in vitro* a strong antibacterial activity against bee pathogens. In this case, it is known from decades, that inhibition activity is mainly due to the production of antibiotic molecules (lipopeptides and iturin-like lipopeptides) (Alippi and Reynaldi 2006; Lee et al. 2009; Sabaté et al. 2009; Yoshiyama and Kimura 2009). However, as mentioned above, it must be again emphasized that *P. larvae* itself, as spore forming bacteria, produces antibiotics molecules which help the pathogen during infection to defend its niche and dominate the larval gut environment towards resident microorganisms.

The "Probiotic Concept" in honey bee

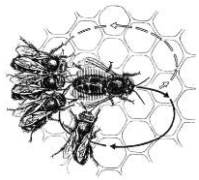
It is clear that a balanced gut microbiota offers a wide range of metabolic, trophic and protective functions, which confer health benefit to honey bees. In this perspective the FAO/WHO probiotic definition (FAO/WHO 2002), which encompasses strain specificity (Sanders et al. 2014), is more than appropriate. However, the transfer of the probiotic concept from vertebrates to invertebrates still requires further considerations and several questions still need to be investigated and debated. In particular, beyond the health aspect, probiotic microorganisms fulfil a list of biological requirements and safety criteria, *e.g.* to be non-toxic and non-pathogenic, to have an accurate taxonomic identification, to be normal inhabitants of the targeted host-species, to adhere to the gut epithelium (Hooper and Gordon 2001; Gaggìa et al. 2010). For these reasons, in the present review authors will refer to "beneficial microorganisms" rather than to probiotic microorganisms, since honey bee gut symbionts characterization is far to be completed. From our and general experience in humans and animals, biotic and abiotic stresses could negatively affect the composition of the gut microbiota and therefore induce specific changes in the microorganism activities at gut level (Gaggìa et al. 2010). The analysis of the honey bee microbial gut community in colonies suffering from Colony Collapse Disorders (CCD) evidenced a variation of some microbial phyla in healthy colonies compared to diseased ones (Cox-Foster et al. 2007); in affected colonies a decrease of *Firmicutes* and *Alphaproteobacteria* was observed. We can deduce that this alteration could reflect physiological changes due to the incoming infection or support the hypothesis that the low presence of beneficial species could weaken host defence. Anyway, we have to ask ourselves if any kind of microbiota modulation, by the administration of selected strains, could restore this perturbation, reduce bee mortality and/or improve honey bee health. In other studies, by introducing a given stress, no perturbation was observed (Babendreier et al. 2007; Hui-Ru et al. 2016). In particular, Hui-Ru et al. (2016) did not evidence significant difference in the microbial gut community of honey bees, under laboratory conditions, following exposure to sub-lethal dose of the neonicotinoid Imidacloprid, whose adverse effects on honey bees have been already documented (Medrzycki et al. 2003; Dively et al. 2015). Nevertheless, it has been also verified how exposure to sub-lethal concentration of pesticides could significantly enhance bee susceptibility towards pathogens (Alaux et al. 2010a; Vidau et al. 2011; Doublet et al. 2015), thus weakening honey bee health and compromising the gut microbiota. Attempts of gut microbiota modulation have been already performed in some insect species (Wittebolle et al. 2009; Ben Ami et al. 2010; Robinson et al. 2010), showing the importance of the endogenous gut microbial community. In the next section, a description of the main application of beneficial microorganisms in honey bees will be reported and commented, including assays in larvae and adults both under laboratory and field conditions.

Application of beneficial microorganisms: state of the art

Beneficial microorganisms in honey bee are mainly applied to fight the most widespread pathogens affecting both larvae and adults (Table 2). Most of the bacterial strains used in these studies are isolated from honey bee crop or gut, whose selection derives from *in vitro* tests based on direct antagonism towards target pathogens. However, some other applications rely on the use of bacterial strains isolated from the environment or on

formulation for animal and human consumption. With respect to the AFB, Forsgren et al. (2010) used a mixture of twelve isolates from honey crop - *L. kunkeei*, *L. mellis*, *L. kimbladii, L. kullabergensis, L. helsinborgensis, L. melliventris, L. apis, L. mellifer, B. asteroides* and *B. coryneforme* - with a final concentration of 10^7 bacteria/ml. The exposure assay was performed by rearing 1st instar honey bee larvae, infected with two different spores concentration of *P. larvae*. The LAB mixture was supplemented with sugar syrup, both in combination with *P. larvae* at the time of spore inoculum and 48 hours post infection. Results showed the positive effect of LAB supplementation only in the group challenged with the highest dose of *P. larvae* with a significant reduction of larvae mortality. However, these results are of little biological relevance because the reduced larvae mortality, from 70% to 55%, is not enough to combat a notifiable epizootic and the colony will probably succumb to the disease, although it might take one week longer. Recently, a probiotic mixture, based on two spore forming bacteria (SFB; *Bacillus thuringiensis* HD110 and *Brevibacillus laterosporus* BMG65) in association with *Saccharibacter* spp., has been developed for the protection of bee larvae against the AFB (Hamdi and Daffonchio, 2011). The efficacy of the invention was tested on *P. larvae* infected-larvae and the experiments showed that the addition of the bacterial mix to the diet decreased the mortality level from 70% in the control to 22% in larvae fed with the microorganisms' mix. Although the mortality reduction is encouraging, the invention should be investigated in infected apiaries in open field to assess the biological relevance of the microorganisms-based product. Concerning EFB, a single laboratory assay has been performed in *Apis mellifera* (Vásquez et al. 2012). The same LAB strains isolated from honey crop and used by Forsgren et al. (2010) were orally administered to honey bee larvae challenged with *M. plutonius* at three concentrations (10^7, 10^6 and 10^5 bacteria/ml). Irrespective of the infectious dose, mortality was significantly reduced in groups treated with the LAB mixture. However, as outlined in Forsgren et al. (2010), these data does not prove the efficacy of these microorganisms since the reduced mortality between 10-20%, although significant, is biologically irrelevant. Based on these results, it could be interesting to investigate the efficacy of the LAB mixture in infected larvae with a lower dose of the pathogen and perform the treatments as preventive measure before the infection step. The native microbial community inhabiting the honey crop is mainly involved in the production of the bee bread nourishing the brood and constitute the first defence line against potential brood pathogens acquired from the floral environment (Vásquez et al. 2012). Therefore, an application of beneficial microorganisms prior to infection to boost the gut microbiota composition could be more successful in contrasting brood pathogens. However, no data are actually available.

An interesting observation from this study is the antibiotic susceptibility of the LAB strains towards oxytetracycline and tylosin, two antibiotics used in apiculture to fight *P. larvae* and *M. plutonius*. All LAB strains were highly sensitive to tylosin, while *L. kunkeei* Fhon2, *L. apis* Hma11, *L. melliventris* Hma8 and *L. mellis* Hon2 showed resistance to oxytetracycline. Antibiotic resistance is an important concern for insects and human health, if we look at the risk of an increased antibiotic resistance among bee pathogens and accumulation in the hive products. These are some of the reasons leading to the ban of antibiotics in apiculture in EU. Unfortunately, this regulation has not yet been adopt in non-EU countries.



With respect to adult honey bees, beneficial microorganisms are targeted against the emergent pathogen *Nosema* spp., in particular *Nosema ceranae*, which multiplies within gut cells and no relevant symptoms can be detected during infection (see details in Higes et al. 2010) (Table 2). The microsporidium is prevalent in southern Europe (Fernandez et al. 2012; Porrini et al. 2016) and it has been associated with reduced honey bee life span and colony weakening (Goblirsch et al. 2013). However, according to the investigation of Fernandez et al. (2012) in Spanish apiaries, *N. ceranae* does not necessarily kill honey bee colonies and does not influence beehive production. Almost all the reported experiments are performed in plastic cages under laboratory conditions with newly emerging honey bees. Many issues can be argued about the use of cage experiments. Although the laboratory assessment allows the standardization of the variables and the direct observation of the introduced perturbations (e.g. diet change, pathogen inoculation, beneficial microorganisms, pesticides), most of the behavioural and social interactions both inside and outside the hive are lacking. Moreover, this confinement can also introduce stress factors and influence the experiment itself.

The trial performed by Corby-Harris et al. (2016) showed an improve resistance to *Nosema* spp. in honey bee adults individually challenged with 10^4 spores and originating from larvae fed with pollen patty mixed with an inoculum of *Parasaccharibacter apium* C6. *P. apium* (Corby-Harris et al. 2014), of *Acetobacteraceae* family, is particularly abundant in honey crop, hypopharyngeal glands, royal jelly and larval gut through nurse worker bees feeding behaviour. However, spore load reduction was always biological irrelevant since the decrease was less than 40% compared to the control group. Similarly, Baffoni et al. (2016), observed a significant decrease of *N. ceranae* in infected honey bees orally fed with *Lactobacillus* and *Bifidobacterium* strains. The ~1 log reduction observed in challenged and treated insects could be considered irrelevant since the spore number remained high and honey bees would surely die. However, Baffoni et al. (2016) also evidenced a significant reduction in spore load from 2.04±0.91 and 0.78±0.81 (mean Log spores/bee±sd) in honey bees exposed to a low natural infection and treated with the microorganisms; in this particular case a hypothetical protective effect, contrasting the low infection rate, might be

Table 2 Overview of beneficial microorganism applications for the treatment of the main honey bee microbial infections

Honey bee disease	Infection dose	Microorganisms/metabolites	Source	Reported effect(s)	References
***P. larvae* - AFB**	10^3 and 10^4 spores/ml	*L. kunkeei*, *L. mellis*, *L. kimbladii*, *L. kullabergensis*, *L. helsinborgensis*, *L. melliventris*, *L. apis*, *L. mellifer*, *B. asteroides* and *B. coryneforme* (10^7 bacteria/ml)	Honey crop	Reduced larvae mortality	Forsgren et al. 2010
	Not described	*B. thuringiensis* HD110, *B. laterosporus* BMG65.	Honey bee gut	Reduced larvae mortality	Hamdi and Daffonchio 2011
***M. plutonius* - EFB**	10^7-10^6-10^5 bacteria/ml	*L. kunkeei*, *L. mellis*, *L. kimbladii*, *L. kullabergensis*, *L. helsinborgensis*, *L. melliventris*, *L. apis*, *L. mellifer*, *B. asteroides* and *B. coryneforme* (10^7 bacteria/ml)	Honey crop	Reduced larvae mortality	Vasquez et al. 2012
N. ceranae	1st trial: 10^4 spores/µl 2nd trial: natural infection	*L. kunkeei* Dan39, *L. plantarum* Dan91 and *L. johnsonii* Dan92, *B. asteroides* DSM 20431, *B. coryneforme* C155, *B. indicum* C449. (10^6-10^7cfu/ml of sugar syrup)	Honey bee gut	Reduced spore detection	Baffoni et al. 2016
***Nosema* spp.**	10^3 spores/µl	*P. apium* C6 (10^6 cfu/500 µl)	2nd instar larvae	Reduced spore detection	Corby-Harris et al. 2014
	Diseased bees	*L. johnsonii* CRL1647 (10^5 cfu/ml)	Honey bee gut	Reduced spore detection	Audisio et al. 2015
	Diseased bees	10^5 spores/mL of *Bacillus subtilis* Mori2 spores	Honey	Reduced spore detection	Sabaté et al. 2012

considered of biological relevance. Sabaté et al. (2012) and Audisio et al. (2015) observed a decrease in the amount of spores in field conditions in honey bees orally fed for several months with strains isolated from the gut of healthy insects, namely *B. subtilis* Mori2 and *L. johnsonii* CRL1647. In both cases, the biological relevance of the reduction (less than 1 log) is still questionable since the spore numbers are still high. The decrease in *Nosema* incidence observed by Sabaté et al. (2012) is only evident in September and October when a slight spore increase can be observed in the control group. When the control group showed a physiological decrease in the spore number, no relevant reduction is observed in the treated groups. From these data, firm evidences on the positive effect of beneficial microorganism administration against *Nosema* spp. cannot be drawn. Conversely, Andrearczyk et al. (2014) found an increase of *Nosema* spp. infection, following administration in both winter and summer bees of a probiotic product recommended for animals. Likely, Ptaszyńska et al. (2016) observed an increased mortality rate in *Nosema*-infected honey bees fed with the probiotic microorganism *L. rhamnosus*, both as preventive measure and along the infection. The authors argued that the increased infection was associated with a pH reduction of the honey bee midgut, because of the metabolic activity of the supplemented microorganism. However, this consideration relies on previous data (Ptaszyńska et al. 2013), where this association is not clearly, and statistically demonstrated and further investigations are envisaged to better understand such interactions. Moreover, the honey bee midgut is a multi-niche environment, harbouring a complex microbial community and fermentation products (as lactic and acetic acids) may be taken up and utilized by some components of this community or by the bee host (Kwong and Moran 2016), thus limiting their contribution to the reduction of gut pH. An interesting approach to study *N. ceranae*-host interactions comes from Gisder and Genersch (2015). The authors developed a cell culture model by using the lepidopteran cell line IPL-LD 65Y, from *Lymantria dispar*, which was susceptible to *N. ceranae* infection and could support the entire microsporidium life cycle. By this approach, the authors tested several molecules for cytotoxicity and inhibition of *N. ceranae* intracellular development and demonstrated the efficacy of the synthetic antibiotics metronidazole and tinidazole, while a surfactin from Sigma-Aldrich did not show any inhibition and at low concentration was also cytotoxic for the cells.

Microbial gut symbionts could be useful to sustain honey bee health and productivity since, as already described, bacteria from honey bee crop and gut are highly specialized in performing thousands of metabolic activities necessary to honey bee for a normal development (Table 3). However, most of the published data are still not very convincing and experiments should have more replicates. An improved wax gland cells development was observed by Pătruică et al. (2012), following the supplementation of organic acids and two probiotics for human consumption. In particular, lactic acid and a probiotic product containing *Lactobacillus* and *Bifidobacterium* spp., both individually and in combination, positively influenced the number, the morphology and the diameter of the wax cells. Audisio and Benítez-Ahrendts (2011) performed two different trials to assess colony health and performance on honey bee hives treated with a cell suspension (10^5 ufc/ml sugar syrup) of *L. johnsonii* CRL1647 (every 15 days for three months and a monthly administration for one

year). All the parameters analysed (open and operculated brood area, bee number, honey storage), with some fluctuations every month, were significantly higher in the treated groups. Sabaté et al. (2012) obtained comparable results with the supplementation in field conditions of spores of *B. subtilis* Mori2, isolated from honey, once a month for eight consecutive months. Alberoni et al. (2015) found a significant increase in honey supers production following the administration of a mixture of lactobacilli and bifidobacteria in hives before the linden (*Tilia* spp.) honey flow. Moreover, authors investigated at the end of the 4 weeks treatments the composition of the honey bee microbial gut community by NGS; surprisingly, lactobacilli showed a significant decrease, whereas a significant increase was observed for bifidobacteria and *Acetobacteraceae* compared to non-treated hives. The bifidobacteria increase confirmed the results obtain under laboratory conditions (Baffoni et al. 2016). The increase of the *Acetobacteraceae* in the treated group could be considered a promising result since many members of the family have recently emerged as important endosymbionts for honey bees (Crotti et al. 2010). However, further investigations are envisaged to better understand if and how these compositional changes can affect the host-gut microbe interaction.

Overall, data are too sparse and weak to support the hypothesis that beneficial microorganisms have a role in improving honey bee health. Moreover, the introduction within the hive of biological agents, even if beneficial, should be carefully treated, in particular for spore-forming bacteria (SFB). Notably, the use of SFB into the hive pose a serious issue regarding the finding of such bacteria in the stored honey. The European Food Safety

Table 3 Overview of beneficial microorganism applications for the support of honey bee health

Microorganisms	Field/laboratory	Duration	Reported effect(s)	References
Enterobiotics and Enterolactis Plus (1.2-2.5g/1.4 L syrup)	Field	1 application a week for 3 weeks	Improved wax gland cells	Patruica et al. 2012
10^5 cfu/ml of *L. johnsonii* CRL1647 in syrup	Field	1st trial: 3 months (1 application every 15 days) 2nd trial: 13 months (1 application a month)	Increase of open and opercolated brood Increased honey production	Audisio et al. 2011a
10^5 spores/mL of *Bacillus subtilis* Mori2 spores in syrup	Field	1 application a month for 8 months	Increase of open and opercolated brood Increased honey production	Sabaté et al. 2012
L. kunkeei Dan39, *L. plantarum* Dan91 and *L. johnsonii* Dan92, *B. asteroides* DSM 20431, *B. coryneforme* C155, *B. indicum* C449. (10^6-10^7cfu/ml of sugar syrup)	Field	1 application a week for 1 month	Increased honey production Decrease of *Lactobacillus* spp. Increase of Acetobacteraceae and *Bifidobacterium* spp.	Alberoni et al. 2015
Biogen-N (1 mg in 100 g of pollen substitute) and Trilac (7 capsules in 100 g of pollen substitute)	Laboratory	1st trial: every day for 14 days 2nd trial: two consecutive applications in 14 days	Better bee survival Higher dry mass and crude fat level No differences in total protein No correlation with feeding duration	Kaznowski et al. 2005
Biogen-N (0.5 mg-2 mg in 100 g of pollen substitute) and Trilac (0.724-2.534 mg in 100 g of pollen substitute)	Laboratory	1st trial: every day for 20 days 2nd trial: every day for 20 days	No increase in feed intake Decreased death rate of bees Stimulation of fat body growth	Kazimierczak-Baryczko a Szymas 2006
Biogen-N (0.5 mg-2 mg in 100 g of pollen substitute) and Trilac (0.724-2.534 mg in 100 g of pollen substitute)	Laboratory	Every day for 14 days	Better bee survival Higher dry mass and crude fat level Greater quantities of peritrophic membranes	Szymas et al. 2012

Authority (EFSA) is requested to verify, through the qualified presumption of safety (QPS) assessment, the safety of a broad range of biological agents in the context of notifications for market authorization (EFSA Journal 2015), including SFB. The chemical composition in natural honey make the growth of microorganisms difficult (Snowdon and Cliver 1996); however, SFB can survive and may become a risk for human health. Actually, no data are available on the microbiological quality of honey, following SFB application into the hive.

The use of pollen substitute and its fortification with probiotic microorganisms have been also investigated in different trials (Kaznowski et al. 2005; Kazimierczak-Baryczko and Szymaś 2006; Szymaś et al. 2012), although such alternatives required more studies due to the harmful effect of their components on honey bee gut and the few available data. All three studies used a mixed of protein ingredients (fish meal, egg powder, soybean flour etc.) with two different probiotic products, for animal ((Biogen-N; Biogen Idec Sp. z.o.o, Poland) and human consumption ((Trilac®; Allergon Health Care, Sweden). Overall, the trials showed an improved condition of bees, confirmed by lower mortality, more developed pharyngeal glands, higher dry matter, and fat body content. A positive influence was also assessed on the morphological changes in the midgut epithelium. After 14 days, midgut analysis evidenced a high epithelium, cytoplasm slightly vacuolized and the presence of considerable quantities of peritrophic membranes, which are associated with duration of the feeding, presence of beneficial bacteria and protection towards harmful compounds (Szymaś et al. 2012).

Finally, we are still far to conclude that beneficial microorganisms could actually limit pathogen widespread, support honey bee health and the hive productivity, even if a starting point has been set. Research activities are still sparse and further implementations are envisaged.

Conclusions

The preservation of the European honey bee *Apis mellifera* is imperative; the beekeeping sector and the ecosystems depending on pollinators are suffering from missed pollination and lack of productivity with an associated loss of biodiversity in the long run (Aizen et al. 2009; Klein et al. 2007). Nowadays beekeepers too often rely on subspecies hybrids, with the false hope to increase disease resistance, but the resistance mechanisms against bee pathogens/parasites are usually a result of a co-evolution in local ecosystems (Ruottinen et al. 2014). Overall, the described applications offer to some extent a picture of the favourable influence of beneficial microorganisms on bee health, in particular their potential activity against some pathogens. However, information is scarce and limited to specific investigations. It could be useful, as in human and animal applications, to define some guidelines in order to standardize the studies and drawn up appropriate protocols. The dose, the timing, the duration of the administration and the number of strains may influence the efficacy of the treatments. The number of experimental replicates and the repetition along the years should be accurately established. Moreover, investigation methods (*i.e. N. ceranae* spore number detection) ought to be uniformed in order to improve as major as possible the output accuracy and the trial comparison. It is necessary to address the study towards gut symbionts isolated from healthy honey bee gut possessing the QPS status, and omit the use of probiotics for human and animal consumption. This is in authors' opinion a key factor, since the main issue, which stand out from this review and from the literature, is the specificity of each microbial strain within its gut niche. In particular, metagenomic and transcriptomic studies are envisaged to better describe the bacterial strain(s) and their interaction with the host, following the supplementation. A deep investigation about the effect on host immunity, physiology and composition of the honey bee gut microbiota could improve the rationale of such supplementation. This is finalized to build a robust

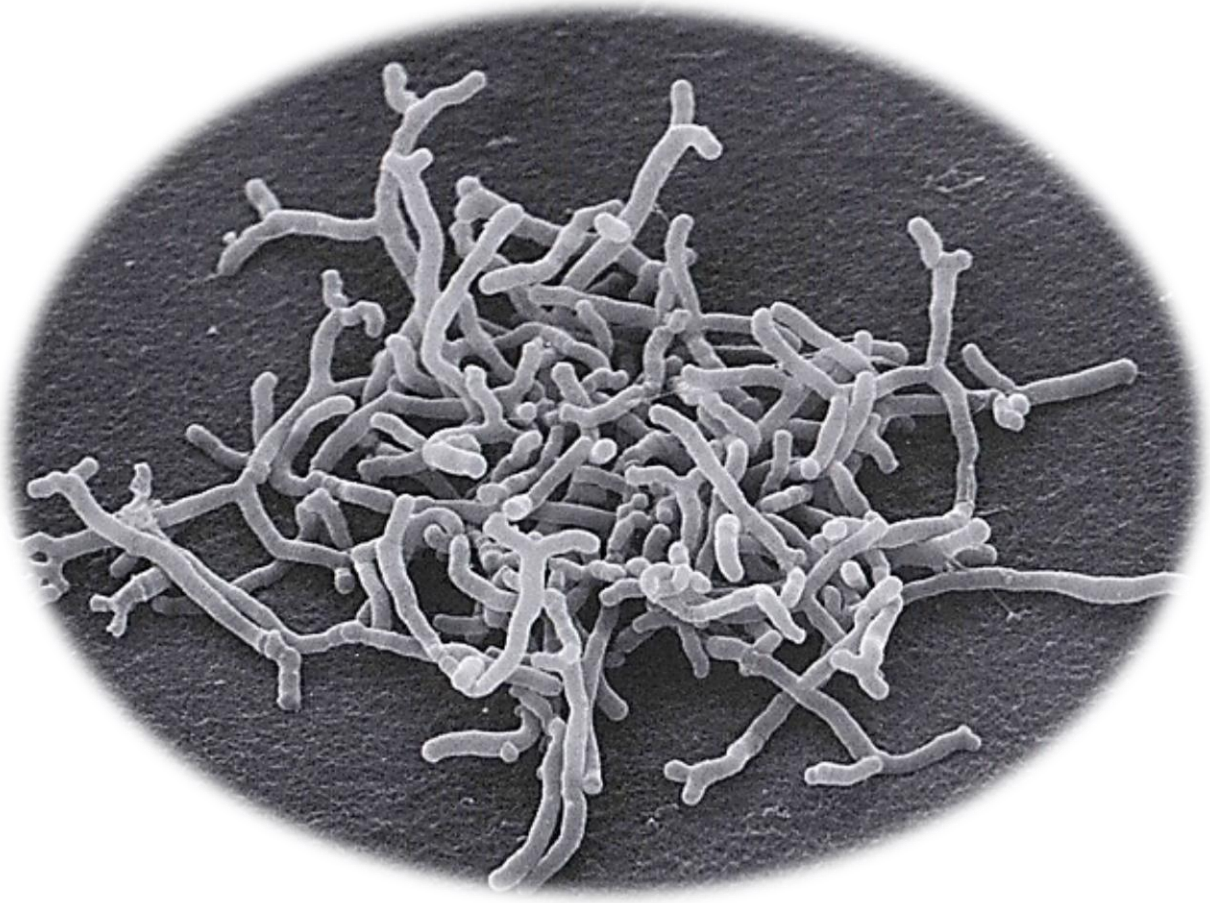
experimental structure, to minimize risk associated with bio-treatments and to analyse results in a comparable way. Finally, this will allow the realization of microorganisms-based products with a reliable scientific literature, which will be more appreciated by beekeepers who are constantly looking for high quality products combined with an excellent ratio quality/price. The beekeeping sector includes operators having a particular feeling towards honey bees, but sometimes a deep knowledge on their biological activities, including the wide world of gut symbionts, is lacking.

This article does not contain any studies with human participants or animals performed by any of the authors

Conflict of interest: the authors declare that they have no competing interests.

CHAPTER 2

Isolation of gut microorganisms from Hymenopetra



Isolation of gut bacteria from *Apoideae* and *Vespulae*, and related habitat.

The aim of this work was to isolate microbial strains from different Apoideae and Vespulae sources and their related environment in order to explore the biodiversity of the cultivable microorganisms in the studied samples and to obtain a large number of strains that can be potentially used in further researches. A collection of the isolated strains has in fact been created at the Department of Agricultural Science, University of Bologna, and the strains will be available to all researchers as soon as the related results have been published.

Isolation source and procedure:

Isolation of bacteria was carried out from the gut of the following Hymenoptera:

- *Apis mellifera* spp. *ligustica* (Italian Golden Bee);
- *Apis mellifera* spp. *ruttneri* (Maltese Bee);
- *Osmia cornuta* (European Orchard Bee);
- *Osmia bicornis* (Red Meson Bee);
- *Xylocopa violacea* (Carpenter bee);
- *Polystes gallica* (Paper Wasp);
- *Vespa crabro* (European hornet).

A further isolation was carried out from other sources related to *Apis mellifera* spp. *ligustica:*

- wax,
- fermented pollen,
- honey
- dead larvae obtained from diseased hives of the Bologna area, but also collected at national level with the BeeNet monitoring programme (False AFB – paper 1)

The samples obtained were serially diluted (serial 10-fold dilutions), and plated on the suitable agarized medium of interest. After picking up colonies, they were inoculated again on the same isolation medium, stroked on plates and these steps were repeated until strain purity was reached. The isolation media used are listed in Table 1.

Table 1: Media used for the isolation of bacteria and further purification of the isolated strains.

Base medium *	Additives	Abbreviation
Tripton Pepton Yeast extract (Mazzola et al., 2015)	-	TPY
Tripton Pepton Yeast extract	Cycloheximide	TPY + Chx
de Mann, Rogosa and Sharpe (BD, Milan, Italy)	-	MRS
de Mann, Rogosa and Sharpe	Chloramphenicol	MRS + Chp
de Mann, Rogosa and Sharpe	Cysteine	MRSC
de Mann, Rogosa and Sharpe	Cysteine and Fructose	MRSCF
Brain Heart Infusion (BD, Milan, Italy)	-	BHI
Nutrient (BD, Milan, Italy)	-	N
Columbia Blood (Oxoid, Rodano, Milan, Italy)	-	CB

Glucose, Yeast, Calcium Carbonate (Gosseld et al. 1983)	-	GYC
Mueller-Hinton, Yeast, Peptone; Glucose, sodium Pyruvate (Dingman, D.W. and Stahly 1983)	-	MYPGP
Starch, Yeast, Peptone; Glucose (Bailey and Ball 1991)	-	SYPG

*1.5% agar added when necessary

During the 3-year PhD work, a total of 625 strains were isolated from 22 different sources as listed in Table 2. Fingerprinting of the isolated strains was obtained with different standard fingerprinting techniques. An approach relaying on multiple fingerprinting techniques is necessary because often fingerprinting techniques are failing in discriminating genetic variability as shown in Figure 1.

Table 2: Isolation Source of Bacteria.

Isolation Source	Number of Stocks/Pools	Pools composed of:
Apis mellifera spp. *ligustica* flying	1	100 Honey bee guts
Apis mellifera spp. ruttneri flying	3	30 Honey bee guts
Osmia cornuta edged	1	10 Orchard Bee guts
Osmia cornuta 30 days fly	1	10 Orchard Bee guts
Osmia bicornis edged	1	10 Meson Bee guts
Osmia bicornis 30 days fly	1	10 Meson Bee guts
Xylocopa violacea flying	1	1 Carpenter Bee gut
Polystes gallica flying	1	15 Paper Wasp guts
Vespa crabro flying	1	3 European Hornet guts
Wax	1	-
Fermented Pollen	1	-
Honey	4	-
Dead Larvae	5	-

Fingerprinting techniques used in this study are described below:

1- **BOX-PCR analysis**

Bacterial isolates were fingerprinted by BOX-PCR. The reaction was carried out in a 30 µl volume containing 1 U Taq DNA polymerase (AmpliTaq Gold, Applied Biosystems, Foster City, CA, USA), 3 µl 10X PCR Gold Buffer (Applied Biosystems), 200 nM of each dNTPs (Fermentas International Inc., Thermo Fisher Scientific Inc., Waltham, MA, USA), 2 mM $MgCl_2$ (Applied Biosystems), 0.4 µM of primer BOXA1R (5'-CTACGGCAAGGCGACGCTGACG-3') (Eurofins Genomics, Ebersberg, Germany), 0.1% (wt/vol) Bovine Serum Albumin (BSA, Fermentas), 2 µl of DNA template, and sterile MilliQ water. The PCR reaction was performed on a TGradient Biometra thermocycler (Biotron, Göttingen, Germany) using the following thermocycling conditions: 7 min of initial denaturation at 95 °C, 30 cycles of 94 °C for 1 min, 53 °C for 1 min, 72 °C for 3 min followed by a final elongation step of 72 °C for 10 min.

2- **RAPD-PCR analysis**

RAPD (Randonly Amplified Polymorphic DNA)-PCR was carried out with a reaction mixture composed of 10µL HotStarTaq Master Mix (QIAGEN GmbH, Hilden,

Germania), 2 µL of 20 µM primer M13 (5'-GAGGGTGGCGGTTCT-3') (Andrighetto et al., 2001), 1.2 µL of template DNA and water to the total volume of 20 µL.
The PCR reaction was performed on a SymplyAmpl thermicycler (Thermo Fisher Scientific, U.S.A) under the following thermocycling program: 10 min of initial denaturation at 95 °C, 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 3 min followed by a final elongation step of 72 °C for 10 min.

3- ERIC-PCR analysis

ERIC-PCR were carried out using the same protocol except for the primers ERIC-1 (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC-2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') (Versalovic et al., 1991) added at the PCR mixture as 0.5 µL for a concentration of 20 µM each primer.
The PCR reaction was performed on a SymplyAmpl thermicycler (Thermo Fisher Scientific, U.S.A) under the following thermocycling program: 10 min of initial denaturation at 95 °C, 30 cycles of 94 °C for 1 min, 57 °C for 1 min, 72 °C for 3 min followed by a final elongation step of 72 °C for 10 min.

PCR products were loaded on 2% w/v agarose gel, and run at 75 V for four several hours. Gels were stained with ethidium bromide and visualized with the gel documentation system Gel DocTM X⁺R (Bio-Rad), See Figure 2.

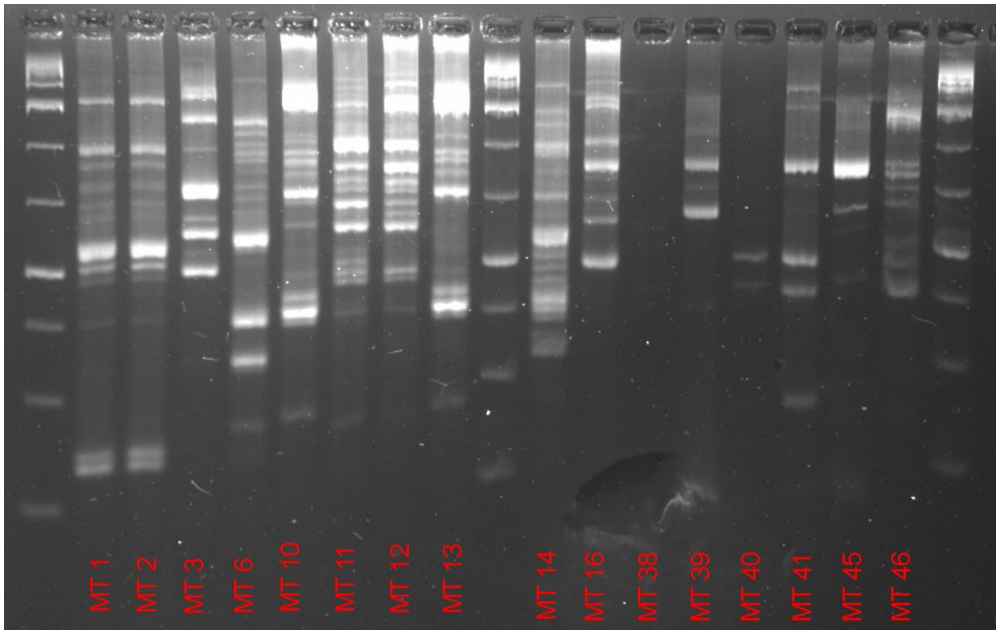


Figure 2 Example of a fingerprinting Gel visualized with Gel DocTM X⁺R (Bio-Rad).

A dendrogram was constructed using the Dice similarity coefficient and the UPGMA (unweighted pair group method with arithmetic mean) algorithm with GelCompar II software, version 6.6 (Applied Maths, Sint-Martens-Latem, Belgium) Figure 1.

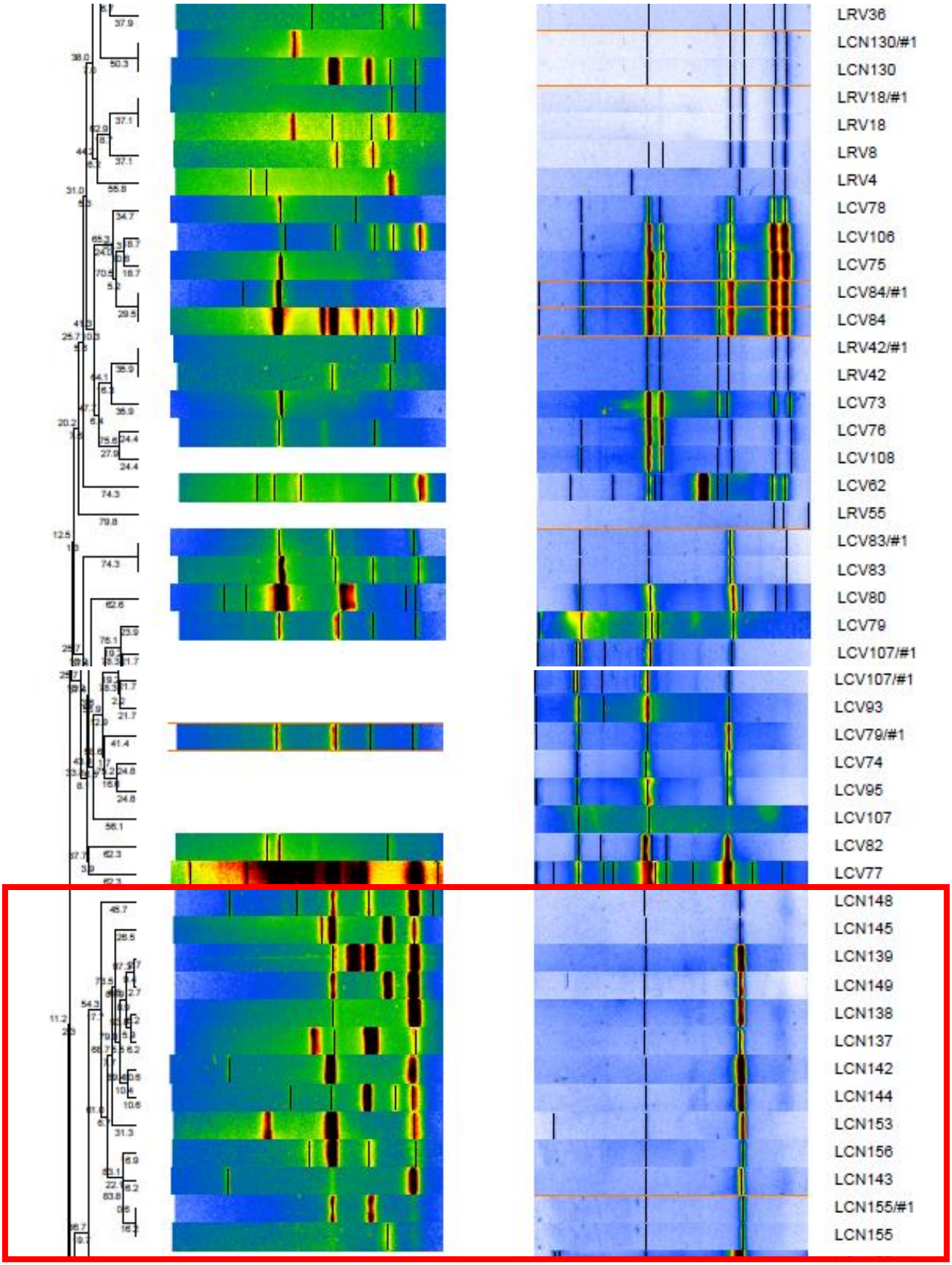
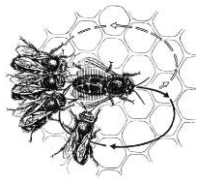


Figure 1: Example of a dendrogram where two fingerprinting techniques are compared for the same samples. It is interesting to point out that, in some circumstances, identical profiles on the left (RAPD-PCR) correspond to completely different profiles in the left column (BOX-PCR) for the same samples, E.g.: Red Box.

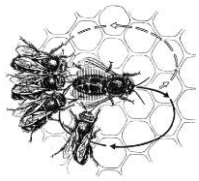
DNA of unique strains according to their combined fingerprinting profile analysis was sequenced for the conserved region coding for the small ribosomal unit (16S rRNA). Primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACT-3') were used for the PCR reaction (Lane, 1991) according to Gaggìa et al. (2013). Amplicons were sequenced by a commercial sequencing facility (Eurofins MWG, Edersberg, Germany). Sequence chromatograms were edited and analyzed using the software program Finch TV version 1.4.0 (Geospiza Inc., Seattle, WA, USA) and deposited in the GeneBank nucleotide database after classification using the RDP classifier (Wang et al., 2007). Identified strains and GeneBank Accession Number are reported in Table 3 and 4.

Table 3: Strains isolated during the Ph.D. activity and related taxonomic identification Strain reference, sample collection date and GeneBank Accession Number are also indicated.

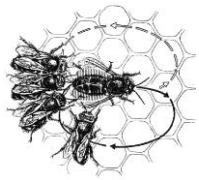
Microorganism	Isolation Source	Strain reference	Collection Date	GeneBank Accession Number
Achromobacter xylosoxidans	Gut of *Apis mellifera* subsp. *ligustica*	X1X	21-mar-2014	MG650022
Acinetobacter nectaris	Gut of *Osmia cornuta*	Sp45	1-apr-2016	MG645298
Acinetobacter nectaris	Gut of *Osmia cornuta*	Sp82	1-apr-2016	MG645306
Acinetobacter nectaris	Gut of *Osmia cornuta*	Sp84	1-apr-2016	MG645307
Acinetobacter nectaris	Gut of *Osmia bicornis*	Sp93	1-apr-2016	MG645309
Acinetobacter nectaris	Gut of *Osmia bicornis*	Sp100	1-apr-2016	MG645310
Acinetobacter nectaris	Gut of *Osmia bicornis*	Sp106	1-apr-2016	MG645311
Actinomyces odontolyticus	Gut of *Osmia cornuta*	OCN12	1-apr-2016	MG597264
Arthrobacter oryzae	Unfermented Pollen	XB1	01-may-2017	MG649992
Bacillus aerius	Gut of *Apis mellifera* subsp. *ligustica*	Sp11	21-mar-2014	MG650014
Bacillus aerius	Gut of *Apis mellifera* subsp. *ligustica*	Sp12	21-mar-2014	MG650015
Bacillus aerius	Dead Honey Bee Larvae	SP38	01-oct-2015	MG650038
Bacillus amyloliquefaciens	Honey Bee fermented pollen	Sp16	21-mar-2014	MG650017
Bacillus amyloliquefaciens	Dead Honey Bee Larvae	Sp20	01-oct-2015	MG650040
Bacillus amyloliquefaciens	Honey Bee fermented pollen	Sp25	01-oct-2015	MG650044
Bacillus amyloliquefaciens	Gut of *Apis mellifera* subsp. *ligustica*	SP43	01-oct-2015	MG650055
Bacillus aryabhattai	Gut of *Apis mellifera* subsp. *ligustica*	SP36	01-oct-2015	MG650052
Bacillus aryabhattai	Gut of *Apis mellifera* subsp. *ligustica*	SP41	01-oct-2015	MG650053
Bacillus cereus	Dead Honey Bee Larvae	Sp21	01-oct-2015	MG650041
Bacillus cereus	Dead Honey Bee Larvae	Sp23	01-oct-2015	MG650042
Bacillus cereus	Dead Honey Bee Larvae	Sp24	01-oct-2015	MG650043
Bacillus cereus	Honey	Sp35	01-jul-2015	MG650051
Bacillus cereus	Honey bee wax	CE1	01-may-2017	MG649990
Bacillus licheniformis	Gut of *Apis mellifera* subsp. *ligustica*	Sp1	21-mar-2014	MG650010
Bacillus licheniformis	Gut of *Apis mellifera* subsp. *ligustica*	Sp2	21-mar-2014	MG650011
Bacillus licheniformis	Gut of *Apis mellifera* subsp. *ligustica*	Sp9	21-mar-2014	MG650013
Bacillus licheniformis	Debrides	F2	01-oct-2015	MG650060
Bacillus mojavensis	Honey Bee fermented pollen	Sp17	21-mar-2014	MG650018
Bacillus pumilus	Honey Bee fermented pollen	Sp26	01-oct-2015	MG650045
Bacillus pumilus	Gut of *Osmia bicornis*	Sp114	1-apr-2016	MG645313
Bacillus safensis	Gut of *Osmia bicornis*	Sp122	1-apr-2016	MG645314
Bacillus simplex	Gut of *Osmia bicornis*	LRV34	1-apr-2016	MG645295
Bacillus simplex	Gut of *Osmia cornuta*	Sp57	1-apr-2016	MG645300
Bacillus simplex	Dead Honey Bee Larvae	Sp37	01-oct-2015	MG650039
Bacillus subtilis	Gut of *Apis mellifera* subsp. *ligustica*	Sp8	21-mar-2014	MG650012
Bacillus thuringiensis	Honey Bee fermented pollen	Sp15	21-mar-2014	MG650016
Bacillus toyonensis	Honey Bee fermented pollen	Sp27	01-oct-2015	MG650046
Bacillus toyonensis	Gut of *Apis mellifera* subsp. *ligustica*	Q1Q	21-mar-2014	MG650020
Bifidobacterium actinocoloniiforme	Gut of *Xylocopa violacea*	XV11B	1-mar-2016	MG597283



Bifidobacterium asteroides	Gut of *Apis mellifera* subsp. *ligustica*	Dan1	5-dec-2014	MG601136
Bifidobacterium asteroides	Gut of *Osmia bicornis*	LRV11	1-apr-2016	MG645294
Bifidobacterium asteroides	Gut of *Apis mellifera* subsp. *ligustica*	BB5	21-mar-2014	MG650000
Bifidobacterium asteroides	Gut of *Apis mellifera* subsp. *ligustica*	BB6	21-mar-2014	MG650001
Bifidobacterium asteroides	Gut of *Apis mellifera* subsp. *ligustica*	BB7	21-mar-2014	MG650002
Bifidobacterium asteroides	Gut of *Apis mellifera* subsp. *ligustica*	BB8	21-mar-2014	MG650003
Bifidobacterium asteroides	Gut of *Apis mellifera* subsp. *ligustica*	BB10	21-mar-2014	MG650004
Bifidobacterium asteroides	Gut of *Apis mellifera* subsp. *ligustica*	BB23	21-mar-2014	MG650006
Bifidobacterium asteroides	Gut of *Apis mellifera* subsp. *ligustica*	Dan10	21-mar-2014	MG650023
Bifidobacterium asteroides	Gut of *Apis mellifera* subsp. *ligustica*	Dan44	21-mar-2014	MG650024
Bifidobacterium asteroides	Gut of *Apis mellifera* subsp. *ligustica*	Dan19	21-mar-2014	MG650026
Bifidobacterium coryneforme	Gut of *Osmia cornuta*	OCV28	1-apr-2016	MG597271
Bifidobacterium coryneforme	Gut of *Osmia bicornis*	ORV8	1-apr-2016	MG597273
Bifidobacterium coryneforme	Gut of *Osmia bicornis*	ORV23	1-apr-2016	MG597275
Bifidobacterium coryneforme	Gut of *Osmia bicornis*	ORV27	1-apr-2016	MG597276
Bifidobacterium coryneforme	Gut of *Apis mellifera* subsp. *ligustica*	BB11	21-mar-2014	MG650005
Bifidobacterium coryneforme	Gut of *Apis mellifera* subsp. *ligustica*	BB25	21-mar-2014	MG650007
Bifidobacterium coryneforme	Gut of *Apis mellifera* subsp. *ligustica*	BB26	21-mar-2014	MG650008
Bifidobacterium coryneforme	Gut of *Apis mellifera* subsp. *ligustica*	BB33	21-mar-2014	MG650009
Bifidobacterium coryneforme	Gut of *Apis mellifera* subsp. *ligustica*	Dan103	21-mar-2014	MG650025
Bifidobacterium coryneforme	Gut of *Osmia bicornis*	ORV30	1-apr-2016	MG597277
Bifidobacterium coryneforme	Gut of *Apis mellifera* subsp. *ligustica*	BB1	21-mar-2014	MG649993
Bifidobacterium sp.	Gut of *Xylocopa violacea*	XV2B	1-mar-2016	MG597278
Candidatus Bifidobacterium xylocopae				
Bifidobacterium sp.	Gut of *Xylocopa violacea*	XV10B	1-mar-2016	MG597282
Candidatus Bifidobacterium aemilianum				
Bifidobacterium sp.	Gut of *Xylocopa violacea*	XV16B	1-mar-2016	MG597285
Ewingella americana	Gut of *Osmia bicornis*	Sp112	1-apr-2016	MG645312
Fructobacillus fructosus	Gut of *Osmia bicornis*	LRV37	1-apr-2016	MG645296
Fructobacillus fructosus	Gut of *Osmia cornuta*	Sp61	1-apr-2016	MG645301
Fructobacillus fructosus	Gut of *Osmia cornuta*	Sp63	1-apr-2016	MG645302
Fructobacillus fructosus	Gut of *Osmia bicornis*	Sp125	1-apr-2016	MG645315
Fructobacillus fructosus	Gut of *Osmia cornuta*	LCV 67	1-apr-2016	MG645282
Gilliamella apicola	Gut of *Apis mellifera* subsp. *ruttneri*	MT1	1-apr-2016	MG601164
Gilliamella apicola	Gut of *Apis mellifera* subsp. *ruttneri*	MT6	1-apr-2016	MG601169
Hafnia alvei	Gut of *Apis mellifera* subsp. *ligustica*	AC1	21-mar-2014	MG649994
Klebsiella oxytoca	Gut of *Apis mellifera* subsp. *ruttneri*	MT11	1-apr-2016	MG601168
Lactobacillus apis	Gut of *Apis mellifera* subsp. *ligustica*	Dan63	5-dec-2014	MG601154
Lactobacillus apis	Gut of *Osmia bicornis*	LRV 4	1-apr-2016	MG645292
Lactobacillus apis	Gut of *Osmia bicornis*	LRV55	1-apr-2016	MG645297
Lactobacillus apis	Gut of *Apis mellifera* subsp. *ruttneri*	MT61	1-apr-2016	MG601166
Lactobacillus apis	Gut of *Apis mellifera* subsp. *ruttneri*	MT76	1-apr-2016	MG601165
Lactobacillus bombi	Gut of *Xylocopa violacea*	XV2L	1-mar-2016	MG597279
Lactobacillus bombi	Gut of *Xylocopa violacea*	XV5L	1-mar-2016	MG597280
Lactobacillus bombi	Gut of *Xylocopa violacea*	XV8L	1-mar-2016	MG597281
Lactobacillus bombi	Gut of *Xylocopa violacea*	XV17L	1-mar-2016	MG597286
Lactobacillus graminis	Gut of *Osmia cornuta*	LCV 62	1-apr-2016	MG645281
Lactobacillus helsinborgensis	Gut of *Apis mellifera* subsp. *ligustica*	Dan16	5-dec-2014	MG601141
Lactobacillus helsingborgensis	Gut of *Apis mellifera* subsp. *ligustica*	Dan4	5-dec-2014	MG601138
Lactobacillus helsingborgensis	Gut of *Apis mellifera* subsp. *ligustica*	Dan51	5-dec-2014	MG601148
Lactobacillus helsingborgensis	Gut of *Apis mellifera* subsp. *ligustica*	Dan56	5-dec-2014	MG601150
Lactobacillus helsingborgensis	Gut of *Apis mellifera* subsp. *ligustica*	Dan61	5-dec-2014	MG601152
Lactobacillus helsingborgensis	Gut of *Apis mellifera* subsp. *ligustica*	Dan70	5-dec-2014	MG601155
Lactobacillus helsingborgensis	Gut of *Apis mellifera* subsp. *ligustica*	Dan75	5-dec-2014	MG601156
Lactobacillus helsingborgensis	Gut of *Apis mellifera* subsp. *ligustica*	Dan101	5-dec-2014	MG601159
Lactobacillus helsingborgensis	Gut of *Apis mellifera* subsp. *ligustica*	Dan102	5-dec-2014	MG601160
Lactobacillus jhonsonii	Gut of *Apis mellifera* subsp. *ligustica*	Dan92	5-dec-2014	MG601158
Lactobacillus kimbladii	Gut of *Apis mellifera* subsp. *ligustica*	Dan46	5-dec-2014	MG601146



Lactobacillus kimbladii	Gut of *Apis mellifera* subsp. *ligustica*	Dan47	5-dec-2014	MG601147
Lactobacillus kimbladii	Gut of *Apis mellifera* subsp. *ligustica*	Dan62	5-dec-2014	MG601153
Lactobacillus kullabergensis	Gut of *Apis mellifera* subsp. *ligustica*	Dan21	5-dec-2014	MG601142
Lactobacillus kullabergensis	Gut of *Apis mellifera* subsp. *ligustica*	Dan23	5-dec-2014	MG601143
Lactobacillus kullabergensis	Gut of *Apis mellifera* subsp. *ligustica*	Dan25	5-dec-2014	MG601144
Lactobacillus kullabergensis	Gut of *Apis mellifera* subsp. *ligustica*	Dan54	5-dec-2014	MG601149
Lactobacillus kullabergensis	Gut of *Apis mellifera* subsp. *ligustica*	Dan59	5-dec-2014	MG601151
Lactobacillus kullabergensis	Gut of *Apis mellifera* subsp. *ligustica*	Dan104	5-dec-2014	MG601161
Lactobacillus kullabergensis	Gut of *Apis mellifera* subsp. *ligustica*	Dan6	5-dec-2014	MG601139
Lactobacillus kullabergensis	Gut of *Apis mellifera* subsp. *ligustica*	Dan14	5-dec-2014	MG601140
Lactobacillus kunkeei	Gut of *Osmia bicornis*	LRV 5	1-apr-2016	MG645293
Lactobacillus kunkeei	Gut of *Apis mellifera* subsp. *ruttneri*	Gh1	1-apr-2016	MG601170
Lactobacillus kunkeei	Gut of *Apis mellifera* subsp. *ruttneri*	Gh2	1-apr-2016	MG601171
Lactobacillus kunkeei	Gut of *Apis mellifera* subsp. *ruttneri*	Gh3	1-apr-2016	MG601172
Lactobacillus kunkeei	Gut of *Apis mellifera* subsp. *ruttneri*	MT39	1-apr-2016	MG601167
Lactobacillus kunkeii	Gut of *Apis mellifera* subsp. *ligustica*	Dan39	5-dec-2014	MG601145
Lactobacillus mali	Gut of *Vespa Crabro*	VC29	01-may-2017	MG650029
Lactobacillus melliventris	Gut of *Apis mellifera* subsp. *ligustica*	Dan2	5-dec-2014	MG601137
Lactobacillus melliventris	Gut of *Apis mellifera* subsp. *ruttneri*	MT53	1-apr-2016	MG601163
Lactobacillus plantarum	Gut of *Apis mellifera* subsp. *ligustica*	Dan91	5-dec-2014	MG601157
Lactobacillus sp.	Gut of *Xylocopa violacea*	XV13L	1-mar-2016	MG597284
Candidatus Lactobacillus xylocopus				
Lactococcus lactis subsp. cremoris	Gut of *Polystes gallica*	PG29	01-may-2017	MG650033
Lactococcus lactis subsp. hordniae	Gut of *Osmia cornuta*	Sp79	1-apr-2016	MG645305
Lactococcus lactis subsp. hordniae	Gut of *Polystes gallica*	PG69	01-may-2017	MG650032
Leuconostoc mesenteroides	Gut of *Osmia cornuta*	LCV 77	1-apr-2016	MG645284
Leuconostoc mesenteroides	Gut of *Osmia cornuta*	LCV 79	1-apr-2016	MG645286
Leuconostoc mesenteroides	Gut of *Osmia cornuta*	LCV 83	1-apr-2016	MG645288
Leuconostoc mesenteroides	Gut of *Osmia cornuta*	LCV 100	1-apr-2016	MG645289
Leuconostoc mesenteroides	Gut of *Osmia cornuta*	LCV 78	1-apr-2016	MG645285
Leuconostoc mesenteroides	Gut of *Vespa Crabro*	VC32	01-may-2017	MG650027
Leuconostoc mesenteroides	Gut of *Vespa Crabro*	VC30	01-may-2017	MG650028
Leuconostoc pseudomesenteroides	Gut of *Osmia cornuta*	Sp89	1-apr-2016	MG645308
Macrococcus equipercicus	Honey bee wax	CF1	01-may-2017	MG649989
Microbacterium azadirachtae	Gut of *Apis mellifera* subsp. *ligustica*	T1T	21-mar-2014	MG650021
Micrococcus yunnanensis	Honey bee wax	PA1	01-may-2017	MG649988
Obesumbacterium proteus	Gut of *Apis mellifera* subsp. *ligustica*	AC9	21-mar-2014	MG649997
Obesumbacterium proteus	Gut of *Apis mellifera* subsp. *ligustica*	AC10	21-mar-2014	MG649998
Paenibacillus alvei	Honey	Sp30	01-jul-2015	MG650049
Paenibacillus alvei	Honey	Sp33	01-jul-2015	MG650050
Paenibacillus chitinolyticus	Honey Bee fermented pollen	Sp18	21-mar-2014	MG650019
Paenibacillus dendritiformis	Dead Honey Bee Larvae	PA(B)	01-oct-2015	MG650034
Paenibacillus dendritiformis	Dead Honey Bee Larvae	PA(C)	01-oct-2015	MG650035
Paenibacillus dendritiformis	Dead Honey Bee Larvae	PA(A)	01-oct-2015	MG650036
Paenibacillus humicus	Honey	Sp29	01-jul-2015	MG650048
Paenibacillus larvae	Dead Honey Bee Larvae	Sp19	01-oct-2015	MG650037
Paenibacillus larvae	Honey Bee fermented pollen	Sp28	01-oct-2015	MG650047
Paenibacillus larvae	Dead Honey Bee Larvae	SAN	01-oct-2015	MG650056
Paenibacillus peoriae	Gut of *Osmia cornuta*	OCV20	1-apr-2016	MG597268
Paenibacillus taichungensis	Honey	596op	01-oct-2015	MG650057
Paenibacillus xylanilyticus	Gut of *Apis mellifera* subsp. *ligustica*	SP42	01-oct-2015	MG650054
Paenibacillus yonginensis	Debrides	F3	01-oct-2015	MG650058
Propionibacterium acnes	Gut of *Osmia cornuta*	OCN3	1-apr-2016	MG597262
Propionibacterium acnes	Gut of *Osmia cornuta*	OCN10	1-apr-2016	MG597263
Propionibacterium acnes	Gut of *Osmia cornuta*	OCV2	1-apr-2016	MG597265
Propionibacterium acnes	Gut of *Osmia cornuta*	OCV18	1-apr-2016	MG597267
Propionibacterium acnes	Gut of *Osmia cornuta*	OCN2	1-apr-2016	MG597261
Propionibacterium acnes	Gut of *Osmia cornuta*	OCV21	1-apr-2016	MG597269

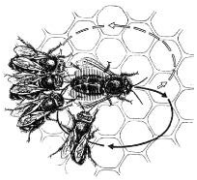


Propionibacterium acnes	Gut of *Osmia bicornis*	ORV20	1-apr-2016	MG597274
Propionibacterium granulosum	Gut of *Osmia cornuta*	OCV7	1-apr-2016	MG597266
Propionibacterium granulosum	Gut of *Osmia cornuta*	OCV26	1-apr-2016	MG597270
Rosenbergiella epipactidis	Gut of *Osmia cornuta*	Sp65	1-apr-2016	MG645303
Rosenbergiella epipactidis	Gut of *Osmia cornuta*	Sp69	1-apr-2016	MG645304
Serratia liquefaciens	Gut of *Apis mellifera* subsp. *ligustica*	AC4	21-mar-2014	MG649995
Serratia marcescens	Gut of *Apis mellifera* subsp. *ligustica*	AC8	21-mar-2014	MG649996
Serratia nematodiphila	Debrides	1A	01-oct-2015	MG650059
Staphylococcus epidermidi	Gut of *Osmia cornuta*	LCN 137	1-apr-2016	MG645275
Staphylococcus epidermidis	Gut of *Osmia cornuta*	LCN129	1-apr-2016	MG645273
Staphylococcus epidermidis	Gut of *Osmia cornuta*	LCN 138	1-apr-2016	MG645276
Staphylococcus epidermidis	Gut of *Osmia cornuta*	LCN 142	1-apr-2016	MG645278
Staphylococcus epidermidis	Gut of *Osmia cornuta*	LCN 148	1-apr-2016	MG645279
Staphylococcus epidermidis	Gut of *Osmia cornuta*	LCN 148	1-apr-2016	MG645280
Staphylococcus epidermidis	Gut of *Osmia cornuta*	LCN122	1-apr-2016	MG645271
Staphylococcus epidermidis	Gut of *Osmia cornuta*	LCN127	1-apr-2016	MG645272
Staphylococcus epidermidis	Gut of *Osmia cornuta*	LCN 141	1-apr-2016	MG645277
Staphylococcus hominis	Honey bee wax	CD1	01-may-2017	MG649991
Staphylococcus lugdunensis	Gut of *Apis mellifera* subsp. *ligustica*	BB2	21-mar-2014	MG649999
Streptomyces pseudogriseolus	Gut of *Osmia cornuta*	Sp49	1-apr-2016	MG645299
Vagococcus entomophilus	Gut of *Osmia cornuta*	OCV32	1-apr-2016	MG597272
Vagococcus entomophilus	Gut of *Vespa Crabro*	VC23	01-may-2017	MG650030
Vagococcus entomophilus	Gut of *Vespa Crabro*	VC21	01-may-2017	MG650031
Weissella cibaria	Gut of *Osmia cornuta*	LCN136	1-apr-2016	MG645274
Weissella cibaria	Gut of *Osmia cornuta*	LCV 68	1-apr-2016	MG645283
Weissella cibaria	Gut of *Osmia cornuta*	LCV 81	1-apr-2016	MG645287
Weissella cibaria	Gut of *Osmia cornuta*	LCV 117	1-apr-2016	MG645290
Weissella cibaria	Gut of *Osmia cornuta*	LCV 118	1-apr-2016	MG645291

NOTE: Most of GeneBank Accession Numbers have a programmed release for the public set for January 2019.

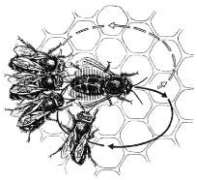
Table 4: Table of isolates; identified species compared with total number of the same isolate and strains number.

Scientific Name	Total isolates	Different fingerprinting pattern	Representative Strain reference according to fingerprinting analysis
Achromobacter xylosoxidans	***1***	***1***	"x"
Acinetobacter nectaris	***13***	***7***	sp 43; Sp45; sp84; Sp92; Sp93; Sp100; Sp106;
Bacillus aerius	***4***	***3***	Sp11; Sp12; Sp38
Bacillus amyloliquefaciens	***6***	***4***	Sp16; Sp20; Sp25; Sp43
Bacillus aryabhattai	***2***	***2***	Sp36; Sp41;
Bacillus cereus	***4***	***4***	Sp21; Sp23; Sp24; Sp35
Bacillus licheniformis	***3***	***3***	Sp1; Sp2; Sp9
Bacillus mojavensis	***1***	***1***	Sp17
Bacillus pumilis	***2***	***1***	Sp26;
	1	***1***	Sp114;
Bacillus safensis	***1***	***1***	Sp122;
	1	***2***	LRV15; LRV34
Bacillus simplex	***1***	***1***	Sp57:
	1	***1***	Sp37
Bacillus sp.	***1***	***1***	F2
Bacillus subtilis	***10***	***2***	Sp8; K84
Bacillus thuringiensis	***1***	***1***	Sp15
Bacillus toyonensis	***2***	***2***	Sp27; "o"
Bifidobacterium actinocoloniiforme	***12***	***2***	XV 11 Bif; XV 16 Bif;



	2	*2*	LRV11
Bifidobacterium asteroides	*11*	*11*	Dan1; Dan10; Dan19; Dan44; Dan66; BBee10; BBee23;BBees5; BBees6; BBees7; BBees8;
	2	*2*	OCV28; OCN12
Bifidobacterium coryneforme	*12*	*4*	ORV8; ORV23; ORV27; ORV30
	5	*5*	Dan103; BBees11; BBees25; BBees26; BBees33
Bifidobacterium sp. 1	*1*	*1*	XV 2 Bif
Bifidobacterium sp. 2	*1*	*1*	XV 10 Bif
Ewingella americana	*8*	*1*	Sp112;
Fructobacillus fructosus	*8*	*2*	LRV37; LCV 67
	9	*3*	Sp61; Sp63; Sp125;
Hafnia alvei	*1*	*1*	AC1
Lactobacillus apis	*4*	*2*	LRV 4; LRV55;
	1	*1*	Dan63
Lactobacillus bombi	*16*	*3*	XV 2 Lac; XV 5 Lac; XV 8 Lac; XV 17 Lac
Lactobacillus graminis	*1*	*1*	LCV 62
Lactobacillus helsingborgensis	*30*	*8*	Dan4; Dan16; Dan51; Dan56; Dan70; Dan75; Dan101; Dan102
Lactobacillus jonsoni	*1*	*1*	Dan92
Lactobacillus kimbladii	*3*	*3*	Dan46; Dan47; Dan62
Lactobacillus kullabergensis	*33*	*7*	Dan6; Dan14; Dan21; Dan25; Dan54; Dan59; Dan104
	14	*1*	LRV 5
Lactobacillus kunkeei	*70*	*10*	Gh1; Gh2; Gh3; Gh4; Gh5; Gh6, Gh7; Gh8; Gh9; Gh10
	1	*1*	Dan39
Lactobacillus mali	*4*	*1*	VC28;
Lactobacillus melliventris	*1*	*1*	Dan2
Lactobacillus plantarum	*1*	*1*	Dan91
Lactobacillus sp.	*1*	*1*	XV13 Lac
Lactococcus lactis subsp. ***cremoris***	*5*	*1*	PG29
Lactococcus lactis subsp. ***hordniae***	*2*	*1*	Sp79;
	1	*1*	PG69
Leuconostoc mesenteroides	*18*	*5*	LCV 77, LCV 78, LCV 79; LCV 83; LCV 100;
	4	*2*	VC32; VC30;
Leuconostoc pseudomesenteroides	*2*	*1*	Sp89;
Mellissococcus plutonius	*1*	*1*	PA(MP)
Microbacterium azadirachtae	*1*	*1*	"[]"
Paeniacillus xylanilyticus	*2*	*1*	Sp42
Paenibacillus alvei	*3*	*3*	PA(A); Sp30; Sp33
Paenibacillus chitinolyticus	*1*	*1*	Sp18
Paenibacillus dendritiformis	*3*	*3*	PA(P); PA(B); PA(C);
Paenibacillus humicus	*1*	*1*	Sp29
Paenibacillus larvae	*3*	*3*	SAN-AFB; SP19; SP28
Paenibacillus peoriae	*1*	*1*	OCV20
Paenibacillus Sp.	*1*	*1*	F3
Paenibacillus taichungensis	*1*	*1*	596op
Pantoea brenneri	*1*	*1*	Sp78;
Propionibacterium acnes	*11*	*1*	ORV20
	22	*7*	ORN3; OCN2; OCN3; OCN10; OCV2; OCV18; OCV21
Propionibacterium granulosum	*1*	*2*	OCV7; OCV26
Rosenbergiella epipactidis	*6*	*2*	Sp65; Sp69,
Serratia liquefaciens	*7*	*1*	AC4
Serratia marcescens	*9*	*1*	AC8
Serratia nematodiphila	*2*	*2*	F1A; F1B
Staphylococcus epidermidis	*25*	*8*	LCN122; LCN127; LCN129; LCN 137;LCN 138; LCN 141;LCN 142; LCN 148
Streptomyces pseudogriseolus	*1*	*1*	Sp47:
Vagococcus entomophilus	*2*	*1*	OCV32
	2	*2*	VC23; VC21;
Weissella cibaria	*8*	*5*	LCV 68; LCV 81; LCV 117; LCV 118; LCN136;

Table 5: Table of isolation sources in relation to diversity detected.



ISOLATION SOURCE	BACTERIAL SPECIES ISOLATED
Gut of *Apis mellifera* subsp. *ligustica*	*Achromobacter xylosoxidans* *Bacillus aerius* *Bacillus amyloliquefaciens* *Bacillus aryabhattai* *Bacillus licheniformis* *Bacillus subtilis* *Bacillus toyonensis* *Bifidobacterium asteroids* *Bifidobacterium coryneforme* *Hafnia alvei* *Lactobacillus apis* *Lactobacillus helsingborgensis* *Lactobacillus jhonsoni* *Lactobacillus kimbladii* *Lactobacillus kullabergensis* *Lactobacillus kunkeei* *Lactobacillus melliventris* *Lactobacillus plantarum* *Microbacterium azadirachtae* *Obesumbacterium proteus* *Paenibacillus xylanilyticus* *Serratia liquefaciens* *Serratia marcescens* *Staphylococcus lugdunensis*
Gut of *Apis mellifera* subsp. *ruttneri*	*Gilliamella apicola* *Klebsiella oxytoca* *Lactobacillus apis* *Lactobacillus kunkeei* *Lactobacillus melliventris*
Gut of *Osmia bicornis*	*Bacillus pumilus* *Bacillus safensis* *Bacillus simplex* *Bifidobacterium asteroids* *Bifidobacterium coryneforme* *Ewingella Americana* *Fructobacillus fructosus* *Lactobacillus apis* *Lactobacillus kunkeei* *Propionibacterium acnes* *Vagococcus entomophilus*
Gut of *Osmia cornuta*	*Acinetobacter nectaris* *Bifidobacterium coryneforme* *Fructobacillus fructosus* *Lactobacillus graminis* *Lactococcus lactis subsp. hordniae* *Leuconostoc mesenteroides* *Leuconostoc pseudomesenteroides* *Paenibacillus peoriae* *Propionibacterium acnes* *Propionibacterium granulosum* *Rosenbergiella epipactidis* *Staphylococcus epidermidis* *Streptomyces pseudogriseolus* *Weissella cibaria*
Gut of *Xylocopa violacea*	*Bifidobacterium actinocoloniiforme*

	Candidatus Bifidobacterium xylocopae
	Candidatus Bifidobacterium aemilianum
	Lactobacillus bombi
	Candidatus Lactobacillus xylocopus
Dead honeybee larvae	*Bacillus aerius*
	Bacillus amyloliquefaciens
	Bacillus cereus
	Bacillus simplex
	Paenibacillus dendritiformis
	Paenibacillus larvae
Honeybee fermented pollen	*Bacillus amyloliquefaciens*
	Bacillus mojavensis
	Bacillus pumilus
	Bacillus thuringiensis
	Bacillus toyonensis
	Paenibacillus chitinolyticus
	Paenibacillus larvae
Honey	*Bacillus cereus*
	Paenibacillus alvei
	Paenibacillus humicus
	Paenibacillus taichungensis
Honeybee wax	*Bacillus cereus*
	Macrococcus equipercicus
	Micrococcus yunnanensis
	Staphylococcus hominis
Gut of *Vespa crabro*	*Lactobacillus mali*
	Leuconostoc mesenteroides
	Vagococcus entomophilus
Gut of *Polystes gallica*	*Lactococcus lactis subsp. cremoris*
	Lactococcus lactis subsp. hordniae

A virtual machine (Microsoft Azure Services https://azure.microsoft.com/it-it/) consisting of 120 GB of RAM memory and 16 Processors was used for any bioinformatic analysis.

Phylogenetic three representing the diversity of isolates was obtained with Qiime 2 (Figure 3), using the Python scripts:

```
align_seqs.py -i Seq_PhD_all.fasta -m pynast -a uclust -e 50% -p 0.50 -o
dani_aligned/
```

where:
-i, --input_fasta_fp
-m, --alignment_method
-a, --pairwise_alignment_method
-e, --min_length
-p, --min_percent_id
-o, --output_dir

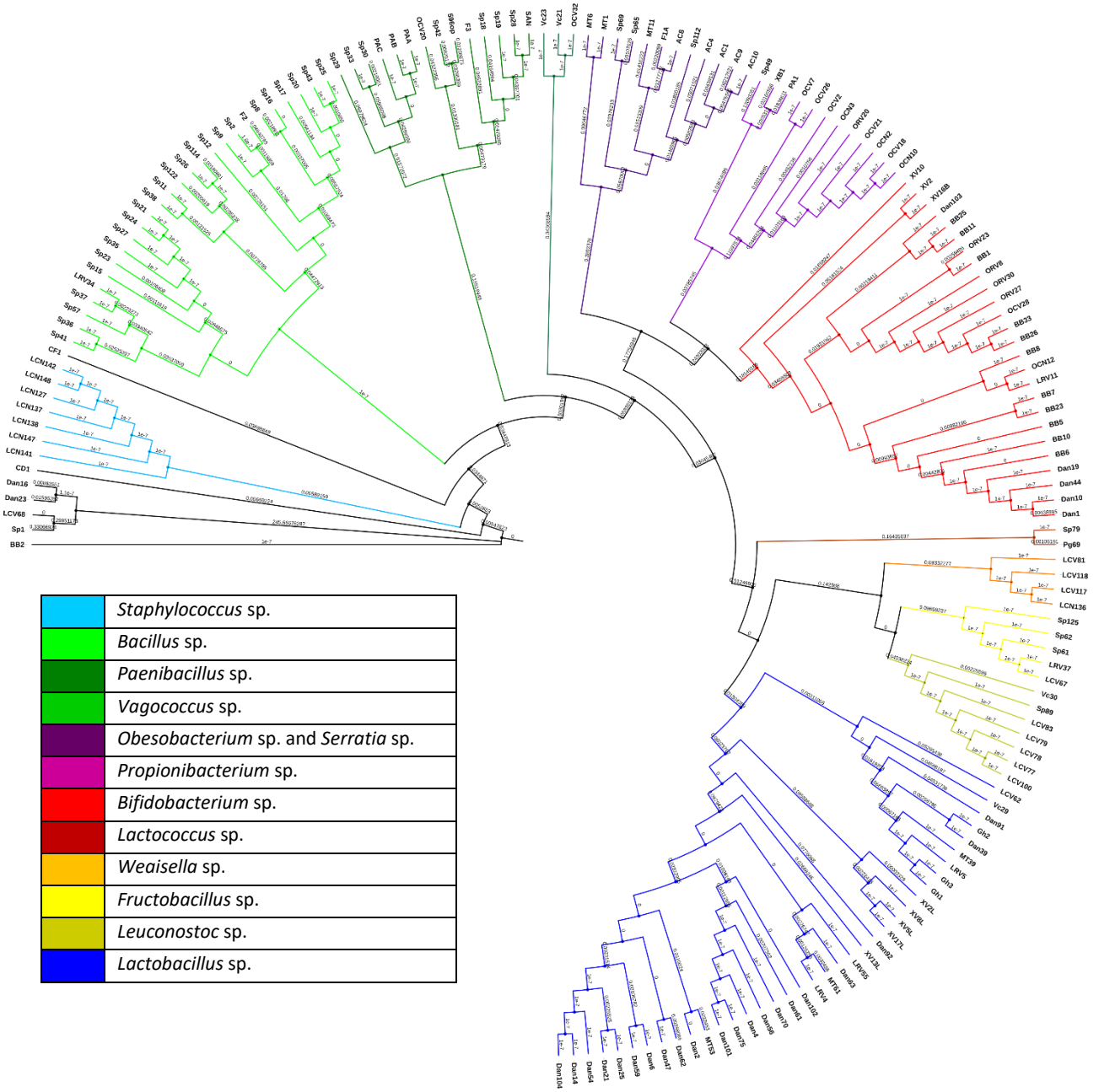
```
make_phylogeny.py -i /media/data/Tree/dani_aligned/Seq_PhD_all_aligned.fasta -t
fasttree -o dani2.tre -r midpoint
```

where:

-i, --input_fp

-t, --tree_method
-o, --result_fp
-l, --log_fp
-r, --root_method

Final phylogenetic three Figure 3 was obtained loading the Qiime 2 output in MEGA6.



	Staphylococcus sp.
	Bacillus sp.
	Paenibacillus sp.
	Vagococcus sp.
	Obesobacterium sp. and *Serratia* sp.
	Propionibacterium sp.
	Bifidobacterium sp.
	Lactococcus sp.
	Weaisella sp.
	Fructobacillus sp.
	Leuconostoc sp.
	Lactobacillus sp.

Figure 3: philogenetic three of isolates, colors reported rapresent the main bacterial family in the colored clade

In this isolation work, a total of 4 novel species were detected:

- *Bifidobacterium* sp. Xv2

- *Bifidobacterium* sp. Xv10

- *Lactobacillus* sp. Xv13L

- *Bacillus* sp. F2

The full characterization of Xv2 and Xv10 as novel species is described in the following chapter, whereas characterization of strains Xv13L and F2 is still ongoing, and it was decided not to consider it from this thesis work.

Bifidobacterium xylocopae sp. nov. and *Bifidobacterium aemilianum* sp. nov., from the carpenter bee (*Xylocopa violacea*) digestive tract

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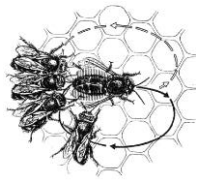


Abstract:
Social bees, including honeybees, harbor a community of gut mutualistic bacteria, among which bifidobacteria occupy an important niche. Recently four novel species have been isolated from guts of different bumblebees, thus allowing us to suppose that a core bifidobacterial population may also be present in wild solitary bees. To date there is only sparse information about bifidobacteria in solitary bees such as *Xylocopa* and *Osmia* spp., this study is therefore focused on the isolation and characterization of bifidobacterial strains from solitary bees, in particular carpenter bee (*Xylocopa violacea*), builder bee (*Osmia cornuta*), and red mason bee (*Osmia rufa*). Among the isolates from *Osmia* spp., strains belonging to *Bifidobacterium asteroides* and *Bifidobacterium coryneforme* species have been detected whereas among *Xylocopa* isolates four strains (XV2, XV4 XV10 and XV16) belonging to putative new species were found. The newly isolated strains are gram-positive, non-spore-forming, lactate- and acetate-producing and possess the fructose-6-phosphate phosphoketolase enzyme. Full genome sequencing and genome annotation were performed as well as phenotypic tests. Phylogenetic relationships were determined using complete 16S rRNA sequences and hsp60 restriction analysis. The presence of genes involved in sugar utilization, hemolytic activity and vitamin biosynthetic pathway are discussed. The results support the proposal of two novel species *Bifidobacterium xylocopae* sp. nov. whose type strain is XV2 (=DSM 104955^T = LMG 30142^T), reference strain XV16 and *Bifidobacterium aemilianum* sp. nov. whose type strain is XV10 (=DSM 104956^T = LMG 30143^T), reference strain XV4.

Introduction:
Bifidobacteria are commensal anaerobic bacteria of the human and animal gut, where they exert important functions for the host. In-depth studies of bifidobacteria type-strain genomes support the hypothesis of co-evolution with the host with both DNA acquisition and loss events (Milani et al., 2014). This taxon constitutes one of the most numerous groups of beneficial bacteria in the gut contributing to the intestinal microbiota in different percentages depending on the host species, age and diet (Turroni et al., 2008). Different molecules have been described and characterized to date as mediators of the *Bifidobacterium* cross-talk with the host (Ruiz et al., 2016; Grimm et al., 2014, Ventura et al., 2012) and accountable for a number of positive effects in host development and physiology (Bottaccini et al., 2014; Sommer and Bäckhed, 2013). As mammals, insects rely on a mutualistic gut microbial community. In some insects, such as several ant species, the microbiota seems to be acquired from the food and the environment (Engel and Moran 2013), whereas in honeybees and bumble bees it looks more host-specific (Kwong and Moran 2015). These specialized bacteria may influence host nutrition, as they contain genes involved in carbohydrate digestion (Engel et al. 2012), and contribute to host defense and physiology (Mohr et al. 2006; Hamdi et al. 2011). Whether gut microbes are environmentally acquired or host-specific, they are extremely important for the host health status and bifidobacteria represents an important gut taxon to be investigated for its beneficial properties. Bifidobacterial population has been characterized in the sixties in some pollinating insects, including the honeybees *Apis mellifera* and *Apis cerana*, and isolated strains were classified as new species: *Bifidobacterium coryneforme*, *Bifidobacterium asteroides* and *Bifidobacterium*

indicum (Scardovi and Trovatelli, 1969). Later studies, aimed at the identification of dominant and recurring honeybee-associated gut microorganisms, confirmed bifidobacteria as stable colonizers/inhabitants of honeybee gut (Sabree et al. 2012). Honeybees are not the only pollinators harboring bifidobacteria: in the last decade four novel species have been identified from *Bombus* spp. gut: *Bifidobacterium bombi*, *Bifidobacterium actinocoloniforme* and *Bifidobacterium bohemicum* from the digestive tract of *Bombus lucorum* and *Bifidobacterium commune* from *Bombus lapidarius* (Killer et al. 2009 and 2011; Praet et al. 2015). However, no information is available in the literature on the presence of bifidobacteria in solitary bees such as *Xilocopa* spp., known as carpenter bees for their ability to burrow into hard plant material, and *Osmia* spp., known as mason bees for their habit of using mud or other "masonry" products to construct their nests. These bees exert a highly efficient non-contracted pollination service (Woodcock et al. 2013). *Xylocopa* species are known to pollinate several crops, such as legums, aubergine, broccoli etc. (Thapa, 2006; Vicidomini 1997) and several fruit crops, especially *Prunus* spp. (Vicidomini 1997, Dar et al., 2013), thus assuming great value for crop pollination strategies. *Osmia* spp., especially *Osmia cornuta*, are also of utmost importance for orchard pollination, especially for *Rosaceae* family plants, like pear and apple and also for almond (Maccagnani et al., 2003; Bosh, 1994). The health status of solitary bees is crucial for the maintenance of the pollination service. In the last century, many wild bee populations, including solitary bees, have become reduced in number and the loss of their genetic diversity makes them more vulnerable to infectious diseases and other stressors such as pesticides (Graystock et al. 2013; Sgolastra et al., 2017). Moreover, the spread of parasitic infections from managed bees to wild bees has been reported and recently reviewed by Graystock et al. (2013), and represents a potential threat for wild bees' population. The gut of insects may harbor one of the largest reservoirs of a yet unexplored microbial diversity. A deeper knowledge of the gut microorganisms of alternative pollinators, such as *Osmia* and *Xilocopa*, could be of great importance, since their health status depends on the presence and activity of commensal microorganisms as for any other animal. Novel strains might show a potential as beneficial bacteria for pollinator insects, reinforcing attempts to establish a beneficial bacteria strategy for bee health (Baffoni et al. 2016; Alberoni et. al., 2016; Alberoni et. al., 2018). This work is therefore aimed at increasing the knowledge on the presence and diversity of cultivable bifidobacteria in solitary bees, in particular in the genera *Xilocopa* and *Osmia*.

Materials and methods and results:

In spring 2016, worker carpenter bees were collected in a flowery meadow in Spilamberto (Modena, Italy), whereas builder and red mason bees were collected in a rearing field located in Cadriano (Bologna, Italy) and promptly transferred to the laboratory. All bees were anesthetized, and their gut content was extracted, weighted, serially diluted and plated on two different media for bifidobacteria isolation: Tryptone, Phytone, and Yeast extract (TPY) agar medium (Mazzola et al., 2015) supplemented with mupirocin (200 mgL^{-1}) and de Man Rogosa Sharpe (MRS) agar medium (Scharlau Chemie, Gato Perez, Spain) supplemented with fructose (10 gL^{-1}), cysteine (1 gL^{-1}) (Olofsson et al., 2014) and cycloheximide (0.01 mgL^{-1}). Antibiotics were purchased from Sigma Aldrich (Steinheim, Germany). Plates were incubated 5 days at 35 °C, in both anaerobic and microaerophilic atmosphere generated in

jars with GasPak™ EZ (Becton, Dickinson Co., Sparks, MD, USA). Morphologically different colonies were randomly picked from both media, re-streaked on the corresponding agar medium and purity checked prior to further analysis. A total of thirty-three, thirty-four, twenty-four bifidobacteria isolates were obtained from *Xylocopa violacea*, *Osmia cornuta* and *Osmia rufa* (synonym *Osmia bicornis*), respectively. Pure cultures were prepared for cryoconservation in stocks at -80°C. Chromosomal DNA was extracted from each isolate with Wizard® Genomic DNA Purification Kit (Promega, Madison, Wi, USA). Isolates were characterized with two PCR-dependent fingerprinting techniques. Randomly amplified polymorphic DNA (RAPD) PCR was carried out in a reaction mixture composed of 10 µL HotStarTaq Master Mix (QIAGEN GmbH, Hilden, Germania), 2 µL of 20 µM primer M13 (5'-GAGGGTGGCGGTTCT-3') (Andrighetto et al., 2001), 1.2 µL of template DNA (50 ng/µl) and water to the total volume of 20 µL. Enterobacterial repetitive intergenic consensus (ERIC) sequence PCR were carried out using the same protocol except for the primers ERIC-1 (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC-2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') (Versalovic et al., 1991) (0.5 µM of each primer). The amplification products were run on a 2% (w/vol) agarose gel, containing 0.05 µL/mL SYBR Safe, in a Tris-borate-EDTA (TBE) buffer, at 120 V for 4 hours. Gel images were captured with GelDoc (BioRad Laboratories, Hercules, CA, USA). Fingerprinting profile patterns were analyzed with GelCompar II 6.6 (Applied Maths, Kortrijk, Belgium) using the DICE coefficient and the UPMGA clustering algorithm. In cluster analysis the cut-off value was defined obtaining 5 clusters in fingerprinting profiles of microbial isolates from *X. violacea* (Figure S1), and 9 clusters in fingerprinting profiles of microbial isolates from *O. cornuta* and *O. rufa* (Figure S2).

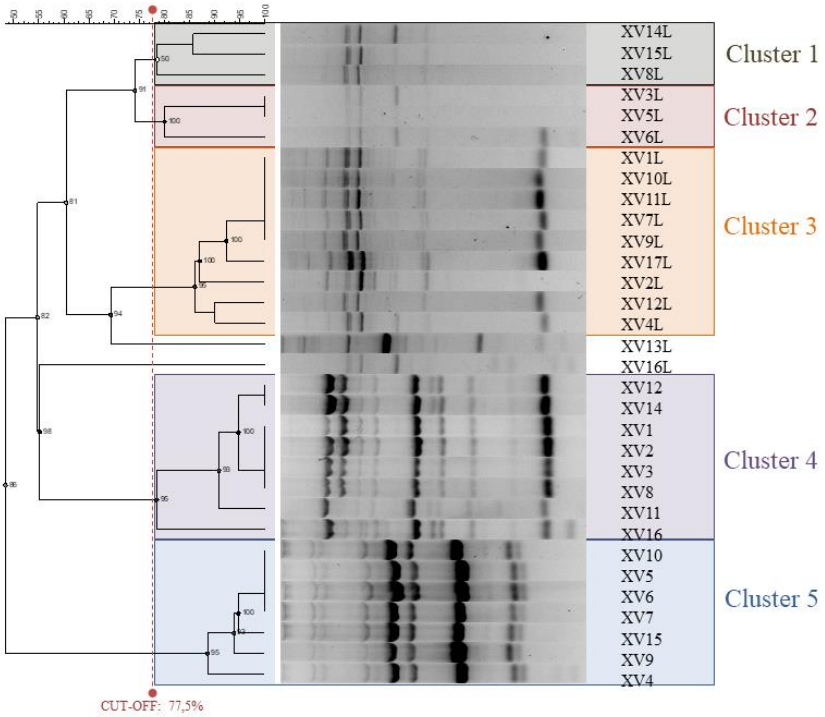


Figure S1 ERIC-PCR on isolates from *Xylocopa violacea*. Cluster analysis of ERIC-PCR fingerprinting on bacterial isolates from *Xylocopa violacea*.

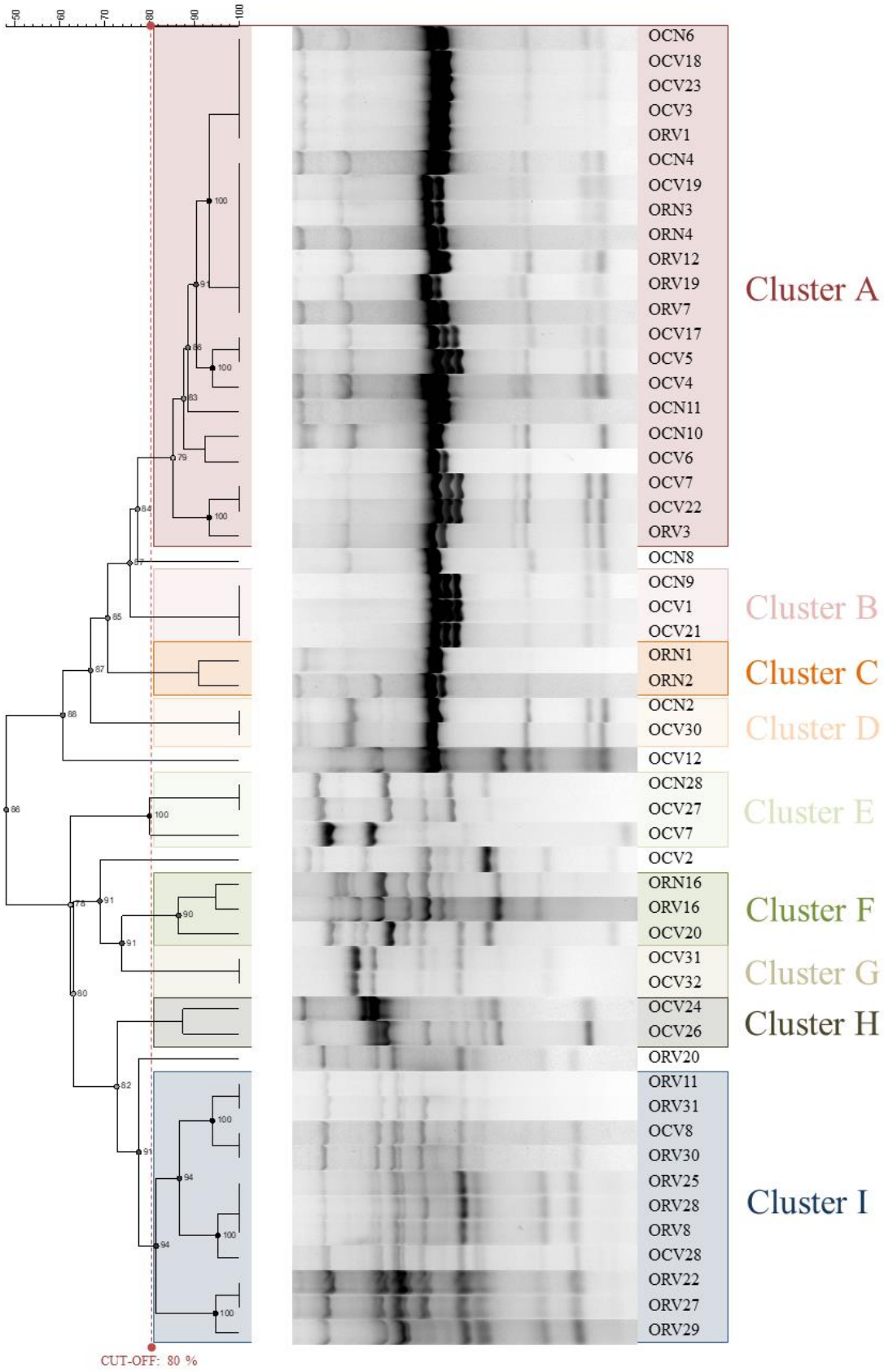
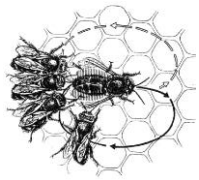


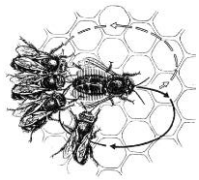
Figure S2 ERIC-PCR on isolates form *Osmia cornuta* and *Osmia rufa*. Cluster analysis of ERIC-PCR fingerprinting on bacterial isolates from *Osmia cornuta* and *Osmia rufa.*



A representative strain for each cluster was further processed. DNA amplification of the 16S rRNA gene was performed with primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACT-3') (Lane, 1991) according to Gaggìa et al. (2013). Amplicons were purified (NucleoSpin® Gel and PCR Clean-up kit; Macherey-Nagel GmbH Co. KG, Germany) and sent to a commercial sequencing facility (Eurofins MWG, Edersberg, Germany). Sequence chromatograms were analyzed using the software program FinchTV version 1.4.0 (Geospiza Inc., Seattle, WA, USA), manually edited, classified using the RDP classifier (Wang et al., 2007) and deposited in GeneBank nucleotide database (accession numbers MG597261- MG597286) (Table 1). Recent studies show how gut microbial population of honeybees (*Apis mellifera*) is transmitted horizontally through contact with nurse bees, fresh faces or hive surfaces (Martinson et al. 2011), therefore it can be defined as a "socially transmitted gut microbiota". On the contrary, *Osmia* and *Xylocopa* genera consist of solitary bees, therefore social transmission at the time of edging is hardly occurring as also evidenced by Lozo et al. (2015). In recent studies (Lozo et al., 2015 and Keller et al., 2013) *Bifidobacterium* species were not detected in mason bees alimentary tract. In our study, on the contrary, isolates from *Osmia* spp. in TPY agar medium were identified as *Propionibacterium acnes*, *Propionibacterium granulosum, B. asteroides*, *B. coryneforme* and *B. indicum* (Table 1), showing a similarity with *Apis mellifera* bifidobacteria core species. One can hypothesize an acquisition of bifidobacteria from food sources (flowers) that might be occasionally shared between solitary and honeybees (McFrederick et al. 2012; Koch et al 2013). Novel species were not detected in *Osmia* spp. gut.

Table 1 Identification of isolates based on 16S rDNA sequences and RDP SeqMatch tool.

Strain	Microorganism	Isolation source	GenBank (Accession number)	S_ab score*
OCN2	*Propionibacterium acnes*	*Osmia cornuta*	MG597261	1.000
OCN10	*Propionibacterium acnes*	*Osmia cornuta*	MG597263	1.000
OCV2	*Propionibacterium acnes*	*Osmia cornuta*	MG597265	0.909
OCV7	*Propionibacterium granulosum*	*Osmia cornuta*	MG597266	0.956
OCV18	*Propionibacterium acnes*	*Osmia cornuta*	MG597267	0.989
OCV20	*Paenibacillus jamilae*	*Osmia cornuta*	MG597268	0.948
OCV21	*Propionibacterium acnes*	*Osmia cornuta*	MG597269	0.993
OCV26	*Propionibacterium granulosum*	*Osmia cornuta*	MG597270	0.969
OCV28	*Bifidobacterium indicum*	*Osmia cornuta*	MG597271	0.946
OCV32	*Vagococcus entomophilus*	*Osmia cornuta*	MG597272	0.998
ORV8	*Bifidobacterium coryneforme*	*Osmia rufa*	MG597273	0.943
ORV20	*Propionibacterium acnes*	*Osmia rufa*	MG597274	0.998
ORV27	*Bifidobacterium coryneforme*	*Osmia rufa*	MG597276	0.934
ORV30	*Bifidobacterium indicum*	*Osmia rufa*	MG597277	0.940
XV2	*Bifidobacterium* sp.	*Xylocopa violacea*	MG597278 Complete: MH043275	0.842
XV2L	*Lactobacillus bombi*	*Xylocopa violacea*	MG597279	0.952
XV4	*Bifidobacterium* sp.	*Xylocopa violacea*	MH043274	0.834
XV5L	*Lactobacillus bombi*	*Xylocopa violacea*	MG597280	0.945
XV8L	*Lactobacillus bombi*	*Xylocopa violacea*	MG597281	0.943
XV10	*Bifidobacterium* sp.	*Xylocopa violacea*	MG597282 Complete: MH043276	0.859
XV11B	*Bifidobacterium actinocoloniiforme*	*Xylocopa violacea*	MG597283	0.932
XV13L	*Lactobacillus* sp.	*Xylocopa violacea*	MG597284	0.864
XV16	*Bifidobacterium* sp.	*Xylocopa violacea*	MG597285	0.928
XV16L	*Lactobacillus bombi*	*Xylocopa violacea*	MH043277	0.942
XV17L	*Lactobacillus bombi*	*Xylocopa violacea*	MG597286	0.944

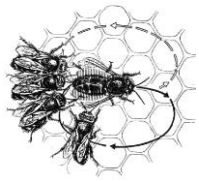


*S_ab score is the percentage of shared 7-mers between two sequences and do not require an alignment

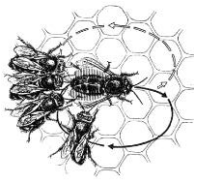
Among TPY and MRS isolates from *X. violacea* (Table 1), XV2 (together with XV16), XV10 (together with XV4), and XV13L 16S rDNA partial sequences gave a similarity score that deserved an in-depth analysis. For this purpose, the web tool EMBOSS-Water (EMBL-EBI release), relaying on the Smith-Waterman algorithm, was used to calculate local alignment and similarity percentages of full 16S rDNA sequences obtained from full genome sequencing. A similarity of 95.7, 97.7 and 96.8% for XV2, XV10 and XV13L respectively was obtained comparing each strain with its closest type strain. These scores are below the threshold of 98.5% and consequently potential novel species candidates (Kim et al., 2014). Other isolates from *Xylocopa violacea* were *Bifidobacterium actinocoloniiforme* and *Lactobacillus bombi*, therefore allowing us to assume a certain affinity for food sources between carpenter bee and *Bombus* spp. *Lactobacillus* strain XV13L needs further characterization, so it will not be discussed in this paper, whereas *Bifidobacterium* sp. XV2 and *Bifidobacterium* sp. XV10 are described and proposed as novel species.
Genomic DNA from XV2 and XV10 strains was sequenced on the Illumina MiSeq NGS platform by BMR Genomics facilities (www.bmr-genomics.it), according to Illumina protocol for Nextera XT DNA library preparation. Reads were assembled into contigs with SPAdes 3.7 according to Bankevich et al. (2012) and assembly quality assessed with QUAST (Gurevich et al., 2013). XV2 fastq output counted 2,634,000 reads whereas XV10 fastq output counted 3,007,626 reads corresponding to an esteemed coverage of 350x and 400x, respectively. The reads assembly output consisted in 242 contigs (1.95 Mbp) for XV2 and 241 contigs (2.12 Mbp) for XV10. Average Nucleotide Identity values were calculated with PYANI (Pritchard et al. 2016), a python3 module, using different methods: ANIb (based on BLAST algorithm), ANIm (based on MUMmer algorithm) and TETRA (Tetranucleotide Signature Frequency Correlation Coefficient) as described by Richter and Rosselló-Móra (2009). ANI and TETRA values were obtained comparing XV2 and XV10 genomes between them and among genomes of bifidobacteria type strains reported in Table S1.

Table S1 General features od *Bifidobacterium* genomes

Taxon N°	*Bifidobacterium* strains	Genome size	Coverage	Sequencing Platform	Isolation source	RefSeq Assembly Accession
01	*B. actinocoloniiforme* DSM22766	1,823,388	278.3x	HiSeq	Bumblebee digestive tract	GCF_000771585.1
02	*B. adolescentis* DSM	2,051,152	unknown	HiSeq	Human adult gut	GCF_000702865.1
03	*B. aesculapii* DSM26737	2,693,486	418x	MiSeq	Faeces of baby common marmosets	GCF_001417815.1
04	*B. angulatum* DSM20098	1,993,784	278.3x	HiSeq	Human faeces	GCF_000771205.1
05	*B. animalis* subsp *animalis* LMG10508	1,915,007	61x	IonTorrent	Rat Faeces	GCF_000741485.1
06	B. *animalis* subsp. *lactis* DSM10140	1,938,483	unknown	unknown	Fermented milk	GCF_000022965.1
07	*B. aquikefiri* LMG28769	2,408,364	96.25x	MiSeq	Household water kefir	GCF_002259795.1
08	*B. asteroides* DSM20089	2,138,592	278.3x	HiSeq	Honeybee hindgut	GCF_000771125.1
09	*B. biavatii* DSM23969	3,262,679	278.3x	HiSeq	Faeces of tamarin	GCF_000771645.1



10	*B. bifidum* DSM20456	2,201,251	181x	illumina	Brest-feed infant faeces	GCF_000466525.1
11	*B. bohemicum* DSM22767	2,054,900	278.3x	HiSeq	Bumble bee digestive tract	GCF_000771605.1
12	*B. bombi* DSM19703	1,912,794	278.3x	HiSeq	Bumble bee digestive tract	GCF_000771085.1
13	*B. boum* DSM20432	2,161,188	278.3x	HiSeq 2000	Bovine rumen	GCF_000687615.1
14	*B. breve* DSM20213	2,331,386	unknown	unknown	Infant intestine	GCF_000158015.1
15	*B. callitrichos* DSM23973	2,867,214	278.3x	HiSeq	Faeces of common marmosets	GCF_000771665.1
16	*B. catenulatum* DSM 16992	2,082,756	31.2x	Ion Torrent	Adult intestine	GCF_000741565.1
17	*B. choerinum* DSM 20434	2,049,941	278.3x	HiSeq	Piglet faeces	GCF_000771425.1
18	*B. commune* R-52791	1,633,662	691x	unknown	Bumble bee gut	GCF_900094885.1
19	*B. coryneforme* LMG18911	1,755,151	182x	IonTorrent	Honeybee hindgut	GCF_000737865.1
20	*B. crudilactis* LMG23609	2,362,816	20x	454	Raw cow milk	GCF_000738005.1
21	*B. cuniculi* DSM20435	2,512,691	278.3x	HiSeq	Rabbit faeces	GCF_000771465.1
22	*B. dentium* DSM 20436	2,668,067	681x	unknown	Oral cavity	GCF_900105745.1
23	*B. eulemuris* DSM 100216	2,913,389	75.23x	MiSeq	Faeces of black lemur	GCF_002259685.1
24	*B. gallicum* DSM 20093	1,989,850	278.3x	HiSeq	Adult intestine	GCF_000771165.1
25	*B. gallinarum* DSM 20670	2,128,566	278.3x	HiSeq	Chicken caecum	GCF_000771505.1
26	*B. hapali* DSM100202	2,834,308	109.83x	MiSeq	Faeces of baby common marmosets	GCF_002259755.1
27	*B. indicum* LMG11587	1,734,546	108x	IonTorrent	Asiatic honeybee hindgut	GCF_000706765.1
28	*B. kashiwanohense* DSM21854	2,307,960	64x	IonTorrent	Infant faeces	GCF_000741605.1
29	*B. lemurum* DSM28807	2,944,293	71.87x	MiSeq	Faeces of the ring-tailed lemur	GCF_002259665.1
30	*B. longum* subsp. *infantis* ATCC15697	2,832,748	unknown	unknown	Intestine of infant	GCF_000020425.1
31	*B. longum* subsp. *longum* LMG13197	2,384,703	34.8x	IonTorrent	Adult intestine	GCF_000741245.1
32	*B. longum* subsp. *suis* DSM 20211	2,389,965	278.3x	HiSeq	Pig faeces	GCF_000771285.1
33	*B. magnum* DSM20222	1,819,235	278.3x	HiSeq	Rabbit faeces	GCF_000771365.1
34	*B. merycicum* DSM 6492	2,274,683	508x	HiSeq	Bovine rumen	GCF_900129045.1
35	*B. minimum* DSM 20102	1,863,807	278.3x	HiSeq	Sewage	GCF_000771245.1
36	*B. mongoliense* DSM 21395	2,154,054	278.3x	HiSeq	Fermented mare's milk	GCF_000771525.1
37	*B. moukalabense* DSM 27321	2,515,335	82.0x	Ion Torrent	Faeces of wild western lowland gorilla	GCF_000522505.1
38	*B. myosotis* DSM 100196	2,944,195	57.03x	MiSeq	Faeces of baby common marmosets	GCF_002259745.1
39	*B. pseudocatenulatum* DSM 20438	2,284,490	278.3x	HiSeq	Infant faeces	GCF_000771445.1
40	*B. pseudolongum* subsp. *globosum* DSM 20092	1,915,040	278.3x	HiSeq	Bovine rumen	GCF_000771145.1
41	*B. pseudolongum* subsp. *pseudolongum* DSM 20099	1,901,000	278.3x	HiSeq	Swine faeces	GCF_000771225.1
42	*B. psychraerophilum* DSM 22366	2,621,124	278.3x	HiSeq	Pig caecum	GCF_000771565.1

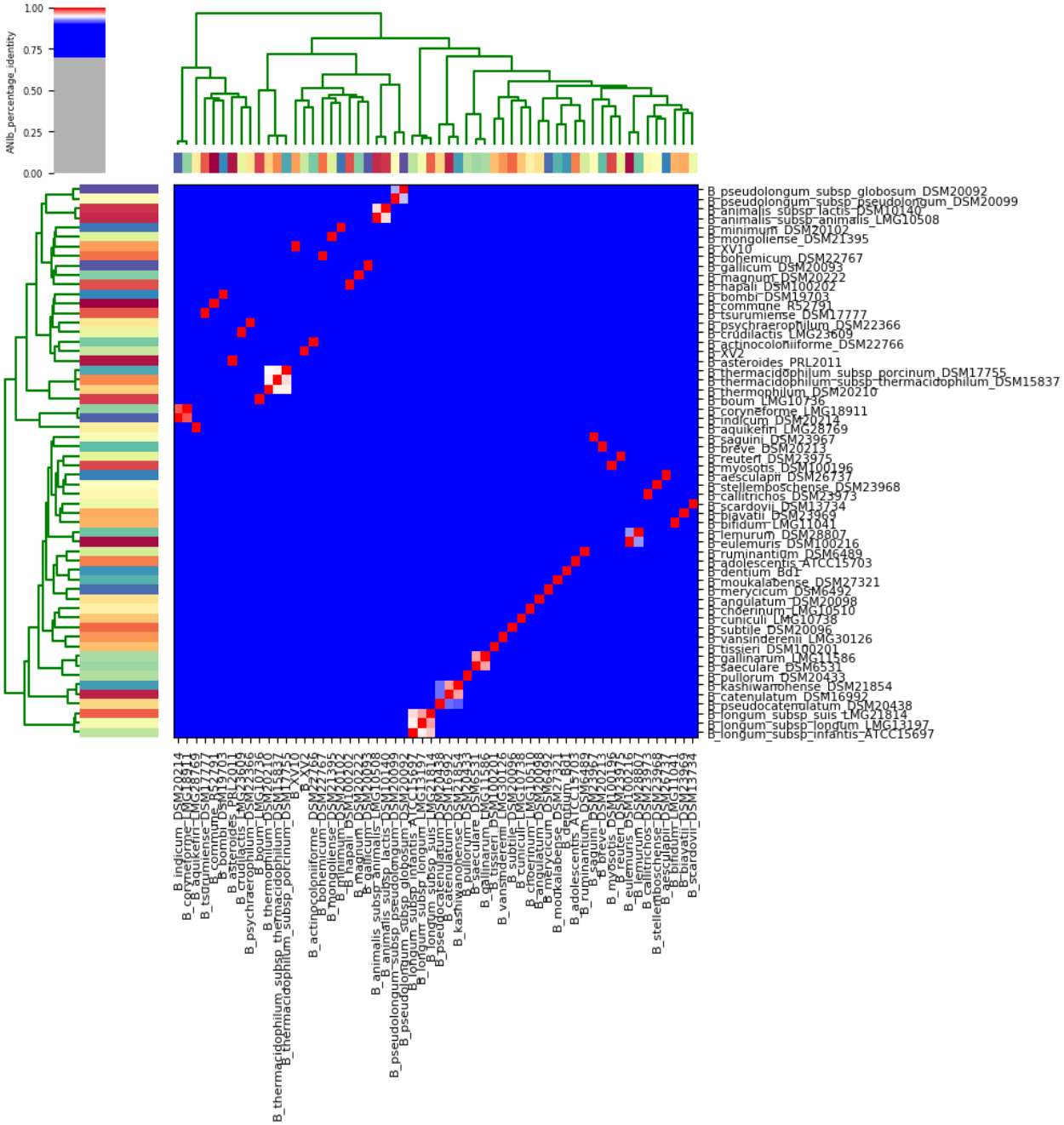


43	*B. pullorum* DSM 20433	2,100,948	278.3x	HiSeq	Faeces of chicken	GCF_000771405.1
44	*B. reuteri* DSM 23975	2,836,004	278.3x	HiSeq	Faeces of common marmosets	GCF_000771685.1
45	*B. ruminantium* DSM 6489	2,230,329	278.3x	HiSeq	Bovine rumen	GCF_000770925.1
46	*B. saeculare* DSM 6531	2,251,259	278.3x	HiSeq	Rabbit faeces	GCF_000770965.1
47	*B. saguini* DSM 23967	2,773,425	278.3x	HiSeq	Faeces of tamarin	GCF_000771625.1
48	*B. scardovii* DSM 13734	3,143,954	736.0x	MiSeq	Human blood	GCF_001005065.1
49	*B. stellenboschense* DSM 23968	2,812,864	109x	Ion Torrent	Faeces of tamarin	GCF_000741785.1
50	*B. subtile* DSM 20096	2,761,997	278.3x	HiSeq	Sewage	GCF_000771185.1
51	*B. thermacidophilum* subsp. *porcinum* DSM 17755	2,062,568	278.3x	HiSeq	Piglet faeces	GCF_000771045.1
52	*B. thermacidophilum* subsp. *thermacidophilum* DSM 15837	2,220,989	278.3x	HiSeq	Anaerobic digester	GCF_000771005.1
53	*B. thermophilum* DSM 20210	2,224,837	278.3x	HiSeq	Bovine rumen	GCF_000771265.1
54	*B. tissieri* DSM 100201	2,873,483	66.77x	MiSeq	Faeces of baby common marmosets	GCF_002259645.1
55	*B. tsurumiense* DSM 17777	2,164,426	unknown	HiSeq	Hamster dental plaque	GCF_000429745.1
56	*B. vansinderenii* Tam10B	3,111,005	138x	MiSeq	Faeces of emperor tamarin	GCF_002234915.1
57	*Bifidobacterium* sp. XV2	1,879,339	350.6x	MiSeq	Carpenter bee gut	PDCH00000000
58	*Bifidobacterium* sp. XV10	2,051,263	400.5x	MiSeq	Carpenter bee gut	PDCG00000000

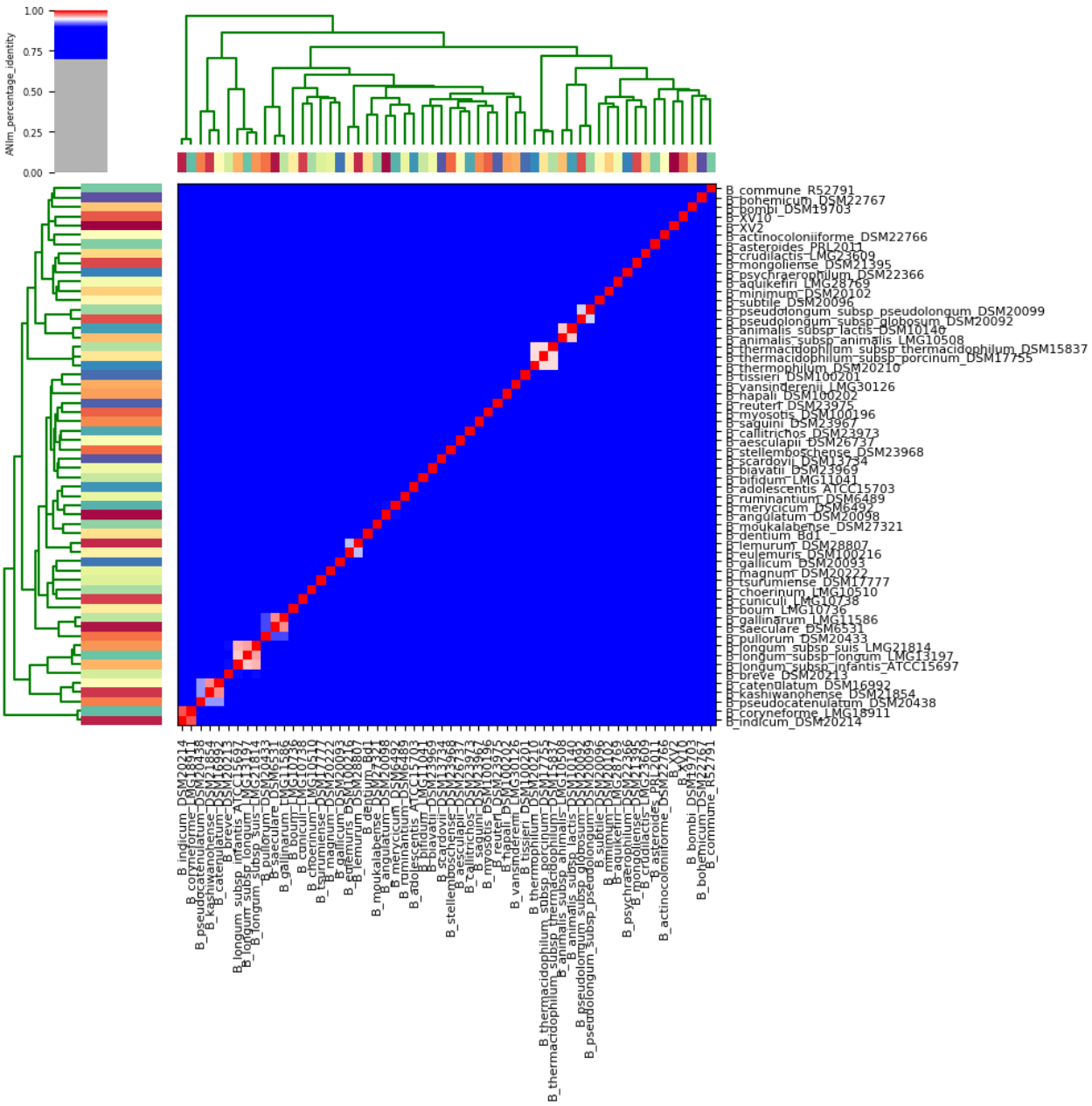
XV2 and XV10 showed the highest identity values between them with 78%, 86% and 95% respectively for ANIb, ANIm and TETRA. This percentages are far below the threshold of 96% for ANIb and ANIm and 99% for TETRA proposed by Richter and Rosselló-Móra (2009), and mirroring the DDH value of 60-70% (Figure 1a, 1b).

Figure 1 Average Nucleotide Identity. Grafical outpur of Python module for average nucleotide identity analyses a) ANIb (BLAST+ algorithm) b)ANIm (MUMmer algorithm).

a)



b)



Three annotation programs based on different algorithms were used for the identification of the ORFs and subsequent functional annotation: i) PROKKA - rapid prokaryotic genome annotation software - (Seemann, 2014) implemented with HMMER package for ribosomal RNA profile annotation (Eddy, 2011); ii) BASys web server for in-depth annotation of raw genomes (Van Domselaar et al. 2005), providing valuable graphical information on genes orientation, as shown Figure 2a, 2b (and Figure S3, S4)

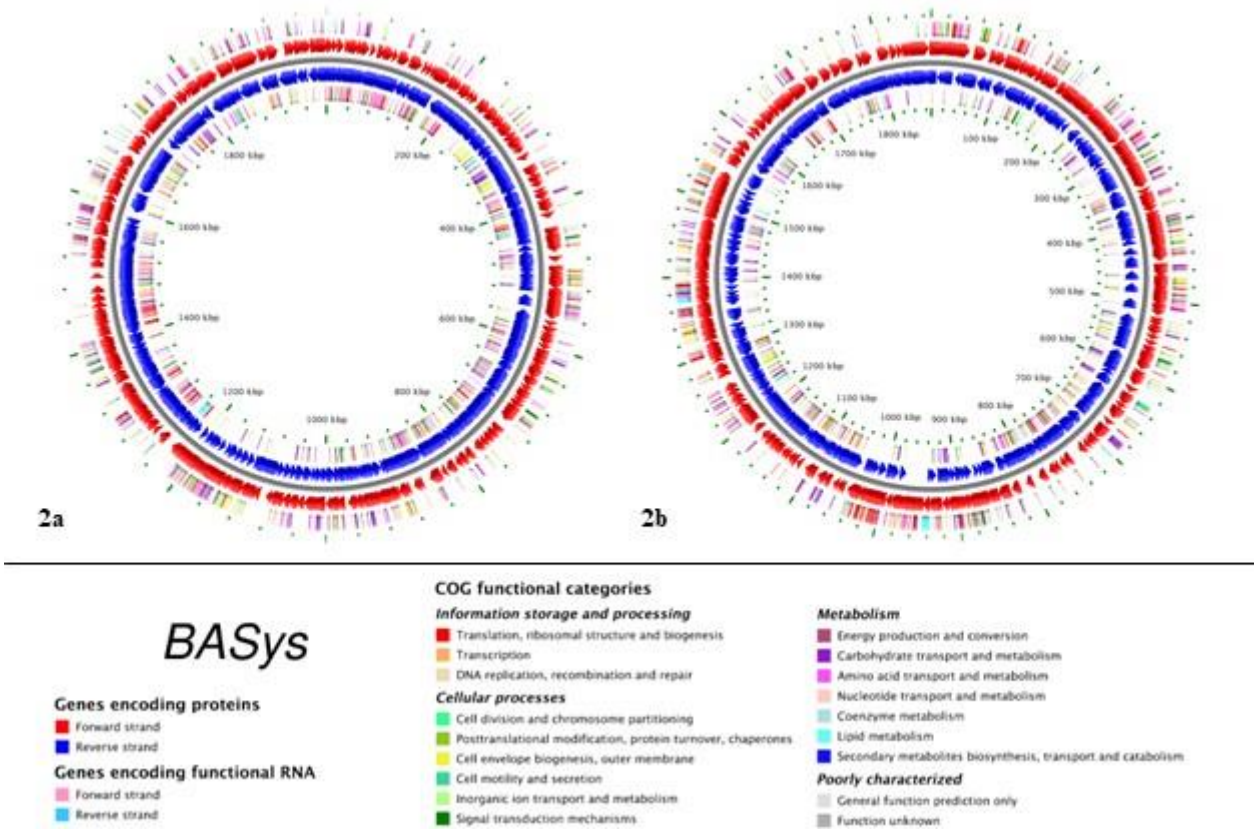
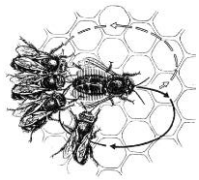


Figure 2 Genome annotation with BASys. Circular representation of the chromosome of a) *Bifidobacterium sp.* XV2 and b) *Bifidobacterium sp.* XV10 annotated with BASys (Bacterial Annotation System) according to van Domselaar et al. (2005). In the representation, red arrows are protein coding genes on the forward strand whereas blue arrows are protein coding genes in the reverse strand. With other colours the Clusters of Orthologous Groups (COG) functional categories of the protein coding genes in the forward (outermost circle) and reverse (innermost circle) directions are colour-coded as designated in the inset.

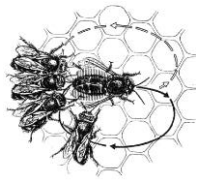
iii) Blast KOALA (KEGG tools), a web tool which performs KO (KEGG Orthology) assignments to characterize individual gene functions and reconstructs KEGG pathways, BRITE hierarchies and KEGG modules to infer high-level functions of the organism or the ecosystem (Kanehisa et al., 2016) (Figure 3a, 3b and Figure S5, S6). Raw reads, contigs assembly and feature tables were deposited on the NCBI database (Accession number PDCH00000000 and PDCG00000000). Genome annotations resulted in the prediction of 1556 genes of which 1457 coding sequences (CDS), 54 genes for RNA and 45 pseudo genes for *Bifidobacterium* sp. XV2 strain, whereas for the *Bifidobacterium* sp. XV10 strain the annotation resulted in 1717 genes of which 1589 CDS, 55 genes for RNA and 73 Pseudo Genes. Both strains have ORFs for two different B vitamin pathways (B6 and B9). Pyridoxin (B6) pathway ORFs are involved only in the "salvage pathway" for pyridoxal 5'-phosphate recycling (through pyridoxal kinase, PdxK). ORFs annotated in the Folate Biosynthesis also regard a “savage pathway” because of the absence of enzymes for precursors formation (the pteridine ring and the *para*-aminobenzoic acid unit *p*ABA). Only XV10 strain contains ORFs annotated as biotin (B7) biosynthesis, however the genes present are only involved in biotin uptake (*i.e.* BioY, component of biotin ECF transporter). Concerning ORFs for antibiotic resistance, a gene for tetracycline resistance has been annotated both in XV2 and



XV10 strains (a ribosome protection type tetracycline resistance gene) and 4-fluoroquinolone resistance mutations in the parC, parE, gyrA and gyrB genes have been found. In the fermentation tests, negative results were obtained for lactose fermentation while the presence of β-galactosidase activity was observed with the use of RAPID ID32A. However, this apparent contradiction is explained by the absence, in both strains, of LacS gene coding for the lactose-galactose permease, as shown in genome annotations. Some genes related to maltose and maltodextrin utilization are present in XV10 strains but also in this case not all genes for the production of the maltose/maltodextrin transporters were shown and indeed fermentation did not occur with this carbon source (Table 2).

Table 2 Characteristic that differentiate the proposed novel species of *Bifidobacterium* from the closest relatives. Strains: 1) *Bifidobacterium* sp. XV2; 2) *Bifidobacterium* sp. XV16; 3) *Bifidobacterium* sp. XV10; 4) *Bifidobacterium* sp. XV4; 5) *B. bombi* DSM 19703^T; 6) *B. asteroides* DSM 20089^T; 7) *B. coryneforme* DSM 20216^T ; 8) *B. indicum* DSM 20214^T ; 9) *B. actinocoloniiforme* DSM 22766^T; 10) *B. bohemicum* DSM 22767^T; 11) *B. commune* LMG 28292 ^{T}; 12) *B. minimum* ATCC 27538^T; 13) *B. subtile* DSM 20096^T. Biochemical test were performed using API CH50 and Rapid ID32 tests (bioMérieux). +, Positive; w, weakly positive; -, negative; ND, not determined;

	Sp. nov.				Insect core bifidobacteria							Other bifidobacteria species	
	XV2	XV16	XV10	XV4	*B. bombi*	*B. asteroides*	*B. coryneforme*	*B. indicum*	*B. actinoco-loniiforme*	*B. bohemicum*	*B. commune*	*B. minimum*	*B. subtile*
Characteristics	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Temperature range for growth (°C)	25-37	25-37	25-37	25-37	10-37	21-42	22-43	22-43	25-37	10-37	10-37	15-35	ND
DNA G+C content (mol%)	61.3	ND	61.9	ND	50.5	59	55	60	52.7	51.2	54.3	61.5	61.5
Growth under microaerophilic conditions	-	+	+	+	-	+	+	+	+	-	+	-	-
Peptidoglycan structure	A4α L-Lys-D-Asp	ND	A4α L-Lys-L-Ala_2-Gly*	ND	ND	A3α L-Lys-Gly	A4α L-Lys-D-Asp	A4α L-Lys-D-Asp	ND	ND	ND	A3α L-Lys-L-Ser*	A4α L-Lys-D-Asp
Acid production from													
L-Arabinose	W	W	W	W	W	-	W	+	-	+	-	-	-
D-Ribose	W	+	+	+	+	+	+	+	W	+	-	-	W
D-Xylose	-	-	+	+	-	-	-	-	-	W	-	-	-
D-Galactose	-	-	-	-	+	-	+	W	-	+	+	-	+
D-Glucose	+	-	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	W	+	+	+	-	-	-	+	+
D-Mannose	-	-	+	+	W	-	-	W	-	+	-	-	-
D-Lactose	-	-	-	-	-	-	+	W	-	-	-	-	W
D-Sorbitol	-	-	-	-	-	-	W	-	-	-	-	-	-
Methyl α-D-glucopyranoside	-	-	W	-	W	-	-	W	-	+	+	-	W
Arbutin	W	W	+	W	+	+	W	+	W	+	+	-	-
Aesculin	+	+	+	+	+	+	+	+	+	+	+	-	-
Salicin	+	+	+	+	+	+	+	+	+	+	+	-	-
Amygdalin	-	-	-	-	+	+	+	-	+	-	+	-	-
D-Cellobiose	-	-	-	-	+	+	-	+	+	-	-	-	-
D-Maltose	-	-	-	-	W	-	+	W	-	W	-	+	+
D-Melibiose	-	+	W	-	+	-	+	+	W	+	+	-	+
D-Sucrose	+	+	+	+	W	+	+	+	-	-	W	-	+
D-Trehalose	-	-	-	-	-	-	-	-	-	-	-	-	-
Inulin	-	-	W	-	-	-	-	-	-	-	-	-	-
D-Raffinose	-	-	+	+	+	-	+	+	-	+	+	-	+



Starch	-	-	w	-	-	-	-	-	-	-	-	+	+
Glycogen	-	-	-	-	-	-	-	-	-	-	-	+	+
D-Turanose	-	-	w	-	-	-	-	w	-	-	-	+	+
Gentiobiose	-	-	-	-	+	+	+	+	+	+	-	-	-
L-Fucose	-	-	-	-	-	-	-	w	-	-	-	-	-
Potassium Gluconate	-	-	-	-	-	-	-	-	-	w	-	-	-
Potassium 5-Ketogluconate	-	-	w	-	-	-	-	-	-	w	-	-	-
Enzymatic activity													
α-Galactosidase	+	+	-	-	+	+	+	+	+	-	+	-	+
β-Galactosidase	+	+	+	-	+	+	+	+	+	-	+	-	+
α-Glucosidase	-	+	+	+	+	+	+	+	+	+	+	+	+
β-Glucosidase	+	+	+	+	+	+	+	+	+	+	+	-	+
α-Arabinosidase	+	+	+	+	+	+	+	+	+	+	+	-	+
N-Acetyl-β-glucosaminidase	-	-	+	+	-	-	-	-	+	-	-	-	-
α-Fucosidase	+	+	-	-	-	+	-	-	-	-	-	-	-
Alkaline phosphatase	-	-	-	-	-	-	-	-	-	+	-	-	+
Arginine arylamidase	+	+	+	+	+	+	+	+	+	+	+	+	+
Proline arylamidase	+	+	+	+	+	+	+	+	+	+	-	+	+
Leucyl glycine arylamidase	+	-	w	-	-	+	+	-	-	-	-	+	+
Phenylalanine arylamidase	+	+	+	+	+	+	+	+	+	+	+	+	+
Leucine arylamidase	+	+	+	+	+	+	+	+	+	+	+	+	+
Tyrosine arylamidase	+	+	+	+	+	+	+	+	-	+	+	+	+
Alanine arylamidase	+	-	w	-	-	+	+	-	-	-	+	+	-
Glycine arylamidase	+	+	+	+	+	+	+	+	-	+	+	+	+
Histidine arylamidase	+	+	+	+	+	+	+	+	-	+	+	+	+
Serine arylamidase	+	+	+	-	+	+	+	-	-	-	+	+	+

*α-carboxyl group of D-Glu substituted by glycine

Genes for arabinose utilization using AraBAD pathway are present in both XV2 and XV10 strains, L-arabinose residues are widely distributed among many heteropolysaccharides of different plant tissues. In *Bifidobacterium* sp. XV10 a full ribose utilization operon and ribose-5-phosphate isomerase enzyme have been annotated, moreover the strain has a xylose transport system permease (protein XylH) together with genes of the XylAB pathway, therefore it is capable of fermenting xylose. As already reported and fully annotated in *B. asteroids* PRL2011 (Bottacini et al., 2012), a simplified respiratory metabolism was also evidenced in *Bifidobacterium* sp. XV2 and XV10 together with enzymes involved in the antioxidant activity such as catalase and superoxide dismutase (the latter present only in XV2 strain). This is consistent with the description of the bee gut as an environment with a higher oxygen concentration compared to humans and other mammals.

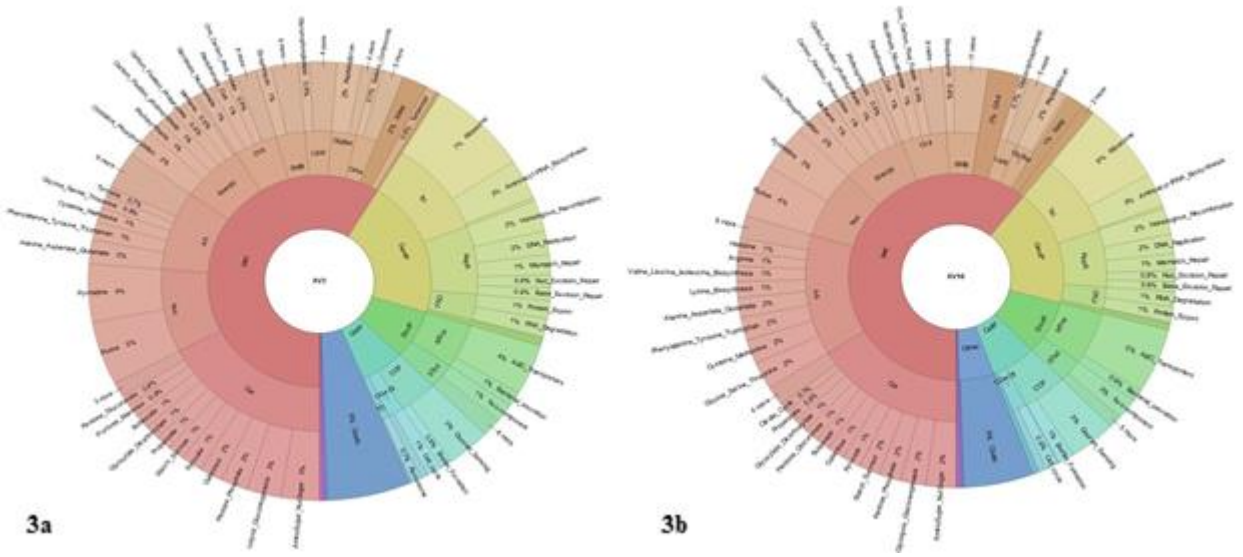


Table of abrreviations for Krona charts

Met	Metabolism	GenIP	Genetic_Information_Processing	Other	Other
Car	Carbohydrate	Tsc	Transcription	Cpath	Common_Pathways
Energy	Energy	Tsl	Translation	DR	Drug_Resistance
Lipid	Lipid	FSD	Folding_Sorting_Degradation	RepR	Replication_Repair
Nuc	Nucleotide	RRep	Replication_Rerepai	IS	Immune_System
AA	Amino_acid	**EnvIP**	**Environmental_Information_Processing**		
OAA	Other_Amino_acid	MTnp	Membrane_Transport		
GlyBst	Glycan_biosynthesis	STsd	Signal_Transduction		
CVit	Cofactors_Vitamins	**CellP**	**Cellular_Processe**		
TeKe	Terpenoids_Polyketides	TC	Transport_Catabolism		
SMB	Secondary_Metabolites_Biosynthesis	CGwDt	Cell_Growth_Death		
Xdeg	Xenobiotics_biodegradation	CCP	Cellular_Community_Prokaryotes		

Figure 3 Krona Charts of a) *Bifidobacterium sp.* XV2 and b) *Bifidobacterium sp.* XV10 annotated with BALST KOALA and elaborated with KronaTools v2.7 (Ondov et al., 2011).

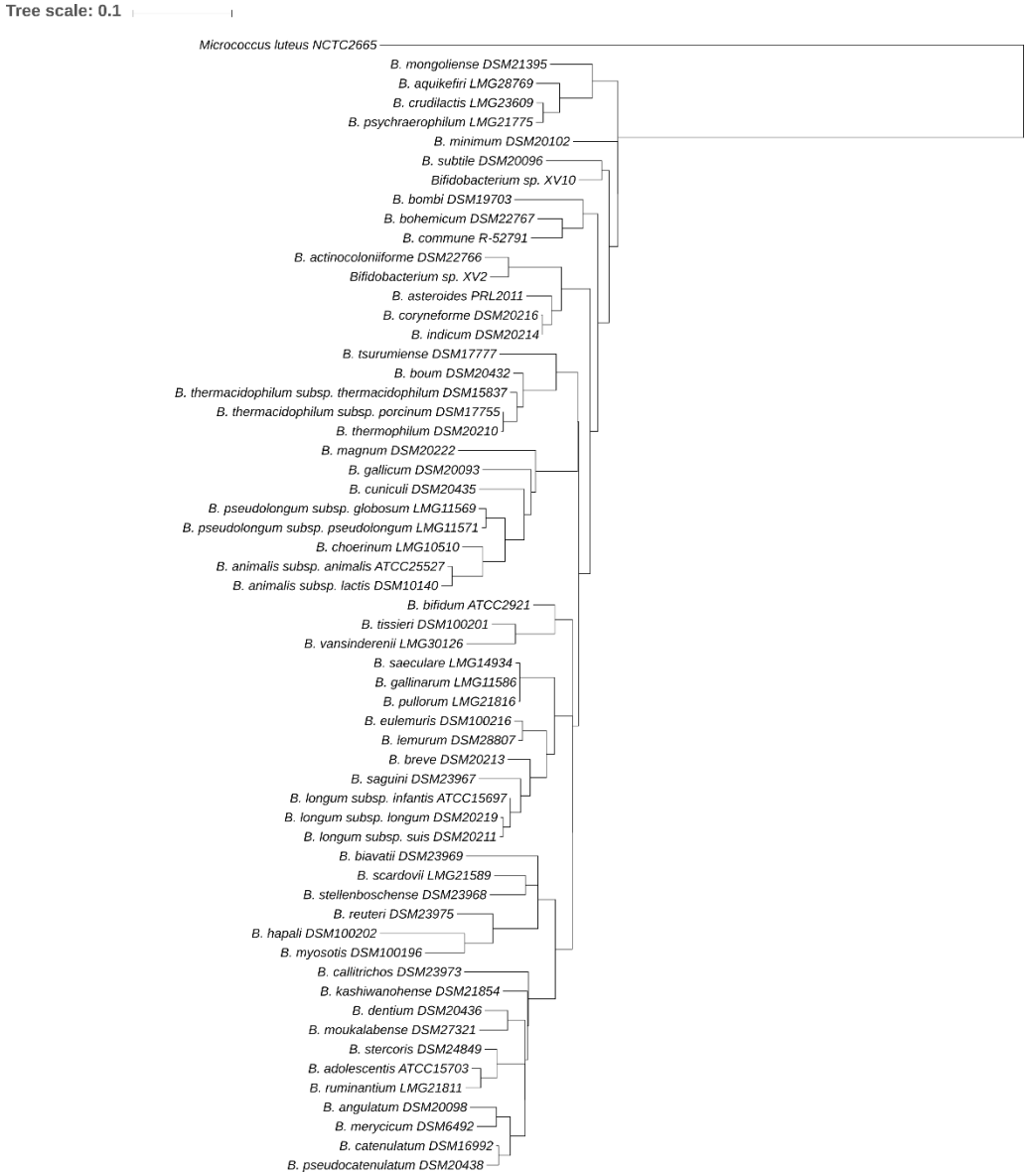
Figure S5 Krona Charts of Bifidobacterium sp. XV2 annotation elaborated with KronaTools v2.7 supplied in html format. [Fig. S5_XV2.html]

Figure S6 Krona Charts of Bifidobacterium sp. XV10 annotation elaborated with KronaTools v2.7 supplied in html format. [Fig. S6_XV10.html]

Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016) and exported in Newick format. The iTOL web-based software (Letunic and Bork, 2016) has been used for the annotation and management of the published phylogenetic trees. Partial 16S rDNA sequences were used to verify the relatedness of XV2 and XV10 with XV16 and XV4 respectively, the tree was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993) (Figure 4b). On the other hand, fifty-nine complete 16S rRNA sequences were retrieved from whole genome sequencing repository database of NCBI (ftp://ftp.ncbi.nlm.nih.gov/genomes/refseq/bacteria/) to infer the phylogeny of the new species (Figure 4a). Seven *Bifidobacterium* species (*B. aerophilum* DSM 100689T; *B. aesculapii* DSM 26737T; *B. apri* CCM 8605T; *B. avesanii* DSM 100685T; *B. callitrichidarum* DSM 103152T; *B. faecale* JCM19861; *B. ramosum* DSM 100688T) were excluded from the 16S rRNA based phylogenetic tree due to only partial 16S rRNA

sequence available. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-10975.2528) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3807)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 65.2412% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 1512 positions in the final dataset. XV2 clustered close to *B. actinocoloniiforme* in the *B. asteroides* group while *Bifidobacterium* sp. XV10 clustered with *B. subtile* DSM 20096, a bacterium isolated from sewage.

a)



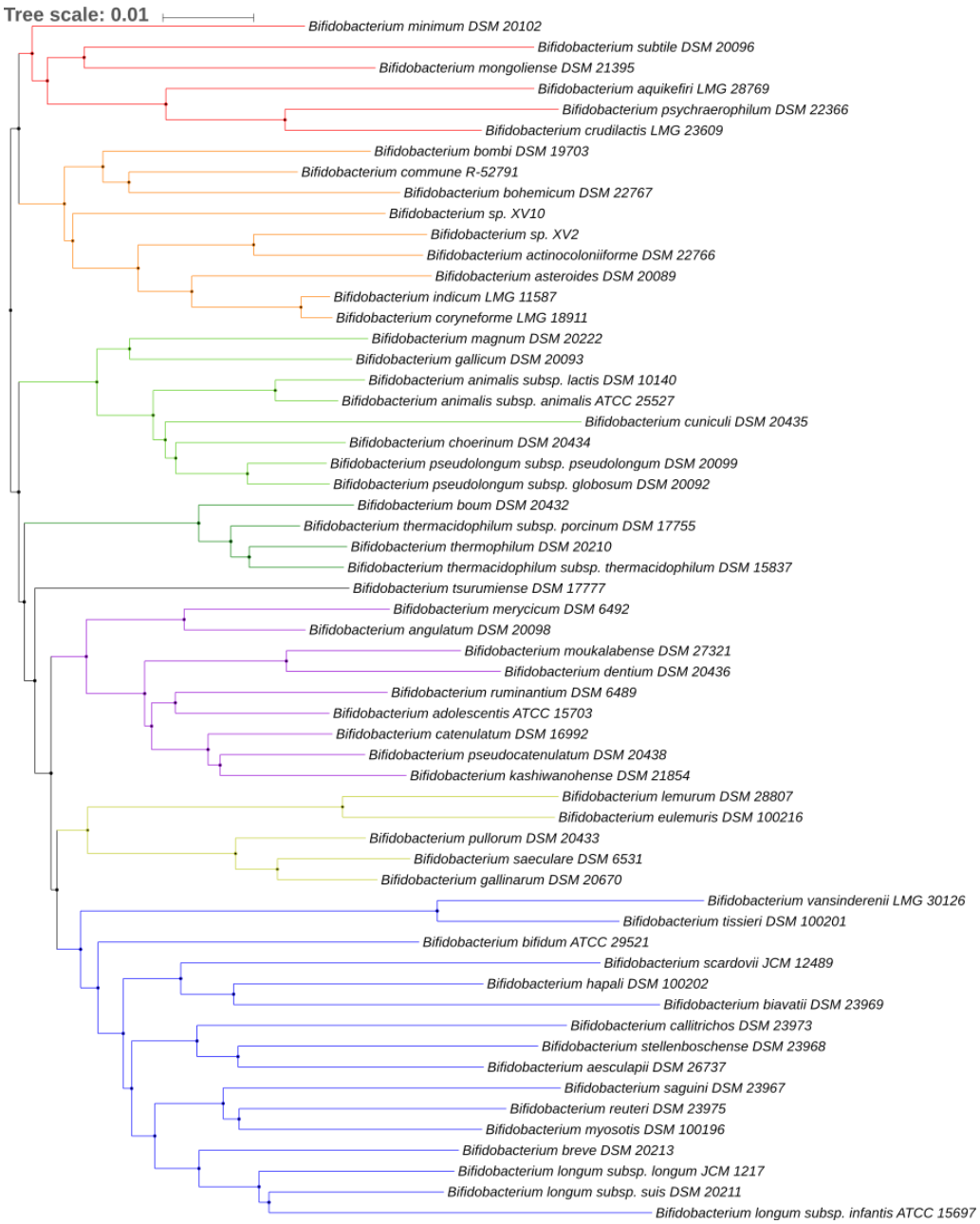
b)

Figure 4 Phylogenetic trees on 16S rDNA. a) 16S rRNA gene complete sequences of recognized *Bifidobacterium* species, *Bifidobacterium* sp. XV2 and *Bifidobacterium* sp. XV10. The analysis involved 59 nucleotide sequences with the *Micrococcus luteus* DSM 20030^T strain as outgroup. b) 16S rRNA gene partial sequences of recognized *Bifidobacterium* species, *Bifidobacterium* sp. XV2, XV4, XV10 and XV16. The analysis involved 61 nucleotide sequences with the *Micrococcus luteus* DSM 20030^T strain as outgroup.

The bifidobacteria core- and pan-genome were analyzed using BPGA (Bacterial Pan Genome Analysis tool) (Chaudhari et al., 2016). The pipeline calculates shared genes after

stepwise addition of each individual genome and plots the trend as core and pan-genome profile curve (data not shown). According to this curve, the pan-genome can be considered as “open”, as also recently evidenced by Lugli et al., 2018. Moreover the pipeline generates a phylogenetic tree based on pan-matrix data (Figure 5a). For the core-tree, after evaluation of core-genes with BPGA, the amino acid sequences of 273 genes were concatenated and aligned to generate the phylogenetic tree using the Neighbor-Joining method (Figure 5b). To better evidence the relationship among *Bifidobacterium* species, no outgroup was used for the pan-genome tree. The phylogenetic analysis highlighted distinct groups (Figure 5a) that were, however, not confirmed in the core-genome tree. In the latter, the presence of an outgroup species restricts the core-genes of the *Bifidobacterium* taxon and probably underestimates peculiarities linked to the adaptation to different ecological niches including horizontal gene transfer events (Yun et al., 2014; Ventura et al., 2012).

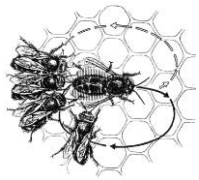
a)



b)

Figure 5 Core- and Pan-genome. a) Phylogenetic tree based on pan-genome analysis. b) Phylogenetic tree based on the concatenation of 273 core amino acid sequence genes of XV2, XV10 and members of the Bifidobacterium genus.

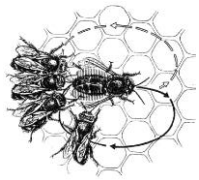
PCR-RFLP analysis of *hsp60* gene allows a rapid and accurate identification of common species of the genus *Bifidobacterium* (Baffoni et al. 2013; Stenico et al. 2014). The restriction of XV2 and XV10 *hsp60* gene, as well as of closely related species, was therefore performed according to Stenico et al. (2014). An *in silico* analysis was preliminary done to obtain the



theoretical restriction profiles for the species that were not previously reported in Stenico et al. 2014, using the *hsp60* sequences retrieved from the GenBank and RefSeq databases and using Webcutterb2.0 (http://rna.lundberg.gu.se/cutter2/) (Table 3). The *in silico* analysis showed two new and distinct restriction profiles for XV2 and XV10 confirming the high discriminating ability of HaeIII enzyme. Moreover, restriction profiles were confirmed on agarose gel for species in bold in Table 3 and for the XV4 and XV16B strains. The restriction profiles of XV4 and XV16B were respectively the same of XV10 and XV2 (data not shown). Estimation of the G+C content was performed by DSMZ Identification Service (Braunschweig, Germany) according to the following protocol. Cells were disrupted by French pressing and DNA purified on hydroxyapatite according to Cashion et al. (1977). The DNA was hydrolyzed with P1 nuclease and the nucleotides dephosphorylated with bovine alkaline phosphatase (Mesbah et al., 1989). The resulting deoxyribonucleosides are analyzed by HPLC (Tamaoka and Komagata, 1984). Lambda-DNA and three DNAs with published genome sequences representing a G+C range of 43-72 mol% were used as standards. G+C values are calculated from the ratio of deoxyguanosine and thymidine. The type strains XV2 and XV10 showed a G+C content of 61.3 and 61.9 mol% G+C, respectively. All values were within the G+C content range of the genus *Bifidobacterium* (52-67 mol%) (Biavati et al., 2000; Killer et al., 2010). Enzymatic activities and substrate fermentation capabilities for the novel strains and the species closely related to them (listed in Table 3) were obtained with API 50 CHL and Rapid ID 32A kits (bioMérieux, Lyon, France) according to the manufacturer's instructions (Table 3) with a slight modification: 10 μL^{-1} of a cysteine sterile solution (1 gL^{-1}) were added to the API 50 CHL medium.

Table 3 Expected fragment sizes obtained with *in silico* digestion of the *hsp60* gene sequences (updated from Stenico et al., 2014)

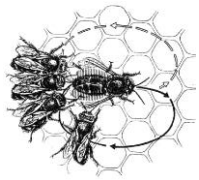
***Bifidobacterium* species**	**Gen Bank entry/ Refseq genome database**	**Predicted fragment sizes**
B. actinocoloniiforme † +	**GCF_000771585.1**	**14-16-16-17-31-42-45-53-75-81-200**
B. adolescentis	AF210319	31-36-81-103-339
***Bifidobacterium* sp. XV10** †	**PDCG00000000**	**9-16-22-31-36-42-59-63-76-78-158**
B. angulatum	AF240568	42-54-59-139-296
B. animalis subsp. *animalis*	AY004273	17-53-86-97-114-223
B. animalis subsp. *lactis*	AY004282	71-86-96-114-223
B. asteroides	AF240570	30-38-75-97-109-242
B. biavatii	AB674321	14-16-42-45-53-123-281
B. bifidum	AY004280	22-31-59-181-297
B. bohemicum † +	**GCF_000771605.1**	**No sites**
B. bombi	EU869281	16-115-178-281
B. boum	AY004285	22-117-200-251
B. breve	AF240566	106-139-139-200
B. callithricos	AB674319	16-22-31-59-462
B. catenulatum	AY004272	53-198-338
B. choerinum	AY013247	36-42-51-52-54-59-97-200
B. commune † ***	**GCF_900094885**	**53-537**
B. coryneforme	AY004275	16-32-54-158-338
B. cuniculi	AY004283	16-42-53-70-128-281



B. dentium	AF240572	22-31-42-68-130-139-158
B. gallicum	AF240575	42-253-297
B. gallinarum	AY004279	16-31-42-81-139-281
B. indicum	AF240574	16-32-36-42-45-123-296
B. longum subsp. *longum* *	AF240578	42-113-138-139-158
B. longum subsp. *infantis* *	AF240577	42-113-138-139-158
B. longum subsp. *suis* *	AY013248	42-113-138-139-158
B. mongoliense	KF751642	16-40-70-78-106-254
B. merycicum	AY004277	22-31-42-59-139-297
B. minimum	AY004284	16-51-60-66-70-327
B. pseudocatenulatum	AY004274	42-53-198-297
B. pseudolongum subsp *pseudolongum*	AY004282	17-22-30-32-42-42-109-297
B. pseudolongum subsp. *globosum*	AF286736	16-17-22-30-32-42-109-323
B. psychraerophilum	AY339132	No sites
B. pullorum	AY004278	16-31-36-42-81-87-297
B. reuteri	AB674318	53-59-139-339
B. ruminantium	AF240571	31-106-114-339
B. saguini	AB674320	53-59-181-297
B. stellenboschense	KF294527	16-42-53-59-123-139-158
B. subtile †	**GCF_000771185.1**	**16-53-240-481**
B. thermacidophilum subsp *porcinum***	AY004276	20-42-53-59-97-139-180
B. thermacidophilum subsp *thermacidophilum***	AY004276	20-42-53-59-97-139-180
B. thermophilum	AF240567	54-59-117-139-222
B. tsurumiense ***	AB241108	53-537
***Bifidobacterium* sp. XV2** †	**PDCH00000000**	**16-112-139-323**

† Sequences obtained from whole genome sequencing (Refseq genome database)
+ *errata corridge* of Stenico et al. 2014
(*, **, ***) species and subspecies not discernible

API 50 CHL inoculated galleries were incubated in anaerobiosis, except for XV10 that was incubated in microaerophilic conditions. Catalase and Oxidase tests were performed according to Modesto et al. 2015. XV2 and XV10 strains tested negative for catalase and oxidase activities; however the genome annotation underlined the presence of a catalase-related coding sequence both in XV2 and XV10 strains. In XV2 a superoxide dismutase CDS was also evidenced, even if XV2 strain resulted more affected by oxygen presence than XV10. It is possible to speculate that the diverse phenotypes can be related to different regulatory mechanisms. According to the protocol described by Schumann, 2011, the cell wall murein composition was determined by DSMZ Identification Service. Peptidoglycan type resulted different for the two strains and is reported in Table 2.
Analyses of cellular fatty acids were carried out at DSMZ Identification Service according to Miller (1982) and Kuykendall et al. (1988) with minor modifications: fatty acid methyl ester mixtures were separated using Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID, Newark, DE 19711 U.S.A.) which consisted of an Agilent model 6890N gas chromatograph fitted with a 5% phenyl-methyl silicone capillary column (0.2 mm x 25 m), a flame ionization detector, Agilent model 7683A automatic sampler, and a HP-computer with MIDI data base. Peaks were automatically integrated and fatty acid names and percentages calculated by the MIS Standard Software (Microbial ID). The gas chromatographic parameters were as follows: carrier gas, ultra-high-purity hydrogen; column head pressure



60 kPa; injection volume 2 µl; column split ratio, 100:1; septum purge 5 ml/min; column temperature, 170 to 270 °C at 5 °C/min; injection port temperature, 240 °C; and detector temperature, 300 °C. Results are summarized in Table 4.

Table 4 Cellular fatty acid profiles of insect related *Bifidobacterium*. Strains: 1) *Bifidobacterium* sp. XV2; 2) *Bifidobacterium* sp. XV10; 3) *B. bombi* DSM 19703^T; 4) *B. actinocoloniiforme* DSM 22766^T; 5) *B. bohemicum* DSM 22767^T. Data are from this study and a previous study of Killer et al. [28]. Relative concentrations (%) of total fatty acids were calculated.

Fatty acid	UPAC name	Common name	1	2	3	4	5
$C_{10:0}$	Decanoic acid	Capric acid	0.11	0.06	-	-	-
$C_{12:0}$	Dodecanoic acid	Lauric acid	0.71	0.46	-	-	-
$C_{14:0}$	Tetradecanoic acid	Myristic acid	1.28	1,43	-	1.97	2.86
$C_{15:0}$	Pentadecanoic acid	Pentadecylic acid	-	-	-	-	-
Iso-$C_{15:0}$	13-Methyltetradecanoic acid	Methylmyristic acid	-	-	-	-	-
$C_{16:0}$	Hexadecanoic acid	Palmitic acid	39.68	21.29	7.14	20.17	15.97
$C_{16:1}$ ω9c	(7Z)-7-Hexadecenoic acid	cis-7-Palmitoleic acid	1.11	0.41	-	-	-
$C_{17:0}$	Heptadecanoic acid	Margaric acid	-	-	4.21	2.11	2.56
$C_{17:1}$ ω9c	(8Z)-8-Heptadecenoic acid	-	-	1.10	-	-	-
Cyclo-$C_{17:0}$	cis-9,10-Methylene-Hexadecanoic acid	-	0.13	-	-	-	-
$C_{18:0}$	Octadecanoic acid	Stearic acid	-	3.85	5.91	7.05	6.56
$C_{18:1}$ ω6c	cis-12-Oleic acid	-	1.19	-	-	-	-
$C_{18:1}$ ω7c	cis-Vaccenic acid	-	2.58	6.6	-	-	-
$C_{18:1}$ ω9c	(9Z)-9-Octadecenoic acid	Oleic acid	19.3	57.92	7.49	9.99	4.69
$C_{18:1}$ ω9c DMA	(9Z)-1,1-Dimethoxy-9-Octadecene	-	-	8.47	-	-	-
$C_{18:2}$ ω6c	(9Z,12Z)-9,12-Octadecadienoic acid	Linoleic acid	6.05	-	7.34	-	-
$C_{20:0}$	Icosanoic acid	Arachidic acid	-	-	7.18	3.25	4.62
Iso-$C_{19:0}$	17-Methylstearic acid	-	0.6	0.74	-	-	-
$C_{22:0}$	Docosanoic acid	Behenic acid	-	-	5.87	-	-
$C_{23:0}$	Tricosanoic acid	Tricosylic acid	-	-	5.38	-	-
$C_{24:0}$	Tetracosanoic acid	Lignoceric acid	-	-	4.68	-	-
$C_{15:1}$ ω^{n}c	(^{n}Z)-^{n}-Pentadecenoic acid	-	-	-	2.47	-	-
-	Summed features 3a*	NA	0.49	-	ND	ND	ND
-	Summed features 3b*	NA	-	0.84	ND	ND	ND
-	Summed features 7*	NA	26.49	1.12	ND	ND	ND
-	Summed features 8*	NA	3.76	-	ND	ND	ND
-	Summed features 10*	NA	-	5.96	ND	ND	ND
-	Summed features 12*	NA	-	0.66	ND	ND	ND

*Summed features are groups of two or more fatty acids that cannot be separated by GLC (MIDI System). Summed feature 3a contained C16 : 1 ω6c and/or C16 : 1 ω7c; Summed feature 3b contained C16 : 1 ω7c and/or C15 : 0 ISO 2-OH. Summed feature 7 contained C19 : 0 CYCLO ω10c and/or C19 : 1 ω6c; Summed feature 8 contained C18 : 1 ω7c and/or C18 : 1 ω6c; Summed feature 10 contained C18 : 1 ω7c and/or unknown ECL 17.834; Summed feature 12 contained unknown ECL 18.622 and/or iso-C19 : 0.

Since the discovery in *Bifidobacterium scardovii* of a weak haemolytic activity (Hoyles et al. 2002), haemolysis should always be checked in new *Bifidobacterium* species. Indeed, haemolytic activity of XV2 and XV10 was tested in three different media: Columbia Blood Agar (BIOLIFE, Milan, Italy), MRS and TPY supplemented with 5% of defibrinated sheep blood. XV2 and XV10 showed α-haemolytic activity in all media as a greenish discoloration that surrounds bacterial colonies in the tested media. In relation to this activity, genome annotation with Prokka as well as the annotation provided by the NCBI pipeline during sequence submission highlighted two distinct ORFs annotated as Haemolysin and Haemolysin III. Haemolysin sequence is a membrane protein of the CCB3/YggT family (IPR003425) and UniProt BLAST results showed its presence in several bifidobacterial strains with identity scores ranging from 85.6% to 57.9% (taking into consideration only the first 47 hits). A YggT protein characterized in *E. coli* seems to be related to osmotic shock

protection (Ito et al. 2009), even if the length of *E. coli* YggY protein is 180 amino acids while bifidobacteria sequences are about 100 aminoacid-long. Concerning the annotation of Hemolysin III, this protein is an integral membrane component of the AdipoR/Haemolysin-III-related (IPR004254) family. This family, among others, groups proteins from pathogenic and non-pathogenic bacteria, including characterized pore-forming proteins. UniProt BLAST results showed the presence of similar proteins in several bifidobacteria strains, the first 50 hits were downloaded, aligned in MEGA6 with MUSCLE and the best ML substitution model was evaluated (WAG+I+F) to generate the tree (Figure S7).

This protein seems to have a high intra-species conservation, moreover proteins from bee species clustered apart, including XV2, while XV10 strains clustered with its respective closest neighbour. Being this protein poorly characterized, it is not possible to infer a correlation with the haemolytic activity. However, a correlation between haemolytic activity and iron availability in the intestine can be discussed. What is clear is that iron is essential for bacterial physiological processes and microorganisms have developed different strategies for iron uptake such as reduction of ferric iron with subsequent transport, iron acquisition from heme or iron-containing proteins of the host and production of siderophores (Caza and Kronstad 2013). Iron is essential also for humans, both heme and non-heme iron present in diet are absorbed preferentially in the duodenum and the absorptive process is strictly regulated in order to avoid an excessive uptake (Sharp and Srai 2007). The intestinal microorganisms and the host are therefore in competition for some nutrients, but the competition is also between the different microbial taxa. This peculiar weak hemolytic activity is therefore not to be seen only as a pathogenic trait. As also shown in the probiotic strain *E. coli* Nissle, although with a different uptake strategy, microorganisms evolve specific tricks/stratagems in order to survive in a harsh environment, and these tricks can, at last, give a greater advantage to harmless bacteria with respect to known pathogenic bacteria (as, for example, *Salmonella enterica* serovar Typhimurium) (Lustri et al., 2017). Concerning iron uptake, genome annotations reported the presence of a ferrous iron transport protein and an iron transporter permease both in *Bifidobacterium* sp. XV2 and XV10.

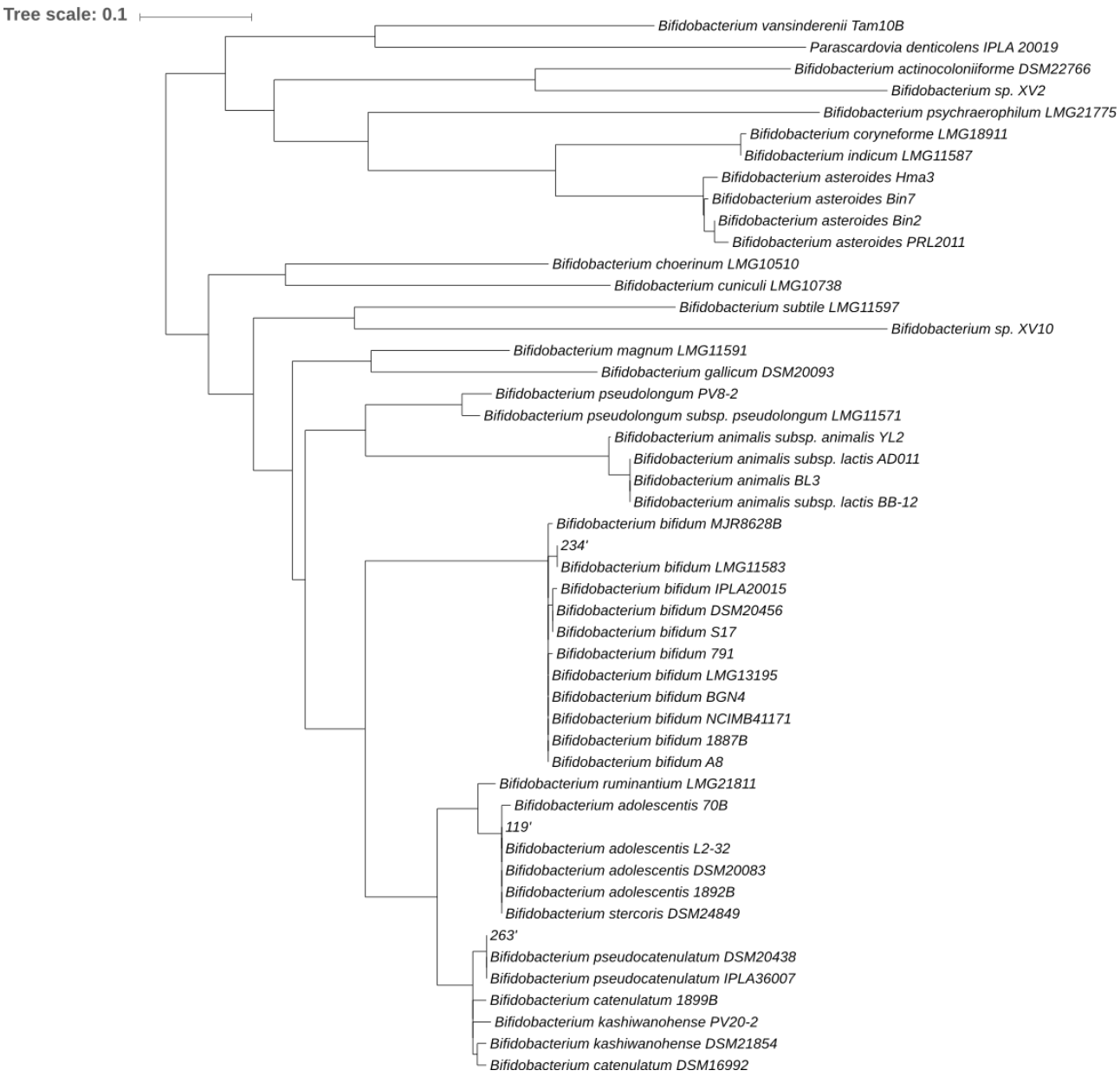


Figure S7 Phylogenetic tree based on *hemolysin III*-related gene sequences of *Bifidobacterium* species, including *Bifidobacterium* sp. XV2 and *Bifidobacterium* sp. XV10. The analysis involved 49 amino acid sequences.

Description of Bifidobacterium xylocopae sp. nov. (XV2)

Bifidobacterium xylocopae [Xy.lo.co.pe N.L. gen. f., of *Xylocopa*, a wood cutter, the genus name of the insect from which the strain was isolated]. Cells are Gram-positive, non-motile, non-sporulating, F6PPK-positive, catalase- and oxidase-negative, indole-negative. XV2 strain grows in anaerobic conditions and cannot survive in microaerophilic conditions. Colonies, grown on the surface of TPY agar plate, are white and circular. The diameter of each colony ranges from 0.5 to 1.0 mm. Strain XV2 grows in the temperature range 25–40 °C; no growth occurs at or below 20 °C. Cells grow in the pH range 4.5-9.0. Optimal conditions for growth occur at pH 6.5 and 35°C. Fermentation profiles of *B. xylocopae* XV2

show that it is able to ferment a narrow range of mono and di-saccharides: D-glucose, D-fructose and D-sucrose. Moreover, the strain hydrolyzes aesculin and salicin, while it displays scarce growth on L-arabinose, D-ribose and arbutin. Positive enzymatic activity is observed for α- and β-galactosidase, β-glucosidase, α-arabinosidase, α-fucosidase, arginine arylamidase, proline arylamidase, leucyl-glycine arylamidase, phenylalanine arylamidase, leucine arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase and serine arylamidase. Whereas, α-glucosidase, N-acetyl-β-glucosaminidase and alkaline phosphatase activities are negative (Table 3). The major fatty acids identified are palmitic acid, oleic and linoleic acid plus a mixture of unresolved fatty acids (referred to as summed features 7 in the related table) (Table 4). The peptidoglycan type is A4α L-Lys – D-Asp. The DNA G+C content is 61.3 %. The type strain XV2 (=DSM 104955^T = LMG 30142^T) and the reference strain XV16 (waiting for DSM and LMG culture collection number) were isolated from gut samples of carpenter bees (*Xylocopa violacea*).

Description of Bifidobacterium aemilianum sp. nov. (XV10)

Bifidobacterium aemilianum [E.mi'lia.num L. neutr. Adj., aemilianum, from the Emilia region, referring to the Italian region where the bacterium was first isolated]. Cells are Gram-positive, non-sporulating, F6PPK-positive, catalase- and oxidase-negative, indole-negative. XV10 strain grows in microaerophilic conditions but it cannot survive in both anaerobic and aerobic conditions. Colonies, grown on the surface of MRS agar plates supplemented with cysteine and fructose, are white and circular. The diameter of each colony ranges from 0.2 to 0.5 mm. Strain XV10 grows in the temperature range 20–40°C; no growth occurs below 20°C. The strain grows in the pH range 5.0-9.0. Optimal conditions for growth occur at pH 6.5 and 35°C. Fermentation profiles of *B. aemilianum* XV10 reveal that it is able to ferment a wide range of mono and di-saccharides:D-ribose, D-xylose, D-glucose, D-fructose D-mannose, D-raffinose and D-sucrose. The strain hydrolyzes aesculin, arbutin and salicin, while it displays scarce growth on L-arabinose, Methyl α-D-glucopyranoside, D-melibiose, inulin, methadone, D-turanose and potassium 5-Ketogluconate. Positive enzymatic activity is observed for β-galactosidase, α- and β-glucosidase, α-arabinosidase, N-Acetyl-β-glucosaminidase, arginine arylamidase, proline arylamidase, phenylalanine arylamidase, leucine arylamidase, tyrosine arylamidase, glycine arylamidase, histidine arylamidase and serine arylamidase. On the contrary, enzymatic activity is negative for α-galactosidase, α-fucosidase, alkaline phosphatase, leucyl glycine arylamidase, alanine arylamidase (Table 3). The major fatty acids identified are palmitic acid, oleic and stearic acid and (9Z)-1,1-Dimethoxy-9-Octadecene (Table 4). The peptidoglycan type is A4α L-Lys-L-Ala with a D-Glu at position 2 substituted by Gly. The DNA G+C content is 61.9 %. The type strain XV10 (=DSM 104956^T = LMG 30143^T) and the reference strain XV4 (waiting for DSM and LMG culture collection number) were isolated from gut samples of carpenter bees (*Xylocopa violacea*).

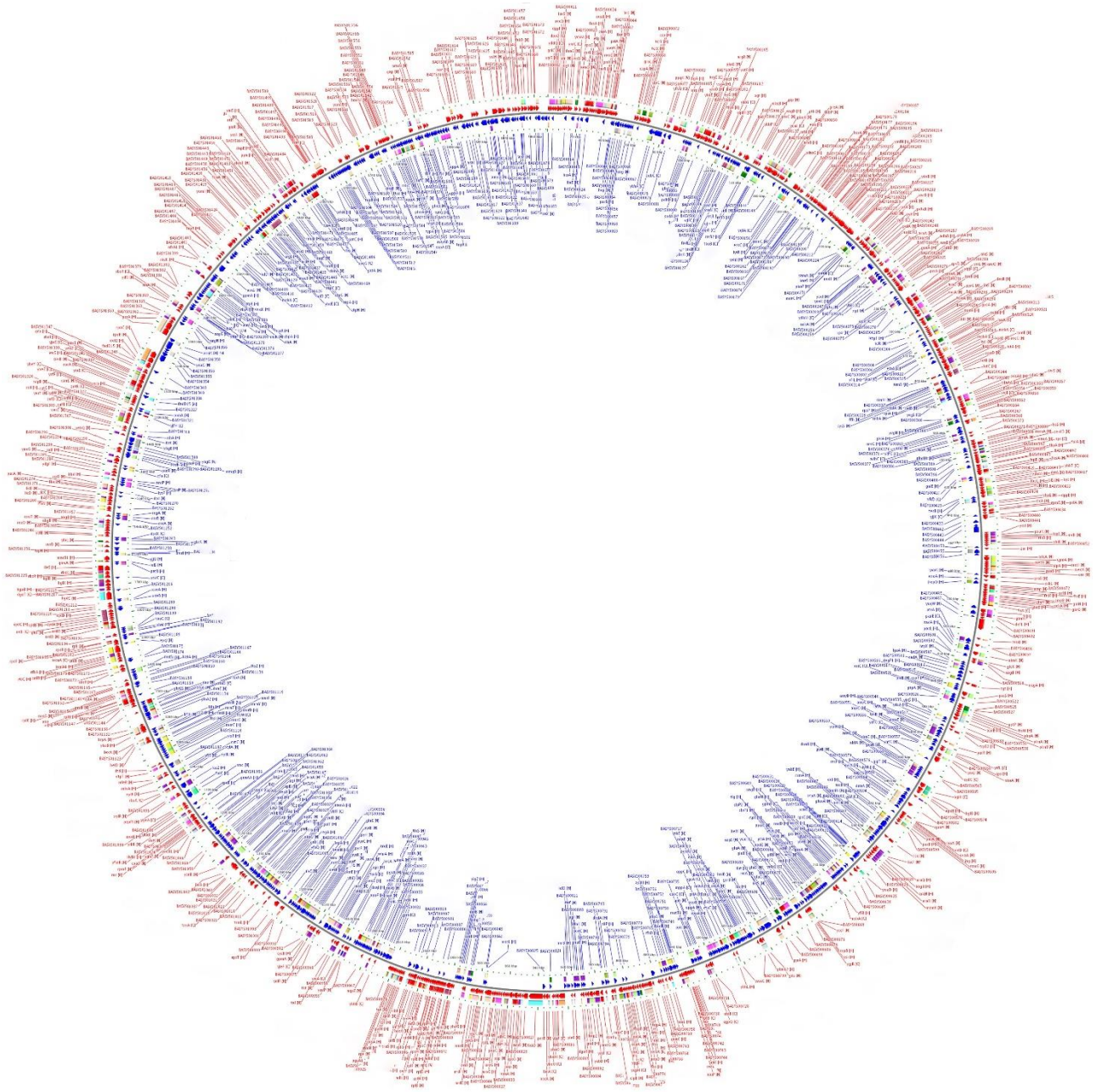


Figure S3: Circular representation of the chromosome of *Bifidobacterium* sp. XV2 in high definition with BASys.

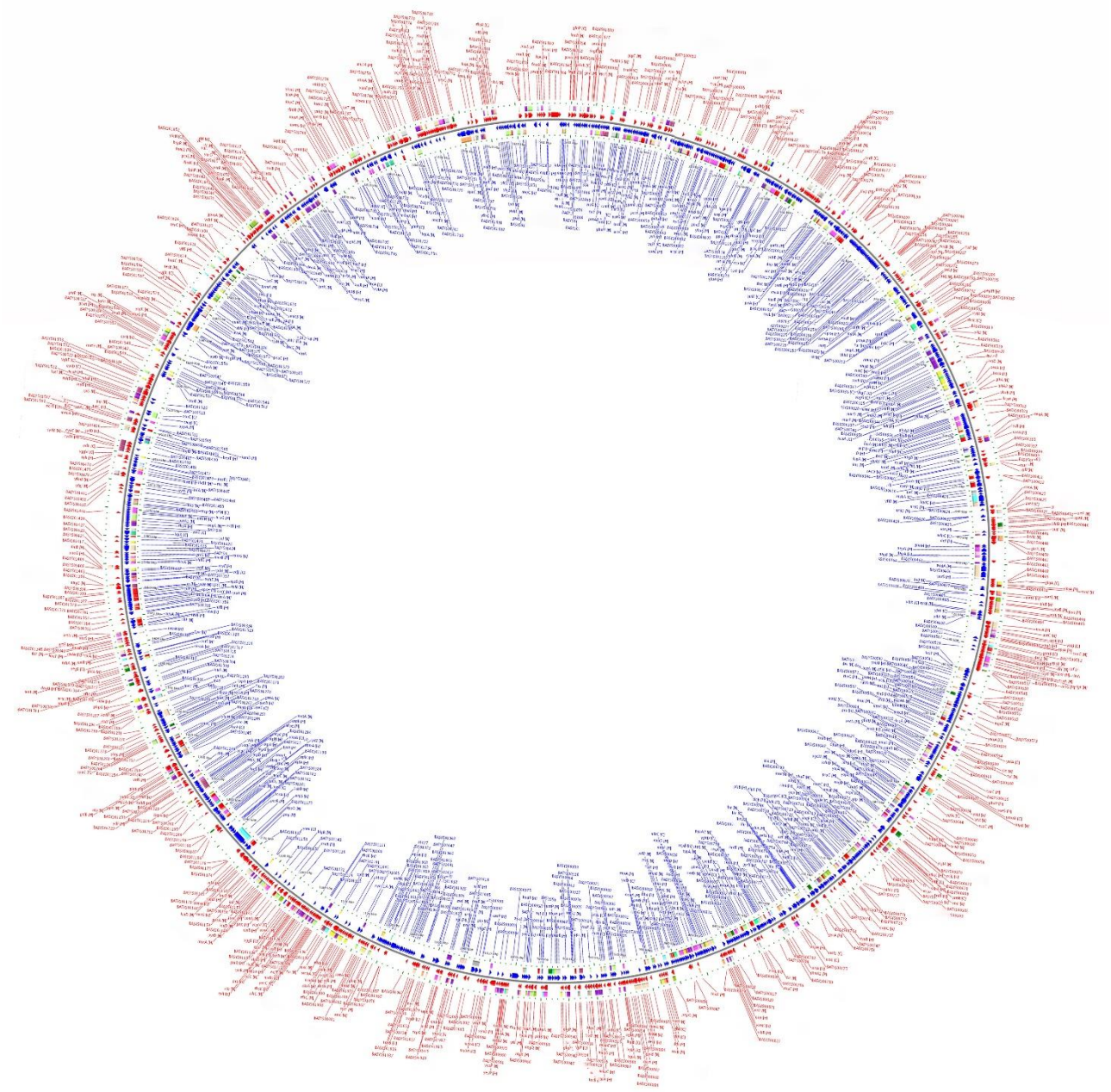


Figure S4: Circular representation of the chromosome of *Bifidobacterium* sp. XV2 in high definition with BASys.

Antimicrobial Activity

The discovery of the first antimicrobial compound by Alexander Fleming in 1929 primed a leading revolution in the approach to diseases. For 60 years a continuous discover of new antimicrobial substances allowed the control of dangerous diseases and the massive production of food items, especially animal derived. Antimicrobial compounds were used as growth promoters for livestock, poultry and swine. The beekeeping sector was not an exception. Chloramphenicol, Erythromycin, Tetracycline, Fumagillin and many other antibiotics are an example of the wide range of medicaments authorized around the world. Often antibiotics in the animal sector, including the beekeeping one, are used not only for the treatment of disease foci but also, and this is the largest use, as "growth promoters". In the beekeeping sector this means to ensure the best honey and royal jelly production, more than the health of bees. Many of these antibiotics are still widely used in several countries, with minor restriction for preventing food contamination, but in Europe they have been banned since 2001 by revocation of commercial licences for honeybee treatments of antibiotic based medicaments. The potential of bacteria in counteracting microbial pathogens is not only expressed by antibiotic production, but also in other ways such as: Production of Short Chain Fatty Acids (SCFA), as acetic acid, or other acidic molecules as Acetic Acid.

- Enhancement of the production of secondary metabolites
- Occupation of ecological niches competing with the pathogenic microorganisms.
- Immune stimulation of the host organism (E.g.: enhanced defencin production)
- Production of bacteriocins

Bacteriocins differ from antibiotics in particular because of their reduced antimicrobial spectrum indirectly leading to a targeted antimicrobial activity. Differences between the two groups of molecules are briefly described in Table 1.

Table 1 Main differences between bacteriocins and antibiotics (according to Gómez-Sala B. 2013)

	Bacteriocins	**Antibiotics**
Composition	Protein	Variable, and only occasionally proteic
Synthesis	Metabolites of ribosomal synthesis, generally as inactivated precursors.	Secondary metabolites Non-Ribosomal synthesis (Multi-enzymatic Complex)
Antimicrobial activity range	Usually reduced to closest phylogenetically related genus.	Variable, Gram negative and/or Gram-positive bacteria.
Action mode	Generally, through pores in the cytoplasmic membrane. Occasionally, by inhibition of the cell wall synthesis.	Inhibiting different cell functions (E.g. Synthesis inhibition of Proteins, DNA and RNA, or cell wall synthesis)
Target cell Requirements	Specific receptors.	Specific receptors.
Self-immunity of the cell producer	Presence of genes coding for resistance.	Undescribed.
Resistance	Modification of cell membrane composition.	Specific resistance genes, modification of specific receptors.

		Transposition of genes for multiple resistances.
Toxicity	Not yet investigated.	Variable according to the kind of antimicrobial.
Application	Food	Clinical

Bacteriocins and antibiotics are divided in sub groups, however their sub-grouping is at present not yet completely defined. There are strong debates on their classification, and even if classification attempts have been made based on their temperature of denaturation, dimension, post transduction modifications, and shape, these classifications are not standardized and internationally recognized.

During my Ph.D. work every isolated strain of potential interest to counteract bee diseases were tested for antimicrobial activity, against indicator strains (including bee pathogenic bacteria) and against themselves to exclude competition if used as a mixture. Tests were carried out according to the agar well diffusion assay (described in the following paper 4 and Figure 1).

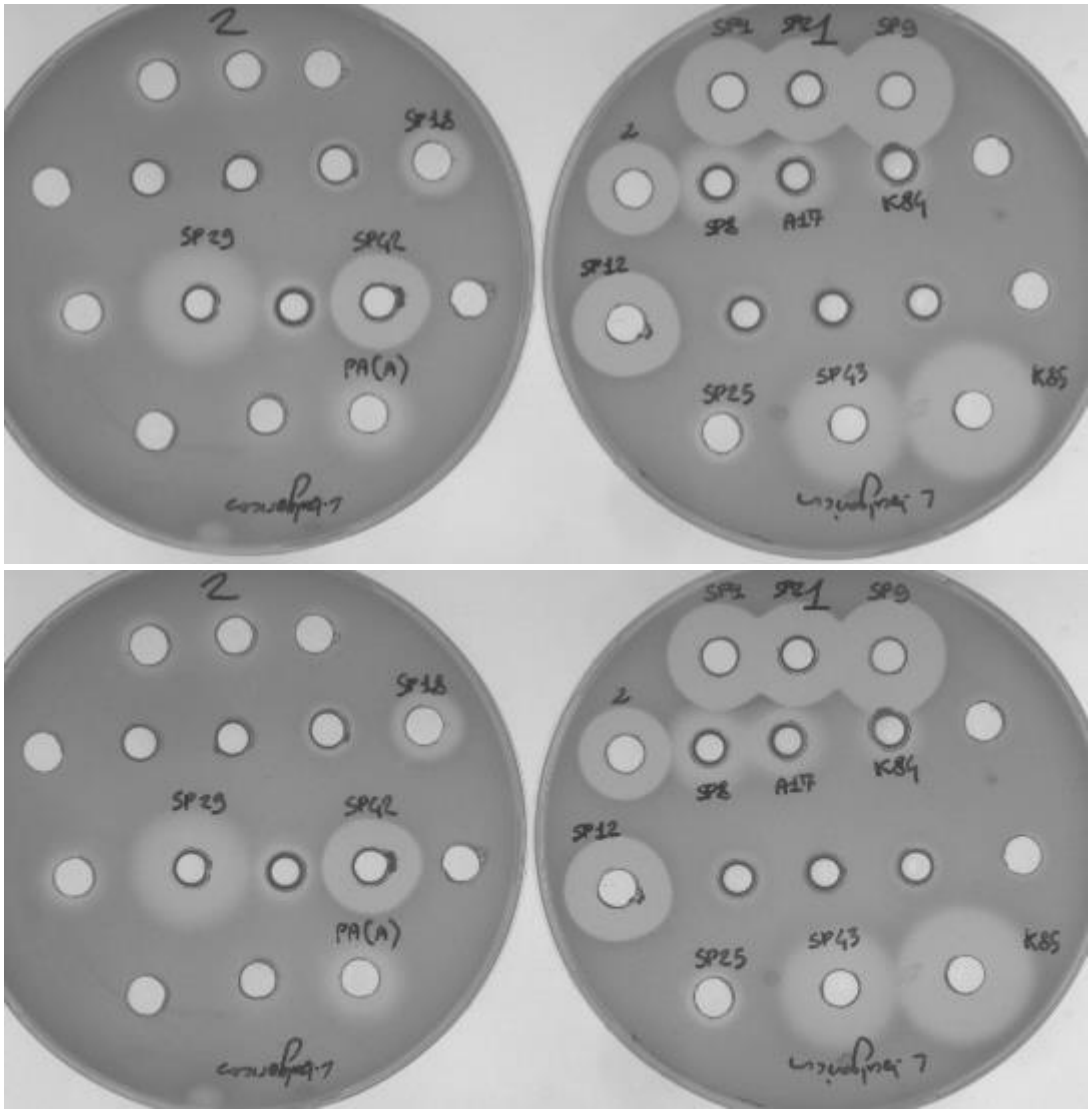
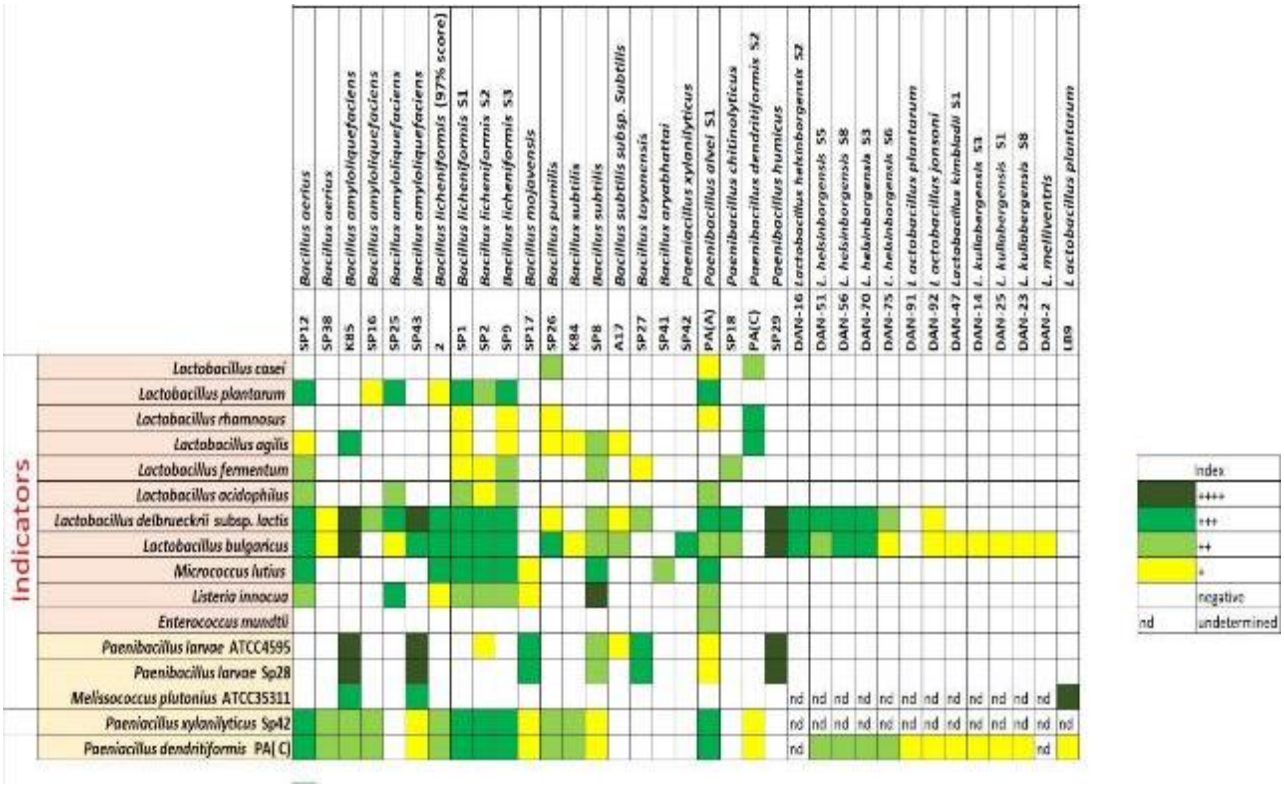


Figure 1: Examples of antimicrobial activity assess with the agar well diffusion assay. Agar plates were inoculated with the indicator strain *Lactobacillus delbrueckrii* subsp. *bulgaricus*. Supernatants containing the antimicrobials were inoculated in the prepared wells in the agar plates. Strain producing bacteriocins or antibiotics active against *Lactobacillus delbrueckrii* subsp. *bulgaricus* inhibited its growth, forming a halo of growth inhibition whose extent depends on the antimicrobial power.

It turned out that most of bacteria can produce antimicrobials, as shown in Table 2. Moreover, most of the antimicrobials detected are bacteriocins (active against closely related strains) rather than antibiotics, this most probably due to their need to protect their ecological niches.

Table 2 An example of antimicrobial activity tested with 35 honeybee environment isolated strains against a selection of 16 indicators including *Paenibacillus larvae* and *Melissococcus plutonius*. Antimicrobial activity: ++++ very powerful; +++ excellent; ++ sufficient; + scarce; non



A test of the ability to produce SCFA or other acidic molecules capable of inhibiting the growth of other strains was carried out with the spot agar test method. A modification of the protocol of Kizerwetter-Swida and Binek (2005) was employed. 70 µl of concentrated overnight cultures of the six selected LABs and bifidobacteria cultures (A_{600} of about 0.1) were spotted on TPY agar plates (Scardovi 1986), and were incubated anaerobically for 24 h at 37 °C. Once growth was reached, plates were overlaid with 10 ml of SYPG agar (Table 5; Bailey and Ball 1991) auditioned with 0,8% agar inoculated with a suspension (MacFarland 0.5) of *Melissococcus plutonius* ATCC 3511. Plates were then incubated under microaerophilic atmosphere at 35 °C, and after 24–48 h the presence of inhibition zones was evaluated. Each assay was performed in triplicate, an example of inhibition results is showed in Figure 2.

All the isolates belonging to the family *Lactobacillales* (*Lactobacillus* sp., *Fructobacillus* sp., *Leuconostoc* sp., etc…) and to the *Bifidobacterium* genus were able to produce acidic molecules capable of counteraction, by acidification, the proliferation of *M. plutonius*, *P. larvae* and *Aschospaera apis*.

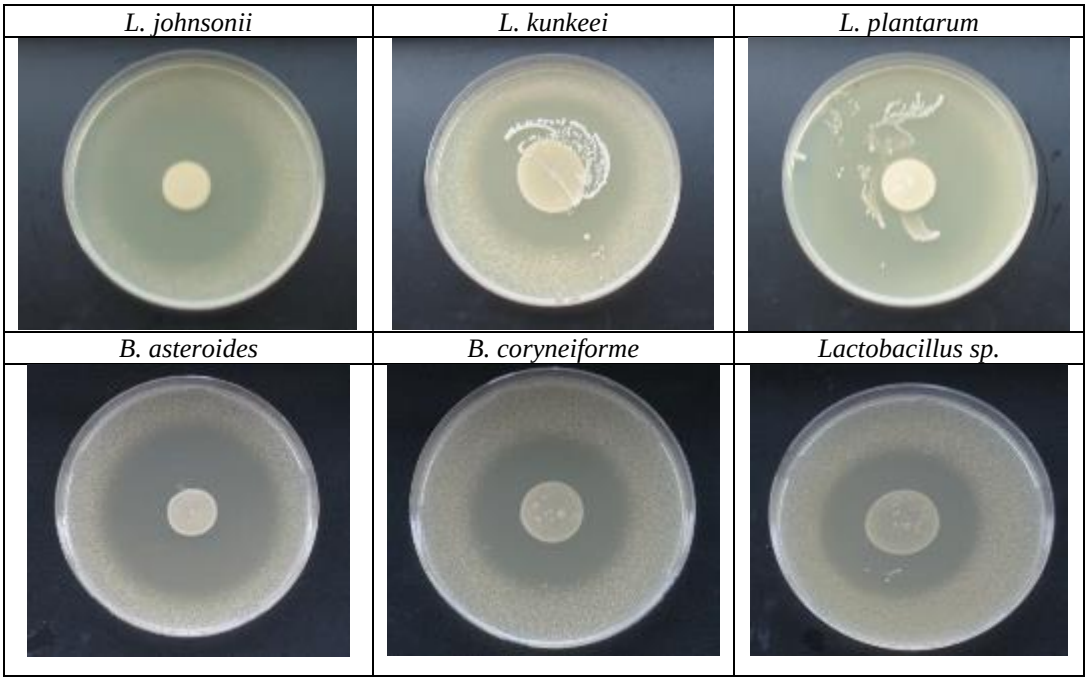
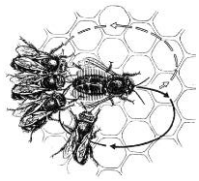


Figure 2: Examples of antimicrobial activity assess with the spot agar test method regarding *L. johnsonii*, *L. kunkeei*, *L. plantarum, B. asteroides, B. coryneiforme, Lactobacillus sp.*. It is visible the central spot of the tested bacteria followed by a growth inhibition of the *Mellissococcus plutonius* pathogen, and then a ring of melissococcus in the border of plates.



PAPER 4

Apis mellifera gut *Lactobacillus* are a source of Helveticin like bacteriolysins.

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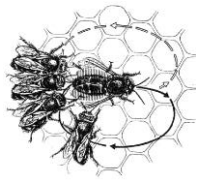
Keywords: *Lactobacillus*, *apis*, *helsingborgensis*, *kimbladii*, *kullabergensis*, Bacteriocin, Bacteriolysin, PFGE, Cloning, Helveticin, SDS-PAGE, Knock out

This paper needs finalization prior to publication in target journal: Environmental Microbiology (SFAM).



Introduction

The genus *Lactobacillus* comprises over 200 species (Sun et al., 2015), populating diverse environmental niches, such as the gut of vertebrates and invertebrates, plants and fermented foods. Their ability to colonize different environments contributed to the development of the wide genetic diversity typical of this genus (Sun et al., 2015). Focusing on invertebrates, metagenomics studies allowed the detection of *Lactobacillus* in different orders of insects. For instance, *Lactobacillus* spp. were detected in the larval gut of Lepidoptera (Tang et al., 2012), in the gut of *Drosophila melanogaster* (Diptera) (Chunli et al., 2007), in the gut of predatory ground beetle, *Poecilus chalcites* (Coleoptera) (Lehman et al. 2009*)* and Imenoptera (Moran, 2012). Recently, the attention of researchers in the isolation and identification of new *Lactobacillus* species has been focused on the insectsuperfamily of *Apoidea*, leading to the discovery of a considerable number of new, extremely host specific, species of *Lactobacillus*. Indeed, the hindgut of honeybees (*Apis mellifera* L.) is colonized by a large community within the *Lactobacillus* genus. 8 novel *Lactobacillus* species from honeybee gut were recently isolated by Olofsson et al., 2014a and Killer et al., 2014a (*L. apis, L. apinorum*, *L. helsingborgensis*, *L. kimbladii*, *L. kullabergensis, L. mellifer, L. mellis*, and *L. melliventris*). These species have been deeply characterized and were shown to comprise multiple strains with highly diverse genetic content (Ellegard et al., 2015) indicating the presence of a extremely specialized microbiota within the bee gut. As well, *Lactobacillus kunkeei* was reported to colonize only the crop of honeybees but also stingless bees (*Melipona* spp.) (Tamarit et al., 2015). Other *Lactobacillus* from *Apoidea* and *Vespidea*, e.g. *L. bombis* (Killer et al., 2014b) and *L. bombicola* (Praet et al., 2015) isolated from *Bombus terrestris, L. vespulae* (Hoang et al., 2015) from *Vespula vulgari,* have been recently described but their potential is to date unexploited.

The *Lactobacillus* microbial gut community is acquired by young insects through trophallaxis with nest mates (Engel and Moran, 2013), or by direct contact with processed feed or feces. Presumably the *Lactobacillus* microbial gut community is also acquired from the environment (McFrederick et al., 2012), but independently from the acquisition root it plays multiple rules. On the nutritional point of view, these insect-adapted *Lactobacillus* can store trehalose, a sugar functioning as an energy storage compound in insects (Ellegard et al., 2015). They are capable of fermenting toxic sugars for bees, like mannose (Ellegard et al., 2015; Lee et al., 2015), helping thus the honeybee gut processing indigested sugars acquired with feed. *Lactobacillus* spp. together with other acidophilic bacteria (*Acetobacteriaceae* and *Bifidobacteriaceae*) cover an important role in the production of short chain fatty acids (SCFAs) such as acetic acid or other acidic compounds such as lactic acid that are assimilated by the insect hindgut, supplementing honeybee nutrition, especially during winter (Martison et al., 2011). Moreover, they could be involuntarily used as a marker of already exploited nectar sources by forager honeybees. Indeed, honeybees avoid the harvest of nectar in flowers colonized by *L. kunkeei* (Good et al., 2014).

To date about 36 *Lactobacillus* species have been accepted as food grade and listed in the QPS list (EFSA 2013), therefore they have such a widespread use in the food industry to produce fermented foods and as protective cultures (Adedokun et al., 2015; Gómez-Sala et al., 2016). The pharmaceutical and nutraceutical applications of *Lactobacillus* spp. is of

interest as well: their ability to stimulate the host immune system and compete with gut pathogenic microorganisms is favourable for their application as probiotics to re-establish the unbalanced gut microbiota in humans and animals. In addition to the acidification properties, *Lactobacillus* spp. can synthetize a wide range of antimicrobial compounds (AC) counteracting pathogenic bacteria like *Staphylococcus aureus* (Cotter et al. 2005). The *Lactobacillus* AC belong to a wide range of categories: lantibiotics, two-peptide bacteriocin and bacteriolysins, (formerly classified as Class III bacteriocins (Cotter et al. 2005) such as Helveticin J (Fremaux and Klaenhammer 1986). Alongside Helveticin J, other two bacteriolysins produced in *L. helveticus* have been identified. Helveticin V-1829 (Vaughan et al. 1992) and a partially characterised Helveticin from *L. helveticus* G51 (Bonade et al. 2001) with all three being narrow spectrum and heat labile (Slattery et al. 2017). Whole genome sequencing technologies allowed the identification of putative Helveticin J or Helveticin like bacteriolysins produced by other *Lactobacillus* species as in the case of *L. acidophilus* and *L. casei* and interestingly, also in 4 honeybee related *Lactobacillus* strains (Butler et al., 2013) (see table 1). This allows us to assume that bacteriolysins in *Lactobacillus* are more popular than expected, even if the reasons for a so energetically expensive bacteriolysins production are still unknown. In this study, we identified 3 new bacteriolysins produced by honeybee gut isolates *L. helsingborgensis* Dan 75, *L. kimbladii* Dan 47 and *L. kullabergensis* Dan 23 respectively named Helveticin H, K1 and K2 showing a homology degree with Helveticin J of 72%, 70%, 70% and a Nisin like bacteriocin showing 60% similarity with Nisin A of *Lactobacillus kunkeei*. Also a Microcin J25 was detected and turned out to be inactivated by incomplete synthesis gene complex. We therefore hypothesize that these new bacteriolysins, acting alone or in combination, prevent the diffusion of exogenous *Lactobacillus* spp. acquired from the environment into the gut lumen, thus preserving the honeybee core lactobacilli microbiota. Confirmation of this theory may open to a new application of insect gut microbes as potential specific probiotic for insects.

Results

Isolation and Fingerprinting results

A total of 170 strains of *Lactobacillus* were isolated on MRS agar. According to Baffoni et al. 2015, the partial bacterial 16S rDNA gene was amplified and sequences from 36 isolates (Accession number KP114138-KP114147 and MG601136-MG601161) were subjected to taxonomical identification using RDP tools classifier and seqmatch. Out of 104 putative *Lactobacillus*, 36 different PFGE fingerprinting profiles were identified (Figure 1), belonging to 8 *Lactobacillus* species (*L. apis, L. helsingborgensis*, *L. kimbladii*, *L. kunkeei*, *L. kullabergensis, L. mellis*, *L. melliventris, L. johnsonii* and *L. plantarum).*

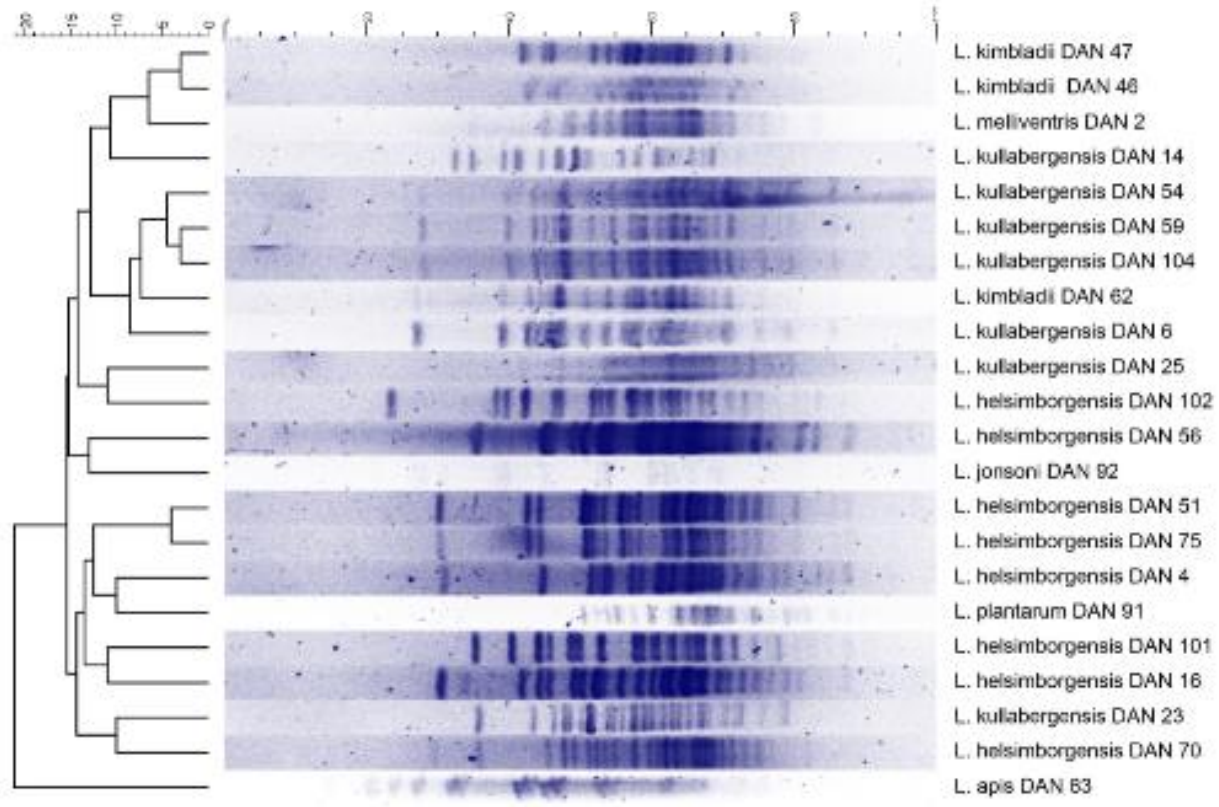


Figure 1: Pulsed Field Gel Electrophoresis (PFGE) analysis of 22 out of 36 *Lactobacillus* strains isolated from honeybee gut.

Antimicrobial activity

Test of antimicrobial activity clearly showed an inhibition halo against indicator strains of *Lactobacillus*. In particular it is worthy noting that the antimicrobial activity showed by Dan 70 against two indicator strains of *L. delbrueckrii* subsp. *lactis* DSM 20072 and *L. delbrueckrii* subsp. *bulgaricus* DSM 20081. Against the same strains, antimicrobial activity of *Lactobacillus kimbladii* Dan47 and *Lactobacillus kullabergensis* Dan 23 was much weaker. This is most probably caused by a low gene expression of the bacteriocin compound by the last two strains.

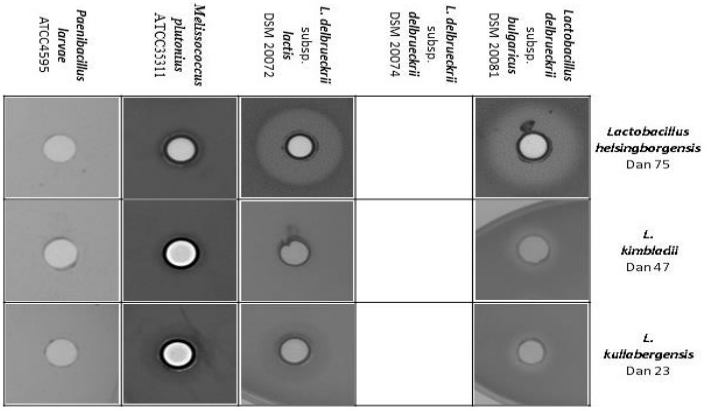


Figure 2: *Antibacterial activity of supernatants. L. delbrueckrii* subsp. *lactis* DSM 20072, *L. delbrueckrii* subsp. *bulgaricus* DSM 20081, *L. delbrueckrii* subsp. *delbrueckrii* DSM 20074 (Savino et al. 2011), *Paenibacillus larvae* ATCC4595, *P. dendritiformis* PA(C), *Melissococcus plutonius* ATCC35311

ORF prediction of antimicrobial proteins in Dan70

ORFs annotation for antimicrobial peptides has been performed with BAGEL web tool. The annotation allowed the identification of three different antimicrobial molecules, putative nisin operon (Figure 3 and 4), microcin (Figure 5) and a helveticin-related peptide (Figure 6). Lantibiotics are class I antimicrobials consisting of a group of post-translationally modified peptides. They are characterized by the presence of unusual amino acids including the thioether amino acids lanthionine and b-methyllanthionine first identified in nisin, the best characterized representative of the group (Mokoena, 2017). The genes for lantibiotics production can be plasmid or chromosome encoded and includes regulatory proteins, transport proteins, specific proteases and immunity proteins. The identified putative nisin-related ORFs seems to include all the different genes for antimicrobial peptide production, post-translational modification proteins and transporters (Figure 3 and 4). The immunity protein was not annotated by the programme, but the manual annotation of the uncharacterized proteins is still in progress. Nisin, as antimicrobial peptide, has an inhibitory activity against Gram positive bacteria like bacilli, lactococci, micrococci and clostridia (Sharma et al., 2014). Its broad spectrum of action allows a great competition for the colonization of ecological niches. Microcin peptides are subdivided in two different classes (Duquesne et al., 2007), they are typically produced as ribosomally synthesized precursors and subsequent modified by additional enzymes. In our case poor characterization of the operon has been obtained and manual annotation of neighbour ORFs should be accomplished to understand if the peptide is actively produced or deactivated. The annotated ORFs showed similarity to Microcin J25 (Figure 5) which belong to class I microcins with lower molecular masses. It was firstly identified in *E. coli* and seems to have antibacterial activity against few genera of enterobacteria.

The third antimicrobial peptides recognized by BAGEL programme has been annotated as putative Helveticin J. Helveticin J belongs to class III bacteriocins, a class consisting in large and heat-labile proteins larger than 30 kDa. In a recent work about bacteriocin identification in *Lactobacillus* Pan-genome, Collins et al. (2017) found a surprising abundance of homologs of helveticin-like bacteriolysins. They suggest that this trait may derive from a common ancestor and then disseminated by horizontal transfer. These peptides are active against a limited number of related lactobacilli probably giving an advantage in the competition for the same niches.

Additional work is needed to understand the regulation of all the annotated antimicrobial peptides that probably play different roles in different situations, according to environmental signals.

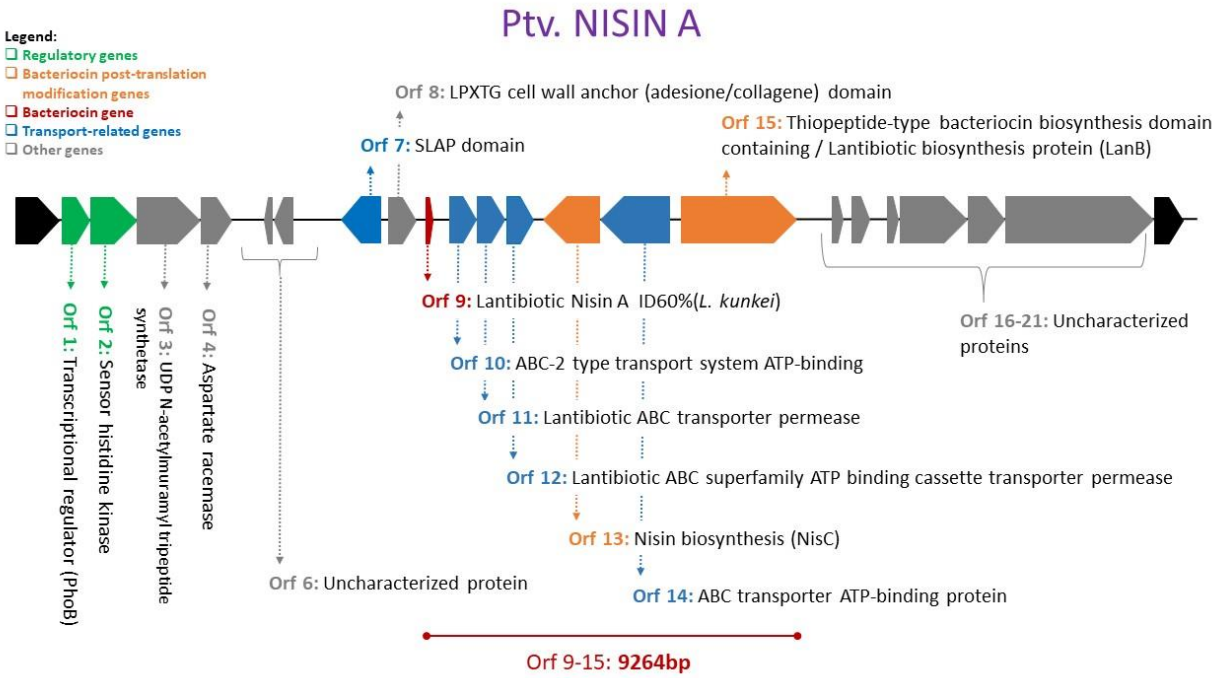


Figure 3: *Lactobacillus helsingborgensis* Dan 70 genome analysis of a 9264bp fragment shows the presence of Nisin A gene (red arrow), near transporters of different kinds (ATP binding permeases) (blue arrows), bacteriocin post-translation modification genes (orange arrows) and finally regulatory genes (green arrows).

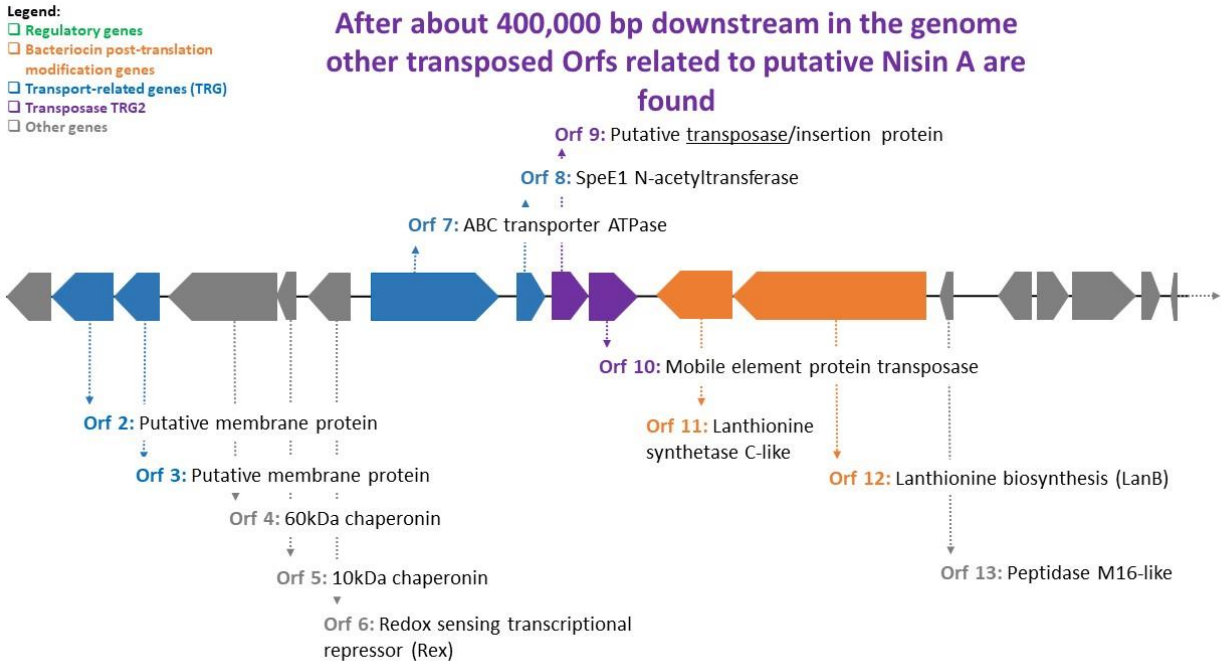


Figure 4: *Lactobacillus helsingborgensis* Dan 70 genome analysis of Nisin A gene complex continues about 400.000 base pairs downstream in the genome, were other Nisin A bacteriocin post-translation modification genes (orange arrows) are identified. Their position so far away from the other Nisin related ORFs is justified by presence of transposases (violet arrows).

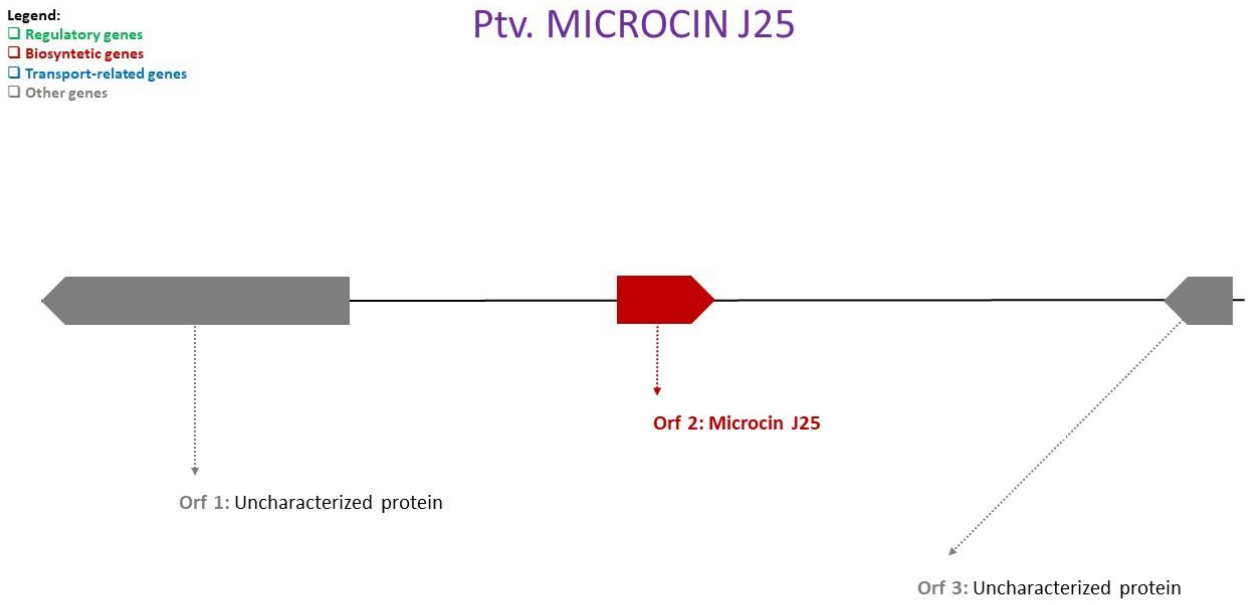


Figure 5: *Lactobacillus helsingborgensis* Dan 70 genome analysis shows a gene coding for Microcin J25. Unfortunately, post translation genes are missing, allowing us to suppose that this gene is deactivated.

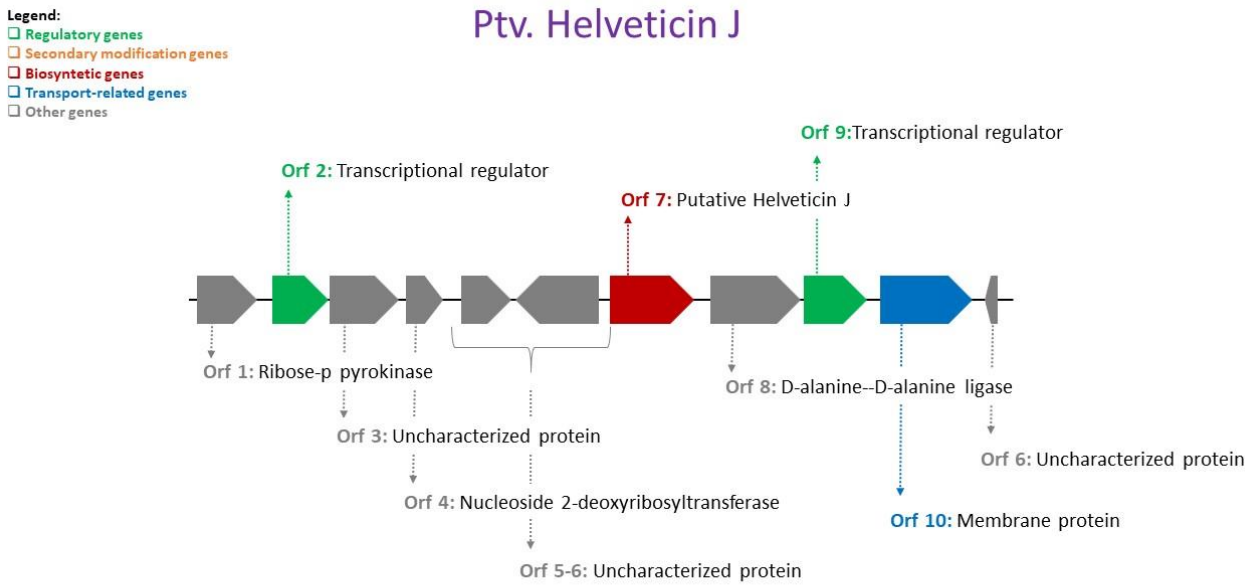


Figure 6: *Lactobacillus helsingborgensis* Dan 70 genome analysis shows the presence of Helveticin J gene (red arrow), near a transporter (blue arrow), and finally regulatory genes (green arrows). Helveticins do not require a complex core of genes for post translation modifications.

Physical and chemical characterization

Physical and chemical characterization of the Bacteriolysin showed its sensibility to high pH (deactivated above pH 7.0) and temperature (deactivated above 55°C) letting suppose that the antimicrobial compound is a large proteinaceous bacteriocin whose secondary and tertiary structure is easily denatured when exposed to high temperatures.

Isolation and characterization of the bacteriocins detected into the genome.

Any attempt performed to date (MALDI-TOF, Cationic and anionic binding in columns, membranes with different kDa cut off, HPLC, SDS-FAGE, UPLC, LPLC, amino acid sequencing) failed in the characterization of bacteriocins.

Further research is necessary to characterize this bacteriocin. The next attempt will be CRISPR-CAS9 on the target regions identified.

Experimental procedure (Materials & Methods)

Lactobacillus isolation

Adults of honeybee (*Apis mellifera*), violet carpenter bee (*Xylocopa violacea*), European hornet (*Vespa crabro*) and paper wasp (*Polystes gallica*) were collected in countryside environments near Bologna and were surface-sterilized (Yoshiyama and Kimura 2009) according to Baffoni et al. 2016. Lactobacilli were isolated and enumerated by surface inoculation on the deMan Rogosa Sharpe (MRS) medium (Becton Dickinson & Co., Mountain View, CA), containing 0.2% (w/v) sorbic acid (Sigma-Aldrich, Milan, Italy) and 0.1% (w/v) cycloheximide (Sigma-Aldrich) to inhibit yeast growth. After 48 h incubation in anaerobic condition at 35±1 °C, putative *Lactobacillus* colonies were picked according to colony colour, shape and growth speed.

DNA extraction

Microbial DNA extraction from pure cultures was performed using the Promega Wizard® Genomic DNA extraction kit (Promega, Madison, USA). The extracted DNA concentration and purity were determined by measuring the absorbance ratio at 260 and 280 nm (Infinite 200 PRO NanoQuant, Tecan, Mannedorf, Switzerland). The extracted DNA was stored at -20 °C until further analysis.

PFGE

Isolated *Lactobacillus* were grown on standard MRS with addition of 2% fructose, 0.1% L–cysteine hydrochloride (Olofsson at al. 2014) and 20 mM/L^{-1} D–threonine (Sygma-Aldrich) to facilitate lysis, according to Guidone et al. 2014. Cells were harvested from 0.5 mL of culture by centrifugation at 13.000 rpm, washed once in 500 µL 10 mM Tris HCl, 1 M NaCl (pH 7.6) buffer, and re-suspended in the 300 µL of the same buffer. The suspension was mixed with an equal volume of 2% of PFGE low melting point agarose (Bio-Rad, UK) before solidification in plugs. Plugs were incubated in a lysis buffer containing Mutanolysin 20 units/mL as suggested by Sympson et al. 2003 and successively treated with proteinase K overnight at 55°C. Successively plugs where exposed overnight to restriction enzymes *Sma* I and *Not* I (New England BioLabs, Hertfordshire, UK) with a final enzyme concentration of 1 UI/µL. DNA fragments were resolved using a CHEF-DR III pulsed-field system (Bio-Rad

Laboratories, Richmond, Calif.) at 6 V/cm for 16 h with pulse time ramped from 1 to 20 seconds. In every gel a Low Range PFGE marker (New England BioLabs, Hertfordshire, UK) was used as a ladder for gel normalization. Gels were stained with 0.5 mg/mL ethidium bromide and gel images were digitized using gel documentation system Gel DocTM $X^{+}R$ (Bio-Rad).

RAPD–PCR and ERIC-PCR fingerprinting

Isolates were also characterized by PCR dependent fingerprinting techniques. RAPD-PCR (Randonly Amplified Polymorphic DNA) was carried out with a reaction mixture composed of 10µL HotStarTaq Master Mix (QIAGEN GmbH, Hilden, Germania), 2 µL of 20 µM primer M13 (5' - GAG GGT GGC GGT TCT - 3') (Andrighetto et al., 2001), 1.2 µL of template DNA and water to the total volume of 20 µL. ERIC-PCR was carried out using the same protocol except for the primers ERIC-1 (5' - ATG TAA GCT CCT GGG GAT TCA C - 3') and ERIC-2 (5' - AAG TAA GTG ACT GGG GTG AGC G - 3') (Versalovic et al., 1991) added at the PCR mix as 0.5 µL of 20 µM each primer. The amplification products were run on an agarose gel 2% (w/vol), stained with 0.05 µL/mL SYBR Safe, 1x TBE as running buffer, at 120 V for 4 hours. Gel images were captured with GelDoc (BioRad Laboratories, Hercules, CA, USA).

Fingerprinting analysis

Profile band patterns were analysed with Gel Compar II 6.6 (Applied Maths, Kortrijk, Belgium). The similarity coefficient was calculated with Pearson correlation. The dendrogram was obtained with Unweighted Pair Group Method (UPGMA), using the algorithm of arithmetic mean of grouping (Clustering). A cut-off coefficient of 11 % was calculated according to Jérôme et al. 2016. The reproducibility of the fingerprinting was investigated on three different strains of isolated *Lactobacillus* and three different strains of type strains *Lactobacillus*. For each isolate, reproducibility was determined as the similarity value among the three replicates. Then, the six values were averaged to obtain the mean reproducibility, used as a threshold to define identical genotypes.

16S rDNA amplification

16S rDNA amplification was performed with primers 27f (5' - AGA GTT TGA TCC TGG CTC AG - 3') and 1492r (5' - GGT TAC CTT GTT ACG ACT - 3') (Lane, 1991) according to Gaggìa et al. (2013). Amplicons were purified (Nucleospin gel and PCR clean-up kit; Macherey-Nagel GmbH & Co. KG, Germany) and sequenced by a commercial sequencing facility (Eurofins MWG Operon), using primers 27f and 1492r. Sequence chromatograms were edited and analyzed using the software program Finch TV version 1.4.0 (Geospiza Inc., Seattle, WA, USA) and the corrected sequences were subjected to taxon classification using RDP classifier, an available tool at the RDP-II website (Wang et al., 2007).

Bacteriolysin production

A modified MRS (mMRS) was used to induce bacteriolysin production from the isolated *Lactobacillus* strains. The mMRS was composed of 2 g/L glucose, 8.7 g/L K_2HPO_4 and 8.0 g/L KH_2PO_4 according to Cho et al. 2010. Moreover also 2 g/L fructose and 1 g/L of L-cysteine hydrochloride were added to the mMRS (Olofsson at al. 2014). *Lactobacillus* strains were incubated at 37 °C for 36 hours, until the stationary phase was reached. Cell free supernatants were obtained by centrifugation of the supernatants at 11000 *g* for 2 minutes.

The pH of the supernatants was corrected to 5.8 with sterile NaOH 3 M, and the supernatant was stored at -20 °C until further analysis.

Agar well diffusion assay

The agar well diffusion essay was carried out as described by Cintas et al. 1995, on soft agar inoculated with indicator strains. The indicator strains used are according to O'Shea et al. 2008 with the exclusion of *Clostridium sp.*, *Enterococcus sp., Listeria monocitogenes* and *Streptococcus sp.* and with addition of *L. delbrueckii* subsp. *delbrueckii* DSM 20074 (Savino et al. 2011), *Paenibacillus larvae* ATCC 4595*, P. dendritiformis* PA(C) and *Melissococcus plutonius* ATCC 35311

HiSeq

The whole genome sequence of the strains giving an inhibition halo in the agar well diffusion assay (*Lactobacillus* sp. Xv13, *L. bombi* Xv6, *L. kimbladii* Dan47, *L. kullambergensis* Dan23 and *L. helsingborgenis* Dan70, *L. apis* Dan63 was obtained by a commercial sequencing facility (MicrobesNG, West Midlands, UK). The genomic DNA was prepared according to Illumina protocols for genomes preparation (MicrobesNG, Birmingham, U.K.). Fragmented genomes where then loaded on an Illumina HiSeq platform.

Genome analysis and primers design

The obtained whole genome sequences of *Lactobacillus* Xv13, Dan47, Dan23 and Dan 70 and the sequences of *Lactobacillus* Bma5, Hma2 and Biut2 deposited by Ellegaard et al. 2015 were run in BAGEL 2, an online open platform for bacteriocin mining (De Jong et al. 2010). Based on the BAGEL 2 outputs, the putative bacteriolysins ORFs were obtained. Multiple consensus sequence of the bacteriolysin ORFs were generated using DNAMAN and loaded on the online platform PRIMER 3 to design specific primers (Untergasser et al. 2012; Koressaar and Remm (2007)).

Antimicrobial compound annotation

Bacteriocin annotation has been performed with the web tool BAGEL. BAGEL is a web server that identifies ribosomally synthesized and post-translationally modified peptides in (meta)genomic data using novel, knowledge-based bacteriocin databases and motif databases. BAGEL can identify biosynthetic gene clusters of antimicrobial peptides, and classify and analyse the putative products (de Jong et al., 2006).

Bacteriocin concentration and partial characterization.

The protein content of the supernatants of Dan 75, Dan 47 and Dan 23 were concentrated up to 30 µg/µL by centrifuging 100 mL of the supernatant in Amicon® Ultra 15 centrifugal filters with 10 kDa cut off cellulose membrane, for 4 hours at 5000 rpm. The concentrates and the flow through were stored at 4°C until further analysis. To confirm the proteinaceous nature of the target antimicrobial, the concentrated supernatants were treated with ethylene acetate and proteinase K. Ethylene acetate was evaporated with rotavapor and the obtained extract was, re-suspended in both MRS and MRS + 20 % DMSO. Retention of antimicrobial activity was tested in indicator plates of *L. delbrueckii* subsp. *delbrueckii* DSM 20074. Heat stability was assessed by exposing supernatant at an increasing temperature gradient, from 40 °C to 100 °C. The workflow is reported in the Figure 7 below:

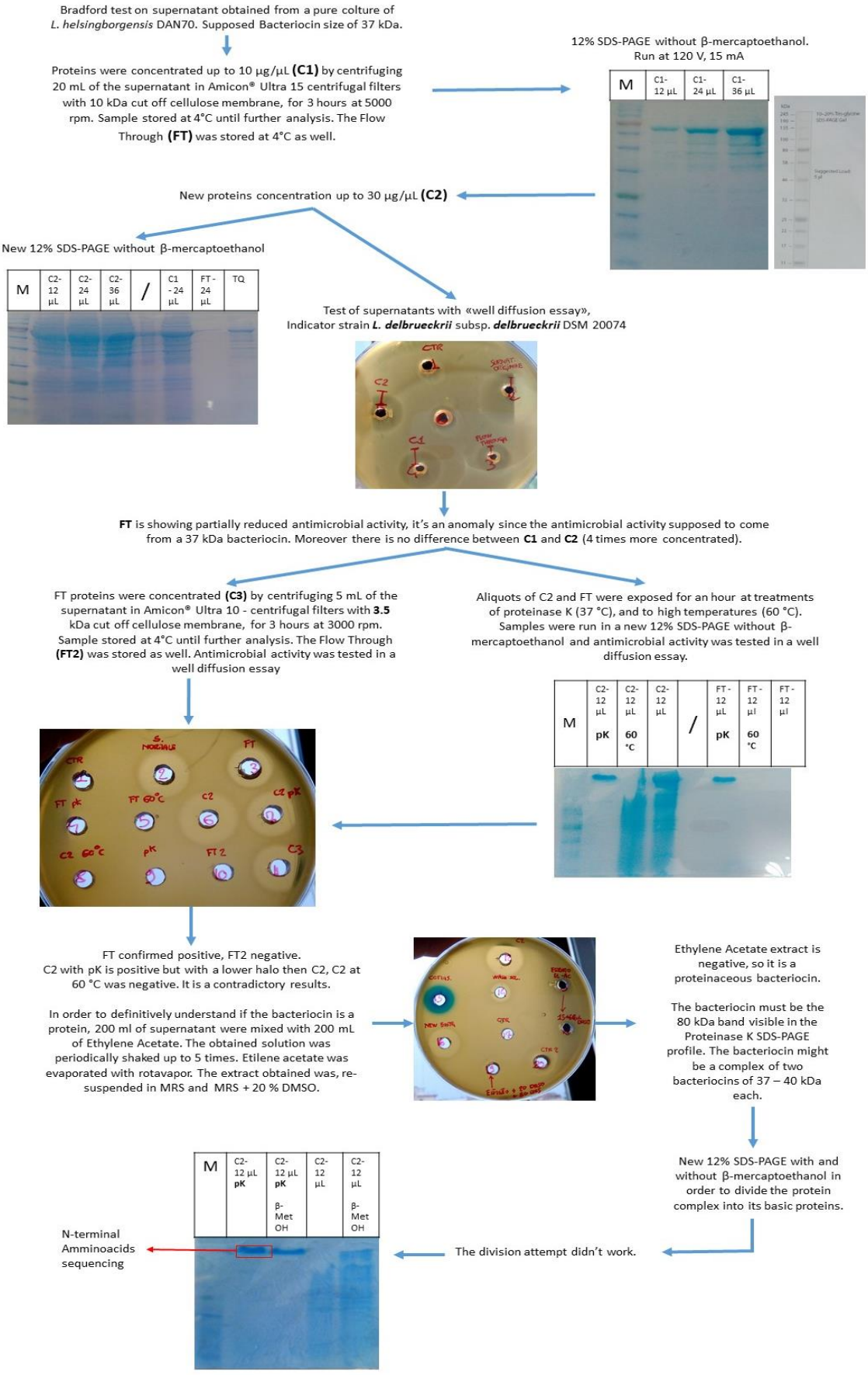
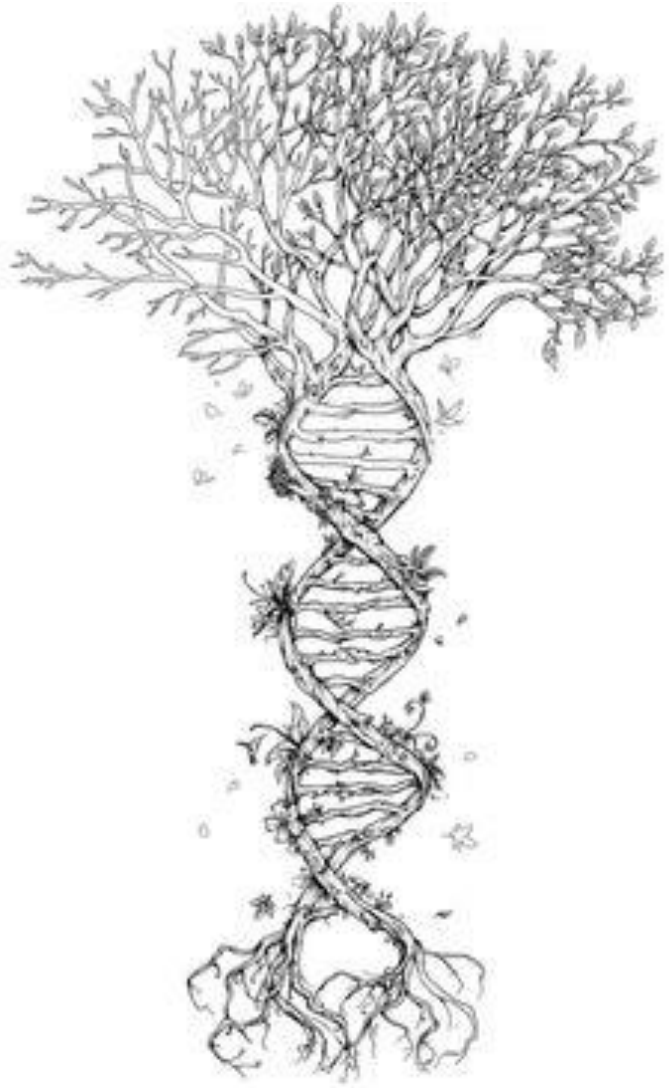


Figure 7: workflow of Helveticin like purification through SDS -PAGE

CHAPTER 3

Honeybee gut microbiota exploited with NGS studies



PAPER 5

Gut microbiota of the Maltese honeybee *Apis mellifera ruttneri SHEP.*

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Keywords: *Lactobacillus*, *Bifidobacterium*, *Nosema ceranae,* Gut Microbiota, Honeybee.

This research needs finalization prior to publication.

Introduction

The fast development of NGS techniques has helped and simplified the study of microbial populations in different ecological niches. Honey bees, in particular their gut microbiota, have been a favourite object of NGS studies, considering their key role in crops pollination and the economic importance of their refined products. Honeybee gut microbiota is simple, distinctive and apparently stable (Moran et al. 2012). Just twenty years ago in Malta a new subspecies of honey bees, *Apis mellifera* subsp *ruttneri,* has been identified by Shepard et al. (1997). It belongs to the African bees subgroup and it is classified close to *Apis mellifera* subsp *intermissa* and *Apis m*ellifera subsp *siciliana* and far from the European subspecies. The Maltese bee shows peculiar characteristics of adaptation to the drought, hotness and the sea wind. It developed these characteristics after thousand years of isolation in the Malta islands, presumably during Pleistocene, colonizing the Maltese islands. In this study, we investigated the gut microbiota of *A. mellifera subsp ruttneri* through culture dependent and independent techniques, hypothesizing some distinctive differences from the European bees, having evolved separately in an isolated niche.

A. mellifera subsp *ruttneri* is an endangered subspecies due to the importation of alien subspecies of *Apis mellifera* subsp *ligustica* and subsp *siciliana*. For a long time, international community of entomologists believed in its total extinction. Luckily, they were wrong, and to date about 100 pure *Apis mellifera* subsp *ruttneri* beehives are present in the Maltese main island, under the protection and coordination of a NGO “Breed of Origin”. This research attempts to characterize the aspects related to the gut microbial community of this endangered subspecies, enriching the scarce existing knowledge. As a curiosity, the Maltese honey bee is, to some extent, still reared in the terracotta hives typical of the Maltese Island (Figure 1), a unique case in Europe to the best of our knowledge.

Figure 1a and 1b Terracotta hives typical of the Maltese island, populated with *Apis mellifera* subsp *ruttneri.* Apiary of Mario Sant, beekeeper promoting Maltese bee preservation.

In the present work, Maltese honey bees were sampled in 3 different localities of the main Maltese island, depicted in Figure 2. The 16S rRNA of the gut microbial community obtained both from single bee gut and from a pool of 10 bee guts per location of interest was sequenced. Then the sum of the OTUs obtained from single samples per location was compared to OTUs obtained from samples pooled per location. In addition to the sequencing

results, the aim was to validate the pooling method as representative of the main status of the gut microbial population of the bee hive analysed.

Materials and Methods

20 bees for culture dependent techniques and 30 bees for microbial culture independent techniques were sampled from 3 different beehives of pure Maltese bees located in Gharghur (GH), Msida (CM)and Zejtun (ZT) (Figure 2). Gut samples were immediately shipped on dry ice (-70°C) to the University of Bologna,

Figure 2 Maltese main Island, geo-localization points where Maltese honey bees gut samples were picked.

Tenfold serial dilution of gut content (0,5 g) stored in glycerol broth and inoculation on MRS, TPY and TSA agar plates was carried out, leading to the isolation of about 300 isolates, nowadays only one hundred analysed. Genomic DNA was extracted from 10 single bee gut per collection locality with Quick-DNA™ Insect Microbe Miniprep Kit - Zymo Research (ZYMO, California, USA). Moreover, a pool of 10 intestines per locality were also extracted with Quick-DNA™ Fecal/Soil Microbe Miniprep Kit - Zymo Research (ZYMO, California, USA). The V3-V4 regions of 16S rRNA were amplified, analyzed via DGGE and sequenced on the MiSeq Illumina platform with an average sequence yield 200.000 seq. per bee. Sequencing results were compared with the results obtained by Moran et al. (2012) on *Apis mellifera* in USA. Bioinformatic analyses were performed with Qiime1, and representative OTUs blasted against the most updated SILVA database (SILVA release 128). OTUs present in less than 0.1% abundance were removed and bar charts generated.

Results and discussion

The Maltese honey bee gut microbial community profile shows the major microbial groups also detected by Moran et al. (2012). Honeybee gut microbiota is surprisingly stable, having

a unique microbial profile typical of honey bees. The same microbial taxa (or phyla) were also detected in *A. mellifera* subsp. *ruttneri* (Figure 3): Firmicutes (with *Lactobacillus* spp.); Actinobacteria (with *Bifidobacterium* spp.); Alphaproteobacteria (with *Commensalibacter* spp and *Bartonella* spp.); *Betaproteobacteria* (with *Snodgrasella* spp.); Gammaproteobacteria (with *Acinetobacter* spp., *Pseudomonas* spp., *Giliamella* spp. and *Frischella* spp.) and *Tenericutes* (with *Spiroplasma* spp.). In addition, *Arsenophonus* sp. was detected, an indicator of the poor health of bees usually due to varroa infestation.

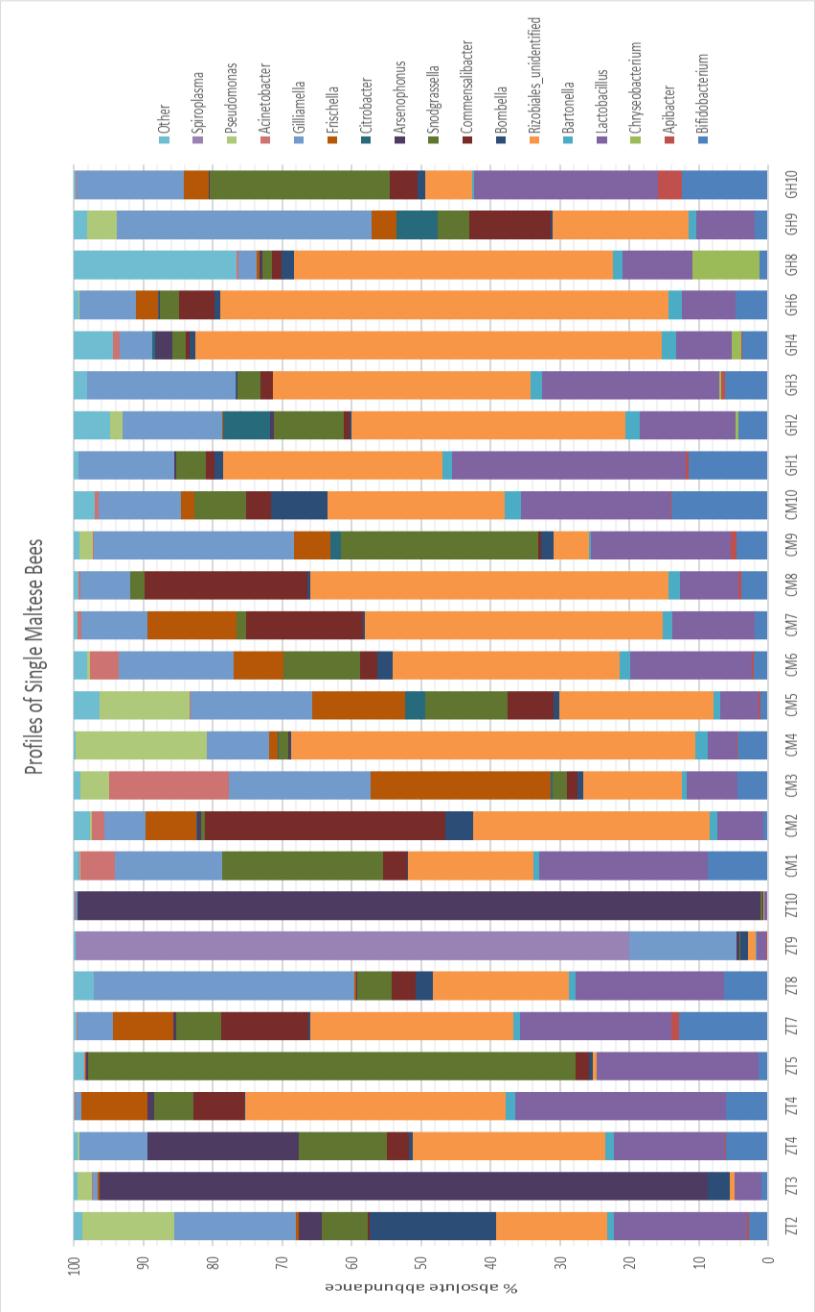

Figure 3 OTUs (Operational taxonomic unit) relative abundance of gut microbiota represented with bar charts, and divided in groups.

Contrarily, two taxa have been evidenced that are distinctive with respect to *A. mellifera*: *Bombella* spp. and *Apibacter* spp. *Bombella* genus, like *Commensalibacter*, belongs to *Acetobacteraceae* family in the *Alphaproteobacterium* phylum, while *Apibacter* genus belongs to *Flavobacteraceae* family in the *Bacteroidetes* phylum. These two taxa are correlated with the African bee subspecies according to the genetic phylogenies of the Maltese bee. In *Apibacter mensalis* genome annotation, Praet et al. (2016) evidenced complete or nearly complete vitamin biosynthetic pathways, together with twogene-abundant subsystems related to protein and amino-acid metabolism. Kwong and Moran (2016b) indicated that different bee hosts may harbour their own specialized species or strains of the genus *Apibacter*. Considering this aspect, a full metagenome analysis could be of particular interest for an in-depth characterization of bee-subspecies-related microbiota. Also the full-genome sequence of *Bombella intestini*, recently analysed by Li et al. (2016) revealed peculiar traits related to bee gut environment and host-microbiota interaction. According to Yun et al. (2014), insect gut bacterial diversity is determined by environmental habitat, diet and host phylogeny. The adaptation of gut symbiont to external factor and host genetic heritage allows microorganisms to play an important role in regulating the host's metabolism, extracting the maximum energy from ingested foods and protecting the host from other potentially harmful microbes.
Statistical analysis comparing the different location has been accomplished with QIME1 using three different metrics of alpha-diversity estimation: chao index, observed_otu and PD_whole_tree (Figure 4).

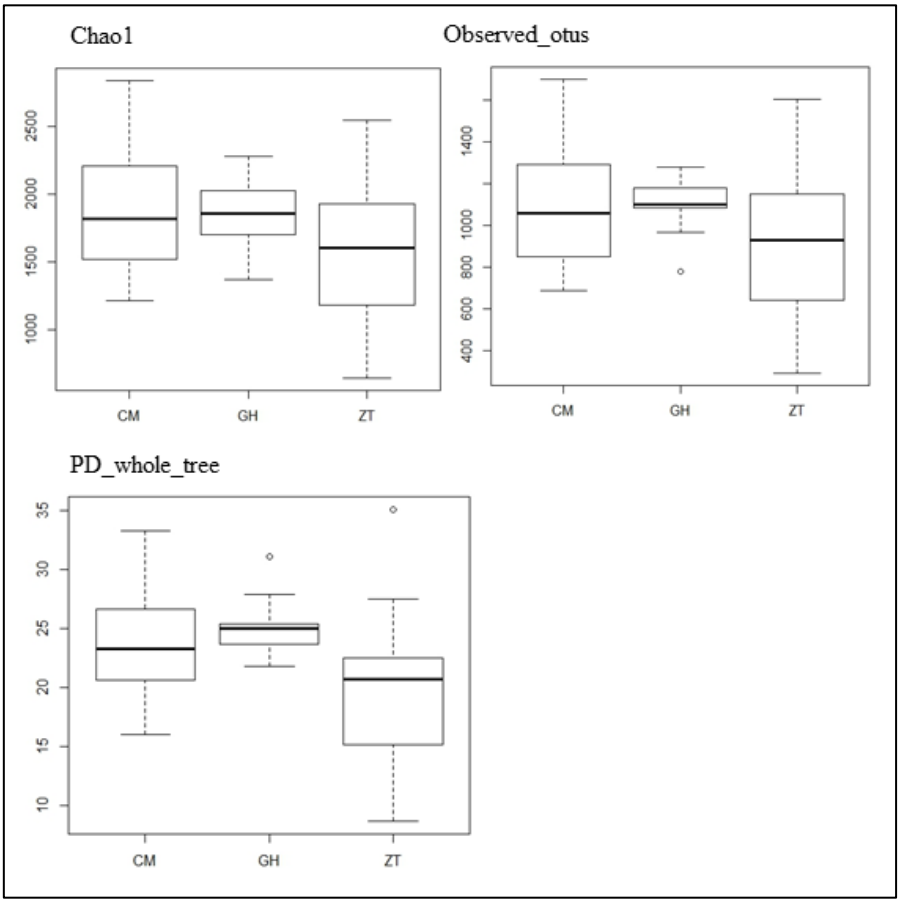

Figure 4: Box plots on statistic of Maltese Honeybee

The chao index estimates diversity from abundance data (importance of rare OTUs), the comparison revealed no significant differences between locations. The observed_otu metric is a richness measure that considers the number of species (or OTUs) observed in the sample; also in this case the no differences have been reported. The PD_whole_tree metric is Faith's Phylogenetic Diversity, and it is based on the phylogenetic tree. Basically, it adds up all the branch lengths as a measure of diversity. So, a new OUT, closely related to another OTU in the sample, will be a small increase in diversity. On the contrary, a new OUT, coming from a totally different lineage than anything else in the sample, will contribute a lot to increase the diversity. Gharghur and Zejtun resulted significantly different (p=0.027). The results evidenced that the three locations are similar for what concern richness and abundance distribution of the estimated OUT, however, a substantial difference among Gharghur and Zejtun has been evidenced when phylogenesis is taken into account. Beta diversity analysis among the different locations has been performed using UniFrac distance metric. It incorporates information on the relative relatedness of community members by taking into account phylogenetic distances between observed organisms in the computation. The unweighted (qualitative) variant of UniFrac considers the presence or absence of observed organisms and is displayed in PCoA plot of Figure 5, underlined again the difference between Gharghur and Zejtun.

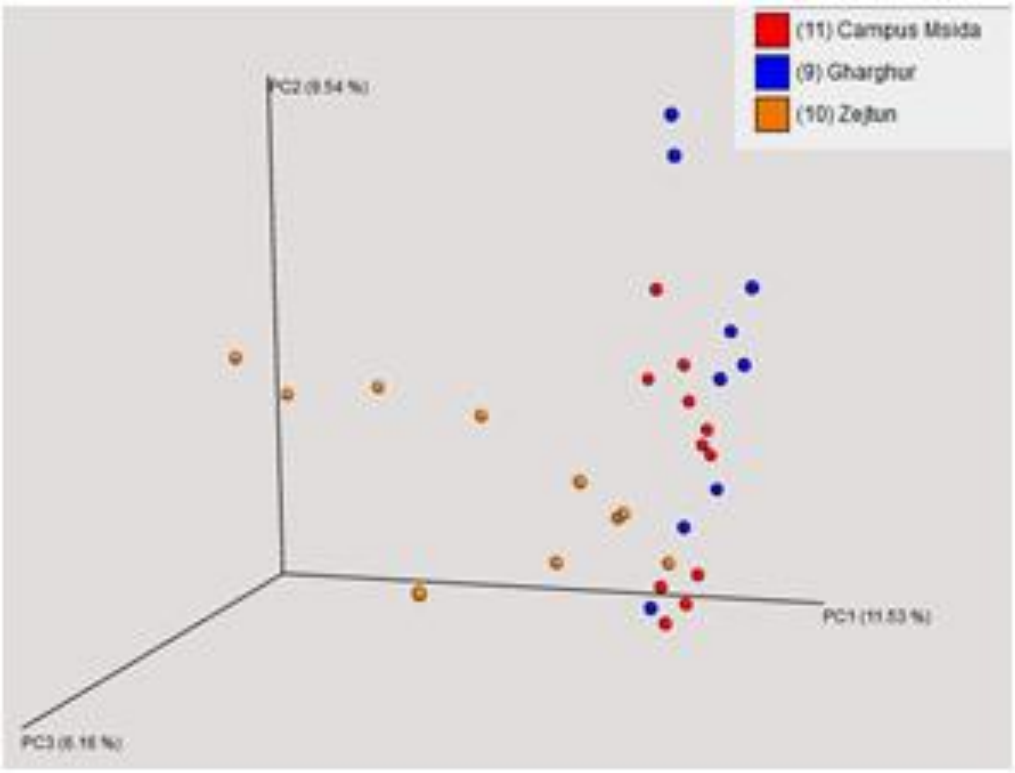

Figure 5: PCoA plot: in red samples from Campus Msida, in blue samples from Ghargur, in orange samples from Zejtun

The use of Qiime1 script to compare single OUT abundance among locations, with Bonferroni correction, revealed significant differences in specific taxa. An OUT, classified as Rhizobiales_unknown, was significantly lower in Zejtun samples. After extracting the reference sequence associated with this OUT, the sequence was blasted using NCBI database (on type material) and classified as *Bartonella apis* (99%, 439nt/440nt), but the second assignment was *Shinella fusca* (99%, 436nt/440nt). *Bartonella apis*, an *Alphaproteobacteria* of the *Bartonellaceae* family, has been firstly isolated from honey bee gut and described by Kešnerová et al. (2016). A comparison of six genomes of *Bartonella apis* (Segers et al., 2017) revealed that *B. apis* encodes a large set of vertically inherited genes for amino acid and cofactor biosynthesis (*i.e.* heme, vitamin B12, vitamin B6, molybdopterine and tetrahydrofolate) and nitrogen metabolism. The nitrogen-limited plant diet of the host may act as selective pressure to retain these vertically inherited biosynthetic capabilities. In Zejtun samples, three OTUs were found to have a higher number of sequences than the other two locations and they were classified as *Chryseobacterium* spp., *Arsenophonus* spp. and *Spiroplasma* spp. As already stated, *Arsenophonus* high number can be an indicator of the poor health of bee. Concerning *Spiroplasma* spp., Shokal et al. (2016) evidenced, in *Drosophila melanogaster*, a possible correlation between *Spiroplasma* presence and the modulation of immune signalling against pathogenic bacteria. *Chryseobacterium* genus has been already detected in insect gut however its role is still unclear, in the gut of the wood-boring beetle *Anoplophora chinensis* it seems to help in cellulose and/or aromatic compounds degradation (Rizzi et al., 2013).
The overall information seem to suggest that bee sampled in Zejtun location were particularly stressed and not in gut health. Further analysis on observed OUT could be appropriate to find correlation between decrease and increase of specific OTUs and bee health status.

The sum of the single bee gut profiles of Figure 3 is shown in Figure 6 and named as “ZT sum; CM sum; GH sum”. These sums were compared with the profiles obtained from a pool of 10 bee guts per location (named as “ZT mix; CM mix; GH mix”, and results showed a non-significant ($p<0.01$) difference among profiles when considering the whole microbial communities composition (Beta-Diversity). However, when abundance of a single genus is compared, a significant difference was highlighted for many genus.

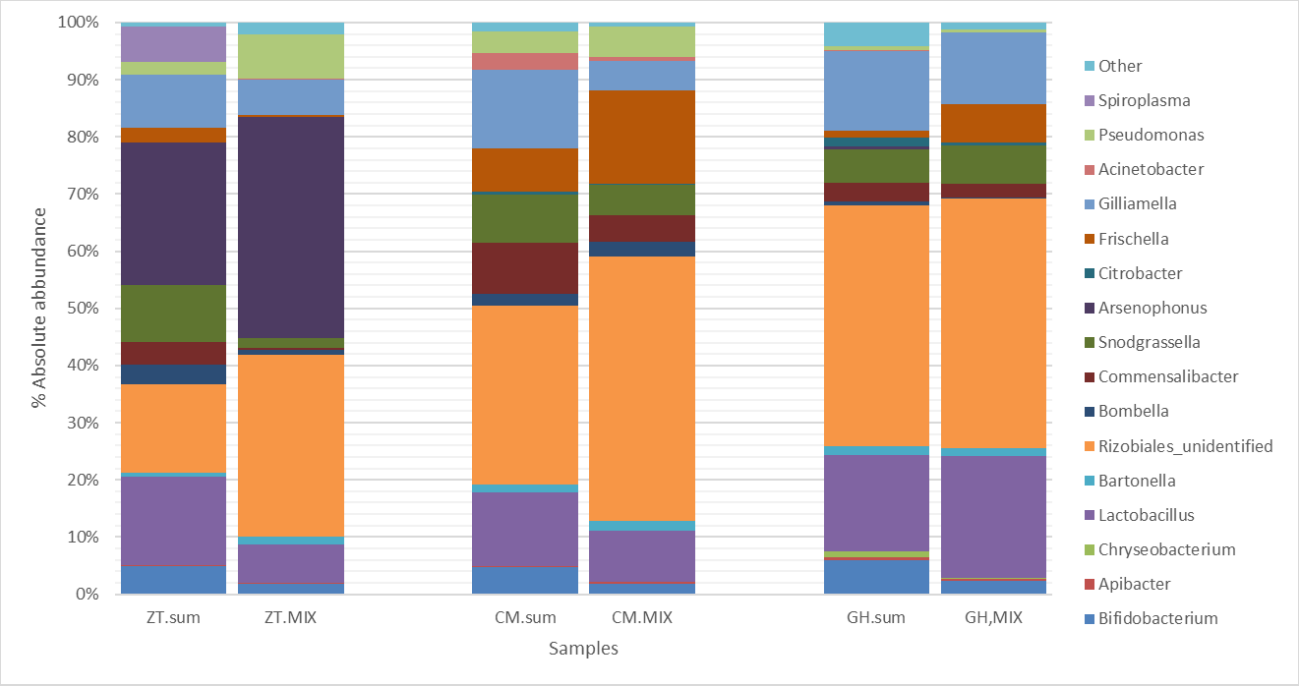

Figure 6 OTUs (Operational taxonomic unit) relative abundance of gut microbiota represented with bar charts summed and compared with pre extraction pools of intestines.

These are preliminary data, research on this topic is going on.

PAPER 6

Impact of antibiotics and natural medicaments on the microbial community of honeybee *Apis mellifera subsp. ligustica SPIN*.

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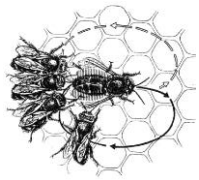
Keywords: *Lactobacillus*, *Bifidobacterium*, *Antibiotics, Sulfaquinoxaline, Tetracicline*, Tylosine, Tymol, Neem Oil, Gut Microbiota, Honeybees.

This paper has not been completed yet and it needs finalization prior to publication.



Introduction

In the last decades honeybees have been afflicted by several gut diseases, such as the American Foulbrood (caused by *Paenibacillus larvae*), the European Foulbrood (*Melissococcus plutonius*) and Nosemosis (*Nosema apis* and *N. ceranae*). The virulence of these diseases is enhanced by synergies with abiotic stressors causing noteworthy significant losses in honeybee hives and honey production. To counteract losses, pharmaceutical companies have developed specific medicaments for microbial mediated honeybee diseases generally classifiable as antibiotics or chemotherapics. Antibiotics are widely used in the beekeeping sector as both growth promoters and therapeutic agents for the main microbial diseases affecting honeybees (*Apis mellifera* spp.). A certain number of antibiotics are used worldwide: Oxitetracicline-HCl (Terramicin®), authorized to control *Paenibacillus larvae*, the causative agent of American Foulbrood (Genersch 2006), Tylosine (Tylovet®), used to control *Melissococcus plutonius*, the first invader of European Foulbrood, and Fumagilin B (Fumidil-B®), the only effective antibiotic known to control *Nosema* spp.. After the ban of antibiotics at European level in 2001, a strong input in finding alternative solutions has stimulated both researchers and companies. Consequently, several new strategies, such as natural oils (e.g. thymol, Neem oil) or probiotics, have been proposed on the veterinary market or suggested by researchers as possible alternatives. However, the use of any oral medicament represents an anthropogenic pressure that can lead to modification of the honeybees' gut microbial community.

To date, the antibiotic shaping effects on the bee microbiota as well as the effects on the beehive ecosystem (e.g.: selective pressure on the yeast and moulds responsible of the beebread fermentation (Gilliam 1979)) is poorly investigated. It is well known that humoral and cellular defences are used by insects to defend themselves from pathogens and parasites. The intestinal protective microorganisms act in synergy with the insect immune system, underlying their importance in modulating the insect defence response to the pathogen. Commensal bacteria can both modulate the innate immune system and contribute to the integrity of the intestinal barrier, acting as a further protection sheath, which partially edges the contact between the epithelial cells and the pathogens. Any unbalance of the microbial populations in the intestine, defined as intestinal dysbiosis (Sartor 2008), is caused by a multitude of factors, including pharmacological treatments. In particular, the use of antibiotics is known to have side effects on humans and animals, including insects.

In this work, we investigated if and how traditional chemotherapeutics strategies and new approaches can shape the honey bee gut microbial community. The effects at the gut level of antibiotic treatments in honey bees is evaluated, comparing it with two eco-friendly strategies involving the use of probiotic strains and oils (a particular focus will be made on beneficial microorganisms in the bee gut such as Lactobacilli and Bifidobacteria. The microbial composition of the bee gut of the new-borns is detected through high-throughput pyrosequencing of 16S rDNA (V3-V4 regions) amplicons. The advantage of this technique is the determination of the sequence data from amplified single DNA fragments, avoiding the need for cloning of DNA fragments.

Materials and Methods

Two different trials have been designed to test eco-friendly and antibiotic/chemotherapeutic strategies, respectively (Figure 1):

1. **In field trial to test beneficial microorganisms and natural oils.**
   For each thesis, six micro hives were prepared and established in an experimental field. The developed thesis were: Thymol + Tween80 [T], Neem Oil + Tween80 [N], Probiotics [P], Tween80 [W] and Control [C]. Regarding the thesis with probiotics, a mixture of 6 strains, belonging to the *Bifidobacterium* and *Lactobacillus* genera, was freshly prepared (Baffoni et al. 2016, Alberoni et al. 2018)

2. **Laboratory trial to test antibiotics.**
   For each thesis, six micro hives were prepared and established in a controlled laboratory environment, i.e., in a thermostat with controlled temperature and humidity (37°C and 60RH). The developed thesis were: Tylosin [TL], Tetracycline [PT], Sulfaquinoxaline [S], Antibiotic Control [CA].

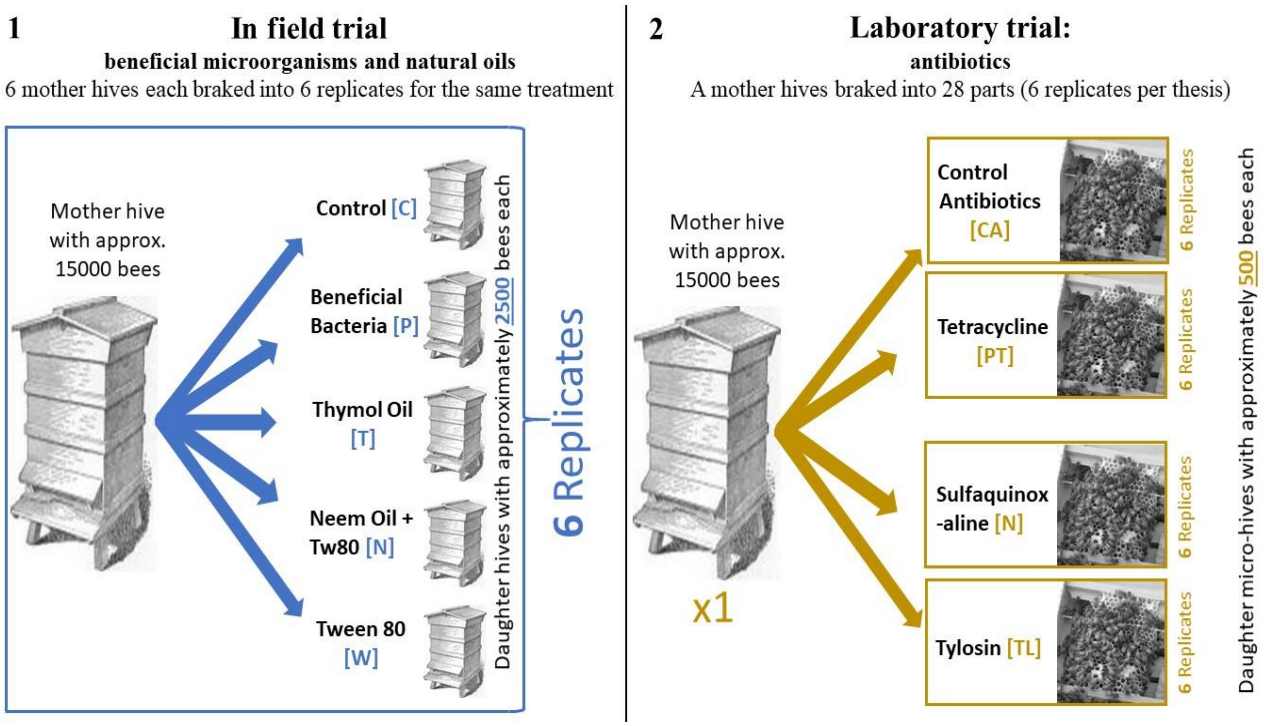


Figure 1 Schematic representation of *in field* (1) and laboratory (2) tests. In the figure are stated also dimensions of the tests and how many bees and beehives have been used to achieve the aim.

All micro hives were treated once a week for three weeks by spraying honeybees (in field) or by nourishment (laboratory cages) with 30 mL of the assayed treatment (antibiotic/oil/beneficial bacteria brought to volume with sugar syrup (1:1 sugar-water)) supplemented with the respective treatment. A weekly administration was decided according to the instructions of veterinary medicament manufacturers to cover all the honeybee workers brood circle (21 days), and simulate as accurately as possible the beekeeping practices. Gut content of 30 bees per replicate was picked, pooled and extracted at the beginning of the experiment (T0) and after 4 weeks (T4). Microbial gut DNA was extracted with Quick-DNA™ Fecal/Soil Microbe Miniprep Kit - Zymo Research (ZYMO, California,

USA) and 16S rDNA was amplified with a primer targeting V3-V4 region. Libraries were prepared and sequenced on Illumina MiSeq platform. Bioinformatic analyses were performed with Qiime1, and representative OTUs blasted against the most updated SILVA database (SILVA release 128). OTUs present in less than 0.1% abundance were removed. Percentage Based Cognitive Illusion Correction was applied to all the data, in order to identify microbials groups that are actually changing in proportion if compared with unvaried microbial taxa (Squartini et al., 2017)

Table 1 Table of medicaments used and doses per treatment preparation per hive. All antibiotics or antimicrobial agents was brought to a total volume of 30mL with sugar syrup and sprayed on bees

Antibiotic/Antimicrobial/Beneficial Microorganisms	Dose per treatment	Reference article
Oxitetraciclin	267 mg	Skinner J.A. et al.; Mutinelli F. 2003
Tylosin	200 mg	Mutinelli F. 2003; Huvepharma®
Sulfaquinoxaline	1 g	Mutinelli F. 2003
Tween 80 + (Essential oil -below specified)	1,5 mL	-
- **Neem Oil**	1,5 mL	-
- **Tymole**	0,01 mL	-
Beneficial bacteria mixture*	0,2 g	Audisio M.C. et al., 2015; Baffoni et al. 2016
Control [CTR]	-	-
Control Antibiotics [CTR-ANT]		-
Control [CTR] + (Tween 80)	-	-

*prepared according to Baffoni et al. 2016

Results and discussion

A total of 35 million sequences were obtained; of these, 16.7 million passed the quality control and Kimera check for an average of 86,074 joint reads per sample.

Analysis on obtained OTUs showed that (Figure 2), beneficial microorganisms' administration on honeybees did not show a significant perturbation of the distribution of the major representative genera. Surprisingly the treatments with oxytetracycline, sulfaquinoxaline and tymol didn't significatively modify the core microbial community. The most significant output of the research was the decrease of *Lactobacillus* and eradication of *Bifidobacterium* member after Tylosin antibiotic administration, that represent the only significant honeybee core gut microbiota modification. Moreover, interestingly PCoA analysis of the distribution of samples according to their microbial profile, showed a clear division between in field and laboratory trials (Figure 3), highlighting the importance of the environmental conditions in shaping the gut microbiota.

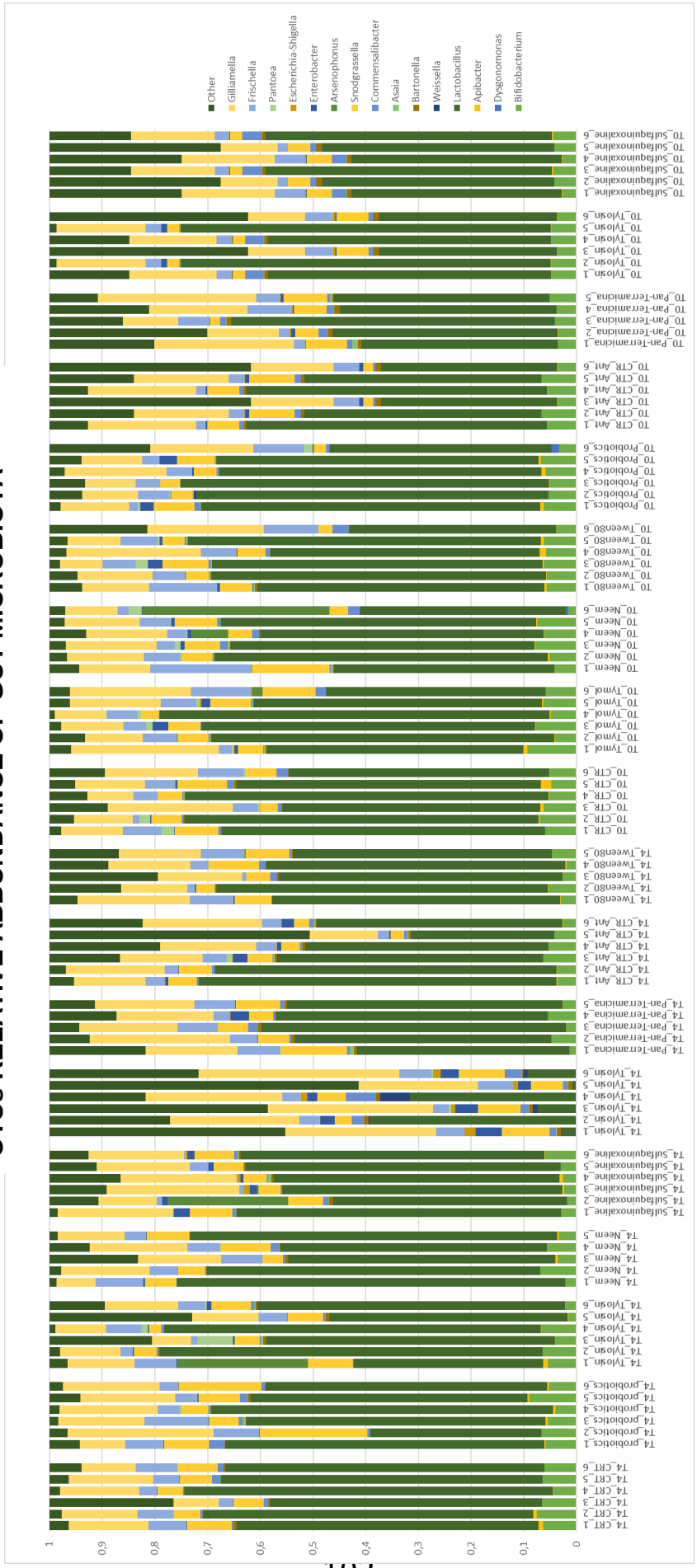


Figure 2 OTUs (Operational taxonomic unit) relative abundance of gut microbiota represented with bar charts, and divided in groups per treatment. In every group is present T0 and T4 profiles.

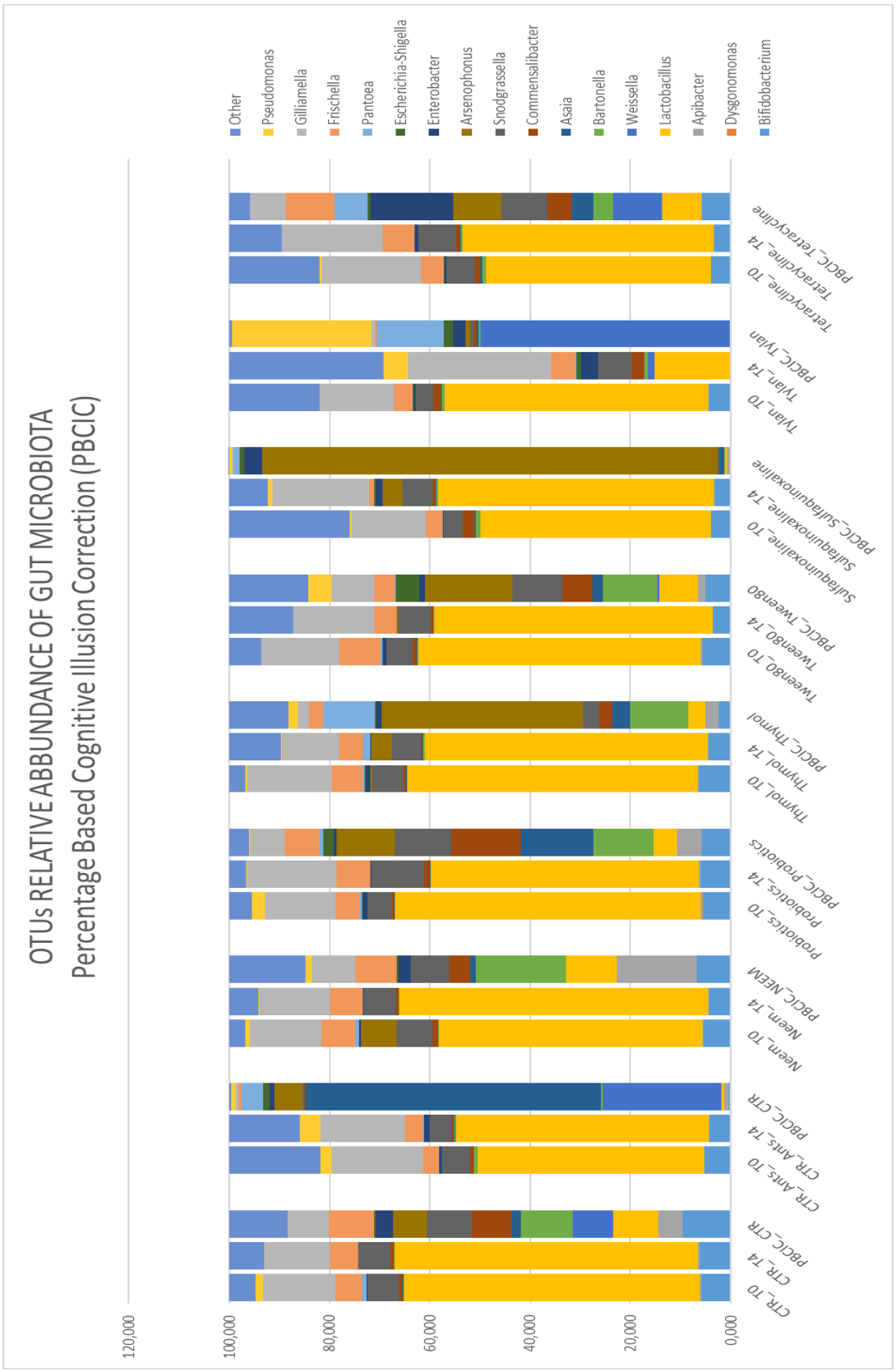


Figure 2 OTUs (Operational taxonomic unit) relative abundance of gut microbiota represented with bar charts, and divided in groups per treatment. In every group is present T0 and T4 profiles. Moreover a third column represent the Percentage Based Cognitive Illusion Correction (PBCIC).

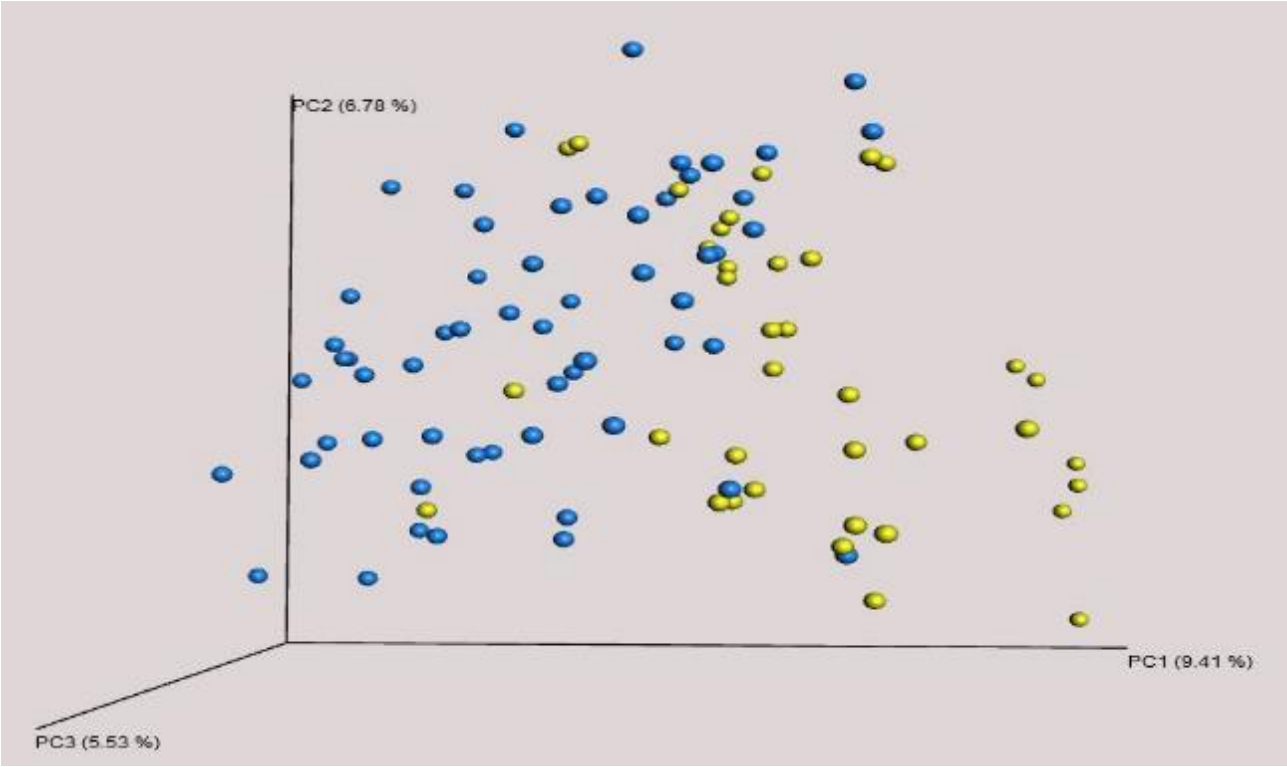


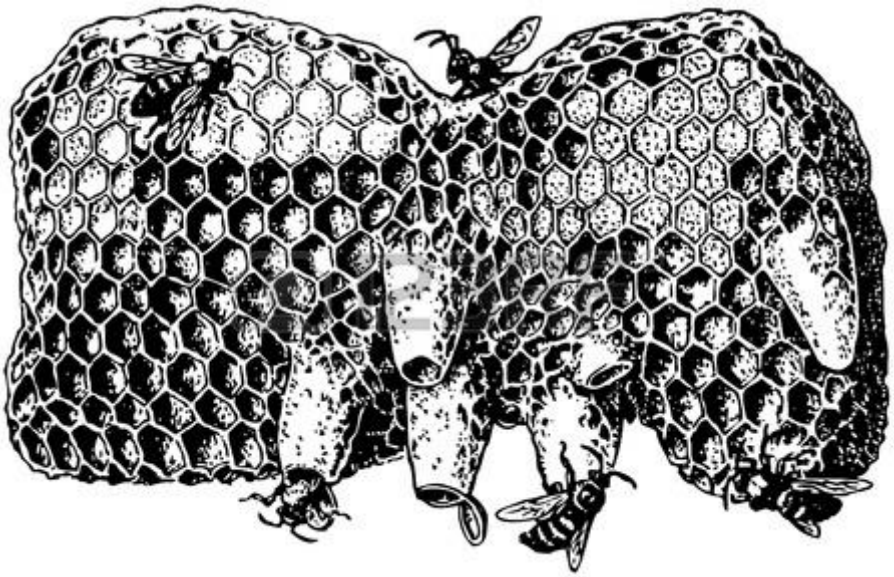
Figure 3 PCoA showing distribution of samples according to their microbial profile. In Yellow samples from laboratory tests; in light blue samples from the *in-field* tests;

Although further elaborations of the results are necessary to draw definitive conclusions, it can be preliminary outlined that the gut microbial community of honeybees shows a great resilience to most of the treatments applied after 3 weeks of administration plus 1-week wash-out. This is somehow different from the achievements of Raymann et al. (2017), which showed that tetracycline can strongly shape the gut microbial community after a few days from administration.

These are preliminary data, research on this topic is ongoing.

CHAPTER 4

Counteracting diseases for a honeybee wealth and health



PAPER 7

Effect of dietary supplementation of *Bifidobacterium* and *Lactobacillus* strains in *Apis mellifera* L. against *Nosema ceranae.*

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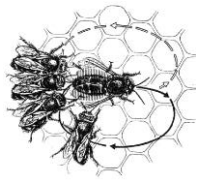
Abstract

Nosema ceranae is a widespread microsporidium of European honeybee *Apis mellifera* L. affecting bee health. The ban of Fumagillin-B (dicyclohexylammonium salt) in the European Union has driven the search for sustainable strategies to prevent and control the infection. The gut microbial symbionts associated to the intestinal system of vertebrates and invertebrates and its impact on host health, are receiving increasing attention. In particular, bifidobacteria and lactobacilli, which are normal inhabitants of the digestive system of bees, are known to protect their hosts via antimicrobial metabolites, immunomodulation and competition. In this work, the dietary supplementation of gut bacteria was evaluated under laboratory conditions in bees artificially infected with the parasite and bees not artificially infected but evidencing a low natural infection. Supplemented bacteria were selected among bifidobacteria, previously isolated, and lactobacilli, isolated in this work from healthy honeybee gut. Four treatments were compared: bees fed with sugar syrup (CTR); bees fed with sugar syrup containing bifidobacteria and lactobacilli (PRO); bees infected with *N. ceranae* spores and fed with sugar syrup (NOS); bees infected with *N. ceranae* and fed with sugar syrup containing bifidobacteria and lactobacilli (NP). The sugar syrup, with or without microorganisms, was administered to bees from the first day of life for 13 days. *N. ceranae* infection was carried out individually on anesthetized 5-day-old bees. Eight days after infection, a significant ($P<0.05$) lower level of *N. ceranae* was detected by real-time PCR in both NP and PRO group, showing a positive effect of supplemented microorganisms in controlling the infection. These results represent a first attempt of application of bifidobacteria and lactobacilli against *N. ceranae* in honeybees.

Keywords: honeybee pathogens, gut microbiota, real-time PCR, beneficial bacteria



Introduction

Pollination by honey bee is widely recognized as an important service for wild plant communities as well as for the agricultural ecosystems. Besides the economic benefits due to crop pollination, bees should also be considered for the contribution to the global biodiversity (Hamdi *et al.*, 2011). Unfortunately, in many European and North American countries honey bee population have registered a significant decrease (Aizen and Harder, 2009) as a result of habitat destruction, pesticide use, pathogens and climate change or some combination of these factors (Vanengelsdorp and Meixner, 2010). The microsporidium *Nosema ceranae* is becoming a serious threat, affecting honey bee health, particularly in countries with temperate climate. It has been found to be highly virulent in field, and a direct correlation has been shown between *N. ceranae* infection and the death of honey bee colonies in a study performed in Spain (Higes *et al*., 2010). *N. ceranae* belongs to the fungal phylum Microsporidia, a group of obligate intracellular single-cell spore-forming parasites that can infect a variety of insects, including honey bees. Infection and replication cycles take place in the midgut once spores germinate by extrusion of a polar tube, injecting the sporoplasm inside the epithelial cells (Higes *et al*., 2007). Within three-four days, the host epithelial cells are filled with offspring spores and the cells burst to release a new generation of primary spores (Gisder *et al*., 2011), which can germinate again infecting more cells or leave the insect with feces. The severe energetic stress observed in *N. ceranae*-infected bees is probably due to the parasite itself, which develops exploiting the host cell mitochondria (Chen *et al*., 2009; Higes *et al*., 2007) and competing directly for key nutrients and energy resources. The infection firstly causes increased food consumption (Martin-Hernández *et al.*, 2011), immune suppression (Antùnez *et al*., 2009), degeneration of gut epithelial cells and shortened life spans (Higes *et al.*, 2007). Consequently, a decrease on population size can be observed, associated with a loss of adult bees. Evidences show that the microsporidium, due to epithelial lesions, increases the susceptibility to other pathogens, in particular facilitating trans-enteric viral infection (Higes *et al.*, 2008). In addition, the exposure to sub-lethal concentration of neonicotinoids in immature bees significantly enhanced the number of spore production per bee (Vidau *et al.*, 2011). Nowadays, the antibiotic Fumagillin-B (dicyclohexylammonium salt) is the only available compound to treat *N. ceranae* infection; however, it is no longer licensed in the EU states and recent reports provide controversial results about its efficacy and its effects related to residues in honey (Lopez *et al.*, 2008; Williams *et al.*, 2008). Considering that a multi-factorial approach is suggested to face out this hard issue, a possible role of gut microbial symbionts, commonly isolated from the bee digestive system, could be envisaged to contain and/or reduce *N. ceranae* spread and provide an eco-sustainable tool to beekeepers. Bifidobacteria and lactobacilli are known to confer health benefits to their host (Vasquez *et al.*, 2012). Recently, they have received special attention as a new option for the management of the natural bee microbiome (Hamdi *et al.*, 2011; Pătruică *et al.*, 2013). Commensal gut bifidobacteria and lactobacilli, whose beneficial effect is commonly exploited in vertebrates and invertebrates, can modulate the innate immune system and strengthen the epithelial barrier, limiting pathogenic contact with the epithelium by the secretion of antimicrobial compounds (bacteriocins) or direct competition (Crotti *et al.*, 2012; Hamdi *et al.*, 2011). In the present study, the effect of orally administered bifidobacteria and lactobacilli strains,

isolated from the gut of healthy honey bee adults and partially identified in this work, was investigated on *N. ceranae* artificially and naturally infected honey bees (*Apis mellifera* L.). Real-time PCR on DNA extracted from honey bee gut was performed to evaluate the effective probiotic colonization and *N. ceranae* load.

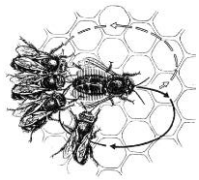
Materials and Methods

Bacteria isolation and culture growth conditions

Bifidobacterium asteroides DSM 20431, *Bifidobacterium coryneforme* C155 and *Bifidobacterium indicum* C449 were previously isolated from hindgut of bee (Scardovi and trovatelli, 1969) and available at the BUSCoB collection (Bologna University Scardovi Collection of Bifidobacteria) of the Department of Agricultural Sciences (Bologna). All three strains were grown on Tryptone Peptone Yeast extract (TPY) medium (Scardovi, 1986) in anaerobic atmosphere at 37 °C±1 for 24-48 h. To isolate lactobacilli, adult bees were collected from the apiary at CRA-API (Bologna, Italy) and surface-sterilized as suggested by Yoshiyama and Kimura (2009). The gut content of 100 honey bees was pooled, diluted in phosphate-buffered saline (PBS) and tenfold serial dilutions were prepared. Lactobacilli were isolated and enumerated in anaerobic condition at 35±1 °C by surface inoculation on the Man Rogosa Sharpe medium (MRS) (Becton Dickinson & Co., Mountain View, CA), containing 0.2% (w/v) sorbic acid (Sigma-Aldrich, Milan, Italy) and 0.1% (w/v) cycloheximide (Sigma-Aldrich) to inhibit yeast growth. Analysis were performed in triplicate. Following incubation, the number of colony forming units (cfu/g) of gut content was recorded, and mean Log values and standard deviations were calculated. Ten isolated colonies were selected and according to size, color and morphology, they were re-streaked and purified for further characterization. For long-term storage, isolates were stored at - 80 °C.

Pure cultures DNA extraction, 16S rDNA amplification and sequencing

Genomic DNA extraction was carried out using the Promega Wizard® Genomic DNA extraction kit (Promega, Madison, USA) from 2 ml of an overnight culture of the bacterial isolates, according to the manufacturer's instructions. The purity and concentration of extracted DNA were determined by measuring the ratio of the absorbance at 260 and 280 nm (Infinite 200 PRO NanoQuant, Tecan, Mannedorf, Switzerland). 16S rDNA amplification was performed with primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACT-3') (Lane, 1991) according to Gaggìa *et al.* (2013). After electrophoresis (1.5% w/v agarose gel at 75 V), gels were stained with ethidium bromide and visualized with the gel documentation system Gel DocTM XR (Bio-Rad, Hercules, CA, USA). Amplicons were then purified (Nucleospin gel and PCR clean-up kit; Macherey-Nagel GmbH & Co. KG, Germany) and sequenced by a commercial sequencing facility (Eurofins MWG Operon), using primers 27f and 1492r. Sequence chromatograms were edited and analyzed using the software program Finch TV version 1.4.0 (Geospiza Inc., Seattle, WA, USA) and the corrected sequences were subjected to taxon classification using RDP classifier, an available tool at the RDP-II website (Wang *et al.*, 2007).



N. ceranae spore suspension

N. ceranae-infected bees were obtained from an apiary located in Castello di Serravalle (Bologna, Italy), where infection is endemic. Twenty bees were crushed in saline solution (0.85% w/v), filtered through nylon mesh, and the resulting suspension was washed twice in distilled water, centrifuged (5,000 *g*, 5 min) and re-suspended in distilled water. Spore suspension was then overlaid on 40 ml of a 95% (w/v) Percoll® solution (Sigma P1644). Following 75 min of centrifuge at 9,000 *g*, spores, appearing as a cloud floating almost in the bottom of the tube, were sucked with a long pipette, washed and centrifuged three times with PBS to eliminate the residual Percoll® solution. The concentration of *N. ceranae* spores was then determined by haemocytometer count (Cantwell, 1970). Purified spores were suspended in 50 % (w/v) of sucrose solution necessary to yield a final concentration of 13,000 spores/µl. An aliquot was used for DNA extraction and species identification by a multiplex PCR-based method, according to Martìn-Hernández *et al.* (2007).

Experimental design

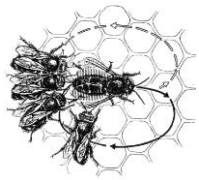
Honey bees were kept in small polyethylene, glass-sided cages measuring 10 × 10 × 20 cm, provided with gravity feeders. Each cage contained 22 bees and was incubated at 33 °C and 65 % RH in the dark (Costa *et al*., 2010). Each treatment was replicated three times, thus yielding 12 cages: bees fed with sugar syrup (1:1 sugar-water) (CTR); bees fed with sugar syrup enriched with bifidobacteria and lactobacilli (PRO); bees fed with sugar syrup and infected with *N. ceranae* at the 5th day of life (NOS); bees fed with sugar syrup enriched with bifidobacteria and lactobacilli, and infected with *N. ceranae* at the 5th day of life (NP).

Lactobacillus and *Bifidobacterium* feed additive preparation

The selected bifidobacteria (*B. asteroides* DSM 20431*, B. coryneforme* C155 and *B. indicum* C449) and lactobacilli (*L.kunkeei* Dan39, *L. plantarum* Dan91 and *L. johnsonii* Dan92) were grown in TPY broth (Scardovi *et al*., 1986) and MRS broth with 2 % (w/v) fructose (Olofsson et al., 2014), respectively. The overnight cultures were harvested and centrifuged to obtain a fresh pellet that was re-suspended in 100 mL of a 1:1 sugar/water sterilized solution. The final concentration of the microorganisms was between 10^6-10^7cfu/ml of sugar syrup. The microbial inoculum was prepared twice, stored at 4 °C and used daily to feed honey bees.

Bees, feeding and infection

Newly emerged honey bees (*Apis mellifera* L.) were obtained from a colony in the apiary at CRA-API (Bologna, Italy). Frames with emerging bees capped into cells close to emergence were brought into the laboratory and incubated at 33 °C and 65 % RH, in the dark. Emerged bees were picked one by one and caged in the plastic boxes. The sugar syrup, with or without microorganisms, was administered to bees via gravity feeders (Costa *et al*., 2010) fitted into each cage from the first day of life for 13 days. *N. ceranae* infection was carried out individually on anesthetized 5-day-old bees (Martin-Hernández *et al*., 2011) with one µl of sugar syrup containing 13,000 spores.



DNA extraction from gut samples

DNA extraction was performed at day eight post-infection (13-day-old bees). Bees were anesthetized with CO_2 and guts were extracted with a sterilized tweezers by stretching their abdomen (Fries *et al*., 2013). For bifidobacteria and lactobacilli quantification, DNA extraction was performed on 200 mg of pooled guts derived from five bees per cage by using the QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK), according to the manufacturer's instructions. The recommended lysis temperature was increased to 95 °C. DNA extraction for *N. ceranae* analysis was carried out from individual gut derived from ten bees per cage (thirty bees per treatment). Each single sample was then processed using the ZR Tissue and Insect DNA MicroPrep extraction kit (Zymo Research, CA, USA) with a modified protocol, including two hours of incubation at 55 °C with 20 µL of 20 µg/µl proteinase K (Sigma-Aldrich). Purity and concentration of extracted DNA were measured with the Infinite 200 PRO NanoQuant. The DNA was stored at -20 °C until use.

Real-time PCR

Quantification of *Bifidobacterium* spp. and *Lactobacillus* spp. was carried out by real-time PCR in both supplemented (PRO, NP) and unsupplemented groups (CTR, NOS) according to Baffoni *et al*. (2012). Obtained data were then transformed to obtain the Log number of bacterial cells/g of gut content, according with the rRNA copy number available at the rRNA copy number database (Klappenbach *et al*., 2001; Lee *et al*., 2009). Quantification of *N. ceranae* was carried out on DNA extracted from individual honey bee guts (ten/cage) from all experimental groups (CTR, PRO, NOS and NP) according to Forsgren and Fries (2010). Data were then transformed to obtain the Log number of spores/bee, according with preliminary evidence for 10 copies of the 16S rRNA gene in the *N. ceranae* genome (J. Evans, personal communication).

Statistical analysis

Real-time PCR data on the experimental groups were analysed using R software (R Development Core Team, 2005) and tested for normality (Shapiro–Wilks test) and homogeneity of variance (Levene's test). In case that one of those conditions was not met, the normalization was attempt with Box-Cox analysis on X'=log(X+0.1) (Box and Cox, 1964). If assumption of normality could not be satisfied by transformation, data were analysed by Kruskal-Wallis test ($P < 0.01$) followed by the pairwise Kolmogorov-Smirnov test. $P < 0.05$ was considered significant.

Results

Enumeration and characterization of isolates by 16S rRNA gene sequence analysis

Presumptive lactobacilli were isolated from honey bee guts on MRS agar plates and count was 8.67±0.03 Log cfu/g of gut content. The partial bacterial 16S rDNA gene sequences from ten isolates (Accession number KP114138-KP114147) were subjected to taxonomical identification by the use of RDP tools classifier and seqmatch (Table 1).

Table 1 Identification of isolates based on 16S rRNA sequences and Ribosomal Database Project tool.

Strain	Closest match (accession number)	Gene Bank (accession)	S_ab score*
Dan6	*Lactobacillus kullabergensis* (S004044228)	KP114138	1.000
Dan10	*Bifidobacterium asteroides* (S002908335)	KP114139	0.981
Dan25	*Lactobacillus kullabergensis* (S004044228)	KP114140	1.000
Dan39	*Lactobacillus kunkeei* (S004079211)	KP114141	0.988
Dan44	*Bifidobacterium asteroides* (S002908335)	KP114142	0.981
Dan47	*Lactobacillus kimbladii* (S004044226)	KP114143	0.995
Dan70	*Lactobacillus helsingborgensis* (S004044231)	KP114144	1.000
Dan91	*Lactobacillus plantarum* (S000118676)	KP114145	1.000
Dan92	*Lactobacillus johnsonii* (S000925461)	KP114146	1.000
Dan101	*Lactobacillus helsingborgensis* (S004044231)	KP114147	0.999

*S_ab score is the percentage of shared words between sequences compared.

Real-time PCR

Results of the bacterial counts are shown in Table 2. Statistical analysis showed that differences in bifidobacteria as well as lactobacilli population were highly significant among groups (Kruskal-Wallis test; $P<0.01$). The pairwise comparison with the Kolmogorov-Smirnov test (CTR vs PRO; NOS vs NP) indicated that bifidobacteria and lactobacilli population PRO and NP groups had a significant increase compared to groups fed with only sugar syrup ($P<0.05$), whereas no significant changes were observed between CTR and NOS ($P = 0.27$), PRO and NP ($P = 0.08$ for bifidobacteria; $P = 0.06$ for lactobacilli).

At day 13, 90% of the bees were still alive in all groups, independently from the treatments, with a significant difference in *N. ceranae* level (Kruskal-Wallis, $P < 0.01$), (Figure 1). A high number of *N. ceranae* was detected in NOS and NP groups (7.75±0.35 and 6.67±1.81 mean Log spores/bee±sd respectively), whereas bees not artificially infected, CTR and PRO, showed a low natural presence of the microsporidia (2.04±0.91 and 0.78±0.81 mean Log spores/bee±sd respectively). Post hoc pairwise comparison indicated a significant difference between NOS and NP, as well as CTR and PRO ($P < 0.05$). Both treated groups NP and PRO showed more than one Log reduction of spore count, resulting in more than 90% reduction of the parasite load. *N. ceranae* detection was positive in all samples, both in NOS and NP groups, with 20.7% of samples presenting a spore count under the inoculated dose in NP. The PRO group showed the absence of *N. ceranae* in 53.3% of insects, whereas in CTR the detection was positive in all samples.

Table 2 *Bifidobacterium* spp. and *Lactobacillus* spp. quantification by real-time PCR

Group	*Bifidobacterium* spp.	*Lactobacillus* spp.
CTR	5.94±0.96	8.41±0.32
PRO	8.13±0.14	8.92±0.09
NOS	6.31±0.20	8.28±0.26

NP	8.00±0.12	8.83±0.09

CTR: control group fed with sugar syrup (1:1 sugar-water); PRO: group fed with sugar syrup enriched with bifidobacteria and lactobacilli; NOS: group fed with sugar syrup and infected with *N. ceranae*; NP: group fed with sugar syrup enriched with bifidobacteria and lactobacilli and infected with *N. ceranae*. Quantification was performed *via* qPCR using SybrGreen chemistry. Results are the mean (±sd) of three independent PCR analysis and expressed as Log cfu/g.

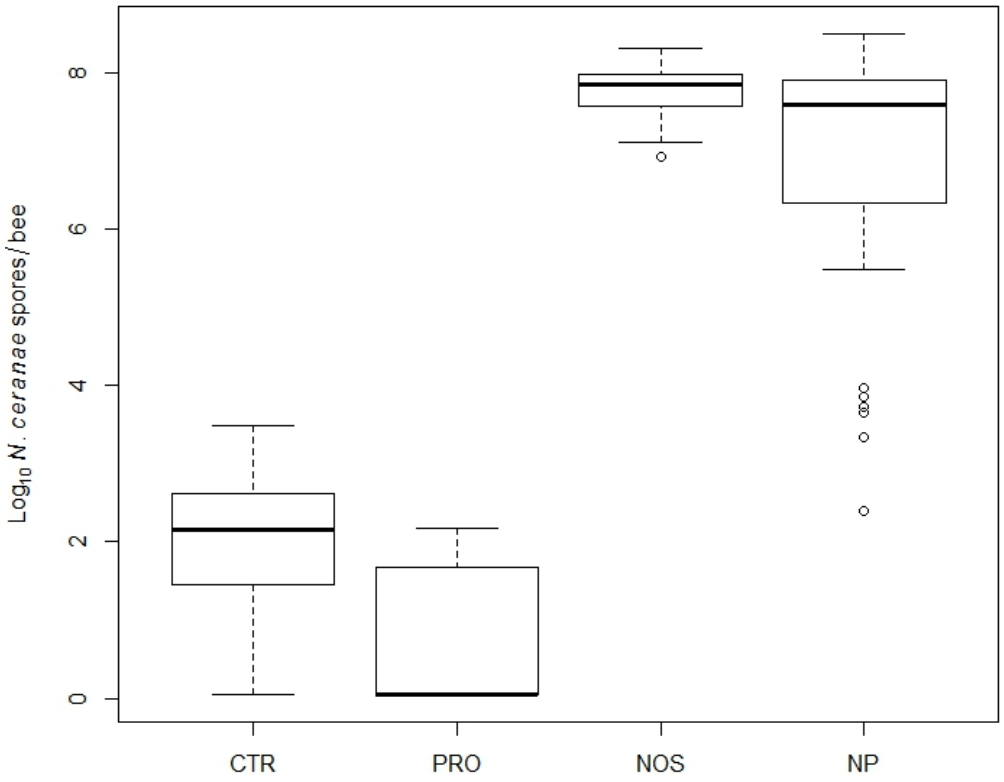
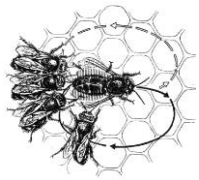


Figure 1 *N. ceranae* quantification by real-time PCR. CTR: group fed with sugar syrup (1:1 sugar-water); PRO: bees fed with sugar syrup enriched with bifidobacteria and lactobacilli; NOS: bees infected with spores of *N. ceranae* and fed with sugar syrup; NP: bees infected with spores of *N. ceranae* and fed with sugar syrup enriched with bifidobacteria and lactobacilli. Results are given as box plots, where the horizontal line indicates the median, the box the first quartile of the data above and below the median.

Discussion

The use of gut microbial symbionts as dietary supplementation has been scarcely investigated in honey bees and only a few studies confirm the hypothesis that such microorganisms (multiple strains or single strain) or their metabolites could improve the health status of bees (Audisio and Benítez-Ahrendts, 2011; Audisio *et al.*, 2015; Pătruică and Mot, 2012; Pătruică *et al.*, 2012). In this work, the administration of multiple strains, isolated from honey bee gut, was preferred to single strain administration. The combination



of several strains with different properties may result more effective in restoring the commensal community of the gut microbiota when a disturbance arises. The supplemented bifidobacteria, which represent the three species of the bee gut so far described, were already available in the BUSCoB collection of our laboratory. Among isolated lactobacilli, *L. johnsonii* was selected considering the results obtained by Maggi et al. (2013), who showed the efficacy of lactic acid obtained from a culture of *L. johnsonii* CRL1647 to reduce the intensity of *N. ceranae* infection. *L. plantarum* was chosen as it is a widely spread species and most of the strains are bacteriocin producers (Nes *et al.*, 1996). *L. kunkeei* is a dominant species in honey bee gut, and a number of characterized strains exhibit antimicrobial activity against some bacterial bee pathogens (Vasquez *et al.*, 2012). Interestingly, the identification of the further strains from plate led to the recovery of *L. kullabergensis, L. kimbladii* and *L. helsingborgensis*, which are the new species recently isolated by Olofsson *et al.* (2014). Since few information is available on those strains, they were not considered for the dietary administration.

Probiotic bacteria, such as bifidobacteria and lactobacilli, find their main application in the prevention of gastrointestinal infection and disease more than a curative approach (Gaggìa *et al.*, 2010); therefore, the supplementation was performed daily from the first day of insect life, whereas *N. ceranae* spore infection was induced in 5-day-old bees, as described by Martin-Hernández *et al.* (2011). Bifidobacteria and lactobacilli daily fed to bees with sugar syrup significantly increased their population in honey bee gut and led to a significant decrease of *N. ceranae* spore load in artificially infected bees, thus showing an antagonistic behaviour to the parasite spread. Moreover, their concentration was not influenced by infection. Interestingly, the low spore number detected in CTR evidenced that bees were naturally infected with the parasite; this number significantly decreased in PRO group, which only differ from CTR in the bacterial supplementation. As reported by Higes et al. (2008), *N. ceranae* is frequently found in both healthy and weak honey bee colonies. Unexpectedly, our results also outlined the importance of a preventive, long-term supplementation in the presence of a lighter infection, as probably occurs in field condition. In that sense, the treatment could be more effective if compared to artificially infected bees where the spore load immediately reaches a high sprawling number and the gut bacteria could only contain the infection. Although the experiments could not be properly compared, Audisio *et al.* (2015) observed a reduced intensity of *Nosema* spp. in hives treated with *Lactobacillus johnsonii* CRL1647. The mechanisms at the basis of the observed spore reduction is far to be elucidated, since many aspects of the *Nosema*-honey bee interactions remain not understood and no studies are available. However, it is known that lactobacilli and bifidobacteria exert an antagonist effect against a wide range of pathogens through the production of antibacterial metabolites, *e.g.* organic acids and bacteriocins (Gaggìa *et al.*, 2011). Recently, this activity has been assessed against *Paenibacillus larvae* and *Melissococcus plutonius* (Audisio *et al.*, 2011; Wu *et al.*, 2013; Yoshiyama and Kimura, 2009), the causal agents of the American and European foulbrood, respectively. Moreover, the increased number of beneficial gut microorganisms due to feed supplementation may provide the host with protection by competitive exclusion, reducing the chance of pathogen contact with the epithelial cells. With respect to *N. ceranae*, considering that germination occurs only at pH values higher than five (De Graaf *et al.*, 1993), it could be also

hypothesized that the natural presence of these bacteria, in certain amount, could lower the intestinal pH and contrast the germination process, leading to spore elimination along with undigested residues. The laboratory trials performed by Maggi *et al*. (2013) support this hypothesis, as mentioned above.

Considering that, beekeepers constantly treat hives with formic, lactic and acetic acids to prevent pathogen infections and parasites, the use of bifidobacteria and lactobacilli, in the light of their metabolites production, may represent a natural tool to protect honey bee from pathogens. In our opinion, this study shows the possible role of beneficial bacteria supplementation to support honey bee health against *N. ceranae* infection as a preventive measure and new tool for the management of the bee microbiome. Further studies are necessary to investigate the efficacy of the supplemented bacteria under field conditions.

Acknowledgement
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PAPER 8

Impact of beneficial bacteria supplementation on the gut microbiota, colony growth and productivity of *Apis mellifera* L.

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Abstract

Honey bees are important pollinators of several crops and for that reason have great biological and economic value. Several threats are seriously affecting honey bees resulting in an increasing need for natural products to sustain the beekeeping sector. This need has been emphasised by the engagement of researchers in recent years towards bee health and in particular towards bee interaction with its own gut microbiota. The modulation of the microbiota has been recognized as a practical and successful approach in the entomological field for the management of insect-related problems. However, to date, only a few studies have attempted to investigate the effect of bacterial supplementation as a strategy to improve the health status of colonies, in terms of productivity and boosting the presence of beneficial microorganisms within the gut of new generation bees. To this purpose, a preparation of sugar syrup containing bifidobacteria and lactobacilli isolated from bee gut was sprayed on the frames of an apiary located in open field. Treated and control hives were monitored for one month for brood extension, honey and pollen harvest. The presence of beneficial gut microorganisms within bee gut was investigated with denaturing gradient gel electrophoresis and next generation sequencing. The administered bacteria led to a significant increase of brood population, pollen and harvestable honey in honey supers; analysis of the gut microbiota on the new generation of bees showed an increase of Acetobacteraceae and Bifidobacterium spp., which are known to be involved in bee nutrition and protection. Although further experiments are envisaged, the research stresses the long-term effects of the microorganism-based approach to sustain bee health.

Introduction

Honey bees are widely recognized as important pollinators of several crops, thus contributing to the diversity and quality of the human diet (Eilers *et al.,* 2011). Moreover, hive products (honey, propolis, royal jelly) are extensively used as dietary supplements for their high nutritional value and antimicrobial properties (Ellis *et al.*, 2015). Considering the ecological and economic value of this insect, threats affecting honey bees (*e.g.* pathogens, parasites, agro-chemicals, climate change, anthropogenic disturbances) have prompted the scientific community to look for new mitigation tools, as alternatives to therapeutic agents, to preserve honey bee health. In the last decade, gut microbial symbionts have received much attention for their contribution to the health status of bees (Anderson *et al*., 2011; Hamdi *et al.*, 2011). As in vertebrates, the honey bee gut microbiota has pivotal functions (Crotti *et al.*, 2012; Martinson *et al*., 2011), involving multiple interactions that depend on microbiota composition, host genotype, and environmental factors (Wong *et al.*, 2014). The core microbiota of honey bee gut (Moran *et al.*, 2012) seems to be highly specialized to guarantee the overall functionality of the microbiome and its interaction with the host (Kwong and Moran, 2016).

Lactobacilli and bifidobacteria are among the microbial groups that provide health benefits to honey bees (Crotti *et al.*, 2010; Vasquez *et al.*, 2012). In particular, they assure enzymatic activities for nutrient absorption, facilitate a vigorous immune response against invading pathogens (Crotti *et al.*, 2010), preserve hive products from spoilage microorganisms and interact with host genome and physiology (Kwong and Moran, 2016). They are present in the gut microbiota of adults bees and larvae, in the honey stomach and in the hive environment (Anderson *et al.*, 2013; Olofsson and Vaquez, 2008; Gaggìa *et al.*, 2015).

Both lactobacilli and bifidobacteria, currently used as probiotics in human and animal nutrition, have been shown to decrease in numbers when exposed to stress (Baffoni *et al*., 2012; Gaggìa *et al.*, 2010). Specific changes in the composition of the microbial gut community and perturbation of gut functionality, referred to as dysbiosis (Crotti *et al.*, 2012), can influence the health status of bees (Hamdi and Daffonchio, 2011). For example, Koch and Schmid-Hempel (2012) reported that gut microbiota changes in *Bombus terrestris* can be responsible for specific immune phenotypes.

The modulation of the gut microbiota has been recognized as a practical approach with strong potential in the entomological field for the management of insect-related problems (Crotti *et al.*, 2012). This is particularly useful within eusocial insects living in a dense population of individuals that make up a colony and are continuously in contact due to grooming and trophallaxis activities. In the present work, we explored the hypothesis that the ingestion of selected gut bacteria can provide great benefits to bees, both improving productivity and boosting the presence of beneficial gut microorganisms. A sugar syrup solution containing bifidobacteria and lactobacilli was sprayed on the frames of an apiary located in open field close to linden trees (*Tilia* spp.) and before their bearing period. In order to assess hive productivity, treated and control hives were monitored for two months for brood extension, honey and pollen harvest. In addition, gut microbiota was studied with denaturing gradient gel electrophoresis (DGGE) and next generation sequencing (NGS) at the end of the experiment.

Materials and Methods

Beehive standardization and environmental conditions.

The experimental apiary was established nearby Bologna, Emilia Romagna (Italy). The apiary was positioned into a local regional park (Parco dei Gessi Bolognesi e Calanchi dell'Abbadessa, Bologna, Italy) at 210 m a.s.l and was composed of 18 hives. The climate is continental, with an average temperature of 23.5 ±2 °C during the experimental period (May 2014) (ARPA, 2014). Frames were obtained from beehives managed with organic beekeeping practices and treated for *Varroa destructor* with oxalic acid (4.2%) during the winter of 2013/2014 (Nanetti *et al.*, 2003). The bee colonies were kept in standard modified Dadant Blatt hives (Zappi-Recordati, 1956) of nine frames each, and all of which were equally treated with regard to food resources, bee population, and sealed/open brood (Delaplane *et al.*, 2013). The beehives were then divided in two equal groups as follows: control (CTR)- hives receiving either a 1:1 solution of sugar syrup (50% sugar and 50% deionized water; treated (TRT)- hives receiving the bacterial supplement suspended in a 1:1 solution of sugar syrup. Both CTR and TRT hives where randomly placed in the experimental apiary; in addition, landmarks and paintings on the hives entrance were used to limit worker drift between colonies according to Von Frisch (1967).

Bacterial strains, supplement preparation and administration

Bifidobacterium asteroides DSM 20431, *B. coryneforme* C155 and *B. indicum* C449 (Scardovi and Trovatelli, 1969), available at the Bologna University Scardovi Collection of Bifidobacteria, were grown anaerobically at 37 °C in Tryptone Peptone Yeast extract (TPY) liquid medium (Scardovi, 1986). *Lactobacillus kunkeei* Dan39 (KP114141), *L. plantarum* Dan91 (KP114145) and *L. johnsonii* Dan92 (KP114146) isolated from bee gut (Baffoni *et al.*, 2016) were grown in de Man Rogosa Sharpe medium (MRS broth) (Becton Dickinson & Co., Mountain View, CA) with 1 % (w/v) fructose (Sigma-Aldrich, Milano, Italy), as suggested by Olofsson *et al.* (2014). Overnight cultures were inoculated in 500 ml flasks in the same conditions mentioned above for 48h. Cells of bifidobacteria and lactobacilli were then harvested by centrifugation (4000g) to obtain fresh pellets that were mixed and re-suspended in five 400 ml flasks of a 1:1 sterilized solution of sugar syrup (50% sugar and 50% deionized water). The final concentration of the microorganisms, assessed between 10^7-10^8 cfu/ml of sugar syrup, was checked in TPY agar for bifidobacteria and MRS agar for lactobacilli by plating 1 ml of ten-fold serial dilutions of the bacterial supplement. The microbial inoculum was prepared weekly before use and administered within 3 hours of its preparation. Tolerance of the selected strains to 1:1 solution of sugar syrup was tested by plating the final suspension after 5-hour-storage at room temperature and after 24 hours at 4°C.

Before the *Tilia* spp. honey flow, CTR and TRT hives received their respective treatment once a week for 4 weeks (the last three weeks of May and the first week of June). Treatments were directly sprayed onto the surface of each frame to reach adult bees and the brood. Each hive received approximately 130 ml of sugar solution.

Bee productivity assessment and sample collection

To monitor the productivity of the colonies, the following parameters were evaluated in the hive brood chambers according to Audisio *et al*. (2015): brood areas, stored pollen and honey. In addition, honey in the hive supers was also monitored. In the brood chambers, data were collected at the beginning of the experiment (T0), after 30 days (T30), and 60 days (T60) by estimating the box surface covered with brood, honey or pollen with the help of a mask frame counting 260 squares of 4 cm^2 each (Figure 1). A picture of each frame (both sides) was taken and then analyzed with Fiji, an open platform for scientific image analysis, freely available online (Schindelin *et al*., 2012). Harvestable honey (from honey supers) was collected at T60 (at the end of the *Tilia* spp. bearing) and extracted according to the beekeeping practice, centrifuged and weighted separately.

A month after the last treatment (T60), 4-5 bees per hive (30 bees from CTR hives and 30 bees from TRT hives), expected to be approximately 16 days old, were captured from the external frames (Moran *et al.*, 2012; Moore *et al.*, 1987); guts were immediately extracted with a sterilized tweezers by stretching the abdomen and conserving on dried-ice until transport to the laboratory.

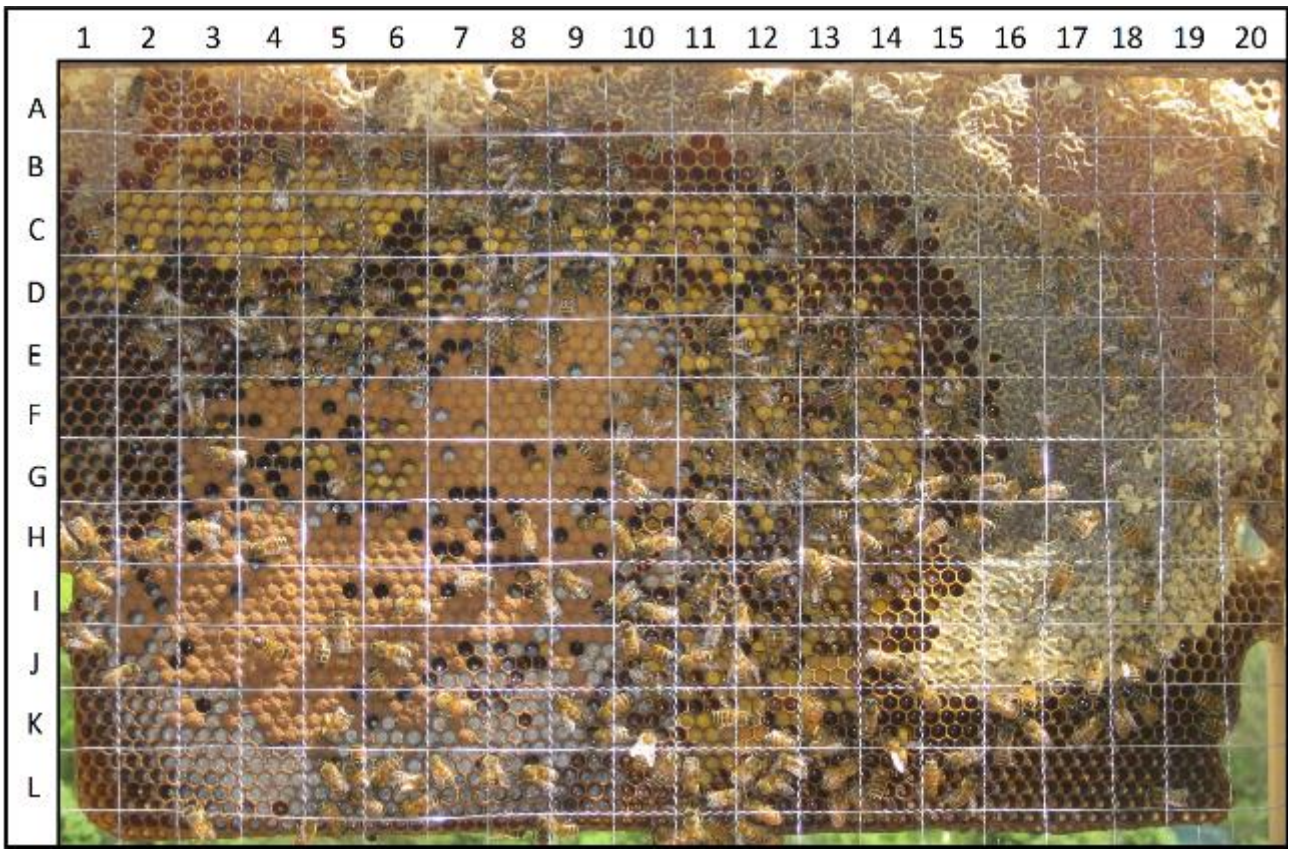

Figure 1 Mask frame to count brood, honey and pollen surfaces

DNA extraction

Pooled digestive tracts were used for total genomic DNA extraction, which was performed in triplicate using the QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK), according to the manufacturer's instructions. The recommended lysis temperature was increased to 95

°C. The quality and quantity of DNA samples were quantified using Infinite® 200 PRO NanoQuant (Tecan, Mannedorf, Switzerland).

PCR-DGGE

Three PCR-DGGE analyses were performed to investigate total eubacteria, *Lactobacillus* and *Bifidobacteriaum* populations. Primers for DNA amplification are listed in Table 1. The PCR programs and DGGE analyses of the amplified products, using the Dcode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA), were performed as described previously (Aloisio *et al.*, 2014; Gaggìa *et al.*, 2015; Gaggìa *et al.*, 2013). Cluster analysis were carried out using the Gel compare II v6.6 (Applied Maths, St. Martens-Latem, Belgium), by the UPGMA algorithm based on the Pearson correlation coefficient with an optimization coefficient of 1%. Relevant bands in the gels were excised from the gels and purified (Gaggìa *et al.*, 2015). Sequencing was carried out by Eurofins Genomic (Ebersberg, Germany) and obtained sequences were assigned to bacterial phyla based on comparisons with the GenBank database by using the BLASTN algorithm (Altschul *et al.*, 1990).

Table 1 DGGE and NGS primers targeting 16S rRNA

Target	Primer Sequence	References
Eubacteria (DGGE)		
HDA1	5'- ACTCCTACGGGAGGCAGCAGT-3'	Walter, 2000
HDA2	5'- GTATTACCGCGGCTGCTGGCA-3'	
GC-clamp (F)	5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG-3'	
Bifidobacterium spp.		
Bif164-F	5'- GGGTGGTAATGCCGGATG-3'	Satokari *et al*., 2001
Bif662-R	5'-CCACCGTTACACCGGGAA-3'	
GC-clamp (R)	5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3'	
Lactobacillus spp.		
Lac1	5'-AGCAGTAGGGAATCTTCCA-3'	Walter, 2001
Lac2	5'-ATTYCACCGCTACACATG-3'	
GC-clamp (R)	5'- CGCCCGGGGCGCGCCCCGGGCGGCCCGGGGGCACCGGGGG-3'	
Eubacteria (NGS)		
Fw	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGG NGGCWGCAG-3'	Illumina, 2017
Rev	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACH VGGGTATCTAATCC-3'	

(F) and (R) refers to the clamped primer: (F) forward, (R) reverse

Preparation of 16S V3 and V4 rRNA amplicons for Illumina MiSeq sequencing

The V3-V4 regions of the 16S gene were amplified using the primers listed in Table 1 as per Illumina 16S metagenomic sequencing library preparation guide (Illumina, 2017). Illumina sequencing adapters and dual-index barcodes were added to amplicons using the Nextera XT index kit (Illumina, San Diego, CA). Amplicons were cleaned and quantified using the Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies, CA, USA) and were pooled in an equimolar mode following library preparation and sequenced on the MiSeq platform. Samples were sequenced on the Illumina MiSeq platform at the Teagasc Food Research Centre Moorepark facility using a 2x300 nucleotide paired-end protocol.

Bioinformatic analysis

Resulting 300 bp paired-end reads were assembled using FLASH (Magoč and Salzberg 2011). Further sequence read processing was performed using QIIME suite of tools, (Caporaso *et al.*, 2010a) including quality filtering based on a quality score of > 25 and removal of mismatched barcodes and sequences below length thresholds. Denoising, chimera detection and clustering into operational taxonomic units (OTUs) (97% identity) were performed using USEARCH version 7 (Edgar, 2010). OTU sequences were aligned using PyNAST (Caporaso *et al.*, 2010b) and taxonomy assignment was determined using the SILVA SSU Ref database release 111. Considering the reclassification of some genera in newly described families (Adeolu *et al*., 2016, Kwong and Moran, 2013), the taxonomy assignment was changed accordingly.

Statistical analysis on bee colonies parameters

Bee colonies parameters (brood, pollen and honey within the frames) were analysed using gls function of 'nlme' package (Pinheiro *et al*., 2016) in R 3.3.1 (R Core Team, 2016). The repeated measure structure in the data sets was included using the grouped data function specifying that observations were repeated within colonies. Models with different variance-covariance structure (compound symmetry, unstructured, autoregressive and autoregressive with heterogeneous variances) were compared. Model comparison was performed using the ANOVA function. Best fitting models were selected according to AIC and logLik values, the unstructured model resulted to be the best one. Data on honey super production were collected at the end of the experiment and analyzed with R software using ANOVA since they showed normality and homoscedasticity.

Results

Bacterial survival in the sugar syrup

Microbial analyses of the inocula demonstrated no influence of 1:1 sugar syrup solution on the viability of bifidobacteria and lactobacilli monitored at T0 (7.73±0.05 and 8.93±0.02, respectively) and after 5-hour-storage at room temperature (7.69±0.01 and 8.90±0.01, respectively). The same test was repeated after 24 hours and the viability count was reduced by 1 log (6.69±0.02 and 7.83±0.02, respectively).

Honey, pollen and brood production

Nine hundred and fifty pictures from the hive brood chambers were obtained and analysed with Fiji software at T0, T30 and T60, respectively, in both CTR and TRT hives. The extension areas were calculated, taking into account separately the different parameters for each hive; results are reported in Figure 2. Brood surface was significantly higher ($p<0.05$; Figure 2A) in TRT hives compared to CTR hives at T30, with a 46.2% increase. Stored pollen was significantly higher in supplemented hives at T60 ($p<0.05$; Figure 2B), while stored honey did not show any significant difference (Figure 2C). With respect to the harvestable honey from hive supers, a remarkable increase ($p<0.01$) in TRT group was observed at T60. The hive supers contained 242 kg and 152 kg of pure linden honey in TRT and CTR hives, respectively, corresponding to 59.21% higher productivity ($p<0.05$) in hives supplemented with beneficial microorganisms (Figure 2D).

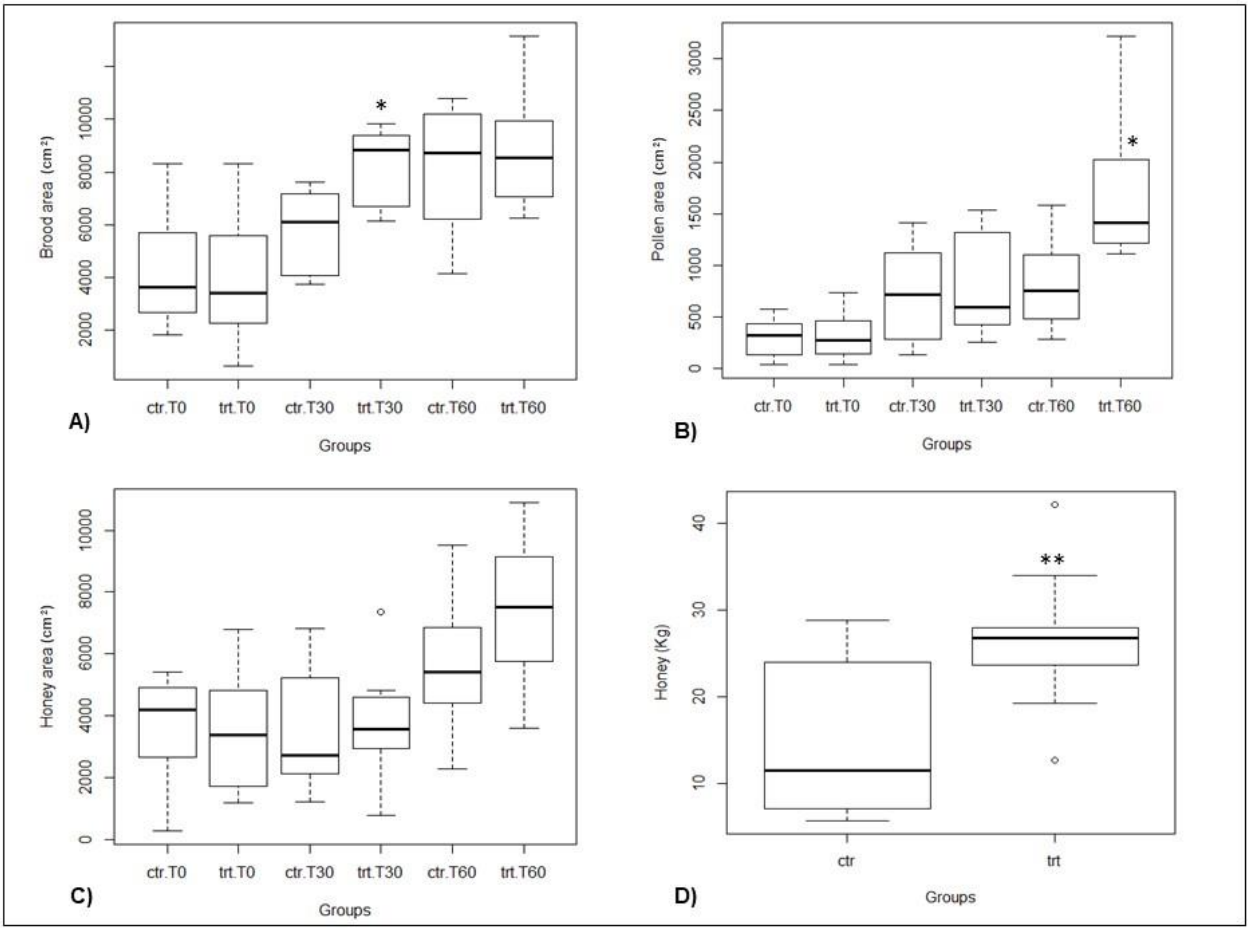

Figure 2 Box plot on productivity parameters. A) Brood; B) Pollen; C) Honey area; D) Honey in hive supers. Asterisk indicates a significant value (*=p<0.05, **p<0.01) of the treated group compared with the control group at the considered time point with respect to the previous time point.

PCR-DGGE

Three different 16S rDNA PCR-DGGE analyses (Eubacteria, *Lactobacillus* group and *Bifidobacterium* spp.) were performed to investigate the structure of the microbial communities associated with the honey bee gut, following the administration of the selected gut microorganisms. Cluster analysis of the three different PCR-DGGE revealed more than 90% similarity between CTR and TRT groups, although the two groups clustered separately (Figure S1). The nucleotide sequences from excised DGGE bands have been deposited in GenBank (Table 2). Three major bacterial species were identified by partial 16S rRNA band sequencing in the PCR-DGGE targeting Eubacteria (Figure 3A): *Giliamella apicola*, *Snodgrassela alvi* and *Commensalibacter intestini*, belonging to the *Orbaceae*, *Nesseiraceae* and *Acetobacteraceae* families respectively. Several species were identified from the PCR-DGGE targeting lactobacilli: *L. kullabergensis, L. mellis, L. kimbladii, L. apis L. melliventris* and *L. kunkeei* (Figure 3B). No bands having the same migration distance of the lactobacilli used in the administration trial (ladder of Figure 3B) were detected. The PCR-DGGE addressed to the identification of *Bifidobacterium* spp. (Figure 3C) led to the recovery of two species, *B. asteroides* and *B. coryneforme*. Some bands assigned to *B. asteroides*

and *B. coryneforme* had the same migration distances of the administered strains both in control and treated samples.

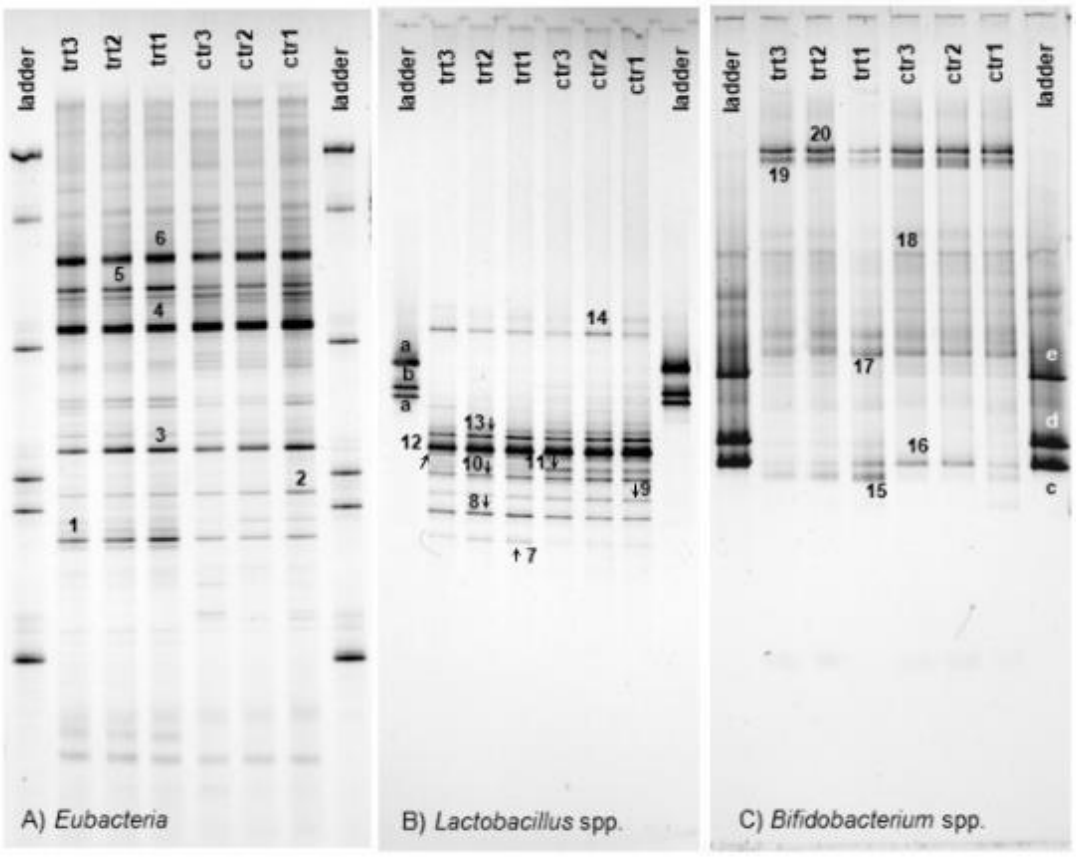

Figure 3 DGGE profiles and bands. A) DGGE bands obtained with universal primers; B) DGGE bands obtained with primers targeting *Lactobacillus* spp., C) DGGE bands obtained with primers targeting *Bifidobacterium* spp. Letters in ladder profiles indicate administered bacteria (a: *L. kunkeei* Dan39 and *L. plantarum* Dan91; b: *L. acidophilus* Dan92; c: *B. asteroides* DSM 20431; d: *B. indicum* C449; e: *B. coryneforme* C155).

Table 2 Best-match phylotypes identification of excised DGGE bands

Band	pb	Closest match	Accession Number	% identity
1	169	*Commensalibacter intestini*	KU764720	99
2	156	*Snodgrassella alvi*	KU764721	99
3	155	*Snodgrassella alvi*	KU764722	100
4	163	*Giliamella apicola*	KU764723	99
5	163	*Giliamella apicola*	KU764724	99
6	159	*Giliamella apicola*	KU764725	99
7	293	*Lactobacillus kullabergensis*	KU764726	99
8	293	*Lactobacillus kullabergensis*	KU764727	99
9	292	*Lactobacillus mellis*	KU764728	100
10	293	*Lactobacillus kimbladii*	KU764729	100
11	293	*Lactobacillus kimbladii*	KU764730	100

12	293	*Lactobacillus apis*	KU764731	100
13	293	*Lactobacillus melliventris*	KU764732	100
14	292	*Lactobacillus kunkeei*	KU764733	100
15	430	*Bifidobacterium* spp.	KU764735	99
16	430	*Bifidobacterium* spp.	KU764736	99
17	431	*Bifidobacterium* spp.	KU764738	99
18	431	*Bifidobacterium* spp.	KU764742	99
19	432	*Bifidobacterium* spp.	KU764743	99
20	432	*Bifidobacterium* spp.	KU764744	99

NGS

MiSeq sequencing yielded a total of 2,949,934 raw reads, which were qualitatively filtered to obtain an average of 184,749 reads per sample. Sequences were then clustered into 92 OTUs, which were assigned to taxa from phylum to genus level (Figure 4). Phylum *Proteobacteria* included the vast majority of OTUs, with 86 and 90% relative abundances for CTR and TRT groups, respectively. *Orbaceae* family participated in the microbial community of Proteobacteria with the highest relative abundance both in the TRT (57%) and CTR (64%) groups. The other two most represented families of *Proteobacteria* were *Neisseraceae* and *Acetobacteraceae* with a relative abundance of 16.3% and 3.6% in CTR group and 15.9% and 8.8% in TRT group. *Firmicutes* and *Actinobacteria* were the second and third most represented phyla with a relative abundance of 10.9% and 1.2% in CTR group and 7.03% and 1.8% in TRT group respectively. OTUs of phylum *Actinobacteria* were totally assigned to family *Bifidobacteriaceae* and genus *Bifidobacterium*. OTU assigned to *Firmicutes* were predominantly of family *Lactobacillaceae* with a 100% assignment to the *Lactobacillus* genus in both groups with a relative abundance of 10.8% and 7% in CTR and TRT groups, respectively.

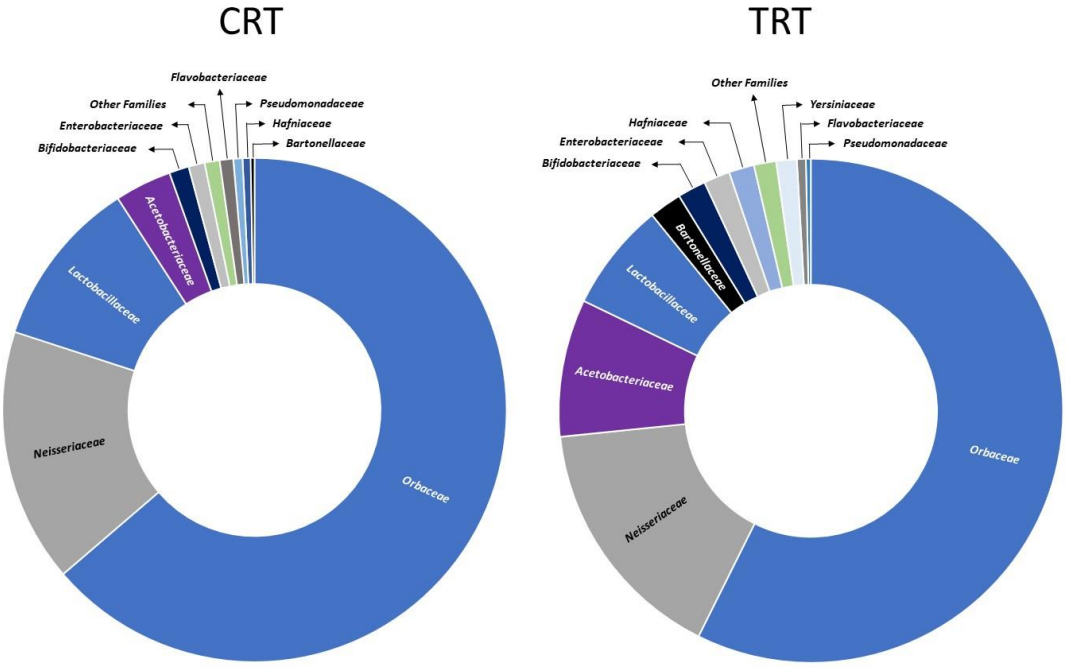

Figure 4 Pie charts representing families relative abundance in CTR and TRT groups

Discussion

This pilot study aimed at evaluating the effects of a microbial supplement on honey bees reared in a natural environment. by monitoring the productivity of the colonies and the gut microbiota composition. The microbial supplement, consisting of bifidobacteria and lactobacilli strains, was chosen according to a previous study (Baffoni et al., 2016). A weekly administration for one month was selected to cover all the edging brood (approximately 21 to 24 days) and the spraying method was adopted relying on the hygienic behaviour of bees. It is already established that a balanced gut microbiota offers a wide range of metabolic, trophic and protective functions, supporting honey bees' activities (Ellegaard et al., 2015; Evans and Pettis, 2005; Hamdi et al., 2011; Lee et al., 2015). The positive effect of beneficial bacteria application has been already reported and reviewed by several authors (Alberoni et al., 2016; Audisio and Benítez-Ahrendts, 2011; Audisio et al., 2015; Corby-Harris et al., 2016; Pătruică and Mot, 2012; Pătruică et al., 2012) and notably host-derived microorganisms are preferred, as also underlined by Ptaszyńska et al. (2016). The administered bacteria lead to a significant increase in brood population, pollen and harvestable honey in honey supers. The significant increase evidenced in stored pollen and harvestable honey at T60 could be associated to brood increase assessed at T30, according to the studies performed by Pankiw *et al*. (2008 and 1998). Authors observed that brood increase was directly proportional to brood pheromone levels, which positively affect pollen foragers and consequently pollen foraging. Moreover, a higher amount of pollen supports both brood and bee health status and the brood increase generates an expansion of bee colonies and, consequently, a higher production of harvestable honey (Di Pasquale *et al*., 2013; Keller *et al.,* 2005). Similar results have been obtained by Audisio and Benítez-Ahrendts (2011) and Audisio et al. (2015), although different experimental settings have been used. The authors performed two different trials with a cell suspension (10^5 ufc/ml sugar syrup) of *L. johnsonii* CRL1647 (every 15 days for three months and a monthly administration for one year). All the analysed parameters (open and operculated brood area, bee number, honey storage and honey yield) were significantly higher in the treated groups. Sabaté *et al*. (2012) obtained comparable results with the supplementation of spores of *B. subtilis* Mori2, isolated from honey. Concerning the analysis of the honey bee gut microbiota, our previous experiment reported a significant increase of lactobacilli and bifidobacteria soon after bacterial administration (Baffoni *et al*., 2016). However, in laboratory conditions, it is not possible to monitor the long-term effects. In this study, the analysis of the gut microbial community on the new generation of bees confirmed the presence of Alpha- (*Acetobacteraceae*), Beta- (*Neisseraceae* with *S. alvi*), Gamma- Proteobacteria *(Orbacea*e with *G. apicola*), Firmicutes and Actinobacteria, which represent the main groups of the honey bee core gut microbiota (Moran *et al.*, 2012; Kwong and Moran, 2013). In particular, the increase of *Acetobacteraceae* in TRT hives is interesting because of its important role in nutrition and protection as reviewed by Crotti *et al*. (2010). For example, the genome sequencing of *C. intestini* (*Acetobacteraceae* family) revealed the presence of different pathways for the degradation of sugars (*e.g.* xylose, arabinose, sucrose) and the sugar rich digestive system of bees could represent an ideal environment to be colonized (Chouaia *et al*., 2014). Moreover, Aceti Acid Bacteria (AAB) tolerate and grow at acidic pH and could

have taken advantage from lactate production by lactobacilli and bifidobacteria which may provide a carbon source for AAB proliferation (Mamlouk and Gullo, 2013; Crotti *et al*., 2010). Concerning lactobacilli and bifidobacteria, a controversial finding has been reported. NGS analysis highlighted in TRT group, an increase of *Bifidobacterium* spp. and a decrease of lactobacilli, compared to CTR group. It could be hypothesised that the micro-niche that bifidobacteria inhabit has, to some extent, the potential to expand as observed Baffoni *et al*. (2016). The decrease of lactobacilli could also be partially due to a competition for nutrient uptake and catabolism with both *Acetobacteraceae* and bifidobacteria. PCR-DGGE analyses did not reveal substantial differences between TRT and CTR groups and no bands related to administered lactobacilli were detected, whereas two administered bifidobacterial strains revealed the same migration distance as the endogenous strains. It should be emphasized that DGGE, as PCR- and Gel-based molecular technique, has some limitations in terms of detection limits and relative quantification of detected populations (Marzorati *et al*., 2008). In conclusion, the administration of beneficial bacteria has improved colony productivity and influenced the composition of the honey bee gut microbiota in new generation of bees. Moreover, the application of molecular techniques gives a broader view of the gut microbiota composition following supplementation. Further investigations are envisaged to understand the impact on host immunity and physiology to improve the rationale for such supplementations.

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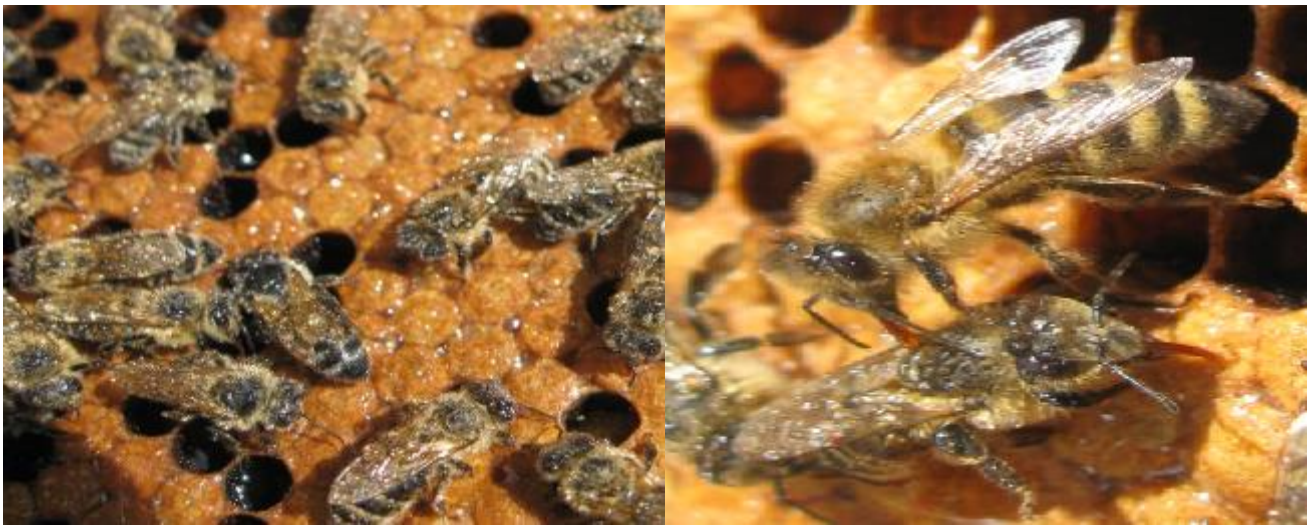

Figure S1 and S2: Honeybees sprayed with sugar syrup. The hygienic behaviour lead honeybees to clean themselves and the frames surface, inducing ingestion and consequent trophallaxis of any medicament/food additive mixed with sugar syrup.

CHAPTER 5

CONCLUSIONS

The increasing interest in the use of beneficial microorganisms in animal rearing basically derives from diverse driving factors:

- The consumer awareness and desire for natural products;
- The application of the EU legislation that bans the use of chemotherapeutic agents at sub-therapeutic levels as growth promoters in animals;
- The increased warming and drought conditions in several countries of the planet.

On the other hand, the proliferation in the world population and the reduction of agricultural production areas due to the changing climatic conditions will require the intensification of production systems. Therefore, it will be essential to have tools as substitutes to antibiotics to help animal and zoonotic pathogens control and thus improve animal and public health. Beneficial microorganisms belong to this approach. The increasing knowledge on the composition and functions of the bee gut microbiota and the link between a balanced gut microbiota and health status of several animals have encouraged researchers on the use of gut microorganisms to improve bee health. The work described in this dissertation was therefore aimed at increasing the knowledge, at present still limited, on the characterization and application of beneficial microorganisms in the beekeeping sector.

The experimental results and data analyses performed in this work has allowed to reach the following achievements:

1. **Awareness of the troubles generated by changing climatic conditions in the Emilia-Romagna region.** The average temperature increase and drought in the Italian region where most of the described work has been performed (Emilia-Romagna) has generated serious troubles to the agricultural production system, including the cultivation of plants and breeding of animals. The impact on bees is dramatic, and honey bees need more and more protection from stressors afterwards.

2. **Awareness of the lethal synergy between disease and the environment.** This sinergy might occur once the immune system of honey bees is compromised due to absence of an adequate source of feed. Perfect examples are synergies between *Nosema* and scarcity of pollen, and the case of honey bee larvae affected by the EFB, which present an atypical *Paenibacillus* species as second invader, conferring a different symptomatology to the diseased brood.

3. **Creation of a collection of microorganisms isolated from bees, bee pathogen and the related environment**. This work includes the characterization of two new *Bifidobacterium* species. This collection is an impressive stock of genetic variability wich shows adaptation to insects gut.

4. **Composition and resilience of the honey bee microbial community.** The community is composed of 12 core bacterial families showing resilience to perturbations caused by biotic and abiotic factors.

5. **Core gut microbial community of honey bees' subspecies**, evolved in isolation like the **Maltese Bee**, does not vary significantly from honey bees' microbial community analysed in Europe or America. Only few bacteria families, of apparently marginal importance might distinguish European subspecies from African subspecies of Apis mellifera.

6. **Potentiality of the use of *Lactobacillus* strains as diet supplements in bees.** This effect is due to their capability of producing bacteriocins**.** Possible detection and selection of active bacteriocins against bee pathogens might increase the potential of beneficial bacteria strains.

7. **The supplementation of *Bifidobacterium* and *Lactobacillus* strains to honey bees helps to control *Nosema ceranae* infection** if honey bees are infected with a low spore content in the gut. *Nosema* proliferation is hypothesized to be controlled by acidification of the gut lumen.

8. **The supplementation of *Bifidobacterium* and *Lactobacillus* strains to honey bees has a positive effect on bees.** It affects colony growth, speeding up brood deposition and consequently productivity, perhaps thanks to immune stimulation and enhancmento of hygienic behivour of the bee superorganism.

However, the attention should be posed on beekeepers and on their real needs: what the market requires are high-quality, cost-effective and easy-to-use products. A lot of work still needs to be done in this field.

A much more extensive and accurate *in field* test has been planned within the **H2020-MSCA-RISE-2017** project entitled "Nourishing PRObiotics to Bees to Mitigate Stressors" (No-PROBleMS) (Grant 777760). The research project is expected to see its official start on January 2018.

Therefore, the research aimed at honeybee health support will continue!

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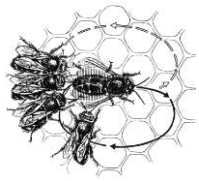

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