UNIVERSITÀ DEGLI STUDI DI MILANO

FACULTY OF AGRICULTURAL AND FOOD SCIENCE

THE DEPARTMENT OF FOOD ENVIRONMENTAL AND
NUTRITIONAL SCIENCES

PHILOSOPHY DOCTORATE SCHOOL IN
MOLECULAR SCIENCE AND AGRICULTURAL, FOOD AND ENVIRONMENTAL
BIOTECHNOLOGY

PHILOSOPHY DOCTORATE COURSE IN
CHEMISTRY, BIOCHEMISTRY AND ECOLOGY OF PESTICIDES

XXVI CYCLE

PHILOSOPHY DOCTORATE THESIS

SYMBIONTS TODAY, PROBIOTICS TOMORROW: MICROBE-BASED STRATEGY FOR IMPROVING HONEYBEE HEALTH

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ACADEMIC YEAR 2012/2013

Cover: Upper picture: Section of a honeybee comb.
Picture below: Optical microscope view of *Bacillus thuringiensis* HD110.
Pictures kindly given by Chadlia Hamdi

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Abstract

Among pollinators the honeybee *Apis mellifera* is the most important one playing an essential role in many crops, fruit and wild plants and contributing in the maintenance of biodiversity. In the last decades, a large-scale loss of honeybee colonies is occurring worldwide. The causes of this decline are yet not completely clear and are believed to stem from the interaction of several biotic and abiotic stress factors, such as insecticides, pathogens and parasites and the ongoing climate changes. In the recent years bacterial gut symbionts have been revealed a very important but yet understudied factor in protecting animal health. Symbionts are microorganisms establishing close interactions with their animal host, including insects and honeybees. They are involved in many aspects of the host physiology, including nutrition, reproduction, immune homeostasis and defense and have played major role in the host evolution. The manipulation and exploitation of the insect microbiota could be effective for the development of strategies for the management of insect-related problems. Indeed, this approach, generally defined as 'Microbial Resource Management' (MRM), was described as 'Symbiont Resource Management' (SMR) when applied to insect symbionts. The importance of the honeybee microbial commensals for the maintenance and improvement of honeybee health is the main topic of this PhD thesis. In particular, this study aims to dissect, first, the microbial diversity associated to Mediterranean honeybee gut, its interaction with the host and a model honeybee pathogen and, finally, to develop a pathogen biocontrol strategy, based on the use of honeybee symbionts, in order to improve the host health and to counter face the pathogen infection.

Using as pathogenic model *Paenibacillus larvae*, the causative agent of the American Foulbrood Disease (AFB), the ability of different intestinal honeybee symbionts has been assessed in order to verify if synergistic activities of different classes of bacteria can occur in preserving host health.

AFB is one of the most virulent disease of honeybee larvae. It was detected in many beekeeping areas, where it causes important economic losses, but little is known about the diversity of the causing agent. Seventy-five isolates of *P. larvae*, identified by biochemical tests and 16S rRNA gene sequencing, were obtained from fifteen contaminated broods showing typical AFB symptoms and collected in different locations in Tunisia. Using BOX-PCR, distinct profiles of *P. larvae* with respect to related *Paenibacillus* species were detected and may be useful for its identification. Some *P. larvae*-specific bands represented novel potential molecular markers for the identification of the species. BOX-PCR fingerprints indicated a relatively high intraspecific diversity. Nonetheless, the *in vivo* evaluation of virulence of three selected *P. larvae* genotypes did not differ significantly one another, suggesting that pathogenicity is not the only effect related to the genotypic and phenotypic diversity.

The microbiota associated to the gut of healthy and *P. larvae*-infected honeybees of different stage was characterized by 16S rRNA gene based Denaturing Gradient Gel Electrophoresis (DGGE). *Proteobacteria* of the α-, β- and γ- subgroups and *Firmicutes* were identified as the major bacterial taxa

associated to *A. mellifera* larvae and adults. Moreover, an increasing intestinal unbalance in the larval microbiome (dysbiosis) associated with the development of the disease was observed. An in-depth analysis of the microbial diversity from 5th instar larvae collected from healthy and AFB infected hives was performed by 16S rRNA gene barcoding pyrosequencing. Data analysis confirmed DGGE results: symptomatic larvae clustered clearly together, separately from healthy ones, and showed dominance of sequences of the order Bacillales, to which *P. larvae* belongs. Conversely, in healthy larvae members of Firmicutes, Alpha and Gammaproteobacteria were detected.
Culture-based methods allowed the isolation of bacteria belonging to different taxa, including Acetic Acid (AAB), Lactic Acid (LAB), and Spore Forming (SFB) Bacteria. In order to evaluate if the isolates may hinder the growth of *P. larvae*, an inhibition test was performed *in vitro* against two strains of *P. larvae*, namely *P. larvae* 20it and *P. larvae* BMG93. The experiments demonstrated that several strains, among which one AAB, one LAB and two SFB, were capable of strongly inhibiting the growth of two pathogen strains.
An *in-vivo* rearing assay was performed. The capacity of the selected symbionts to protect young honeybee larvae from *P. larvae* infection was assessed by challenging the animals with the pathogen after administering to the larvae, reared in 96-well plates, the symbionts through the diet. It was demonstrated the capability of the two SFB strains (BT and BL) to counter face the pathogen, lowering the larvae mortality to the background mortality measured under normal diet. The protection action resulted stronger when the two bacteria were administered together to the larvae.
Different mechanisms mediated by the microbial symbionts are involved in the honeybee protection (Hamdi et al., 2011), among which 1) direct inhibition of pathogen by the release of antimicrobial compounds; 2) stimulation of the immune system; and 3) competitive exclusion. In order to develop a suitable and feasible biocontrol strategy this research focused on the evaluation of the symbionts-mediated mechanisms.
First, the symbiotic ability to inhibit the growth of the pathogen was analysed measuring directly the pathogen inhibition "*in vivo*" by the symbionts. Smashed guts obtained from larvae fed with the probiotics were evaluated for their inhibition capability against the two strains of *P. larvae*, confirming that a direct inhibition activity is produced in the larval gut.
In order to understand whether the candidate probiotic bacteria enhance the honeybee larval immune system, honeybee brood response to bacterial-enriched diets was detected by assessing the expression of innate-immune system-related genes using quantitative Real Time RT-PCR. The main antimicrobial peptides (AMPs) hymenoptaecin, abaecin, and defensin, showed an increase of transcription when larvae were fed with the mixture of BT and BL, confirming a synergistic activity between the two probiotics. Conversely, the lysozyme transcripts were down regulated in all the treatments, in comparison to the larvae fed with the artificial sterile diet. Ultimately, it has been evaluated the capability of the probiotics to outcompete with the pathogen by competitive exclusion. The two probiotics were able to successfully recolonize the larval gut

and by the use of molecular techniques, such as BOX PCR, it was demonstrated that BT and BL were present after six days from the initial administration. The colonization of the probiotics in the larval gut was confirmed *by Fluorescence In Situ Hybridization* (FISH*)*. BT and BL colonized the honeybee midgut, hindering the development of the disease in co-administration experiments.
Finally, experiments to evaluate the efficacy of the probiotic treatment in counteracting the pathogen were performed in real field conditions. An approach to administer the probiotics to honeybee larvae directly on the hives was developed, and a mortality test was coupled for the 7 week-applications of the probiotics. The results confirmed that the treatment with the BT/BL mix significantly decreased the larval mortality, indicating the approach as an effective method to prevent the disease development.
The levels of AMP transcripts (abaecin and hymenoptaecin) of larvae treated or not in field condition with the two probiotic strains and then challenged with the pathogen, were measured by Real Time RT-PCR. A disease prevention response, measured as significant increases of abaecin and hymenoptaecin transcript levels, occurred when the larvae were treated with the probiotic bacteria. When the larvae treated with the probiotic strains were exposed to the pathogen, a decrease in the levels of the abaecin and hymenoptaecin transcripts respect to the non treated larvae occurred at the fifth week of treatments with the probiotic strains, despite such a treatment significantly decreased the larval mortality induced by the pathogen. Such decreases of the two transcript levels induced by the pathogen were abolished at the seventh week of treatment with the two probiotic strains, in coherence with the maintained decreased mortality. This indicates that the influence of the two probiotic strains on the AMP expression, when the larvae were continuously treated with the two strains overtime, prevailed on that driven by the pathogen and that the two probiotics support the immune response homeostasis even in presence of the pathogen challenge.
In summary, the research emphasized the importance of probiotic gut symbionts in the prevention of a honeybee disease and the overall results suggest that probiotics may in general improve host health possibly by helping in protecting it from different kinds of stresses.

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Hamdi C, Balloi A, Essanaa J, Crotti E, Gonella E, Raddadi N, Ricci I, Boudabous A, Borin S, Manino A, Bandi C, Alma A, Daffonchio D, Cherif A. Gut microbiome dysbiosis and honeybee health. Journal of Applied Entomology 2011;135:524-533

Riassunto

Il più importante tra gli insetti impollinatori è l'ape, *Apis mellifera*, il quale svolge un ruolo fondamentale in molti raccolti orto-frutticoli ma anche contribuisce al mantenimento della biodiversità delle colture selvatiche. Nelle ultime decadi è stata registrata una grossa moria di alveari in tutto il pianeta. Le cause di questo fenomeno non sono ancora state del tutto chiarite e sembra esserci alla base una interazione di fattori biotici e abiotici, come il largo uso di agrofarmaci (es. neonicotinoidi), patogeni, parassiti e i cambiamenti climatici che stressano la salute dell'animale.
Negli ultimi anni le ricerche hanno dimostrato che i batteri simbionti sembrano essere determinanti nel proteggere la salute animale ma purtroppo il loro ruolo non è ancora stato studiato in modo approfondito. I simbionti sono dei microrganismi che stabiliscono delle forti interazioni con il loro animale "ospite", e tra questi anche gli insetti (compresa l'ape). Tali sono coinvolti in molti aspetti della fisiologia dell'ospite, come la nutrizione, la riproduzione, l'omeostasi delle difese immunitarie, svolgendo un ruolo fondamentale nell'evoluzione dell'ospite stesso. La manipolazione e l'utilizzo del microbiota batterico può essere utile per lo sviluppo di strategie per la gestione di problemi legati agli insetti. Infatti, questo approccio, generalmente definito come 'Microbial Resource Management' (MRM), è stato descritto anche come 'Symbiont Resource Management' (SMR), quando applicato ai simbionti. L'importanza dei commensali microbici dell'ape per il mantenimento e il miglioramento della salute dell'ape è il tema principale di questa tesi di dottorato. In particolare, questo studio ha come obiettivo, inizialmente, di approfondire la diversità microbica associata all'apparato digerente delle api dell'area Mediterranea, la sua interazione con l'ospite e con un modello patogeno e, infine, sviluppare una strategia di biocontrollo basata sull'uso dei simbionti microbici così da migliorare la salute dell'ospite e non permettere lo sviluppo della malattia.
Usando il patogeno *Paenibacillus larvae*, agente eziologico della Peste Americana (AFB), è stata determinata la capacità di diversi batteri simbionti dell'ape in modo da verificare se possano co-esistere differenti attività sinergiche tra le varie classi batteriche nel preservare la salute dell'ape. L'AFB è una delle malattie più virulenti della larva dell'ape. E' stata identificata in numerose aree, provocando enormi perdite economiche, ma purtroppo sono ancora poche informazioni sulla diversità del fattore eziologico. Settantacinque isolati di *P. larvae* ottenuti da larve sintomatiche e collezionate in differenti siti della Tunisia sono stati analizzati tramite test biochimici e sequenziamento del gene ribosomiale 16s. Tramite la metodologia della BOX-PCR, sono stati definiti dei profili specifici di *P. larvae* (comparandoli a quelli di altre specie di *Paenibacillus*) e che possono essere utili per la loro identificazione. Alcune bande, specifiche di *P.larvae*, possono rappresentare dei nuovi modelli molecolari per la identificazione delle specie. Il pattern ottenuto dalla BOX-PCR ha identificato una certa diversità intraspecifica. Ciononostante, le analisi di virulenza effettuate *in-vivo* di tre genotipi di *P.larvae* selezionati non hanno

differito fortemente tra di loro, suggerendo che la patogenicità non è l'unico effetto connesso alla diversità genotipica e fenotipica.
Il microbiota associato all'intestino di api di diversa età sia sane che affette dal patogeno è stato studiato tramite l'analisi del gene 16s ribosomiale tramite la tecnica della elettroforesi con gradiente denaturante (DGGE). I taxa più rappresentativi identificati sono α-, β-, γ- Proteobatteri e Firmicutes sia nelle api adulte che nelle larve. Inoltre, è stato osservato un crescente sbilanciamento del microbioma all'avanzare della malattia. Uno studio più approfondito della diversità microbica delle larve del quinto stadio di crescita è stata fatta con l'analisi del gene ribosomiale 16s tramite la metodologia del pirosequenziamento. I dati hanno confermanto i risultati ottenuti dalle DGGE: le larve sintomatiche creano un unico cluster, separatamente dalle larve sane, mostrando anche una dominanza di sequenze dell'ordine dei Firmicutes, dalla quale fa parte *P.larvae*. Contrariamente, nelle larve sane α-, γ- Proteobatteri e Firmicutes sono gli ordini dominanti.
I metodi cultura-dipendenti hanno permesso di isolare differenti gruppi batterici, tra i quali batteri acetici (AAB), batteri lattici (LAB) e batteri sporigeni (SFB). Quindi, allo scopo di valutare se questi isolati potessero inibire la crescita del patogeno, è stato effettuato un test di inibizione *in vitro* contro due ceppi di *P.larvae*; in particolare *P.larvae* 20it e *P.larvae* BMG93. Gli esperimenti hanno dimostrato che numerosi ceppi tra gli AAB, LAB e SFB sono stati capaci di inibire fortemente lo sviluppo del patogeno.
Successivamente alcuni di questi ceppi sono stati saggiati *in-vivo*: la capacità dei simbionti selezionati nel proteggere le giovani larve (prima fase larvale) dall'infezione patogena è stata determinata confrontando la mortalità degli insetti con il patogeno a cui è stato somministrato il simbionte attraverso la dieta. E' stata dimostrata la capacità dei due SFB di fermare l'invasione di *P.larvae*, abbassando la mortalità fino alla mortalità basale (cioè la mortalità delle larve alimentate con la sola dieta sterile). L'effetto di protezione sembra essere addirittura maggiore quando i due SFB erano somministrati alle larve contemporaneamente.
Alla base della protezione mediata dai batteri simbionti esistono diversi meccanismi, come sostiene Hamdi et al. (2011), tra i quali: 1) inibizione diretta del patogeno con il rilascio di molecole antimicrobiche; 2) induzione del sistema immunitario; 3) esclusione competitiva.
In modo da sviluppare una strategia di biocontrollo efficace e praticabile, questa ricerca ha focalizzato l'attenzione sullo studio di tali meccanismi svolti dai simbionti.
In primis, l'abilità dei simbionti di inibire la crescita del patogeno è stata dimostrata con un test di inibizione *in vitro* usando come surnatante competente al patogeno *P.larvae*, intestini omogeneizzati ottenuti da larve alimentate con i probiotici. I risultati hanno confermato che esiste un effetto diretto dei surnatanti intestinali nei confronti del patogeno.
Quindi, per valutare la capacità di aumentare l'espressione del sistema immunitario dell'ape, larve alimentate con diete arricchite con i probiotici sono state analizzate per l'espressione del sistema immunitario innato, tramite l'uso

della tecnica della RT-Real Time-PCR quantitativa. Il trascritto mRNA di alcuni dei peptidi antimicrobici (AMPs) espressi dal sistema immunitario dell'ape; imenoptaecina, abaecina e defensina, hanno mostrato un aumento della trascrizione quando le larve erano alimentate in presenza del mix BT e BL, confermando un'attività probiotica sinergica dei due simbionti. Al contrario, il trascritto del gene lisozima ha mostrato di essere sotto espresso in tutti i trattamenti, rispetto alle larve alimentate con la sola dieta sterile. Infine, è stata valutata la capacità dei due probiotici di spiazzare il patogeno tramite competizione esclusiva. E' stato difatti dimostrato che i due probiotici sono capaci di colonizzare l'intestino dell'ape. Questo è stato dimostrato tramite l'uso di tecniche molecolari come la BOX-PCR, che ha evidenziato come BT e BL erano presenti dopo 6 giorni dalla somministrazione. La colonizzazione dei probiotici è stata quindi confermata tramite la tecnica della ibridazione fluorescente *in situ* (FISH). BT e BL sembrano colonizzare l'intestino della larva, rallentando lo sviluppo della malattia quando co-somministrati in presenza del patogeno PL.
Inoltre, ulteriori esperimenti sono stati effettuati per dimostrare l'efficacia dei trattamenti probiotici nello prevenire l'attacco patogeno anche "in campo". E' stata quindi definita una modalità di somministrazione del prodotto probiotico direttamente sugli alveari, ed è stato effettuato anche un test di mortalità affiancato a ciascuna applicazione in campo. I risultati hanno confermato che i trattamenti con il mix BT/BL diminuivano fortemente la mortalità larvale, indicando che questo rappresenta un metodo efficace per prevenire lo sviluppo della malattia.
Inoltre, tramite l'uso della RT-Real time PCR quantitativa, sono stati analizzati i livelli dei trascritti di AMPs (abaecina ed imenoptaecina) di larve trattate in campo confrontate con quelle non trattate con il prodotto probiotico e sottoposti al patogeno. Una risposta immunitaria preventiva è evidente, misurando gli aumenti dei trascritti dei geni abaecina ed imenoptaecina quando le larve erano in presenza del prodotto probiotico. Quando le larve trattate erano invece in presenza del ceppo patogeno, una diminuzione dei livelli di trascritto di abaecina e di imenoptaecina rispetto alle larve non trattate avviene dopo il quinto trattamento, nonostante un forte abbassamento della mortalità larvale mostrato dagli esperimenti di mortalità. Questa diminuzione dei trascritti dei due geni erano invece non più osservati dopo il settimo trattamento con i ceppi probiotici, coerentemente con quanto mostrato dall'esperimento di mortalità. Questo indica che l'influenza dei due ceppi probiotici nella espressione degli AMP, quando la larva era trattata con i due ceppi per lungo tempo, sembra prevalere sull'influenza guidata dal patogeno e che inoltre i due probiotici favoriscono l'omeostasi immunitaria quando in presenza del patogeno.
Concludendo, la ricerca enfatizza l'importanza dei batteri simbionti intestinali dell'ape con un effetto probiotico nella prevenzione di una patologia e soprattutto suggerisce che i probiotici possono migliorare lo stato di salute dell'ospite implementando la protezione contro i diversi tipi di stress a cui l'ospite è sottoposto.

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Aim of the work

Recent honeybee colony losses and consequent economic damages, push scientific researchers to develop new strategies to contrast honeybee diseases. Several of them are focusing their attention to the selection of honeybee genetic stock showing resistance or social immune response against pathogens (Harbo and Harris, 1999); others to natural antibacterial substances, like essential oils or propolis; others to the biocontrol activity by antagonistic bacteria.
In particular, recent successful experiments, by using non pathogen bacteria as biocontrol agents, suggest this as the most promising solution to counteract honeybees infections (Evans and Lopez 2004). However, there is still a paucity of *in vivo* experimental data (Forsgren et al. 2010) and the mechanisms implicated in this process are far to be clarified.
According to this, the principal aim of the present PhD doctoral thesis is to shed light on the importance of honeybee intestinal symbionts to actively counteract *in vivo* bee pathogens and parasites, to enhance bee immunity and thus to enhance the fitness of the hive.
The first chapter will discuss about the importance of microbiota manipulation in order to develop strategies for the management of insect-related problems, introducing the concept of 'Symbiont Resource Management' (SMR).
In the second chapter of this volume, a deeper review on the current knowledge of the importance of honeybee symbionts for the maintenance and improvement of the insect health is presented. In particular, the microbiomes' involvement in the stimulation of the insect immune system and homeostasis, with a special focus on the gut dysbiosis, and how gut dysbiosis may be related to the use of pesticides, the spread of viruses and the occurrence of parasites are discussed.
In the third chapter, it is presented one of the main disease affecting honeybees, the American foulbrood (AFB), caused by the bacterium *P. larvae* (Genersch et al, 2006; Alippi et al, 2007). In particular, the genetic and biochemical diversity related to a collection of *P. larvae* isolates, derived from Tunisian diseased broods, have been studied using the combination of molecular typing, and phylogenetic and biochemical approaches.
Then, **the fourth chapter** describes a strategy based on the use of honeybee symbionts, isolated from Italian and Tunisian honeybees, able to counteract the development of *P. larvae* and, hence, to exert a general improvement of the honeybee health. To evaluate the microbial composition and structure of the honeybee microbial community Denaturing Gradient Gel Electrophoresis (DGGE)-PCR, 16S rRNA barcoding pyrosequencing and phylochip were performed on bee broods (5th instar) with and without symptoms of the disease.

Once verified the microbiota's composition and structure of asympomatic larvae, a collection of bacterial isolates belonging to different taxa, including Acetic Acid (AAB), Lactic Acid (LAB), and Spore Forming (SFB) Bacteria was obtained from asymptomatic larvae, using specific growth media. The isolates were then screened for the capability to inhibit *in vitro P. larvae.* Thus, selected antagonistic strains were employed in *in vivo* larval rearing assays to assess the larval susceptibility against the pathogen, with or without a previous exposure to the antagonistic strains. Bacteria were tested both singularly and in mix in order to evaluate the bacterial synergies in enhancing honeybee protection.

Different mechanisms mediated by the microbial symbionts could be involved in the honeybee protection, among which 1) the direct inhibition of pathogen by the release of antimicrobial compounds; 2) the stimulation of the immune system; and 3) the competitive exclusion (Hamdi et al., 2011). To shed light on the different mechanisms exerted by the probiotic symbionts, several experiments were performed: a) *in vitro P.larvae* inhibition assays by the use of smashed guts of larvae reared in the presence of the probiotics (singularly and mix); b) immune system analysis by RT qPCR, evaluating the transcripts of four genes involved in the immune system, after the larval exposure to probiotics; c) evaluation of colonization capability of the probiotic bacteria, re-isolating them from the colonized larvae and detecting them by Fluorescence In Situ Hybridization (FISH).

Moreover, field trials of the bacterial mixtures were also performed to prove the concrete effectiveness of the treatments by administering, for 7 consecutive weeks, the probiotics to larvae directly on the bee hives, and evaluating the larval mortality after the exposure, in laboratory conditions, to *P. larvae*. To determine whether, also in this case, the probiotics acted on the immune system, the transcript levels of selected immunity-related genes (abaecin and hymenoptaecin) were measured by Real Time RT-PCR, analyzing larvae treated or not with the probiotic strains and then challenged with the pathogen.

Finally, **the Conclusions chapter** summarizes general conclusions of this Ph.D. thesis and suggests new aims for future work.

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Chapter I

Microbial symbionts: a resource for the management of insect-related problems

Published by: Crotti E, Balloi A, Hamdi C, Sansonno L, Marzorati M, Gonella E, Favia G, Cherif A, Bandi C, Alma A and Daffonchio D. Microbial Biotechnology 2012;5(3):307-17.

Summary

Microorganisms establish with their animal hosts close interactions. They are involved in many aspects of the host life, physiology and evolution, including nutrition, reproduction, immune homeostasis, defence and speciation. Thus, the manipulation and the exploitation the microbiota could result in importantpractical applications for the development of strategies for the management of insect-related problems. This approach, defined as 'Microbial Resource Management' (MRM), has been applied successfully in various environments and ecosystems, as wastewater treatments, prebiotics in humans, anaerobic digestion and so on. MRM foresees the proper management of the microbial resource present in a given ecosystem in order to solve practical problems through the use of microorganisms. In this review we present an interesting field for application for MRM concept, i.e. the microbial communities associated with arthropods and nematodes. Several examples related to this field of applications are presented. Insect microbiota can be manipulated: (i) to control insect pests for agriculture; (ii) to control pathogens transmitted by insects to humans, animals and plants; (iii) to protect beneficial insects from diseases and stresses. Besides, we prospect further studies aimed to verify, improve and apply MRM by using the insect–symbiont ecosystem as a model.

Introduction

Microbes and humans are strictly linked in every facet of the society (evolution, economy, behaviour and lifestyle). These interactions can bring about alternative effects from a human perspective. For instance, malaria (caused by *Plasmodium* parasites) is one of the major worldwide health emergences, and this disease represents a strong selective force on human populations. Indeed, in different malaria endemic areas, exposed populations developed genetic adaptations that confer resistance to the infection (Shi and Su, 2011). Moreover, the recent *Escherichia coli* outbreak in Germany (Nature Editorial, 2011, Vol.

474) underlined yet again how microbes can influence our lifedetermining public health emergencies even in developed countries (Fislage, 2011). On the contrary, there are several examples of beneficial interactions of microbes with plants, animals and humans, even in extreme conditions. For instance some bacteria are able to degrade contaminants and clean up polluted ecosystems (Balloi *et al*., 2010), plant endophytes or rhizobacteria promote soil fertility and a safe plant growth even under environmental stresses (Hayat *et al*., 2010), or animal gut symbionts are positively involved in the stimulation of the host's immune system and contribute to increase nutrient availability (Kinross *et al*., 2011). Although humans have unconsciously learnt to harness several microbial processes from the dawn of history, for example in the preparation of food (leavening of dough), beverage (fermentation of wine and beer) and tissues (soaking of linen), only from the second half of 1800 the development of microbiology slowly built up the awareness that it was possible to exploit the microbial metaboliccapabilities for humans' benefit (Rittmann *et al*., 2006).

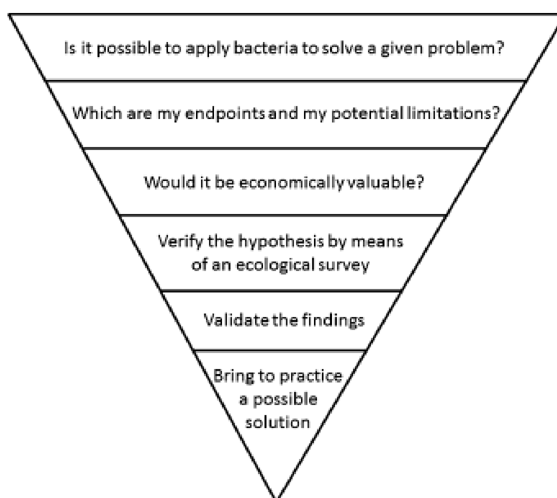

Fig. 1. MRM conceptual flow as adapted from Read andcolleagues (2011).

In 2007, Willy Verstraete theorized this concept and defined the Microbial Resource Management (MRM) as the human ability to manage complex microbial systems and their associated metabolic capabilities in order to solve practical problems (Verstraete, 2007). This led to the development of three parameters – Richness (Rr), Dynamics (Dy) and Functional organization (Fo) – to describe the complex microbial community and to ansie questions like 'who is there?', 'who is doing what?', 'who is with whom?' (Marzorati *et al*., 2008). This approach, originally designed for the ecological interpretation of raw fingerprinting patterns (e.g. DGGE, LH-PCR, t-RFLP), has been recently

updated to be applied to the new molecular technologies (i.e. pyrosequencing), thus allowing to provide a more accurate picture of the complexity and variability of the microbial communities (Read *et al*., 2011). Besides proposing a series of parameters to assess the 'nature' of a given microbial community, Read and colleagues (2011) also proposed a practical mind-set and a flow sheet based on the economical value of the approach, a clear determination of the end-points, and an ecological survey to determine the proper microbial weapons, in order to logically identify the correct direction to proceed when implementing the big picture of MRM (Fig. 1). This new approach inaugurated a more consciousphase of the microbial ecology, no longer dominated by the inductive method and based on empirical observations, but by the application of microbial ecology theories, capable to explain and predict the behaviour of a given microbial community. The aim was to establish the base for the control and the steering of microbial resources. A typical example is the change in prospective in the case of probiotics. At the beginning of the 20th century, Elia Metchinkoff, in his book *The Prolongation of Life*, hypothesized that the presence of lactic acid bacteria (LAB) in human intestinal tract could positively affect health and longevity. He based the hypothesis on the observation of the longevity of populations used to eat high amounts of yogurt (such as Baltic populations). Following this intuition, the concept of probiotic developed as the use of bacteria that could improve host health. However, the scientific literature presents many studies in which bacteria have been provided to humans with promising but often uncertain effects (Dunne *et al*., 1999). Just to mention a few examples, the effect of an oral probiotic bacteriotherapy with *Lactobacillus rhamnosus* GG– previously shown to be effective in alleviating intestinal inflammation associated with food allergy in small children (Majamaa and Isolauri, 1997) – gave no beneficial effects once administrated to apple and birch-pollen-sensitive teenagers and young adults, who manifested intermittent symptoms of allergy and mild asthma (Helin *et al*., 2002). The same LAB was shown to reduce the duration of viral diarrheal illness in European and North African children from 1 month to 3 years of age (Guandalini *et al*., 2000), but not in Brazilian patients with similar traits (Costa-Ribeiro *et al*., 2003). These and similar studies clearly show that the effectiveness of probiotics can be related to the patient traits, dietary habits (Hehemann *et al*., 2010) and age (Biagi *et al*., 2010) and that different people may have different needs. These examples show that, even if MRM was initially conceived as a practical approach for the development of an elaborative system that would describe and drive the management of the resources associated to a given microbial community, the practical implementation for many environments is still complex (Read *et al*., 2011). This is mainly due to our limited understanding of

those key factors that shape the composition and the activity of a microbial community in a complex environment. Despite these limitations, there is a specific area in which MRM has been successfully applied nowadays. In fact, the recent literature in the entomological field (a simplified environment as compared with the human gut) can provide several examples in which the MRM concept has been used to practically solve real problems. The present work, after briefly discussing the biological role, sometime essential, of microbial simbionts in insects, aims to review these cases classifying them according to the purpose of the microbiota management: (i) for the control of insect pest for agriculture; (ii) for the control of insect-transmitted pathogens; (iii) for the protection of beneficial insects from Fig. 1. MRM conceptual flow as adapted from Read and colleagues (2011). diseases and stresses. Moreover, this review will conclude analysing the possibility to develop future studies aimed to verify, improve and apply the MRM concept by using the insect–symbiont ecosystem as a model.

MRM of the insect microbiota

One of the environmental hot topics in MRM is the gastrointestinal tract (GIT), defined as an ‘outside world inside the living animals’ (Verstraete, 2007). The microbiota associated to the GIT is an highly complex community in which microbial cells outnumber, in the case of humans, prokaryotic cells by a factor of 10, comprising more than 1000 microbial taxa, most of which are unique to each host individual (Dethlefsen *et al*., 2007; Ley *et al*., 2008; Costello *et al*., 2009; Qin *et al*., 2010). This vast and diverse animal microbial ecosystem is a complex biological ‘superorganism’, whose components co-evolved with the host, and play an essential role for the host’s health and the metabolic regulation. With regards to the invertebrate gut, the microbial communities are generally less complex if compared with those of mammals, with one or two orders of magnitude less in terms of richness. However, remarkable differences could be found among species (Dillon and Dillon, 2004; Dunn and Stabb, 2005; Behar *et al*., 2008;Hongoh, 2010; Robinson *et al*., 2010; Wong *et al*., 2011). For instance, termite’s microbiota is more complex than fruit fly’s one. In fact, the former harbours several tundre species of gut microbes unique to termites, comprising protists, bacteria and archea (Hongoh, 2010), while the fruit fly *Drosophila melanogaster* less than 10 (Wong *et al*., 2011). Despite these differences microbes exert important and crucial functions for the survival and benefit of the host also in insects. In particular, the interactions established between bacteria and insects, or arthropods in general, have been known since long to go beyond pathogenesis (Dale and Moran, 2006). Cellular and humoral defences are deployed by insects to defend themselves from pathogens and parasites. Inherited protective microbes act as an additional exogenous immune

system, highlighting their great relevance in preserving insect health (Hurst and Hutchence, 2010). Commensal bacteria can modulate the innate immune system and strengthen the epithelial barrier, limiting pathogenic bacterial contact with the epithelium by inducing the secretion of antimicrobial compounds or competing with them (Hamdi *et al*., 2011). For instance, in the case of aphids, we can find several examples of symbiontmediated protection. Besides the obligate mutualistic symbiont *Buchnera aphidicola*, the aphid *Acyrthosiphon pisum* harbours one or more facultative symbionts, i.e. *Hamiltonella defensa*, *Regiella insecticola* and *Serratia symbiotica.* They explicate a role of protection of the aphid against natural enemies, such as entomopathogenic fungi and parasitoid wasps, or against heat stress (Oliver *et al*., 2010). Also *Drosophila* in nature is commonly defended by protective symbionts. *Wolbachia* infection in the fruit fly results in a strong resistance to RNA virus infection (Hedges *et al*., 2008; Teixeira *et al*., 2008). To exploit gut microbes in a MRM approach, firstly, the healthy intestinal microbiome must be understood, in terms of diversity and functionality. The diversity of the gut microbiota is linked to the genotype, diet, developmental stage, sex and physiological conditions of the host (Dethlefsen *et al*., 2007; Sharon *et al*., 2010). In the case of *Drosophila melanogaster,* it has been shown that the gut microbiome was constituted by *Lactobacillus*, *Enterococcus* and *Acetobacter* members, in several studies performed on the same species by different authors (Corby-Harris *et al*., 2007; Cox and Gilmore, 2007; Ren *et al*., 2007; Ryu *et al*., 2008; Crotti *et al*., 2010). This is in analogy with the human gut in which recently it has been identified a 'core' microbiome (Turnbaugh *et al*., 2009). Studies performed on honeybees collected from different geographic regions, such as South Africa (Jeyaprakash *et al*., 2003), Germany (Mohr and Tebbe, 2006) and Switzerland (Babendreier *et al*., 2007), gave a similar picture: the presence of a core bacterial microbiota conserved worldwide (Hamdi *et al*., 2011). On the other side, in the case of the cabbage white butterfly, the bacterial community shows temporal instability at the species level and conservation at phylum level (Robinson *et al*., 2010). These examples show how in different species, nature apparently selected for different mechanisms of adaptation. The essential factor is to maintain the overall functionality of a community rather than to conserve the presence of particular members (Robinson *et al*., 2010). Cases in which the gut functionality is disrupted by specific changes in the composition of the resident microbial community are known as dysbiosis. This is often referredto as a perturbation of the intestinal microbe–host homeostasis and it can be implicated with a pathological state, explicating a role in the occurrence of a disease. An example of insect dysbiosis has been reported by Cox- Foster and colleagues (2007). By the use of a metagenomic survey, it has been demonstrated that in the

microbiota of healthy bees there is a predominance of *Alphaproteobacteria* and *Firmicutes*, which are not found when bee specimens affected by colony collapse disorder (CCD) are analysed. A phenomenon of dysbiosis occurs in this case and the restoration of a healthy microbiota could counteract the microbial disequilibrium. In humans, such conditions are normally treated by means of therapeutic approaches – such as bacteriotherapy (Borody*et al*., 2004) and bioecological control (Bengmark, 2005) – which make use of pre- and probiotics (or a combination of the two – 'synbiotics') in order to modulate the intestinal microbial community and improve the human health. In the next paragraph we will evaluate how this modulation can be translated in the insect world.

Symbiont management in insect pests for agriculture

An elegant example of the manipulation of the insect microbiota is the management of the bacterial community associated to the Mediterranean fruit fly, *Ceratitis capitata* (Ben Ami *et al*., 2010; Gavriel *et al*., 2011). One of the strategies, commonly used to control this invasive pest, is the sterile insect technique (SIT) that foresees, firstly, a mass rearing of overwhelming numbers of male individuals, followed by insect sterilization by gamma irradiation and finally their release in the target area. After releasing, the sterile males compete with the native males for the mating with wild females and, in a successful scenario, the reduction of the next fly generation is expected. However, several studies have emphasized that irradiated males are less competent in attracting and mating with wild females than wild males. As demonstrated by molecular tools by Ben Ami and colleagues (2010), gamma irradiation influences the fly's gut microbial community leading to a dramatic reduction of *Klebsiella* sp. and to a problematic increase of *Pseudomonas* sp. Therefore, a clear case of dysbiosis due to the irradiation process affects phenotypically the sterile male performances. In order to restore the original microbial community, Ben Ami and colleagues (2010) fed the insects with the fly symbiont *Klebsiella oxytoca*. The administration of *K. oxytoca* led to its stable colonization and a decrease of potentially pathogenic *Pseudomonas* spp., resulting in a higher mating competitiveness as compared with wild males. Furthermore, other experiments performed on captured wild medflies had showed that the administration of high levels of a mix of bacteria belonging to the *Enterobacteriaceae* family – previously isolated from the fly community – and in which one of the members was *K. oxytoca*, extended the fly's longevity (Behar *et al*., 2008). This approach could be applied in order to extend the life span of sterile male insect and to enhance the success of SIT programs. The reported examples show that the manipulation of the insect microbiota by the administration of members of the fly's community can positively influences several aspects of the insect life.

InMRMterms, these experiments showed that within a plan of biological control strategy against a pest, it is of key importance to consider the role of the whole microbiota of the target insect. In the three mentioned studies (Behar *et al*., 2008; Ben Ami *et al*., 2010; Gavriel *et al*., 2011), the authors were able to reach successful results by applying an MRM approach: they use molecular tools in order to: (i) evaluating the microbial community structure, satisfying the question 'who is there'; (ii) defining the key microorganisms, satisfying the question 'who is doing what'; and (iii) planning the strategy to restore the suitable climax community, satisfying the question 'who is with whom'. Another strategy proposed for the control of *C. capitata* foresees the use of cytoplasmic incompatibility (CI)- inducing *Wolbachia* endosymbionts as a novel environmental-friendly tool (Zabalou *et al*., 2004). *Ceratitis capitata* is generally not infected by *Wolbachia*,although a few records referred to the presence of this symbiont in some Brazilian medflies (Rocha *et al*., 2005; Coscrato *et al*., 2009). *Wolbachia* transinfections from a closely related species of the medfly, *Rhagoletis cerasi*, allow obtaining *Wolbachia*-transinfected lines of *C. capitata*, stably infected with the bacterium with rates of 100% and able to express the CI phenotype. Results obtained by Zabalou and colleagues (2004) evidenced that for the suppression of the insect pest a release of *Wolbachia*infected medflies could be successfully and efficiently used, as demonstrated by laboratory cage trials. This study is an example of a more general application of *Wolbachia* or of other CI-inducing agents in strategies defined 'Incompatible Insect Technique' (IIT). The introduction of *Wolbachia* into pest and vector species of economic and hygienic relevance could be a powerful tool to suppress or modify natural populations. For a successful implementation of IIT it is mandatory to employ an efficient sexing strain of the insect pest, in order to release only the males. Thus, a medfly line infected with CI-inducing *Wolbachia* and carrying the selectable marker *temperature sensitive lethal* (*tsl*) for the male-only production has been developed by Zabalou and colleagues (2009). Insect mass rearing for SIT is widespread all over the world. In 2002, it has been estimated that more than 1.4 billion sterile male-only pupae were produced per week in different facilities around the world. The SIT programs contributed to the eradication of some insect species from specific regions, such as the New World Screwworm eradicated from Libya or the tsetse fly from Zanzibar (Lindquist *et al*., 1992; Reichard, 2002). The sterile insect technique is applied on different insect species and its economic and social benefits have been demonstrated in various cases (Vargas-Terán *et al*., 2005). The process of implementing SIT requires seven components: suppression of density, mass rearing, sterilization, shipment, release, evaluation, and quality control. The application of thisMRMapproach

for SIT or IIT could contribute to the implementation of these techniques for the production of males more competitive than wild ones or with *Wolbachia*-induced CI trait for other species of insect. A microbial tool widely used in biocontrol programs of specific insect species is represented by the use of the entomopathogenic bacterium *Bacillus thuringiensis* (Bt). Bt has been widely studied for its ability to produce parasporal crystalline protein inclusions, usually indicated as crystals, which explicate interesting and exploitable insecticidal activities. Bt ability has been used worldwide for the biocontrol of insect pests and for the development of transgenic crops (van Frankenhuyzen, 2009). Recently, the '*B. thuringiensis* toxin specificity database' has been designed to collect information on the biological specificità of the individual crystal proteins available in literature (K. van Frankenhuyzen and C. Nystrom, http:// www.glfc.forestry.ca/bacillus, January 2008; van Frankenhuyzen, 2009). Nowadays, Bt has become the leading biological insecticide and, along with *Bacillus sphaericus,* it has also been successfully used to control the mosquito vectors of diseases, such as dengue and malaria (Becker, 2000). The use of biopesticides as a component of integrated pest management (IPM) have been gaining acceptance over the world. However, in some cases, the lack of proper strategy and effective application methods are among the reasons why the usage of Bt is not successful, as it has been recorded for Bt ssp. israelensis in Malaysia (Lee *et al*., 2006). The application of the MRM mind-set in this field could enhance the exploitation of this microbial insecticide, which has proven to possess interesting features such as the safety for non-target organisms, high specificity, easy productivity of the commercial formulates and realistic market positioning.

Symbiont management in insect vectors to control the carried pathogens

Still nowadays infectious diseases pose real and several problems, especially in developing countries, with diseases like malaria, trypanosomiasis, lymphatic filariasis and onchocerciasis, which are vectored by arthropods. In order to eliminate or block the diffusion of a pathogen, one of the recently proposed strategies is based on the exploitation of mutualistic symbiotic bacteria, which are associated to the host vector or to the pathogenic agent and which are essential for the host survival or pathogen reproduction. In this respect, they can be considered as the final target for 'chemotherapy treatments'. An explicative example is again on the *Alphaproteobacterium Wolbachia*. Generally, *Wolbachia* is not a primary symbiont since it is not essential for the insect survival, though exceptions have been found, like in the case of the *Drosophila* parasitoid, *Asobara tabida*, where *Wolbachia* is necessary for the wasp oogenesis (Dedeine *et al*., 2001). On the other hand, in nematodes as *Brugia malayi*, *Wuchereria bancrofti* and *Oncocherca volvulus* (agents of lymphatic

filariasis and river blindness) *Wolbachia* is a primary obligate symbiont, essential for the host development and survival. The principle of treating filarial diseases through antibiotic treatment exploits this strict association with the host. The therapeutic approach has been attested by multiple studies in which the anti-filarial effects of antibiotics such as doxycycline or rifampicin on nematodes have been evaluated in laboratory conditions and by several clinical trials in humans (Bandi *et al*., 1998; 1999; Taylor *et al*., 2005; Bazzocchi *et al*., 2008; Hoerauf, 2008; Supali *et al*., 2008; Coulibaly *et al*., 2009; Mand *et al*., 2009; Specht *et al*., 2009; Wanji *et al*., 2009). Nowadays, mass drug administration (MDA) is used worldwide for the elimination of filariasis, but the employed drugs only temporarily clear the juvenile stage of nematodes without killing all adult specimens (Gyapong *et al*., 2005). The antibiotic-based treatments against *Wolbachia* are among the top research priorities with new promising insights. The Anti-*Wolbachia* Consortium, A-WOL, was thus established with the aim to discover and develop new anti-*Wolbachia* drugs and application, with therapies compatible with MDA (Taylor *et al*., 2010). This is a clear example of how the manipulation of the host microbiota, with the elimination of an essential primary endosymbiont, results in the impairing of a highly virulent and pathogenic parasite. Essential for the transmission of a pathogen is that the pathogen spends a period of extrinsic incubation into the vector, in order to be transmitted. This means that only the vectors from a defined age are able to transmit thepathogen, that is to say that only the oldest part of the vector population transmit the pathogen. *Wolbachia* strain *w*MelPop, a symbiont of *Drosophila*, is a life-shortening strain, therefore able to reduce adult life span of its natural host and, as a consequence, to reduce pathogen transmission (McMeniman *et al*., 2009). A recent strategy proposes to transfer this strain in vectors of medical and agriculture importance. In order to get this achievement in mosquito-transmitted diseases, scientists firstly adapted *w*MelPop from *Drosophila* in a mosquito cell culture for 3 years and then they microinjected the adapted *w*MelPop strain into naturally uninfected embryos of the major mosquito vector of dengue *Aedes aegypti*. Strain *w*MelPop halved the life span of the mosquito, inducing CI and maintaining high maternal inheritance, with no differences in fecundity (McMeniman *et al*., 2009). *Wolbachia* is a powerful tool for the control of vector-borne diseases. In this standpoint different scenario can be pictured: (i) *Wolbachia* can be used as a ‘gene driven agent’, able to ‘drive’ refractory genes into the vector population (Rasgon *et al*., 2006); (ii) *Wolbachia*-infected males can be released into the insect population and, through *Wolbachia*-induced CI, it could be obtained a reduction of vector population (see previous paragraph); (iii) insect vectors with virulent or pathogenic strains of *Wolbachia* can be released, as the case of the

aforementioned *w*MelPop strain, able to shorter the host life span (McMeniman *et al*., 2009). Moreover, it has been observed that *Wolbachia* is able to exert an interference with transmitted pathogens, being able to inhibit *Plasmodium falciparum* oocysts in mosquito midgut, or the devel- opment of the infectious stage of filarial nematodes (Kambris *et al*., 2009; Hughes *et al*., 2011). Formulations based on entomopathogenic fungi have been proposed as powerful tools in the control of vectorborne diseases. *Metarhizium anisopliae* and *Beauveria bassiana* have been shown to efficiently infect and kill mosquito larvae in laboratory trials (Scholte *et al*., 2005). Also recombinant strains of *M. anisopliae*, ex pressing molecules whose targets were *Plasmodium* sporozoites, in a variation of the so called ‘paratransgenesis approach’, resulted in a high inhibition of the malaria protozoan (Fang *et al*., 2011). Specific formulations have been developed in order to prepare a more useful and persistent product under field conditions for the control of malaria-transmitting anophelines (Bukhari *et al*., 2011). It is not only important to evaluate the effective agent for the foreseen application, but also to consider the best carrier for the delivery of a product and the best delivery way (where, when and how) in order to scale up the procedure from the laboratory condition to the open field. Paratransgenesis was firstly introduced with the study carried out on the triatomine *Rhodnius prolixus*, the vector of the parasitic protozoan *Trypanosoma cruzi*, the causative agent of the Chagas disease (Beard *et al*., 2001). A member of its microbial community, *Rodhococcus rhodnii*, essential for the growth and development of the host, has been genetically modified (GM) to express trypanocidal genes and then it has been ‘re-introduced’ into the host. A formulation based on GM bacteria, named CRUZIGARD, has been developed, at a laboratory scale, in order to introduce GM symbionts into its host, resulting in a successful application method. Similarly, in the tsetse fly *Glossina morsitans*, vector of *Trypanosoma brucei*, the etiological agent of the sleeping sickness, its secondary symbiont *Sodalis* has been proposed as a paratransgenic tool to block the transmission of the disease. *Sodalis* shows a wide tropism in the tsetse body, being mainly localized at the midgut level (Rio *et al*., 2004) and within the cytoplasm of the secretory cells (Attardo *et al*., 2008). Promising tools in the control of disease-transmitting mosquitoes like *Anopheles* are the acetic acid bacterial symbionts of the genus *Asaia* (Favia *et al*., 2007; Crotti *et al*., 2010). *Asaia* is tightly associated to different organs and tissues of the *Anopheles* body, including salivary glands and midgut that represent ‘key spots’ for the development and the transmission of the malarial pathogens. Moreover, several features of *Asaia* account for making it a powerful instrument in a applications ofMRMapplied to the insect microbiome: (i) the high prevalence and relative abundance in the mosquito individuals and populations (Favia *et al*., 2007;

Chouaia *et al*., 2010); (ii) the versatility to be transmitted by horizontal (via co-feeding or venereal) and vertical routes (maternal or paternal; Damiani *et al*., 2008; Crotti *et al*., 2009; Gonella *et al*., 2011); (iii) the ability to efficiently spread through insects populations supported by the capacity of the bacterium to colonize and cross-colonize phylogenetically related or distant hosts (Crotti *et al*., 2009); and (iv) the ease to be transformable with exogenous DNA (Favia *et al*., 2007; Crotti *et al*., 2009). Similarly, very recently it has been proposed another symbiont of *Anopheles*, the *Gammaproteobacterium Pantoea agglomerans* as a potential carrier of antagonistic factors against *Plasmodium* (Riehle *et al*., 2007). By using suitable heterologous secretion signals several anti- *Plasmodium* effector proteins could be efficiently secreted by the strain without apparently affecting the growth rate in the mosquito midgut (Bisi and Lampe, 2011). Another microorganism with a potential for the control of mosquito-borne diseases is the *Saccharomycetales* yeast, *Wickerhamomyces anomalus,* previously known with the name of *Pichia anomala* (Ricci *et al*., 2011a,b). *Wickerhamomyces anomalus* has been identified in several *Anopheles* and *Aedes* species as a stably associated symbiont in the host midgut and reproductive systems. Great attention is placed towards the use of a paratransgenesis approach based on genetically modified yeasts that, as eukaryotic organisms, could allow solving translation and folding biases of eukaryotic recombinant proteins. Insect-transmitted plant pathogens are another area in which the MRM approach could be applied with success. More precisely, research has been conducted on phytoplasmas, vectored by leafhoppers, *Liberibacter* pathogens transmitted by psyllids, and the *Gammaproteobacterium Xylella fastidiosa,* spread by the glassy-winged sharpshooter *Homalodisca vitripennis.* All these microorganisms are responsible of plant diseases that cause devastating yield losses in diverse low- and high-value crops worldwide. Disease control is commonly based on the control of the insects, i.e. by spraying various insecticides, and on practices that consist in the removal of symptomatic plants. However, some first steps of MRM applications have been already carried out on the vectors, with the aim of defining the microbial community composition and functionality in the insects (Marzorati *et al*., 2006; Miller *et al*., 2006; Crotti *et al*., 2009; Raddadi *et al*., 2011). The final aim is to propose a biocontrol approach based on the management of the microbial symbionts associated to the vectors in order to counteract directly the pathogen or to reduce the vector competence. An example is represented by the Pierce's disease of grape caused by the above mentioned *X. fastidiosa*. A culturable bacterial symbiont of the *X. fastidiosa* vector *H. vitripennis* has been isolated from the host foregut. This symbiont, identified as an *Alcaligenes xylosoxidans* ssp. *denitrificans*, was capable of

colonizing the same niche, the foregut, occupied by *X. fastidiosa* indicating that it has312 *E. Crotti* et al. the basic potential of counteracting the pathogen for instance by competitive exclusion during the colonization of the host foregut. By using a variant of the strain transformed with a plasmid for the expression of a fluorescent protein, it was possible to track the behaviour of the symbiont within the host body. A characteristic potentially very useful for the development of an approach of symbiotic control of the Pierce's disease is the versatility of the strain in colonizing different host type. It has been shown that the specific strain of *A. xylosoxidans* ssp. *denitrificans* is capable to behave as a plant endophyte in grape. Such a feature could be positively exploited to increase the exposure of the transmitted pathogen to antagonistic factors expressed by the bacterial symbiont not only at the level of the insect body but in the target plant species too (Bextine *et al*., 2004; Bextine *et al*., 2005; Miller, 2011).

Symbiont management in the protection of beneficial insects

When people think to insects, or arthropods in general, they have the idea of 'pests' or 'disease vectors'. However, most of the insects are useful for human and environmental benefit. Some of them (bees, wasps, butterflies and ants) are pollinators, others reduce the population of harmful insects, representing a real alternative to chemical application. Others produce useful substances for human activities, as honey, wax, lacquer and silk. Lastly, in many countries, insects are a part of people's diets and edible insects, such as caterpillars and grubs, are important sources of protein. Nowadays, a serious environmental problem is the decline of pollinators and a number of firms are working in the perspective of producing insect species for pollination management in the field, orchards and greenhouses at the flowering time. Honeybees and bumble-bees are sold worldwide and guidelines and operative protocols are provided to farmers for an optimal application. However, these beneficial insects are coping with severe stresses, including both abiotic and biotic ones (e.g. parasites, fungi, bacteria and viruses), which are seriously affecting their wellness, activity and productivity. Management of microbial symbionts could represent a mean to enhance the defences of beneficial insects from pathogens' attacks. Some microbial groups, as LAB or acetic acid bacteria (AAB), have been reported as able to enhance innate immune system of bees or fruit flies (Evans and Lopez, 2004; Ryu *et al*., 2008). Indeed, LAB and AAB are gene rating a lot of interest in apiculture, the former for the potential probiotic activity, the latter because it has been shown to be abundant and prevalent symbionts in healthy insects with sugar-based diets (Crotti *et al*., 2010). LAB and AAB own specific features that make them efficient colonizers of the bee midgut in comparison to acidsensitive pathogens. For instance they are able to tolerate low pH, to produce organic

acids and to utilize a wide range of sugars, interfering with the potential establishment of pathogenic bacteria. Other commensals of the honeybee gut like those of *Bacillus* and related genera have beenrecently shown to have an antagonistic effect against*Paenibacillus larvae*, the causative agent of AmericanFoulbrood disease (AFB, Cherif *et al*., 2008; Hamdi *et al*.,2011). In general, we can say that this could open the possibility – in MRM terms – of acting on the microbial structure and functionality of a specific niche in order to re-establish a good balance of the microbiota with a benefit for the host. Recently, by using artificial microcosms, it has been proved that microorganisms, once present in a suitable climax community, guarantee a high functionality of the system even during stressing events (Wittebolle *et al*., 2009). In the case of the gut microbiota, this functionality contributes to the host protection against pathogen infections (see the review of Hamdi *et al*., 2011). In particular in a recent work, it was demonstrated that structural changes in the midgut bacterial communities of cabbage white butterfly (*Pieris rapae*) larvae, due to variations in the diet, enhanced the susceptibility to biological invasion. Two different experiments were conducted. In the first trial, the community of a pool of larvae fed with an artificial diet was compared with other two pools of larvae fed with the same diet, but enriched with Brussels sprouts or sinigrin respectively (both exert an anti-microbial activity). In the second trial, larvae were fed with a sterile artificial diet both in the presence and in absence of antibiotics. Subsequently, the larvae were exposed to bacteria, commonly present within the larval microbiota, but exogenous to the diet. At the end of the treatment, the microbial community of all the larvae was characterized by using 16S rRNA gene clonal library technique. The study revealed that, compared with the microbiota of the larvae reared with the sterile artificial diet, those exposed to antibiotics, Brussels sprouts and sinigrinwere altered in their structure, resulting to be more susceptible to the invasion (Robinson *et al*., 2010). This study, which provides clear evidences on the importance of the native community structure in preventing exogenous invasions, results in particular interest when the MRM parameters are applied to describe the degree of the perturbation of the microbiota organization in the different treatments. Of particular utility are the Ecological Pareto value (Ep), which describes the optimal microbial community organization for a specific environment, and the Community distortion factor (Cd) that calculates the degree to which the Community organization (Co) is different from the Ecological Pareto value (Read *et al*., 2011). In both proposed experiments we can consider as the EP value the one referred to the structure of the microbiota of the control community (sterile diet) and as Co the value of the microbiota subjected to changes in the diet. In both experiments, the Cd factors resulted in a value

different from the one of EP, indicating that the communities have a low resistance to the applied perturbations (Co values were -24.04, -24.03 and -33.72 for the communities of the larvae fed with sinigrin, Brussels sprouts and antibiotics respectively). These results numerically support the observation that perturbations can decrease the resistance of the communities to invasion.

Future perspectives

In this review, we have evaluated the different possibilities in which the manipulation of the microbial community associated to the insects can be carried out in order to obtain multiple benefits. However, this is just the 'top of the iceberg' and many other possibilities lay in the future. The influence of the microbial partners on the biology and evolution of a eukaryotic host is nowadays well recognized but the main drivers are frequently unknown. This can be highly appreciated in relation to the 'hologenome theory of evolution' (Rosenberg and Zilber-Rosenberg, 2011). This theory considers the holobiont (the host organism and its symbiotic microbiota) with its hologenome (the sum of the genetic information of the host and its microbiota) acting in a consortium as a dynamic entity and a unit of selection in which some microorganisms multiply and other decrease in number as a function of local condition within the holobiont (Rosenberg and Zilber-Rosenberg, 2011). Due to such a close relationship, the possibility of managing the microbial community opens several perspectives in terms of MRM in relation to the comprehensive characterization of the microbiota and the determination of its role in health and disease. The understanding of these principles and the definition of general ecological rules are of key importance to implement MRM to practice. For instance, this is the aim of the Human Microbiome Project that has been initiated by the NIH Roadmap (http://nihroadmap.nih.gov/hmp/).However, mammals are far too complex for basic ecological studies. On the contrary, this is not the case for insects that, in comparison to humans, are a more simplified system. This leads to a double opportunity for the insects. On the one side, due to their relatively easy growth under controlled conditions, the possibility to manipulate both hosts and symbionts, the ability to determine precisely the kind of interactions between the partners and the possibilità to measure the effects of these interactions, insects can be a more handy holobiont to study specific teorie of microbial ecology and develop new aspects of MRM approach. On the other side, extra work has to be conducted to further exploit the MRM approach in the insect world. For example, the already developed MRM parameters (Marzorati *et al*., 2008; Read *et al*., 2011) do not take in consideration the role of the communication occurring among cells within the microbiota and between cells host and microbiota. The cellular communicative strategies, inter- and intra-taxa, are quite complex, comprising conjugation systems, secretory systems,

systems that use small hormone-like signalling molecules, plasmodesmatas, gap junctions and tunnelling nanotubes and probably other still unknown mechanisms (Dubey and Ben-Yehuda, 2011). This ecological aspect can be a promising field of application of MRM to control and manage the ecosystem symbiont-insect.

Acknowledgements

The authors thank for the financial support by the European Union in the ambit of project BIODESERT (European Community's Seventh Framework Programme CSA-SAREGPOT- 2008-2 under grant agreement no. 245746). C.H. was supported by Short Term Scientific Missions to the University of Milan by Cost Action FA0701: 'Arthropod Symbiosis: From Fundamental Studies to Pest and Disease Management'. C.B., A.C., E.C. and D.D. benefited from travel grants from the same Cost Action.

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Chapter II

Microbial symbionts of honeybees: a promising tool to improve honeybee health

Published by: Crotti E, Sansonno L, Prosdocimi EM, Vacchini V, Hamdi C, Gonella E, Cherif A, Marzorati M, Balloi A. New Biotechnology 2013;30(6):716-722.

Among pollinators, honeybees are the most important ones and exert the essential key ecosystem service of pollination for many crops, fruit and wild plants. Indeed, several crops are strictly dependent on honeybee pollination. Since few decades, honeybees are facing largescale losses worldwide, the causes of which are found in the interaction of several biotic and abiotic factors, such as the use of pesticides, the habitat loss, the spread of pathogens and parasites and the occurrence of climate changes. Insect symbionts are emerging as a potential tool to protect beneficial insects, ameliorating the innate immune homeostasis and contributing to the general insect wellbeing. A review about the microbial symbionts associated to honeybees is here presented. The importance of the honeybee microbial commensals for the maintenance and improvement of honeybee health is discussed. Several stressors like infestations of Varroa mites and the use of pesticides can contribute to the occurrence of dysbiosis phenomena, resulting in a perturbation of the microbiocenosis established in the honeybee body.

Introduction

Nonconventional habitats, among which extreme environments (like hot or cold deserts, inland or coastal saline systems), polluted sites and animal gut, have been less explored in terms of biodiversity, richness and functionality as compared to other wellstudied conventional habitats, such as soil and waterassociated matrices. Nonetheless, they represent a considerable source of compounds and microorganisms with interesting biological and biotechnological potential (Cagnanella et al, 2011; Mapelli et al 2012). Growing attention has been recently directed to the study of these niches and, among these various nonconventional habitats, to the animal gut or, in general, body intended as niches in which microorganisms survive and flourish (Crotti et al, 2012). All metazoans hosting a gut microbiota, including arthropods, establish with their microbes complex and dynamic symbiotic interactions, which recently have been shown to go beyond a mere nutritional complementation of the host diet, embracing a wide set of aspects related to the host physiology,

behavior, reproduction, evolution and immunity (Crotti et al 2012; Douglas, 2011). Insects are the most diverse animal group on earth and during their evolutionary history they adapted to feed on a variety of substrates and matrices, ranging from wood or phloem sap to blood. These nutritionally unbalanced diets are exploited and/or complemented through insect microbiota (Dale and Moran, 2006). Microorganisms also played a major role in insect adaptation and evolution (Rosenberg and Zilber-Rosenberg, 2011). Among insects, honeybees are of great importance worldwide due to their pollination activity for crops, fruit and wild plants. They offer a key ecosystem service, essential for a sustainable productive agriculture and for the maintenance of the nonagricultural ecosystem. Pollination services are mandatory for the production of crops like fruits, nuts and fibers, whereas the results of many other agricultural crops are significantly improved by pollination. It has been estimated that without pollinators a decrease by more than 90% of the yields of some fruit, seed and nut crops could occur (Southwick and Southwick, 1992). In the case that wild bees do not exert their pollination service in a specific agricultural crop, managed honeybees, which are versatile, cheap and convenient, represent the only solution to ensure pollination (Klein et al., 2007). The dependence of worldwide crops on pollinators is extremely deep and during 2005 the global economic value of insect pollination was estimated to be s153 billion a year, which corresponds to 9.5% of the total economic value of agricultural crops for human consumption (Gallai et al., 2009). Since few years, concerns are rising over honeybee health and, consequently, over its impact on economy (Plotts et al., 2010). Largescale losses have been reported worldwide and related to several causes, i e, the habitat loss of pollinators, the increasing use of agrochemicals, the outbreak of diseases, the attacks of parasites, the alarm related to climate change, the introduction of alien species and the interaction among all of these factors (Plotts et al., 2010). Managed honeybees are facing increasing threats of diseases, pests and reluctance among younger generations to learn the skills of beekeeping. In the last past years, to define and to calculate the vulnerability of world agriculture pollinator decline have become a primary point of action (Klein et al., 2007; Gallai et al., 2009; Gallai and Vaissière,2009). Recently, Colony Collapse Disorder [CCD] has attracted the attention of academic and public opinion, but this poorly understood syndrome is just one cause of the colony losses. Recent studies suggest that several factors are involved in CCD, as parasites, pathogens, pesticides (and other environmental stressors) and, above all, the interactions among them (Johnson, 2010; Nazzi et al, 2012). Honeybee symbionts could be exploited to actively counteract bee pathogens and parasites or to enhance bee immunity, and thus indirectly to increase the protection of honeybees' health. Probiotic

bacteria, such as lactic acid bacteria (LAB), have been administered in laboratory conditions to honeybees, resulting in the stimulation of the innate immune system and the prevention of attacks by pathogen (Evans and Lopez, 2004). Recent studies in the insect model Drosophila emphasize how complex, intimate and multifaceted is the relation subsisting between the host and the microbiota, which, if well balanced, leads to the optimal insect wellness (Douglas, 2011). In this review, we present the current understanding of the importance of honeybee symbionts for the maintenance and improvement of the insect health. In particular, the microbiota involvement in the stimulation of the insect immune system and body homeostasis – with a special focus on the gut dysbiosis – and how this may be related to the use of pesticides, the spread of viruses and the occurrence of parasites is discussed.

Microbial community associated to the honeybee Apis mellifera

Cultivation-dependent and independent approaches have been long used to define the composition and the structure of the honeybee microbiota, analyzing different honeybee developmental stages, such as larvae, pupae, newly emerging adults and adults; different genders, such as females and drones; and different social individuals, such as queens, nurses or foragers (Hamdi et al., 2011). Six phylogenetic groups, i. e. a, b and gProteobacteria, Firmicutes, Bacteroidetes and Actinobacteria, have been found as the major bacterial taxa of the honeybee bacterial community, representing moreover the bacterial core maintained in honeybees worldwide (Hamdi et al., 2011). The recent technological innovations in the genomics and metagenomics fields revolutionized the potential of applications and the throughput of the analyzed data, allowing DNA sequencing of high numbers of nucleotides with low costs and high accuracy. The microbial composition and structure of a specific community can be evaluated with high sensitivity, low cost and short times, thanks to new sequencing technologies and the multiplexing approach (Dowd et al., 2008;Sogin et al., 2006). Also honeybee microbiota has been evaluated by the use of these techniques (Cox-Foster et al., 2007; Moran et al., 2012, Table 1). Interestingly, eight bacterial phylotypes have been retrieved as major constituents of honeybee bacterial community, i. e. Alpha 1, Alpha2, Beta, Gamma1, Gamma2, Firm4, Firm5 and Bifido, which correspond to the six phylogenetic groups mentioned above. The metagenomic survey on honeybees from CCDaffected and not affected hives performed by CoxFoster et al. (2007) revealed that in nonaffected honeybees Firmicutes and aProteobacteria are more abundant than in CCD colonies. Similarly, in the work by Cornman et al. (Cornman et al., 2012), deep sequencing on honeybees showed a high proportion of Alpha1, Alpha2 and Bifido phylotypes in individuals from not affected hives compared to those from CCDaffected hives. Cloning libraries of

16S rRNA by Martinson et al. (Martinson et al., 2011) revealed that the most abundant taxon in A. mellifera samples was represented by Firm5 phylotype. A. mellifera showed a distinctive bacterial pattern, made up of the eight typical phylotypes, some of which are also present in closely related corbiculate bees of the genera Apis and Bombus. Lately, pyrotag analysis, quantitative PCR (qPCR) and fluorescent in situ hybridization (FISH) confirmed Beta, Firm5 and Gamma1 phylotypes (BFG phylotypes) as main members of *A. mellifera* microbiota, with a characteristic distribution along the gastrointestinal tract (Martinson et al., 2012). The crop resulted poor in microbial species, due to continuous filling and empting for nectar supply, and also the midgut showed a low BFG load, due to the presence of the digestive enzymes and the peritrophic membrane that prevents microbial attachment. By contrast, the ileum and the rectum were rich in microbes. The ileum showed a defined micro bial distribution with Gamma1 phylotype gathered in a thick mat, between Beta phylotypes and the ileum wall, and with Firm5 phylotype located in small pockets along the ileum wall. The rectum showed the majority of BFG phylotypes together with the majority of bacterial diversity (Martinson et al., 2012). A deep sampling of gut microbiota from 40 individuals has been performed by Moran et al. (Moran et al., 2012). Four phylotypes were present in all samples, even if with different frequencies, i. e. one gProteobacterium, classified as Gilliamella apicola (Kwong et al., 2012), one bProteobacterium corresponding to Snodgrassella alvi (Kwong et al., 2012) and two Firmicutes classified in Lactobacillus genus. Yeasts, wide spread microorganisms in the honeybee environment, such as flowers, fruits and plant leaves (Senses-Ergul et al., 2012; Slàvikova et al., 2009), are also important components of the bee microbiota. Recently by the use of molecular tools, sequences related to the genera Saccharomyces Zygosaccharomyces and to the family Saccharomycetaceae have been identified (Cornman et al., 2012), confirming previous results obtained by cultivation-dependent methods that showed the association of yeasts with honeybee (Gilliam, 1997).

Emerging stressors for honeybee health

Currently, a renewed attention has been directed to the relationship between honeybee health and the use of pesticides, the occurrence of parasitic mites and the outbreak of viral disease, emphasizing their interconnection in determining the insect health status (Nazzi et al., 2012; Henry et al., 2012). Pesticides, especially neonicotinoids, which are widely used for their excellent systemic properties, are indicated by scientists to play a role in CCD phenomenon and, in general, in weakening the processes of the colony, interacting with other stressors, such as parasites (Henry et al., 2012). Honeybees are exposed to neonicotinoids at sublethal doses, and this results in insect behavioral

disturbances, orientation difficulties and impairment in social activities (Henry et al., 2012,Laurino et al., 2011). Experiments to prove these difficulties have been performed not only in laboratory conditions – by ingestion tests and indirect contact tests (Laurino et al., 2011) – but also in field trials, where honeybees were exposed to a direct contamination with the pesticides during the foraging activity or to an indirect contamination with the pesticidecontaminated materials stored in the hive or exchanged with the sister bees (Henry et al., 2012). Sublethal doses of pesticides resulted to be dangerous also for bumble bees, inducing a weight loss of the insect, a low number of pupae and a reduced number of queens, thus impacting lastly the bumble bee populations (Whitehorn et al., 2011). The worldwidespread, obligateectoparasitic mite Varroa destructor represents a severe threat for apiculture. It can lead to a colony collapse within a two to threeyear period. Periodic treatments with chemicals increase on the one hand the costs for beekeeping, and on the other hand the risk of the presence of chemical residues in the environment and in the honey(Rosenkranz et al., 2010). Moreover, Varroa mites act as disseminators of viruses between and within bee colonies (Genersh et al., 2010). Recent publications highlighted the multifactorial origin of the honeybee collapse. For instance, Varroa can destabilize Deformed Wing Virus (DWV) dynamics making the virus a rapidly replicating killer (Nazzi et al., 2012). When DWV dynamics are destabilized, a host immunosuppressive status with the downregulation of the transcriptional factor NFkB is recorded. The authors suggest that the DWVmediated immunosuppressive effect shows a DWVthreshold dependency; below a certain threshold, DWV infection is maintained under control. If a stress factor, like Varroa, subtracts the transcriptional factor NFkB, the concentration of the latter becomes too low to keep under control DWV that can finally outbreak, bringing to the collapse the bee population (Nazzi et al., 2012) Pesticides, mites and viruses have a serious impact on the health of honeybees, but in all these studies there is a missing actor, represented by the gut microbial community. We will show in the next paragraphs how deeply correlated is the insect health with the gut microbiota and the immune system. Microorganisms could be a key element in managing and preserving honeybee health status toward different biotic and abiotic stressors.

Roles of the microbial partners

Recent research has shown that the gut microbiota is strictly linked to host homeostasis and metabolic diseases, e. g. diabetes and obesity (Turnbaugh et al., 2006). The gut microbial community is involved in several aspects of the host life, ranging from the nutritional contribution to the energy salvage through fermentation, from influencing mating preferences (e.g. this is the case of the gut bacteria in Drosophila (Sharon et al., 2010)) to immunity (Dale and Moran,

2006)). The animal immune system works synergistically to contain the pathogens and to preserve the symbiotic relationships between host and microbiota. A fine regulation of signaling networks, which control the presence of antimicrobial compounds in the gut, allows the host to tolerate commensals and to block the proliferation of foodborne pathogens (Leulier and Royet, 2009). As presented above, the honeybee microbiota shows a consistency which leads to hypothesize the possibility of a neutral or beneficial involvement of it, or at least with some members of the microbiota, in the honeybee's life. Several of the taxa identified in honeybees are known to produce short chain fatty acids, such as lactic or acetic acid (Lactobacilli, Bifidobacteria, Acetobacteraceae and Simonsiella). These products may act as supplements to honeybee diet. Moreover, gut bacteria could allow to degrade pollen, which is covered by exine layers recalcitrant to most of digestive enzymes, using then the intine as a nutrient source (Roulston and Cane, 2000; Engel et al., 2012). While nutritional symbioses between insects and bacteria are well documented (Dale and Moran, 2006), the correlation that exists between the proper function of insect innate immune system and its microbiota is less explored. Symbionts are recently receiving increasing attention because of their recognition as strong and effective immunomodulators of insects (Ryu et al., 2008). In their work Ryu et al. (2008) found that there is a fine equilibrium between the acetic acid bacterial commensals and the Drosophila innate immune system. The normal flora suppresses the growth of pathogenic bacteria, unless the system is perturbed. If a perturbation of the gut bacterial community occurs, an increased number of pathogenic bacteria could lead to gut apoptosis. In a normal condition the fly's immune system allows the dominance of an Acetobacteraceae strain, which in turn keeps down, by competitive exclusion, the proliferation of the gut apoptosis inducer. Another case study is represented by the tsetse fly and its obligate symbiont Wigglesworthia. The latter complements the deficient diet of the fly with the products of its metabolism. However, the sym biosis at the base of tsetse–Wigglesworthia interactions goes beyond the nutritional role: larvae deprived of Wigglesworthia are immuno compromised when they reach the adult stage. Weiss et al. (2012) show that in aposymbiotic tsetse flies the cellular innate immune system is seriously compromised and consequently the insects are highly susceptible to infections. When hemocytes from wild type indivi duals are transplanted in aposymbiotic adults or Wigglesworthia cell extracts are administered to the aposymbiotic mothers, the innate immune system functionality is restored. Another study that highlights the multidimensionality of symbionts–host interactions has been performed on the Hawaiian squid Euprymna scolopes and the luminous bacterium Vibrio fisheri (McFall-Ngain et al., 2012). V. fisheri is the exclusive partner of the squid light

organ and the symbiosis follows a dynamic balance of symbionts expulsion and regrowth. The wellknown mediators involved in animal– microbe interactions, called ‘microbe associated molecular patterns’ (MAMPs), specifically lipid A component of lipopolysaccharide (LPS) and peptidoglycan component, interplay synergistically with the luminescence of symbionts to sustain the host development.

Researchers found that MAMPs and luminescence interactions are both crucial for the maintenance of the symbiosis. All these findings contribute to state that a finely regulated dialog exists among the symbiotic partners to reach a symbiostasis. This is done through the regulation of pathways implicated in the substrate availability and pathways that govern host/symbionts population dynamics. Recently, artificial microcosms have been employed to prove that the high functionality of a specific system could be maintained, even during stress events, if microorganisms are distributed in a suitable climax community (Wittebolle et al., 2009). In the case of the microbiota associated to the digestive system, the maintenance and improvement of the host health against pathogens infection depends on the functionality of the system, which lastly relies on the presence of a suitable climax community (Hamdi et al., 2011; Cox Foster et al., 2007). Cox Foster et al. (2007) showed that CCD nonaffected honeybees are mainly colonized by Firmicutes and aProteobacteria, while in CCDaffected bees a high abundance of gProteobacteria is measured. This could be related to a case of dysbiosis, i. e. an unbalance of the gut microbiota, with the consequent loss of the proper functionality, which in turn negatively impacts the health status. Further studies are needed to unveil the strict and dynamic interplay existing between host and symbionts.

Case study	Origin	Sample	Method	Total *n* sequences	% known bee species groups[a]	Alpha-1[a]	Alpha-2[a]	Beta[a]	Gamma-1[a]	Gamma-2[a]	Firm-4[a]	Firm-5[a]	Bifido[a]	Other bacteria
Cultivation-independent techniques														
Jeyaprakash et al. (2003)	South Asia	Dissected guts	Sanger	8	n/a	+ (3)	+ (1)	+ (2)	+ (2)	-	-	+ (1)	+ (1)	[b]
Mohr and Tebbe (2006)	Germany	Dissected guts	Sanger	13	n/a	-	+ (1)	+ (1)	+ (2)	-	-	-	-	[b]
Babendreier et al. (2006)	Switzerland	Midgut and hindgut	Sanger	27	n/a	+ (3)	+ (2)	+ (6)	+ (8)	+ (1)	+ (2)	+ (4)	-	[b]
Disayathanoowat et al. (2012)	Thailand	Midgut	Sanger	17	n/a	-	-	+	+ (1)	-	-	+ (2)	+ (1)	[b]
Cox Foster et al. (2007)	Australia, USA, Hawaii	Pooled whole bees	Pyrotags 454	428	97.4	1.9	3.2	16.9	60.9	9.6	0.6	2.8	1.7	2.6
Martinson et al. (2011)	Arizona	Single whole bees	Sanger	271	98.5	0.0	1.1	11.1	11.8	0.0	10.0	63.8	0.7	1.5
Martinson et al. (2011)	Arizona,	Bacterial cells isolated from pooled guts	Sanger	267	98.5	0.7	0.0	3.7	9.7	0.0	10.5	60.7	13.1	1.5
Martinson et al. (2012)	Arizona,	Dissected gut sections	Pyrotags 454	96,505	99.9	0.0	0.3	20.3	10.1	24.2	0.2	44.0	0.8	0.1
Sabree et al. (2012)	Massachusetts	Dissected guts	Pyrotags 454	106,344	94.8	0.0	0.0	6.74	49.10	1.12	11.05	21.36	5.41	5.2
Moran et al., (2012)	Arizona, Maryland	Dissected guts	Pyrotags 454	329,550	99.1	1.0	1.0	9.1	11.9	2.0	45.4	23.2	5.4	0.9
Engel et al. (2012)	Arizona	Hindguts of worker bees	Illumina sequences	76.6 Mb[d]	82.4	13.8	3.4	4.9	23.9		9.7		3.4	17.6
Cultivation-dependent techniques[c]														
Evans and Armstrong (2006)	USA	Individual larvae	Sanger	11	n/a	-	-	-	-	-	-	+ (1)	-	[b]
Olofsson and Vásquez (2008)	Sweden	Guts	Sanger	17	n/a	-	-	-	+ (3)	+ (1)	+ (1)	+ (4)	+ (5)	[b]
Vásquez and Olofsson (2009)	Arizona	Guts	Sanger	11	n/a	-	-	-	+ (1)	-	+ (1)	+ (2)	+ (4)	-
Sabaté et al.(2009)	Argentina	Pooled intestines	Sanger	1	n/a	-	-	-	-	-	-	-	-	[b]
Loncaric et al. (2011)	Austria	Honey sac	Sanger	11[e]	n/a	-	-	-	-	-	-	-	-	[b]
Carina Audisio et al. (2011)	Argentina	Intestines	Sanger	5	n/a	-	-	-	-	-	-	-	-	[b]
Vásquez et al. (2012)	Sweden and Kenya	Dissected honey crops	Sanger	137[e]	n/a	-	-	-	-	-	+ (4)	+ (7)	+ (29)	[b]

TABLE1. Actual knowledge on the bacterial species associated to the honeybee *Apis mellifera* according to cultivation-independent and-dependent methods.Data from cultivation-independent studies and some data from cultivation-dependent studies are from Sabree *et al.* (2012). Other cultivation-dependent data are from studies that identified the isolates by partialorcomplete16SrRNAgenesequencing.

n\a: indicates not available

[a] For studies with deep sequencing methods, percent values of phylotype abundance are indicated. In those studies where methods do not allow percent representation, "+" indicates the presence of a phylotype. Figures in parentheses indicate the numbers of sequences associated to a bacterial group. "-" indicates no presence of a phylotype.

[b] Sequences of other bacteria, besides the phylotypes presented in the table, have been retrieved but the frequencies cannot be calculated due to the methods employed in these case studies.

[c] Cultivation-dependent methods do not allow to represent all bacteria in the gut.

[d] These numbers are from a dataset of metagenomic data.

[e] These numbers are from a dataset including also, but not only, sequences from *A. mellifera.*

Microbial involvement in the general insect health status
Recent publications highlighted that in different Drosophila strains two taxonomically different bacteria, i. e. Acetobacter pomorum and Lactobacillus plantarum, modulate the insulin signaling and TOR pathway, respectively, through different bacterial products (Douglas, 2011; Shin et al., 2012; Storelli et al., 2012). In A. pomorum, the acetic acid produced by the activity of the pyrroloquinoline quinonedependent alcohol deydrogenase (PQQADH) modulates the insulin signaling which in turn controls several host homeostatic programs, as the developmental rate, the body size, the energy metabolism and the intestinal stem cell activity (Shin et al., 2012). By contrast, L. plantarum promotes protein assimilation from the diet, regulating dietderived branchedchain amino acid (BCAA) levels in the hemolymph. BCCA activates TOR signaling: (i) in the fat bodies, which results downstream into the promotion of growth rate and (ii) in the protoracic glands, which has an impact downstream on the length of growth phase (Storelli et al., 20129. In fat bodies TOR pathway normally acts stimulating the systemic production of insulinlike peptides and thus promoting the growth. It has been hypothesized that (1) the stimulation of the insulin signaling in presence of commensals could be the result of the evolution conflict between the host and its microbiota; (2) bacterial metabolites are cues for the host to be informed on the environmental nutritional availability for the host development (Hamdi et al., 2011) Thus according to this second hypothesis the host would exploit its microbiota to sense the environment. Bacteria are known to communicate through quorum sensing which allows the regulation of their activity and physiological processes. Quorum sensing outcomes in important advantages for bacteria, i. e. host colonization, formation of biofilms, defense against competitors, and adaptation to changing environments. The kind of interaction here hypothesized implies a higher level of interaction between symbionts and hosts. The molecular mechanisms that regulate the host microbe crosstalk are still poorly understood. However, all these studies highlight the key role of microbial partners in influencing the systemic growth of the host and preserving its health. As in Drosophila, it is possible to hypothesize that commensals in honeybee could have a higher level of interaction with the host, acting on the growth regulation of the insect. Components of Drosophila microbiota, as Lactobacillales and Acetobacteraceae members, are widespread in A. mellifera. LAB have been shown to exert a probiotic effect on honeybee larvae, eliciting the innate immune system to overcome pathogen attacks (Evans and Lopez, 2004), and have been indicated as major modulators of honeybee health (Vàzquez et al., 2012). Like LAB, wellknown for their ability to produce antimicrobial factors, other symbionts such as sporeforming bacteria are

indicated as producers of peptide antibiotics and antibioticlike compounds, which in some case possess antagonistic activity (Cherif et al., 2008; Li et al., 2012). Finally, acetic acid bacteria (AAB), widespread in nature (Kommanee et al., 2008), can compete with the pathogen along the host epithelia, physically occupying the available niches and nutritionally competing with the pathogens. Moreover, acid and exopolysaccharide production may contribute to AAB successful colonization of the insect gut (Crotti et al., 2010; Kounatidis et al., 2009).

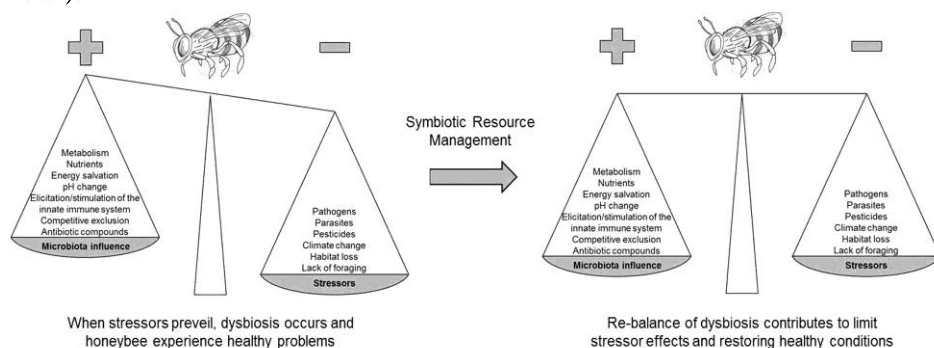

Fig. 1. A graphical representation of the ecological concept of Symbiont Resource Management (SRM)

Perspectives

There is increasing evidence that there is a strict interconnection between the intestinal microbiota balance and the health status of the host (Douglas., 2011). Commensal microbiota drives immune and health which foresees the management of the insect gut microbiome to improve host health. homeostasis by mechanisms that are yet poorly understood and a great effort has to be done in this direction. Insect symbionts are indeed emerging as a potential tool in biocontrol programs to protect beneficial insects, ameliorating the innate immune homeostasis and contributing to the general insect wellbeing (Douglas et al., 2011). The employment and exploitation of microorganisms in a defined environment or niche to solve practical problems have been termed as Microbial Resource Management (MRM) and MRM concepts are applicable to the maintenance and promotion of insect health (Crotti et al., 2012). A novel MRM application, the Symbiont Resource Management (SRM), can be defined as the application of microbial symbionts to manage insectrelated problems (Crotti et al., 2012; Fig. 1). Symbiotic microorganisms can exert their beneficial contribution toward the host to sustain its health in different ways, i.e. by competitive exclusion, production of antibiotic compounds, activation/stimulation of the innate immune system and communication to the

host of the environmental conditions. However, to ecome able to manage these complex microbial communities within the body of the insects it is imperative to understand how they interact with the host. Therefore, further research has to be conducted to clarify the molecular mechanisms at the base of the symbiosis.

Acknowledgements
The authors thank the European Union in the ambit of project BIODESERT (European Community's Seventh Framework Programme CSASA REGPOT20082 under grant agreement no. 245746) and the Ministry for Research (MIUR), in the ambit of projects PRIN 2009 (Interazioni tra insetti vettori. e. microrganismi simbionti: nuove prospettive per il biocontrollo dei patogeni trasmessi alle piante, agli animali. e. all'uomo) for financial support. C.H. was supported for a Short Term Scientific Mission to the University of Milan by Cost Action FA0701: 'Arthropod Symbiosis: From Fundamental Studies to Pest and Disease Management'. M.M. is a Postdoctoral Fellow of the Research Foundation – Flanders (FWO, Belgium).

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Chapter III

Genetic and biochemical diversity of *Paenibacillus larvae* isolated from Tunisian infected honeybee broods

Published by: Hamdi C, Essanaa J, Sansonno L, Crotti E, Abdi K, Barbouche N, Balloi A, Gonella E, Alma A, Daffonchio D, Boudabous A and Cherif A. BioMed Research International 2013;2013:1-9.

Abstract

Paenibacillus larvae is the causative agent of American foulbrood (AFB), a virulent disease of honeybee (*Apis mellifera*) larvae. In Tunisia, AFB has been detected in many beekeeping areas, where it causes important economical losses, but nothing is known about the diversity of the causing agent. Seventy five isolates of *P. larvae*, identified by biochemical tests and 16S rRNA gene sequencing, were obtained from fifteen contaminated broods showing typical AFB symptoms, collected in different locations in the northern part of the country. Using BOX-PCR, a distinct profile of *P. larvae* respect to related *Paenibacillus* species was detected which may be useful for its identification. Some *P. larvae*-specific bands represented novel potential molecular markers for the species. BOX-PCR fingerprints indicated a relatively high intraspecific diversity among the isolates not described previously with several molecular polymorphisms identifying six genotypes on polyacrylamide gel. Polymorphisms were also detected in several biochemical characters (indol production, nitrate reduction, methyl red and oxidase test). Contrary to the relatively high intraspecies molecular and phenotypic diversity, the *in-vivo* virulence of three selected *P. larvae* genotypes did not differ significantly, suggesting that the genotypic/phenotypic differences are neutral or related to ecological aspects other than virulence.

Introduction

American foulbrood (AFB), a severe and highly contagious disease affecting the larval and pupal stages of honeybee (*Apis mellifera*), is caused by the bacterium *Paenibacillus larvae* (Genersch et al., 2006; Alippi et al., 2007). AFB is one of the few diseases capable of killing the honeybee colony (Alippi et al., 2005). Prevention and control of AFB are very difficult because the pathogen produces spores that are resistant to heat and chemical agents and can remain viable for more than 35 years (Heyndrickx et al., 1994; Dobblelaere et al., 2001). AFB is causing considerable economic loss to beekeepers all over the

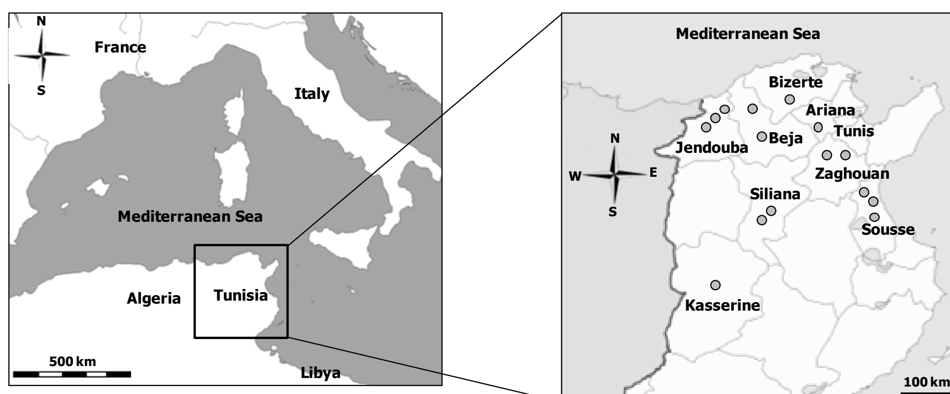

Fig. 1. Location of the 15 sampled AFB contaminated hives in the northern are of Tunisia (○).

world (Antùnez et al., 2004; Kilwinski et al., 2004; Hamdi et al., 2011; Crotti et al., 2012), and it is classified on list B of the World Organization for Animal Health (De Graaf et al., 2006). In many countries, an eradication strategy exists with isolation and destruction of infected colonies and burning of contaminated equipment (De Graaf et al., 2006; Thompson et al., 2007).

Different genotypes of *P. larvae* have been identified in different regions. By using BOX-PCR three genotypes (A, B and C) have been identified within a worldwide isolate collection (Alippi and Aguilar, 1998). In Germany, four different genotypes of *P. larvae* named AB, Ab, ab and αB, have been described by combining BOX A1R and MBO REP1 primers (Genersch and Otten, 2003; Neuendorf et al., 2004). Using the same combination (BOX A1R and MBO REP 1 primers), Loncaric et al. (Loncaric et al., 2009) described five different genotypes (ab, aB, Ab, AB and αb).

After the reclassification of *P. larvae* as one species without subspecies separation, it was proposed the
use of other techniques as ERIC-PCR for subtyping *P. larvae* and four different genotypes (ERIC I-IV) were identified (Genersch et al., 2006). The genotypes ERIC I and II correspond to the former *Paenibacillus larvae* subsp. *larvae* and ERIC III and IV to the former *Paenibacillus larvae* subsp. *pulvifaciens*.

AFB is readily disseminated by honeybees robbing honey from neighboring hives and the larval feeding of spores-contaminated pollen and honey (Evans, 2004), or the reuse of contaminated beekeeping equipments (Thompson et al., 2007). A role in the spread of *P. larvae* has been also attributed to *Varroa destructor* (Rycke et al., 2002) and the hive beetle *Aethina tumida* (Schafer et al., 2010).The spores ingested by the newly hatched larvae germinate in the midgut lumen. The vegetative forms of *P. larvae* penetrate the gut epithelium and spread into the larval tissues (Davison, 1973; Nordhoff et al., 2008).

Despite some studies have investigated the pathogenicity of *P. larvae* and the virulence factors involved in the infection, the picture of the *P. larvae* virulence mechanisms is not yet complete. Dancer and Chantawannakul (Dancer and Chantawannakul, 1997) associated the pathogenicity of *P. larvae* to the secretion of metalloproteases. Antunez et al. (2011) reported the production by *P. larvae* of an enolase that could have a role in the virulence of the pathogen. Recently, *P. larvae* virulence has been associated to an S-layer protein (Poppinga et al., 2012) whose presence determined the difference in the virulence between ERIC I and ERIC II genotypes (Genersch et al., 2005) with the former showing a weaker virulence due to the absence of the specific S-layer (Poppinga et al., 2012). This study evidenced the importance of P. larvae genetic diversity in relation to virulence and highlighted the need of assessing the intraspecies diversity in areas of intensive apiculture.
AFB disease has been reported in Arab countries including North Africa (Hussein, 2000) and in Tunisia it has been detected in many beekeeping areas, where it causes important economical losses. Even though it has been shown that the economic value of pollination in North Africa is among the highest of the African continent (Gallai et al., 2009), very limited knowledge is available on AFB and the genetic diversity of P. larvae.
The aim of the present work was to characterize a collection of *P. larvae* isolated from Tunisian diseased brood and to study the genetic and biochemical diversity related to these isolates.

Materials and methods

P. larvae isolation

Seventy five isolates of *P. larvae* were obtained between 2003 and 2005 from diseased honeybee larvae originating from 15 different hives in the northern part of Tunisia (Figure 1). The isolates were obtained on Columbia blood agar containing 5% horse blood for 48 h at 37°C. This step was preceded by a heat treatment at 80°C for 10 min to eliminate the quick growing bacteria that may outcompete *P. larvae* on the plates. Nine reference strains of seven *Paenibacillus* species phylogenetically related to *P. larvae* were obtained from the Bacillus Genetic Stock Center (BGSC), USA: *Paenibacillus alvei* 33A3 and 33A4, *Paenibacillus polymyxa* ATCC842T, *Paenibacillus popilliae* 2525 and B2519, *Paenibacillus vorticalis* 30A1, *Paenibacillus thiaminolyticus* NRRLB-4156T, *Paenibacillus dentritiformus* T168 and *Paenibacillus macerans* BKM B-51. All these reference strains were routinely cultivated on nutrient broth and agar at 30°C for 24 h.

Phenotypic and biochemical characterization

Cell and colony morphology of all isolates were described and their biochemical profile was determined according to Gordon et al. (1973) with the following tests: catalase test, nitrate reduction, gelatin, starch and casein hydrolysis, tyrosine and urea degradation, acid from glucose, oxidase test, VP test, production of dihydroxyacetone and indol and citrate test. The growth was tested at different temperatures (4°C, 30°C, 37°C and 50°C) and in media containing 2% and 5% of NaCl. All phenotypic tests were made in triplicate and repeated when inconsistent results were observed. Positive and negative results were coded as 1 and 0, respectively, and cluster analysis was carried out by the Unweighted Pair Group Method with arithmetic averages (UPGMA) using the Jaccard coefficient (Sneath and Sokal, 1973).

DNA extraction and PCR conditions

DNA was extracted from bacteria using the TE solution (10 mM Tris HCl, pH 7.4; 1 mM EDTA, pH 8), lysozyme (35 mg ml^{-1}) and proteinase K (10 mg ml^{-1}) (Cherif et al., 2002). The *P. larvae* strains were identified by 16S rRNA gene sequencing and typed by BOX-PCR, a technique widely used for the strain typing of bacteria (Chouaia et al., 2010) including *Bacillus* species (Alippi and Aguilar, 1998;Cherif et al., 2002; Velezmoro et al., 2012).

PCR amplification of the 16S rRNA gene and the BOX gene was performed using the universal primers, S-D-Bact-0008-a-S-20/ S-D-Bact-1495-a-A-20 and BOX A1R respectively (Cherif et al., 2002). PCRs were performed in a final volume of 25 µl containing 0.5 µM of each oligonucleotide primer for the 16S rRNA PCR and 1 µM for the BOX PCR primer, 200 µM dNTPs, 2.5 mM $MgCl_2$ and 1U of DNA *Taq* polymerase. PCR was performed for 35 cycles of 45 s at 94°C, 45 s at 55°C/42°C respectively for 16S rRNA PCR and BOX-PCR and 60 s at 72°C. BOX-PCR products were separated in standard 1.5% agarose gel and in 6% polyacrylamide gel, visualized under UV light and photographed with a Gel Doc digital image capture system (Bio-Rad).

Numerical analysis of BOX patterns was performed using the MVSP 3.1 software (Kovach, 1998). Bands from all the gels were manually detected using as markers the 100 bp (Fermentas) or the 50 bp ladders (Promega), allowing the identification of the different BOX genotypes.

16S rRNA gene sequencing and phylogenetic analysis

The 16S rRNA gene sequencing was performed at the Primm Biotech (Milano, Italy). Partial 16S rRNA gene sequences (*E. coli* coordinates nt 52 to 787) of the isolates were compared with 16S rRNA gene sequences available by the BLAST search (Altschul et al., 1990), in the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments were performed using ClustalW version 1.8 (Thompson et al.,

1994). The method of Jukes and Cantor (1969) was used to calculate evolutionary distances. Phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) and the reliability of the tree topology was evaluated by bootstrap analysis of 500 re-sampled data sets using MEGA 4.1 software (Ettoumi et al., 2009; Forsgren et al., 2010).

16S rRNA gene sequences of thirteen *P. larvae* isolates, BMG 93, BMG 184, BMG 189, BMG 191, BMG 192, BMG 194, BMG 198, BMG 201, BMG 232, BMG 235, BMG 245, BMG 250 and BMG 259, were deposited under Genbank accession numbers FJ649367, FJ649355, FJ649365, FJ649362, FJ649358, FJ649363, FJ649356, FJ649357, FJ649361, FJ649359, FJ649364, FJ649360 and FJ649366, respectively.

Exposure bioassays for investigating the virulence of three P. larvae isolates

The *P. larvae* strains (BMG 93, BMG 184 and BMG 259) used for the artificial larval infection were cultivated on MYPGP-agar, at 37°C for 10 to 14 days as described by Forsgren et al. (Forsgren et al., 2009), with few modifications. The sporulated cultures were centrifuged at 3000 rcf for 15 min and the spores were washed twice with sterile distilled water. The number of spores in the final suspensions was determined by plate count after 80°C heat treatment. The spore solutions were further diluted in larval diet to give final concentrations of approximately 5×10^3 CFU ml^{-1} and 10^5 CFU ml^{-1}.

Honeybee larvae of <24 h (based on body size) were collected from a healthy beehive and reared in U-shaped 96-well plates according to the method of Peng et al. (1992). The grafted larvae were fed with an artificial liquid diet containing 50% of royal jelly, 50% of an aqueous solution of yeast extract, 12% each of D-glucose and D-fructose, both filtered at 0.2 µm (Aupinel et al., 2005). The diet was provided to the larvae with micropipette once a day for six days. For experimental infection and before grafting, each well of the plate was filled with 20 µl of artificial liquid diet supplemented with a final *P. larvae* spore concentrations of 5×10^3 CFU ml^{-1} or 10^5 CFU ml^{-1} for the exposed groups and without *P. larvae* spores for the control group. The larvae were exposed to *P. larvae* spores for 24 h after grafting. Forty-eight larvae per group were used in this exposure test and the experiments were performed three times. After grafting, the plates containing young larvae were incubated at 35°C in presence of a saturated solution of K_2SO_4 to keep the humidity at 96% (Aupinel et al., 2005). During each of the eight days of rearing, the larvae were examined for their vitality and dead and symptomatic individuals were noted both for the larvae exposed to *P. larvae* and the non-treated ones.

Statistical analysis

Statistics were calculated by using Microsoft Excel software (Millar, 2001). Mean and standard deviation were determined for three independent experiments and results were presented as mean ± SD. The Student's t-test was used to test for statistical significance of the difference between the mortality of the three groups of infected larvae with three different strains of *P. larvae*. A *p*-value of less than 0.05 was considered statistically significant.

Results

Biochemical, physiological and morphological characters

P. larvae colonies were small (3 mm in diameter), regular, buttery and greyish. Cells were examined and all isolates were Gram-positive rods with a width of about 1µm and a length of 3-5 µm. Bacteria appeared as single cells or pairs, sometimes as short chains.

All isolates were catalase negative, grew at 30 and 37°C and in 2% NaCl media but not in nutrient broth, at 4°C, at 50°C and 5% NaCl. Citrate was not utilized. Isolates were positive for degradation of casein and gelatin and for acid production from glucose and starch. Tyrosine was not degraded. Most of strains reduced nitrate to nitrite. Variable results were obtained for oxidase and methyl red tests and the strains did not form dihydroxyacetone and indol, and were negative for the Voges-Proskauer test (Table 1).

Table 1. Biochemical characteristics of *P. larvae* isolates and *Paenibacillus* reference strains.

	P. larvae	*Paenibacillus* reference strains (BGSC)								
Biochemical tests	75 isolates of *P. larvae*	*P. alvei* 46-c-3	*P. alvei* 2771	*P. popilliae* 2525	*P. popilliae* B2519	*P. thiaminolyticusa*[a]	*P. dentritiformus* T168[b]	*P. polymyxa* ATCC 842T	*P. vorticalis* 31A1	*P. macerans* BKM B-51
catalase activity	-	+	+	-	-	+	+	+	+	+
oxidase[c]	v	-	+	-	-	+	+	+	+	+
starch hydrolysis	-	-	+	-	-	+	-	+	+	+
casein hydrolysis	+	+	+	-	-	+	-	+	-	-
dihydroxyaceton	-	+	+	-	-	-	-	+	-	-
tyrosin decomposition	-	-	-	-	-	+	-	-	-	-
gelatin liquefaction	+	+	+	-	-	+	-	+	+	+
methyl red[d]	v	-	+	-	-	+	-	+	-	+
indole production[e]	v	-	-	-	-	+	+	-	-	-
urea	-	-	-	+	+	+	+	+	-	-
DNase	-	-	-	-	-	-	-	-	-	-
nitrate reduction[f]	v	-	-	-	-	+	-	+	+	+
Voges Proskauer	-	+	+	-	-	-	-	+	+	+
glucose	+	+	+	-	-	+	+	+	+	+
lactose	+	+	+	+	+	+	+	+	+	+
Gaze	-	-	-	-	-	-	-	-	-	-
H_2S	-	-	-	-	-	+	+	-	-	-
Citrate utilization	-	-	-	-	-	+	-	-	-	-
2% NaCl	+	+	+	+	+	+	+	+	+	+
5% NaCl	-	-	+	-	-	+	-	-	-	-
4°C and 50°C	-	-	-	-	-	-	-	-	-	-
30°C and 37°C	+	+	+	+	+	+	+	+	+	+
Nutrient broth	-	+	+	+	+	+	+	+	+	+

[a]*P. thiaminolyticus* NRRLB-4156T; [b]*P. dendritiformus* subsp. *dendron*; BGSC: *Bacillus* Genetic Stock Center; +, 100% of the strains positive; -, 100% of strains negative; v, variation between strains; [c]7% (-) and 93% (+); [d]1% (+) and 99% (-)

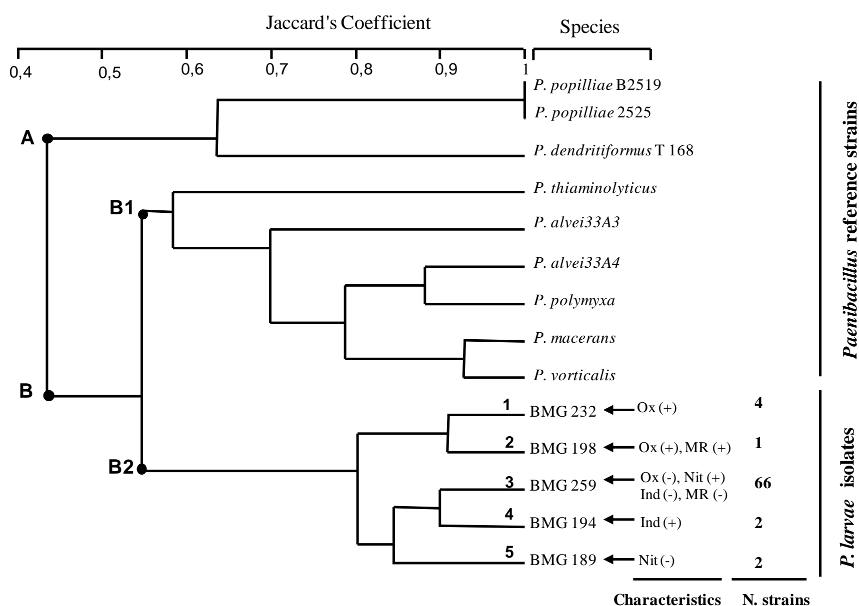

Fig. 2. Dendrogram showing the biochemical profile relationship between *P. larvae* isolates and *Paenibacillus* reference strains. Ox: Oxidase, Nit: Nitrate reduction, MR: Methyl Red, Ind: Indol, +: positive response, -: negative response.

Numerical analysis

The dendrogram of the biochemical results of the isolates and the reference strains discriminated two groups (Figure 2). The first group (A), contained reference strains *P. popilliae* 2525, *P. popilliae* B2519 and *P. dendritiformus* T168. The second group (B) was subdivided into two sub-groups. The first (B1) included the reference strains *P. thiaminolyticus* NRRLB-4156T, *P. alvei* 46-c-3, *P. alvei* 2771, *P. polymyxa* ATCC 842T, *P. macerans* BKM B-51 and *P. vorticalis* 31A1. The second sub-group (B2) contained exclusively the local *P. larvae* isolates (75 strains) well separated from the *Paenibacillus* species reference strains.

16S rRNA gene sequencing

16S rRNA gene sequences of thirteen *P. larvae* isolates (BMG 93, BMG 184, BMG 189, BMG 191, BMG 192, BMG 194, BMG 198, BMG 201, BMG 232, BMG 235, BMG 245, BMG 250 and BMG 259) showed 99% identity with those of *P. larvae* in Genbank. A 16S rRNA gene sequences of 480 bp, were used for the construction of the phylogeny of the isolates and standard strains of *P. larvae* available in Genbank.

The phylogenetic tree of partial 16S rRNA gene sequences (480 bp) grouped all *P. larvae* isolates and strains in branch A that showed two sub-groups (Figure

3). Sub-group A1 contained the reference strain, *P. larvae* DSM 7030. Sub-group A2 showed three branches A2.1, A2.2 and A2.3. Branch A2.1 represented two isolates BMG 194 and BMG 93. A2.2 grouped the reference strains 03-183 (DQ079623) and *P. larvae* (AY030079) and the Tunisian isolates (BMG 191, BMG 235, BMG 184, BMG 192, BMG 245, BMG 232, BMG 250, BMG 198, BMG 201 and BMG 189). A2.3 included only the isolate BMG 259.

BOX-PCR analysis of P. larvae isolates

BOX-PCR distinguished three genotypes out of 75 *P. larvae* isolates named A, B, and C (Figure 4A). *P. larvae* isolates presented a specific banding pattern clearly different from the other *Paenibacillus* species. The presence or absence of bands around 300 and 350 bp distinguished the three genotypes. Genotype A showed six bands of approximate sizes: 280, 300, 350, 650, 700 and 800 bp. Genotype B was characterized by the absence of the 350 bp band and the genotype C showed only four bands of 280 bp, 650 bp, 700 bp and 800 bp.

Eleven polymorphic bands in the 200-1000 bp range were detected within the BOX-PCR profiles separated by 6% polyacrylamide gel electrophoresis (Figure 4B), some of which could not be seen on agarose gel (Figure 4A). Six BOX-PCR genotypes (G1 to G6) were distinguished for the 75 isolates (Table 2). Genotypes G2 and G4 represented the most frequent in the collection, including 50% and 20% of the strains respectively, while the remaining 30% of the strains were distributed among the other four genotypes (G1, G3, G5 and G6).

Exposure bioassays for investigating the virulence of P. larvae isolates

One *P. larvae* isolate for each of the three different branches of the 16S rRNA gene phylogenetic tree was selected for testing its virulence against honeybee larvae (Figure 5)

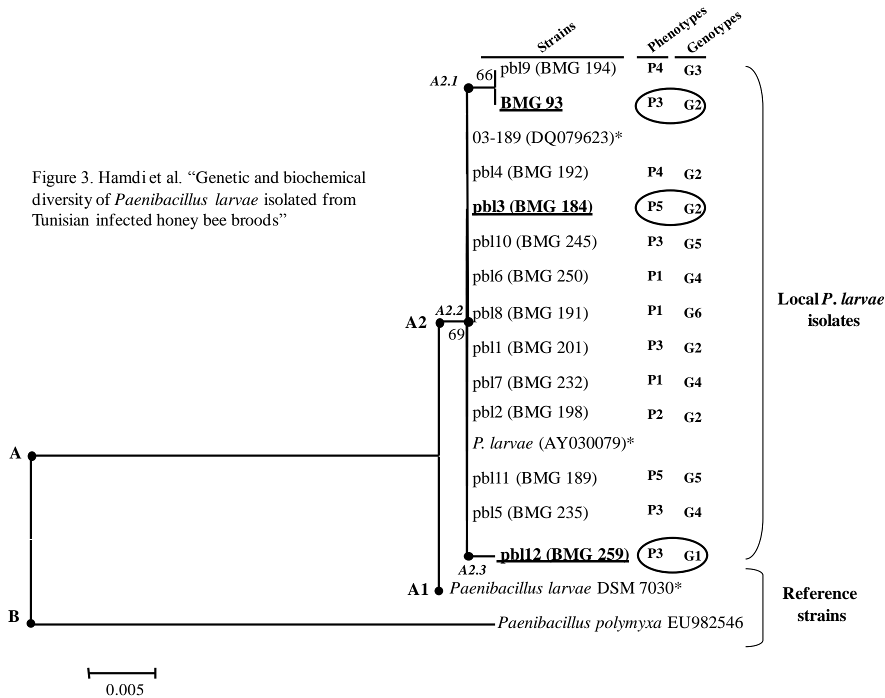

Fig. 3. Neighbour-joining phylogenetic tree of partial 16S rRNA genes sequences of 13 local isolates of *P. larvae* (BMG 93, BMG 192, BMG 194, BMG 198, BMG 201, BMG 232, BMG 245, BMG 235, BMG 250, BMG 259, BMG 184, BMG 189, BMG 191) and three of their closest relatives (indicated by stars). *P. polymyxa* (EU982546) was used as an out-group. The method of Jukes and Cantor was used to calculate evolutionary distances. Bootstrap values (n = 500 replicates) were indicated at the nodes.

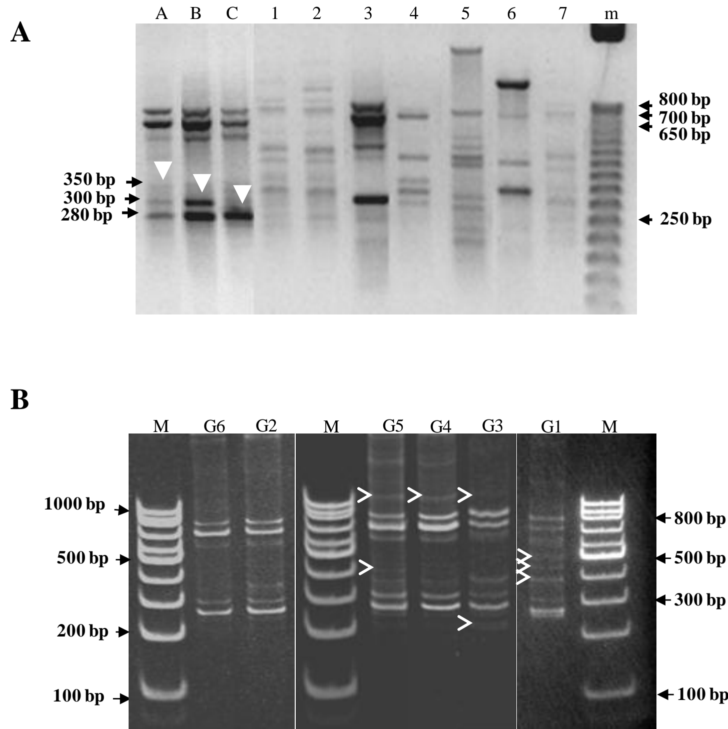

Fig. 4. Rep-PCR using BOX primer. **A.** the relationship between the isolates of *P. larvae* and other *Paenibacillus species* detected on agarose gels: Lane1: *P. macerans*; lane 2: *P. alvei* A4; lane 3: *P. thiaminolyticus*; lane 4: *P. alvei* A3; lane5: *P. dendritiformus*; lane 6: *P. vorticalis*; lane 7: *P. polymyxa.* A, B and C: three BOX haplotypes detected on agarose gel. **B.** BOX-PCR profile of *P. larvae* isolates detected on 6% polyacrylamide gels, six BOX haplotypes were detected for 75 isolates (G1 to G6). m: Marker 50 bp; M: Marker 100 bp; the additional bands detected on polyacrylamide gel were indicated with arrowheads.

All the three isolates, BMG 93, BMG 184 and BMG 259, determined high mortality rates at $5x10^3$ CFU ml^{-1} (50.3±2.05%, 47.33±3.5%, 49±2.6% mortalities, respectively) and at 10^5 CFU ml^{-1} (79±3.8%, 73±1% and 75±1.5 mortalities, respectively). No significant differences were observed between the three isolates basing on the T-test (for the treatment with $5x10^3$ CFU ml^{-1}, BMG 93 vs BMG 184, p=0.29; BMG 93 vs BMG 259, p=0.56; BMG 184 vs BMG 259, p=0.54; for the treatment with 10^5 CFU ml^{-1}, BMG 93 vs BMG 184, p=0.053; BMG 93 vs BMG 259, p=0.09; BMG 184 vs BMG 259, p=0.18). The mortality rate of the uninfected control group was less than 20% in all the three experiments.

Table 2. Identification of six distinct BOX genotypes for seventy five isolates of *P. larvae*, based on the combination of bands size and number on polyacrylamide gel.

Genotypes Bands (bp)	G1	G2	G3	G4	G5	G6	Total
200	-	-	+	-	-	-	
280	+	+	+	+	+	+	
300	+	+	+	+	+	+	
350	-	+	+	+	+	-	
400	+	-	-	-	+	-	
450	+	-	-	-	-	-	
500	+	-	-	-	-	-	
650	+	+	+	+	+	+	
700	+	+	+	+	+	+	
800	+	+	+	+	+	+	
1000	-	-	+	+	+	-	
Number of strains	1	38	2	20	5	9	75

+: presence of band; -: absence of band

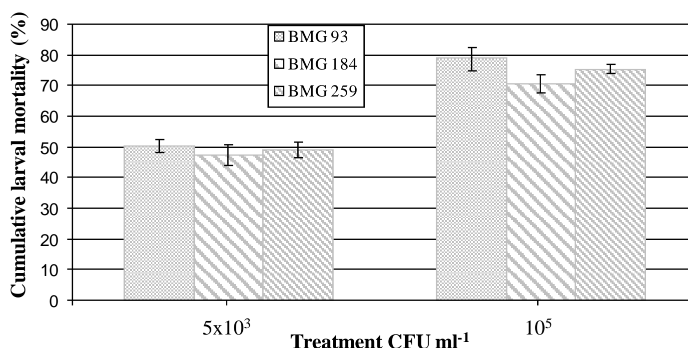

Fig. 5. Larval mortality rate after exposure to the pathogen *P. larvae*. Graphical representation of cumulative mortality percentage of larvae (± SD), fed with artificial diet supplemented with *P. larvae* spores at $5x10^3$ CFU ml^{-1} or 10^5 CFU ml^{-1}, during 8 days. In Y axis are reported the mortality percentage of larvae, in X axis is reported the different *P. larvae* strains used of larval infection tested at the two spore concentrations.

Discussion

In the dendrogram resuming the *P. larvae* isolates relationships according to the biochemical features (Figure 2), five branches corresponding to five biochemical phenotypes (P1 to P5) could be distinguished. This clustering was based on the detected polymorphism in several biochemical properties (nitrate reduction, oxidase production, indol and methyl red test). The isolates in branch 3, representing 88% of the isolates in the collection presented typical

characteristics of *P. larvae* (Gordon et al., 1973) being Gram-, casein- and gelatine-positive, catalase-, oxidase- and starch-negative, capable of using citrate, reducing nitrates to nitrites, acidifying the medium from glucose without gas and H_2S production, and incapable of growing in media containing 5% NaCl or in nutrient broth. The other branches (12% of the collection) presented variability in four tests: nitrates reduction, methyl red test, oxidase and indol production. The isolates in branch 1 (BMG 191, BMG 232, BMG 250 and BMG 257) were oxidase positive while isolate BMG 198 in branch 2 was double positive for oxidase and methyl red. The positive response of *P. larvae* to methyl red and oxidase was not described previously. Isolates BMG 192 and BMG 194 in branch 4 were able to produce indol and isolates BMG 184 and BMG 189 in branch 5 contained isolates unable to reduce nitrates. These results obtained with isolates retrieved from a relatively small area of Northern Tunisia show that *P. larvae* is not a monoclonal species like several other pathogens supporting previous observations (Heyndrickx et al., 1996; Neuendorf et al., 2004) of a certain phenotypic variability highlighted in the former subspecies *P. larvae subsp. larvae* and *P. larvae subsp. pulvifaciens*.
16S rRNA gene sequencing confirmed the assignment of all the strains to *P. larvae* but highlighted certain sequence variability among the isolates confirming the lack of a strict clonality in the species according to the biochemical study. However, it was not possible to identify a clear correspondence in the isolate grouping between the phenotypic and the 16S rRNA gene sequence variability.
A relative intraspecific diversity within the 75 Tunisian isolates was further confirmed by BOX-PCR typing which allowed the distinction of *P. larvae* from the related *Paenibacillus* species. In addition, BOX profiles showed polymorphic bands specific for *P. larvae* that could be useful for its identification as in the case of other pathogenic bacilli like *B. anthracis* (Cherif et al., 2002). Using BOX-PCR, an unexpected genetic variability was revealed for isolates deriving from a relatively small region like the Northern Tunisia. Alippi and coworkers (1998), by typing by BOX-PCR a collection of 100 *P. larvae* originating from a geographic area much larger than Northern Tunisia, detected only three genotypes. BOX-PCR combined to REP-PCR, revealed four genotypes within a collection of 105 strains of *P. larvae* isolated from Germany (Genersch and Otten, 2003; Neuendorf et al., 2004). Similarly, within a collection of 214 *P. larvae* isolates from Austria only five genotypes were identified by PCR typing using BOX A1R and MBO REP1 primers (Loncaric et al., 2009). The results obtained with the present Tunisian isolate collection suggest that the genetic and phenotypic variability of *P. larvae* can be larger that previously estimated.

However, despite the combination of the three approaches, the biochemical, phylogenetic and molecular typing methods highlighted a relatively high intraspecific diversity of the Tunisian *P. larvae* collection, a clear correlation and grouping of the isolates according to the three methods was not evidenced. This may indicate slightly distinct evolutionary pathways within the species that apparently remain neutral and not yet clearly evident in distinct coherent phenotypes.
The attempt to search for a possible effect of the different observed phenotypes/genotypes on the level of virulence supports the considerations that the observed differences have no apparent effects on the pathogenicity against the honeybee larvae, at least in the conditions adopted in the study to test the virulence. Our results showed that the three tested isolates of *P. larvae*, BMG 93, BMG 184 and BMG 259 representing three 16S rRNA gene phylotypes and two BOX-PCR genotypes, presented the same virulence level against honeybee larvae. Such lack of correlation could be due to the procedure adopted, and we cannot exclude that, for instance, the low number of isolates tested in the virulence assays or the limited period (8 days) for observing the mortality may have prevented the observation of virulence differences among the different Tunisian genotypes of *P. larvae*. Also, we do not know the ERIC type of our isolates since all the PCR attempts to get clear fingerprints with the Tunisian isolates failed. For instance, we could be in presence of a collection of isolates representing a single ERIC type and hence a single virulence type (Genersch et al., 2005). Similarly we cannot exclude that in the beehive the virulence behavior of the Tunisian isolates may vary (Hussein, 2000).

Conclusion

By keeping in mind all the above considerations related to the limitations of the adopted experimental conditions, the present data indicate a relatively high biological variability of *P. larvae* in Northern Tunisia, and suggest that the variable phenotypic and genotypic traits observed in the isolate collection apparently have a neutral effect in relation to virulence, or affect other ecological aspects of *P. larvae* non detectable with the experimental approaches used here.

Acknowledgements

The authors thanks for financial support the European Union in the ambit of project BIODESERT (EU FP7-CSA-SA REGPOT-2008-2, grant agreement no. 245746), and the Tunisian Ministry of Higher Education and Scientific research in the ambit of the laboratory projects LR MBA206 and LR11ES31. We are

grateful to Dr. Ziegler from BGSC for providing us the reference strains of *Paenibacillus*.

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Chapter IV

Probiotics help honeybees against *Paenibacillus larvae* infections

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Abstract

In the last years, the central role of the gut microbiome in assuring the healthy state of its host was demonstrated. In humans and in other animals it was clearly established that several pathologies are associated with the alteration of the normal microbial flora. In order to re-balance the microbial disequilibrium, probiotics are widely used to solve dysbiosis-related problems. The honeybee *Apis mellifera* is the most important pollinator worldwide, and a global loss of honeybee colonies is occurring, caused by several biotic and abiotic factors. We propose to apply a probiotic treatment to solve honeybee-related problems. One of the most aggressive pathology is the larval disease American Foulbrood (AFB). Thus, the *Apis mellifera*-AFB pathogenic system was used as a model because of the easy reproducibility and handling of the pathogen in laboratory. At first, to evaluate the microbial composition and structure of the honeybee microbiome a multi-technique approach was applied, confirming also an unbalance of the microbial community comparing honeybee broods with and without AFB symptoms. Afterwards, several bacterial strains among the most representative taxa were isolated and tested for the antagonistic activity against AFB etiological agent, *Paenibacillus larvae*, by *in vitro* and *in vivo* investigations. Two spore-forming bacteria, i.e. *Bacillus thuringiensis* and *Brevibacillus laterosporus*, showed the best performances in preventing the invasion of *P. larvae*, showing a synergistic activity when the bacteria were co-administered. Moreover, those results were confirmed when the probiotic mix was applied “in field” conditions. Several mechanisms, mediated by the probiotics, were involved in the honeybee healthy protection, i.e. direct inhibition with antibiosis, or indirect mechanisms by the elicitation of the immune system or by competitive exclusion.

Introduction

Animals, among which humans, are continuously exposed to pathogens of different nature, such as, bacteria, fungi, viruses, and protozoa. In the establishment of a disease, different phases follow one another, from the initial

contact of the pathogen with the host, to the pathogen multiplication and colonization, up to the pathogen invasion and the following disease outbreak (Hornef et al., 2002; Litchman 2010; Hells et al., 2013; Adler et al., 2009). When a gut pathogen reached the animal gut, it has to compete with the normal microbiota. Naïve microbiota, when present in a suitable climax community, is a powerful barrier to counteract the pathogen establishment, functioning in the prevention of microbiota alteration. Gut symbiotic microbiota, considered as a “super organism” (Aziz et al., 2013), is organized in a specific structure and composition that assures a good functionality to the host, being involved in host health homeostasis (Aziz et al., 2013). However, stressing factors, both abiotic (changes in environmental conditions) and biotic (exposure to a pathogen), can unbalance the relative proportion of the microbial phyla within the community, causing a dysbiosis. The consequences of dysbiosis on host health were studied in several animal models, showing furthermore a link to specific diseases (Turnbaugh et al. 2006; Baffoni et al., 2012). In humans a variety of diseases and pathologies are associated with the alteration of the gut microbiota, such as irritable bowel syndrome (IBS) (Cremon et al., 2010), obesity (Turnbaugh et al. 2006), inflammatory bowel disease (IBD) with ulcerative colitis and Crohn’s disease (Goldszmid and Trinchieri, 2012). Eminent works, conduced both on humans and animals (insects included), proved that, once restored the original climax community, in absence of other risk factors, the host can recover and re-establish its functionality (Hell et al., 2013; Sokol et al., 2008; Ben Ami et al. 2010; Ruth et al., 2006). Under this prospective, counteract the dysbiosis thought a probiotic therapy seems a promising way to prevent specific diseases and pathogenesis (Sartor et al., 2008).

Since few years, honeybees are suffering from a global decline, the causes of which are troubling researchers and beekeepers. As the susceptibility to pathogens (bacteria, viruses and fungi) is one of the important drivers of pollinator decline (among which also abiotic factors are accounted, such as sub lethal doses of pesticides, pollution and global change), researches feel the urgent need to better understand the interaction occurring between bee pathogens and host microbiota and to develop effective control strategies to prevent further mass losses.

Honeybees possess a simplified microbiome, constituted by eight phylotypes, conserved worldwide (Jeyaprakash et al., 2003; Mohr & Tebbe, 2006; Babendreier et al., 2006; Cox Foster et al., 2007; Martinson et al., 2011; Martinson et al., 2012; Moran et al., 2012). Recent works provided the evidence that protective symbionts may play a decisive role as modulators of honeybee health (Vasquez et al., 2012). Moreover, Koch and Schmid-Hempel (2011) reported that, in bumblebees, the microbiome functions as an “extended immune

phenotype” protecting the host against an intestinal and highly virulent trypanosome, *Crithidia bombi*.
One of the most destructive bacterial diseases is the American Foulbrood, which affects bee larvae and causes high economic losses to beekeepers and agriculture all over the world (Genersch et al., 2010). Because of the economic implication and its easily handing and reproducibility in the laboratory, we chose AFB as a bacterial disease model. Since the first isolation of its etiological agent, currently classified as *Paenibacillus larvae* (Genersch et al., 2006), many efforts have been made in defining the development of the disease. *P. larvae* forms tenacious spores and owns a strong virulence machinery, that recently received a great interest (Antúnez et al., 2010; Antúnez et al., 2011; Chan et al., 2011; Poppinga et al., 2012; Garcia-Gonzalez and Genersch, 2013; Fünfhaus, 2013; Poppinga and Genersch, 2013). Due to its high virulence and contagiousness, AFB is a notifiable disease in many Countries: infected hives must be declared to the authorities and destroyed by burning in order to avoid epidemic events (Genersch et al., 2010). At present, curative treatments for AFB are not available, as well as preventive ones. In most of European countries, the use of antibiotics against *P. larvae* is banned due to several problems associated with their use, i.e. presence of antibiotic residues in honeybee-derived products, insurgence of *P. larvae*-resistant strains, and negative effects on honeybee vitality and longevity (Genersch et al., 2010).
In the present work, by employing honeybees and AFB as study model, we performed *in vivo* and field experiments in order to evaluate the bacterial potential in decreasing the larval mortality after the administration of probiotic formulations and the challenge with pathogen. Therefore, after the evaluation by cultivation-independent techniques of the bacterial dysbiosis in AFB symptomatic and asymptomatic honeybee larvae, probiotic bacteria isolated from honeybee gut were selected for their capability to counteract *P. larvae* growth *in vitro*. *In vivo* rearing assays to assess the larval susceptibility to *P. larvae* infection with or without the larval exposure to probiotic bacteria were performed. Probiotics were used both singularly and in mix, in order to evaluate the bacterial synergism in enhancing bee protection. Larval exposure to probiotic strains prevented invasion of the pathogen, decreasing the larval mortality owing to *P. larvae* infection by direct (i.e. antibiosis) and indirect (i.e. exclusive competition and elicitation of the innate immune response) mechanisms. Field trials of the bacterial mixtures were also assayed to prove the concrete effectiveness of the treatments.

Results and Discussion

Bacterial dysbiosis in AFB-symptomatic honeybee larvae

The microbiota associated to 41 honeybee specimens of different developmental stage and health status was investigated by PCR-DGGE (Fig. 1A). Larvae from AFB asymptomatic and symptomatic hives were collected from two different eco-climate zones, one localized near Turin, in Italy (representative of the humid temperate climate zone with some continental characteristics) and the other one in North Tunisia (representative of the Mediterranean climate). The bacterial community profiles of the different individuals showed low complexity with many bands that were rather conserved among the individuals. Conversely to the adult stage, for which a huge number of studies are available in literature (Jeyaprakash et al., 2003; Mohr and Tebbe, 2006; Babiendrieer et al., 2007; Cox Foster et al., 2007; Martinson et al., 2011; Martinson et al., 2012; Moran et al., 2012; Engel et al., 2012; Sabree et al., 2012; Koch et al., 2013), only few studies investigated the bacterial diversity associated to larvae (Mohr and Tebbe, 2006; Martinson et al., 2012). Statistical analysis performed on DGGE profiles of Italian samples clearly clustered asymptomatic larvae separated from symptomatic ones (Fig. S1). Both Italian and Tunisian asymptomatic larvae were characterized by few bacterial species clustering within Firmicutes (*Lactobacillus* sp. and Clostridiales) and α-Proteobacteria (*Acetobacter* sp., *Gluconobacter* sp. and some sequences with 96% nucleotide identity to *Saccharibacter floricola*, Fig. 1A; Tab. S1). Italian asymptomatic samples revealed also the presence of γ-Proteobacteria, related to *Gilliamella apicola* and members of Pasteurellaceae family. On the other hand, in both Italian and Tunisian symptomatic larvae, sequences with 100% of nucleotide identity to *P.larvae* were found, together with sequences that clustered with 94% of nucleotide identity to the fructophilic *Fructobacillus fructosus* in diseased Italian larvae and with Clostridiales and *Leuconostoc* sp. (94% and 94% nucleotide identity, respectively) in Tunisian diseased larvae. Asymptomatic larvae withdrawn in Tunisian hives showed also the presence of *P. larvae* specific band.

16S barcoding parallel pyrosequencing was adopted to further investigate the microbiota of selected Italian larvae from symptomatic (M9, M11, M12, M6, M7, M8) and asymptomatic (S10, S12, S14, S11, S13, S15) hives that were previously analyzed by PCR-DGGE. Tab. S2 reports the total number of good 454-reads for each sample after trimming the chimeras, deleting singletons and reads with a frequency less than 0.1%. Principal coordinate analysis (PCoA) on pyrotag data confirmed PCR-DGGE results (Fig. 1B): symptomatic larvae clustered clearly together, as well healthy larvae. A high number of Paenibacillaceae sequences, to which family *P. larvae* belongs, was retrieved from diseased larvae, confirming the huge larval infection with *P. larvae* (Fig. S2). In asymptomatic larvae, among

the adults previously detected phylotypes (Martinson et al., 2011; Moran et al., 2012), 16S barcoding showed mainly the presence of Firm-4 and Firm-5 phylotypes. Other bacterial taxa detected were Alpha-1, Gamma-1, Beta, Alpha-2.2, and Gamma-2 phylotypes. In symptomatic larvae, besides *P. larvae*, we detected Firm-5, Gamma-1 and Beta phylotypes. Phylochip was also applied to these samples, being in agreement with pyrotag and PCR-DGGE results (see PCoA graph, Fig. 1C).

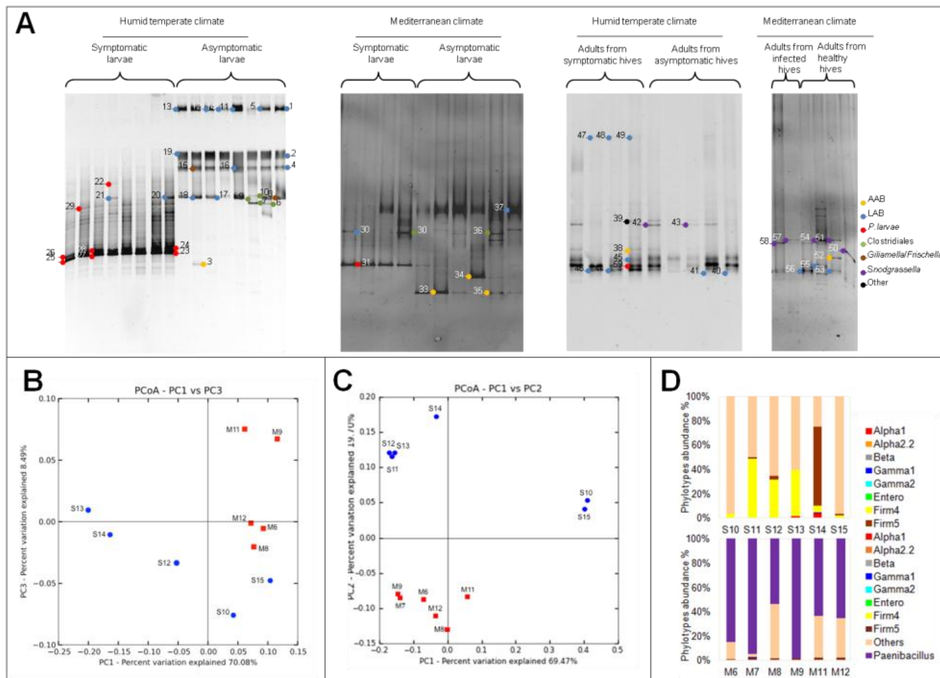


Fig. 1. The microbiome associated to larvae and adults from AFB asymptomatic and symptomatic hives was analysed by Denaturant Gradient Gel Electrophoresis (DGGE)-PCR (A), pyrosequencing of 16S rRNA gene amplicons (B) and Phylochip (C). By DGGE-PCR, the bacterial community profiles of the different individuals showed low complexity with many bands that were rather conserved among the individuals (A). PCoA analysis performed on pyrotag (B) and phylochip data (C) showed a clear separation between infected larvae (red) and healthy (blue) samples. Pyrotag data were then compared to previously reported data (Martinson et al., 2011) to highlight the presence of the phylotypes already described in the above-mentioned publication (D). All the sequences not matching with the described phylotypes are grouped as “others”, from which were subtracted the sequences relative to *Paenibacillus* spp. . Abbreviations: AAB:Acetic Acid Bacteria, LAB: Lactic Acid Bacteria, *P. larvae*: *Paenibacillus larvae*, M: AFB infected larva, S: Healthy larva.

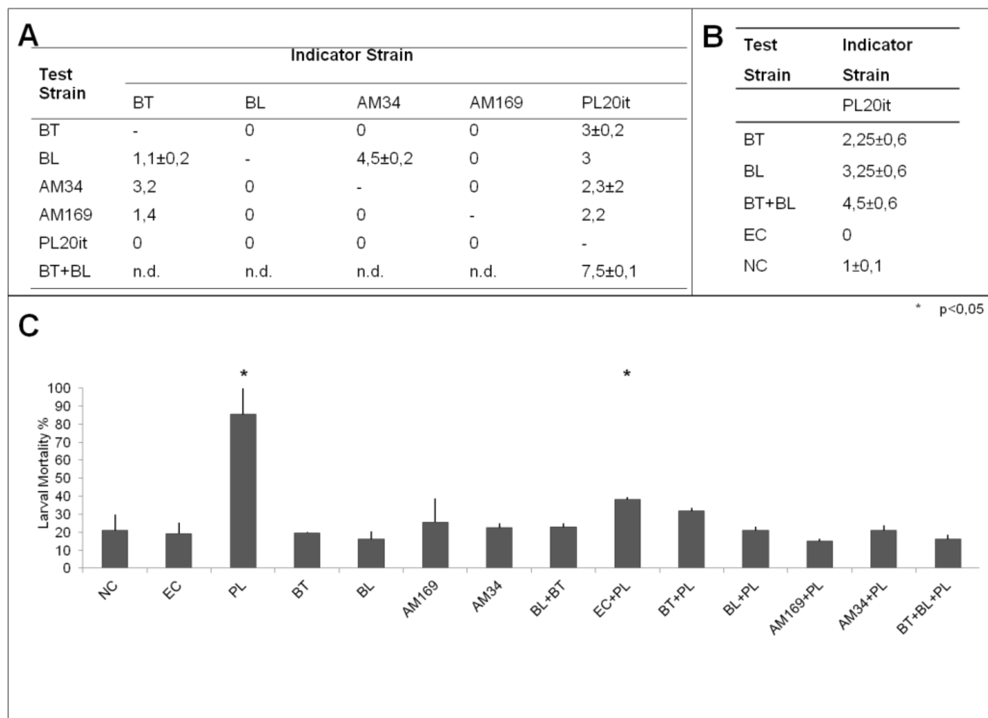
Taken together the molecular tools corroborate the presence of a low complex larval microbiome in the asymptomatic larvae, mainly represented by Lactobacillales, γ-Proteobacteria and Clostridiales. When *P. larvae* invasion is established, an alteration of the healthy microbiome is observed with the collapse of the native bacterial species and the blooming of *P. larvae* over all the other

members of the native bacterial community, recording a phenomenon of dysbiosis. Even thought the adult stage is not affected by *P. larvae* but contributes to the pathogen spreading through the foraging activity (Riessberger-Gallé et al., 2001), honeybee adults sampled both from the Mediterranean and humid temperate climate zones were included in PCR-DGGE analysis to highlight that our procedure confirmed the results previously detected by other groups (Martinson et al., 2011; Martinson et al., 2012). Thus, in accordance with previous works, adults presented a simple, consistent and characteristic microbiota (Martinson et al., 2011; Martinson et al., 2012). Sequencing results of the excised bands highlight the presence of a honeybee "core microbiome" widespread in these two different eco-climate zones, not affected by factors like place, climate and sub-specific feature of the host (Tab. S1; Hamdi et al., 2011). Major components of this adult core microbiome are members of α-Proteobacteria (members of Acetobacteraceae family and *Bartonella tamiae*), β-Proteobacteria (*Snodgrassella alvi*), and Firmicutes (*Lactobacillus* sp. and *Weissella* sp.). In adults collected from hives with or without AFB symptoms, sequences related to *P. larvae* (100% nucleotide identity) were retrieved.

P. larvae antagonistic microbiome to counteract dysbiosis by in vitro inhibition assays

After the characterization of the asymptomatic larvae's microbiota, with the purpose to identify several effective bacteria in counteracting the pathogen, 409 bacterial strains from healthy larval and adult specimens were isolated by using enrichment and rich media (Tab. S3). Specifically, we focused on lactic acid bacteria (LAB) and spore-forming bacteria (SFB), since they have been already described as common inhabitants of animal gut and proved to have some antagonistic effect against PL (Cherif et al., 2008; Carina Audisio et al., 2011). Besides, we considered acetic acid bacteria (AAB) because of their intriguing involvement in the host immune and metabolic homeostasis (Ryu et al., 2008; Shin et al., 2011). They also own some peculiar features, such as the ability to change environmental pH, to prevent the pathogens' colonization of gut epithelia through a massive production of extracellular polysaccharides (Kounatidis et al. 2009; Crotti et al. 2010). After de-replication of the bacterial collection by internal transcribed spacer (ITS)-PCR, near full-length 16S rRNA gene fragments (almost 1500 bp) were amplified from several representatives of each ITS groups and seventy-seven 16S rRNA gene amplicons were partially sequenced and aligned against NCBI database. Among the isolates, five major groups were found. Firmicutes accounted for 56% (no. 230) of the total isolates and was dominated by *Lactobacillus* (no. 135). Gram-positive SFB represented 20.4% of Firmicutes with species frequently found as *Bacillus thuringiensis* (no. 8), *Brevibacillus laterosporus* (no. 5), *Bacillus pumilus* (no. 8), *Bacillus safensis*

(no.5), *Bacillus amyloliquefaciens* (no. 5). Other members of Firmicutes such as *Staphylococcus* (no. 9), *Sporosarcina* (no. 2) and *Bacillus* spp. (no. 1) were found. α-Proteobacteria represented the second abundant group (no. 135) with 33% of total isolates, dominated by AAB members (96.3% of total α-Proteobacteria isolates) with as major representatives *Saccharibacter* sp., *Acetobacter estunensis* and *Gluconobacter cerinus*. γ and β-Proteobacteria groups were respectively represented only by 6 and 8 isolates (1.5 and 2.0% of total isolates), whereas Actinobacteria members accounted for 30% of the total isolates. To test the inhibition capability of the isolates against the AFB causative agent, a pathogenic strain isolated with high frequency from Italian diseased larvae, *P. larvae* strain PL 20it was used.



A

Test Strain	Indicator Strain				
	BT	BL	AM34	AM169	PL20it
BT	-	0	0	0	3±0,2
BL	1,1±0,2	-	4,5±0,2	0	3
AM34	3,2	0	-	0	2,3±2
AM169	1,4	0	0	-	2,2
PL20it	0	0	0	0	-
BT+BL	n.d.	n.d.	n.d.	n.d.	7,5±0,1

B

Test Strain	Indicator Strain
	PL20it
BT	2,25±0,6
BL	3,25±0,6
BT+BL	4,5±0,6
EC	0
NC	1±0,1

Fig.2. *In vitro* and *in vivo* inhibition tests. In panel A and B, values detected for well-diffusion inhibition assays are reported. The indicator strain was cultured in a soft agar layer, while the 48 hrs-supernatant of the test strain (A) or the smashed guts from larvae reared with BT, BL, and EC (B) were added. The measuration of the halos around the wells indicates the inhibition (in cm). Experiments were performed three times in triplicate. Mortality levels for *in vivo* rearing experiments are indicated in panel C. Larvae were fed with one or more symbionts in the presence or in the absence of the pathogen PL. For each treatment, the average of the larval mortality percentage is shown (C); PL and EC+PL treatments showed a statistical difference in comparison to the NC mortality. Abbreviation: NC, sugar-based diet; BT, *Bacillus thuringiensis* HD 110; BL, *Brevibacillus laterosporus* BMG65; PL, *Paenibacillus larvae* 20it; EC, *Escherichia coli* SC110; AM169, *Saccharibacter sp.* AM169; AM34, *Lactobacillus alvei* AM34.

The strain was genotyped via ERIC-PCR (Genersch et al., 2006; Fig. S3), exhibiting ERIC1 profile when compared to the profiles considered the two most important *P. larvae* genotypes, ERIC1 and ERIC2 profiles, represented by the two reference *P. larvae* strains DSM 7030^T and DSM 16115, respectively (Genersch et al., 2006). Moreover, by the detection of the recently identified putative S-layer protein of *P. larvae*, the S-layer protein A (SplA), (Poppinga et al, 2012), it was confirmed the identification of PL 20it as an ERIC1 profile's strain (Fig. S3). Inhibition capability of the isolates against PL 20it was evaluated by measuring (in cm) the distance existing between the hole's edges and the first line of *P. larvae* growth after 24 hours (Fig. 2A). Thirty-three isolates, out of a sub-collection of 285 isolates, consistently inhibit *P. larvae* and specifically, 27 strains among which 21 *Saccharibacter* sp., 1 *Lactobacillus alvei*, and 2 *Brevibacillus laterosporus*. Among them, 3 strains showed the best inhibition performance: *Brevibacillus laterosporus* BMG65 (hereafter indicated as BL), *Saccharibacter* sp. strain AM169 (hereafter indicated as AM169) and *Lactobacillus alvei* AM34 (hereafter indicated as AM34). Reference strain *Bacillus thuringiensis entomocidus* HD110 (hereafter indicated as BT, Cherif et al., 2008) was also included in the inhibition assays, since it showed a consistent inhibition halo on PL-containing plates. Cross-inhibition assays were performed to verify cross-inhibition reactivity among the selected bacteria (Fig. 2A). No cross-inhibition effect has been reported; exception is represented by BT that was lighted inhibited by BL, AM169 and AM34. It is noteworthy the synergistic effect owned by BL and BT in inhibiting PL strains (Fig. 2A): the haloes produced by the combination of the two strains was bigger than the sum of the halos of the two strains measured singularly.

Probiotic microbiome to counteract dysbiosis by* in vivo *rearing experiments

To evaluate if the PL-antagonistic bacteria showed PL-antagonistic activity *in vivo*, larvae were fed in 96-well plates with an artificial diet enriched with the antagonistic bacteria, in combination to PL administration. PL dosage, able to induce a larval mortality upon 50%, was determined as 5×10^4cfu mL^{-1} (Tab. S4, Tab.S5), and was used in the following larval mortality test. BT, BL, AM169 and AM34, together with *E. coli* SC110 (EC) as control, were administered to the larvae, followed by PL exposure (Tab. S6); variation of the larval mortality were monitored along the experimental time course (12 days). A basal larval mortality of 20,9% has been measured (NC treatment, Tab. S7). Mortality rates of larvae treated with antagonistic bacteria were not significantly different from the control value (NC) as confirmed by T-test values ($p>0.05$; Tab. S7; Fig. 2C). Interestingly, the treatment with BT+BL+PL (average mortality was 16,2%) hugely reduced the mortality of larvae (average mortality percentage of PL treatment was 85,5%; Tab. S7). Moreover also the administration of AM169 and

AM34 resulted in a decrease of mortality in comparison to PL treatment: AM169+PL gave a 15% mortality rate, whereas AM34+PL resulted in a 21% mortality rate. Taking together the results of *in vitro* inhibition assays against PL and the *in vivo* challenging experiments with PL, the best performances in exhibiting a protective effect against PL infection were accounted for these three bacterial treatments: BT+BL+PL, AM34+PL and AM169+PL.
To shed light on the stimulation of the host innate immune system, larvae were fed as above described (Tab. S6) with the following bacterial suspensions: BT+BL, AM34, AM169, including as controls EC, and PL. Larvae were collected 6 days after the treatments, and subjected to total RNA extraction. Transcript levels of the antimicrobial peptides (AMPs) abaecin, hymenoptaecin, and defensin, and of the immune factor lysozyme were relatively quantified considering as reference gene the honeybee 5S (Fig. 3C; Pfaffl, 2001). In the case of abaecin, transcript levels significantly increased upon exposure of BT+BL (Fig. 3C; Tab. S8), whereas low expression levels have been measured for AM34 and AM169 treatments. Abaecin transcript levels of BT+BL were 2.3× higher than the control (1×), while in the case of PL treatment they were 0.8× higher than the control. Transcript levels of hymenoptaecin for BT+BL treatment were 8.8× higher than the control (Fig. 3C; Fig. S6). No difference was reported for the transcript levels of defensin that were 0.9× higher than the control in the case of BT+BL treatment (Fig. 3C; Fig. S6). For both hymenoptaecin and defensin the transcript levels after PL exposure were very low, about 0.05× and 0.12× higher than the control, respectively. Interestingly, in the case of lysozyme, any treatment did stimulate higher transcript levels of lysozyme than the control (Fig. 3C; Fig. S6). In their work, Evans and Lopez (2006) reported a significant increase of abaecin transcript levels in 1st instar larvae after 48h from the exposure to a probiotic mixture of lactobacilli and bifidobacteria, while defensin transcripts did not changed significantly. Moreover, abaecin was significantly up-regulated in the first 24 hours after young larval exposure to pathogen spores, whereas in older larvae levels do not change (Evans, 2004). By a proteomic approach an increase of hymenoptaecin and lysozyme after 5 day from PL challenge has been shown by Chan et al (2009). In our case, we measured AMPs and lysozyme transcript levels in 5th instar-larvae that were previously (at 1st instar) exposed to PL. Thus, we found that by the administration of BT+BL mix, the upregulation of abaecin and hymenoptaecin transcripts is recorded even at the fifth larval stage. This could probably imply an improvement of the larval health status, just before the pupation, avoiding also the outbreak of possible secondary infectious.

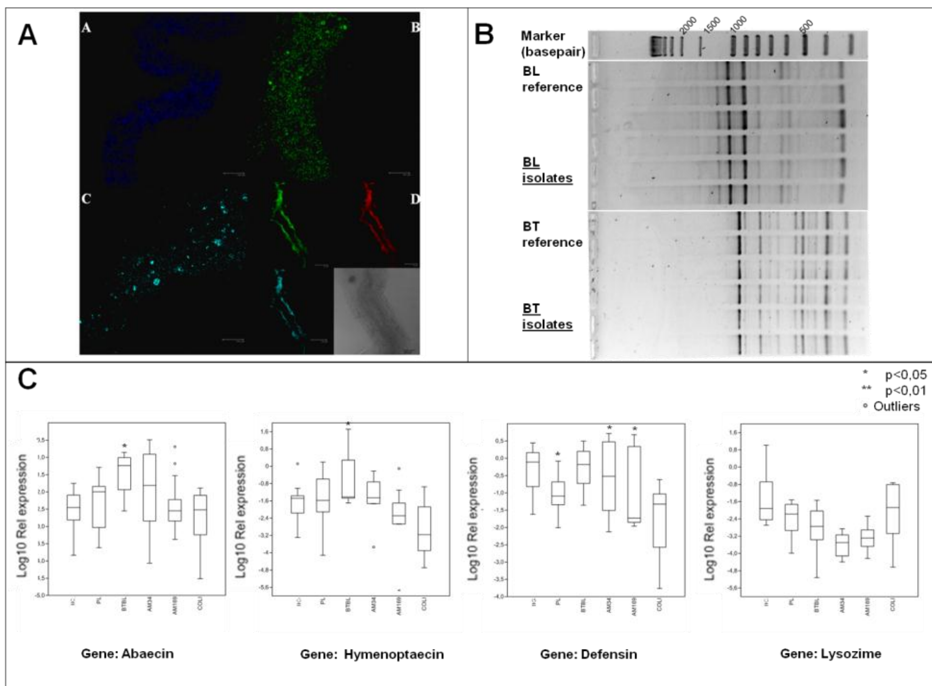


Fig. 3. Symbiont-mediated mechanisms against PL. *Fluorescence in situ hybridization* images showing the localization of the bacterial probiotics in the honeybee gut after the fifth instar stage (A) . Panel A.A) Confocal laser scanning microscopy (CLSM) image of FISH of a larva fed with NC, hybridized with 16s rRNA eubacterial probe (EU-blue). Panel A.B) CLSM image of FISH of a larva fed with BT, hybridized with BT-specific probe (green). Panel A.C) signal of the specific BL probe (cyan) from a larva fed with BL. Panel A.D) CLSM image of FISH of a larva reared with the probiotics in mix and challenged with the pathogen PL, hybridized with BT- (green), BL- (cyan), PL- (red) specific probes, compared with the bright-field image. BOX-PCR profiles of 15 randomly-picked colonies, for each strain, resulting from the plating on TSB (for BT) and NB (for BL) (B). The comparison of the re-isolated strains' profiles with BT and BL reference profiles confirmed the identity of the randomly picked colonies as BT or BL. Transcript levels of 4 genes involved in the honeybee immune system were evaluated from fifteen 5th-instar larvae (C); transcripts levels of abaecin, hymenoptaecin, defensin and lysozime are shown in boxplot (C).

Bacteria-mediated health protection towards PL invasion

Since BT+BL application showed the best performances in enhancing honeybee health (see previous paragraphs), BT+BL protection was evaluated directly in hive. Eight hives were weekly treated with BT+BL suspensions for seven consecutive applications, in duplicate with 8 further hives kept as untreated hives. The day after each treatment, 96 larvae from two treated and untreated hives were collected and half of each amount, from each hive, were challenged with PL (Tab. S9). In general, larval mortality rate showed a reduction along the time of application (Fig. 4A; Tab. S10). It is noteworthy that, when exposed to PL,

treated larvae revealed a significantly reduced mortality in comparison to untreated larvae (Fig. 4A). These results underline that treated larvae received with the weekly treatments an amount of antagonistic bacteria that can prevent a following PL invasion. Indeed, when bacterial treatments were not applied, but larvae were subjected to PL challenge, larvae were not able to respond to the pathogen invasion and high levels of mortality were documented (Tab. S10). Different mechanisms mediated by the symbionts co-operate in the bacterial protection of honeybee (Hamdi et al., 2011). Among them, we can account: i) the direct inhibition of the pathogen by the production of antimicrobial compounds; ii) the competitive exclusion (including the competition of the antagonistic bacteria for nutrients or for the adhesion to the epithelia against the pathogen); and iii) the activation/stimulation of the bee's immune system. We have already verified that BT and BL possessed a direct anti-PL activity (Fig. 2B). Sporeformers, as BT and BL, are known to produce bacteriocins or other antibacterial compounds (Cherif et al., 2006; Singh et al., 2011). However, to confirm their *in vivo* inhibition ability, smashed pooled intestines of larvae fed with BT, BL, BT+BL, EC, and NC suspensions were tested against PL by well diffusion assays (Fig. 2B). Haloes around the wells clearly demonstrated the inhibitory activity of BL, BT and BT+BL suspensions. Particularly, intestines of larvae fed with BT+BL showed a remarkable inhibition activity in comparison to the controls and the smashed guts of larvae fed with BT and BL alone, respectively (Fig. 2B). Interestingly, control intestines showed a small halo, suggesting two hypotheses: i) the inhibition activity can be exerted by native symbionts of the larvae; ii) the dissected guts could contain some antibacterial substances, like honey that is well known for its antibacterial property.
We verified the capability of the antagonistic bacteria to colonize the insect gut, carrying out re-isolation trials from BT-fed and BL-fed larvae (Tab. S11). Colonization ability is indeed a peculiar characteristic of probiotic strains. SFB were re-isolated efficiently from SFB-fed larvae at 6 days after bacterial administration. The strains re-isolated from SFB-fed larvae were typed by BOX-PCR to confirm their identity. To evaluate if SFB can compete with PL by competitive exclusion, FISH were performed (Fig. 3A). Positive controls is shown in Fig. 3A, panel A. FISH micrographs confirmed that BT (Fig. 3A, panel B) and BL (Fig. 3A, panel C) were able to promptly colonize the larval gut. BT and BL are localized in the midgut when BT, BL and PL were co-administered.
To assess if innate immune elicitation occurs in field applications, abaecin and hymenoptaecin transcript levels were measured in treated and untreated larvae collected at time 0 and after the 5th and 7th treatment, followed or not by PL exposure in laboratory. As control, the relative quantity of abaecin and hymenoptaecin transcripts was determined in larvae at time 0 before the bacterial

applications (Fig. 4B; Tab. S12). In the case of no successive PL exposure, both in treated and untreated hives, abaecin and hymenoptaecin mRNA levels increased along the time and a significant increment was detected for larvae exposed to probiotics. In untreated larvae this trend has been explained as a physiological response of the larvae as a consequence of the progression of the development (Evans, 2004). The ratios between mRNA levels of treated and non treated larvae showed that, both for abaecin and hymenoptaecin, the enhancement of the transcript levels was continuous along the time course of treatments (0,83× after 5th treatment and 2,13× after 7th treatment, for abaecin and 0,39× after the 5th treatment and 1,09× after the 7th treatment, in the case of hymenoptaecin, Tab. S12). Observing box plot graphs, a disease prevention response, measured as significant increases of abaecin and hymenoptaecin transcript levels, occurred when the larvae were treated with the probiotic bacteria (Fig. 4B). Conversely, when the larvae treated with the probiotic strains were exposed to the pathogen, decrease in the levels of the abaecin and hymenoptaecin transcripts respect to the non treated larvae occurred at the fifth week of treatments with the probiotic strains, despite such a treatment significantly decreased the larval mortality induced by the pathogen. Such decreases of the two transcript levels induced by the pathogen were abolished at the seventh week of treatment with the two probiotic strains, in coherence with the maintained decreased mortality (Fig. 4A). Measuring the ratios between mRNA levels of treated and untreaed larvae, exposed to PL, showed a little decrease both for abaecin and hymenoptaecin (Tab. S12).

Taking together, this indicates that the influence of the two probiotic strains on the AMP expression, when the larvae were continuously treated with the two strains overtime, prevailed on that driven by the pathogen and that the two probiotics support the immune response homeostasis even in presence of the pathogen challenge. Due to the weak signals reported for defensin and lysozyme in previous experiments, we had not tested these AMPs in field trials.

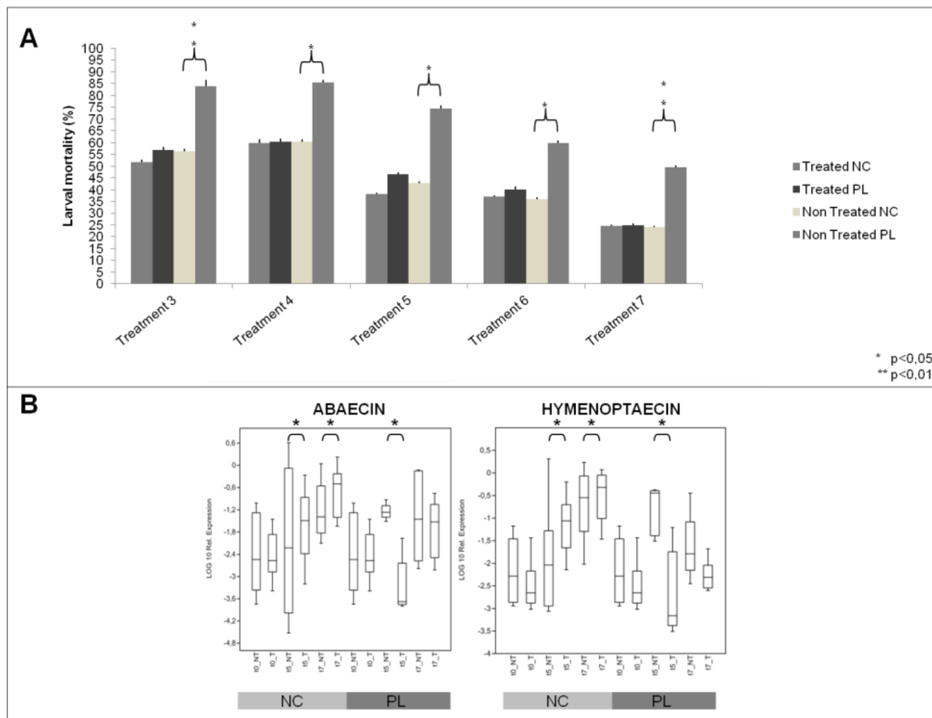


Fig. 4. Larval mortality percentages of bacterial treatments in field trials (A). Eight hives were weekly treated with a bacterial suspension of BT+BL spores for seven consecutive applications, while 8 further hives were kept as controls. The day after each treatment, 96 larvae from two randomly selected treated and untreated hives were collected and reared in 96-well plates. Forty-eight larvae (half of the amount of the withdrawn larvae) from each hive were challenged with the pathogen, while the other 48 were left untreated. Field trials were performed in duplicate. Relative quantity of transcript levels for abaecin and hymenoptaecin from larvae collected during field trials are showed in boxplot (B). Before starting with the application of treatments (treatment 0) and after treatment number 5 and 7, larvae were collected from treated and untreated assays. Transcript levels of these two antimicrobial peptides were measured considering as reference gene the honeybee 5S.
Abbreviation: NC, non challenged with PL, PL, challenged with PL, T: Hive treated with the probiotic, NT: Untreated hive (no probiotic administered).

Conclusion

To define the pelting during *P. larvae* infection is an attracting topic for scientists that recently have spent much effort on it (Yue et al., 2008; Poppinga et al., 2012; Garcia-Gonzales and Genersch, 2013; Fünfhaus et al., 2013). After the initial contact with the pathogen, i.e. the ingestion of *P. larvae* spores, these ones germinate in the midgut lumen, proliferate by a massively multiplication, and then invade the haemocoel by penetrating the midgut epithelium. In the first phase of the infection process, no damage to the epithelial layer can be observed, although *P. larvae* massively proliferates (Yue et al ., 2008). In the second phase

of the infection process, *P. larvae* breaches the epithelial layer and invades the haemocoel. By then, the infected larvae are dead, and *P. larvae* degrades the larval tissues until a ropy mass is left containing only bacteria (Yue et al ., 2008). A pivotal role in the pathogenic development is played by the perithrophic matrix, and the latest studies are focusing on the mechanisms by which the pathogen overcomes this barrier (Garcia-Gonzales and Genersch, 2013). Moreover, recent pathogen produced-virulence factors, such as toxins, were identified (Fünfhaus et al., 2013).
Due to the lack of curative treatments, when a *P. larvae* invasion occurs, there is no chance to revert the progression of the invasion process. To prevent the invasion, acting on the previous phases (ingestion, germination and multiplication), seems to be one possibility to counteract PL invasion. To prevent spore ingestion, hives and apiarian equipments must be safe and, in this perspective, manuals of good practices should be regulated. To remember is that, although worker bees with the "hygienic behaviour" can limit the affection of the disease by discarding PL-infected larvae and by cannibalism, adults are strongly infective since they spread PL spores to the nestmates (Evans and Schwarz, 2011). We also documented by PCR-DGGE the nature of honeybee adults as vectors of PL (Fig. 1A). On the other hand, antagonistic bacteria can contribute hindering germination and multiplication by several mechanisms, i.e. the direct inhibition of the pathogen through antibiosis, the competitive exclusion, and finally the stimulation of the immune system. Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit for the host" (Joint FAO/WHO Working Group, 2002). Even if the exact mechanisms by which probiotics explicate their function remain unclear, several ones were suggested, namely the capability to suppress pathogen growth, the ability to outcompete the pathogen blocking its adherence to the host epithelium, and the involvement in the immunostimulation of the host. BT and BL showed to possess all these capabilities, producing active substances (Fig. 2A-B), reducing larval mortality when hosts are exposed to PL (Fig. 2C), recolonizing efficiently the gut (Fig. 3 A), being alive and in high amount (Fig. 3B), and eliciting the innate immune system (Fig. 3C; Fig. 4B). All of these factors contribute in the prevention of a subsequent PL invasion both *in vivo* and field trials.
An intriguing point to focus on is the possible synergistic effect observed when the two SFB were tested together. The synergy of the two bacteria seemed to be effective in different mechanisms, in relation to the improvement of the healthy state of the host. Beyond the direct inhibition against *P. larvae* demonstrated *in vitro*, the analysis of the transcript levels of 4 immune system-related genes, performed using RT Real Time qPCR, showed an increase of the production of two AMPs transcripts when honeybees were reared in presence of both the two

SFB, in comparison to transcripts of larvae reared with AAB and LAB. Furthermore, a significant inhibition activity was also present when it was measured the pathogen inhibition using the supernatants obtained from the smashed guts of larvae fed with the co-administered probiotics. Moreover, FISH experiments indicated that the probiotic bacteria, although in the presence of massive concentration of *P. larvae*, are able to colonise the gut. It is supposed that a possible cause in hindering the pathogen development is due to competitive exclusion mediated by the symbionts bacteria (Hamdi et al., 2011).
In *in vivo* experiments and field trials the larvae have been exposed to 1×10^5 spores ml^{-1} of PL (Hamdi et al., 2013) to obtain a basal mortality of around 85% in the two *in vivo* experiments and in field trials. Similar values of basal mortality were reported for Genersch et al. (2006) and Hamdi et al. (2013). In nature just ten spores are necessary to achieve a fatal invasion of PL (Genersch et al., 2010). However, even with high number of PL spores, high protection of honeybee larvae was recorded.
Field trials, finally, were useful to understand if the scaling up of a process, effective in laboratory condition, was effective even on field. An advantage in the use of probiotic bacterial spores is the readily availability as veterinary and human dietary supplements (Evans and Lopez, 2004); thus, it would be relatively easy to generate a supply of probiotic treatments for bees. A statistically significant decrease confirmed the effectiveness of probiotics in acting against the pathogen in a very short time, confirming *in vivo* experiments' results. Conversely, AMPs transcript levels of larvae treated or not in field condition with the two probiotic strains and then challenged with the pathogen, were measured by Real Time RT-PCR, showing the clear influence driven by the two probiotic strains on AMPs transcript levels. In fact, when a long-term-administration of the probiotic strains is ongoing, their effects prevails on that driven by the pathogen and the two probiotics support the immune response homeostasis even in presence of the pathogen challenge.
In literature, few reports are available about the characterization of the larval microbiome (Vojvodic et al., 2013). In our study, while evaluating the dysbiosis status in larvae associated to PL invasion, by comparing asymptomatic and symptomatic larvae, it was characterized the structure and composition of the bacterial community by a polyphasic approach with three cultivation-independent techniques, DGGE-PCR, 16S barcoding sequencing and phylochip. Asymptomatic larvae showed to host a simple microbiota (Fig. 1) constituted mainly of Lactobacillales, γ-Proteobacteria and Clostridiales, with other minor represented taxa. To notice is that there is a partial match with the phylotypes previously detected in adults i.e. Firm-4, Firm-5, Alpha-1, Gamma-1, Beta, Alpha-2.2, and Gamma-2. (Martinson et al., 2011; Martinson et al., 2012; Moran

et al., 2012). In general, it is assumed that honeybees acquire their symbionts from the hive (Martinson et al., 2011). However, there are no available data on the transmission routes followed by the bacteria, except for Snodgrassella alvi (Beta) and Gilliamella apidicola (Gamma-1) which are vertically transmitted by colony queens to young gynes (Koch et al., 2013). FISH experiments by the use the eubacterial probe EUB388 on 5th instar larvae, not exposed to probiotic strains or to the pathogen, confirmed the presence of a dense bacterial community in the larva, conversely to the results of Martinson et al. (2012). One hypothesis could be that a reduced larval microbiome is associated to American specimens that were exposed for more years to tetracycline treatments than European ones. In fact, more genes related to tetracycline resistance are present in the bacteria, especially *Snodgrassella alvi* and *Gilliamella apidicola*, more associated to American larvae than European larvae (Tian et al., 2012).
In the present work, it was demonstrated that the honeybee symbionts are not only fundamental in the homeostasis of the host, but moreover they could be determinant in the improvement of the honeybee general healthy state. Further studies are needed to deeply understand the molecular mechanisms by which the probiotics colonize and heal the insect. With the present study we demonstrated, the concrete effectiveness of field application of probiotics treatments.

Materials and Methods

Honeybees

Honeybees (*Apis mellifera*; Hymenoptera: Apidae) were collected from geographically different apiaries localized in Italy (Grugliasco and Caluso, near Turin, representative of the humid temperate climate zone with some continental characteristics, according to Köppen-Geiger classification), and in Tunisia (Manouba, Nabeul, Beja, near Tunisi, representative of the Mediterranean climate, according to Köppen-Geiger classification) from June to August. Larvae and adults were collected from asymptomatic and AFB symptomatic hives and used in cultivation-independent and -dependent experiments. For the isolation of *Paenibacillus larvae*, honeybee larvae with AFB symptoms were collected in Italy from symptomatic hives before their destruction. For *in vivo* experiments first-instar (1-day old) larvae were collected from the Italian apiary and reared using an aseptic artificial diet, controlled temperature (37°C) and high humidity, as reported below.

Characterization of honeybee microbiota by Denaturing Gradient Gel Electrophoresis (DGGE), pyrotag and phylochip

DNA extraction from surface-sterilized larvae and adult body (after removing head, wings and legs) was performed adapting the procedure proposed by Raddadi et al. (2011) to honeybees. After DNA quantification by the use of

NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). PCR-DGGE was performed as followed. A 550 bp fragment of the 16S rRNA gene was amplified by using forward primer GC357F, containing a 40-bp GC clamp (5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCC TAC GGG AGG CAG CAG-3'), and reverse primer 907R (5'-CCG TCA ATT CCT TTG AGT TT-3',Sass et al., 2001). Polyacrylamide gels (7% of a 37:1 acrylamide-bisacrylamide mixture in 1X Tris-acetate-EDTA [TAE] buffer) were prepared according to the manufacturer's guidelines with a denaturing gradient of urea and formamide (100% denaturing polyacrylamide was defined as 7 M urea and 40% formamide) (Muyzer et al., 1993). Denaturant gradients of 38% to 50% for larvae and 38% to 55% for adults were used. DGGE bands were excised by using a sterile scalpel, eluted in 50 µl of MilliQ water and stored at -20°C. PCR for re-amplification was performed by using primers 357F (without GC clamp) and 907R as described in Marzorati et al. (2006). PCR products were then sequenced with primer 357F (Macrogen Inc., Seoul, Korea). Sequences were compared to the sequence database at the National Center for Biotechnology Information by using BLAST (http://www.ncbi.nlm.nih.gov /BLAST) (Altschul et al., 1990).
16S barcoding pyrosequencing was carried on some selected DNAs (i.e. M6, M7, M8, M9, M11, M12, S10, S11, S13, S12, S14, S15) as described by previous literature (Martinson et al., 2012). V1-V3 variable regions were amplified using primers 27F and 518Rmod.
Phylochip microarray was performed on the same honeybee brood samples following the procedure described by Tsiamis and colleagues (2008).

Isolation of gut bacteria and establishment of the bacterial collection

Bacteria were isolated from honeybee larvae and adults, after a pre-treatment to eliminate the microbial contamination of the external insect surface. Specifically, larvae were washed for 1 min in 70% ethanol, for 5 minutes in 5% sodium ipochloride, followed by 5 washes with 0,9% NaCl. Adult guts were carefully dissected by the use of sterile forceps and a Wild Makroskop M5A stereomicroscope (Heerbrugg, Switzerland). Larvae and guts were singularly homogenized in 900 µl of 0,9% NaCl and these suspensions (hereafter indicated with HS "honeybee suspensions") were used to inoculate specific media for the isolation of acetic acid bacteria (AAB), lactic acid bacteria (LAB) and spore-forming bacteria (SFB). TA1 medium (1,0% D-glucose, 0,5% ethanol, 0,3% acetic acid, 1,5% peptone, 0,8% yeast extract, pH 3,5; Lisdiyanti et al., 2001), and ABEM medium (2,0% D-sorbitol, 0,5% peptone, 0,3% yeast extract pH 3,5; Yamada et al., 2000), both added with 100 µg ml^{-1} of cyclohesimide, were used as pre-enrichment media for AAB isolation. One hundred µl of HS was inoculated in 20 ml of TA1 and ABEM media and let grow at 30°C for 3-4 days or at least till the bacterial growth. Then diluted bacterial suspensions were plated on $CaCO_3$-

enriched plates (1,0% D-glucose, 1,0% glycerol, 1,0% ethanol, 1,0% peptone, 0,5% yeast extract, 0,7% $CaCO_3$ and 1,5% agar, pH 6,8) and colonies capable of create $CaCO_3$-clearing haloes were selected and re-streaked for further analysis.

LAB isolation was performed by the use of MRS medium (Applichem, Germany), added with 100 µg ml^{-1} of cyclohesimide. Twenty ml of MRS medium were inoculated with 100 µl of HS and placed at 37°C without agitation for 5 days. After bacterial growth, diluted suspensions were plated in MRS agar plates and incubated anaerobically in GasPak at 37°C up to colony appearance. Finally colonies were randomly selected, re-streaked and subjected to further analysis.

SFB were isolated after pasteurization (10 min, 80°C) on Tryptic Soy Agar (TSA, Difco) plates added with 100 µg ml^{-1} of cyclohesimide. One hundred µl of HS aside with serial dilutions was plated on TSA plates, followed by an overnight incubation at 30°C. When bacterial growth occurred, colonies were selected and re-streaked for identification analysis.

P. larvae was isolated after the pasteurization step from larvae affected by AFB, plating 100 µl of smashed diseased larvae and its serial dilutions on Columbia Agar (Fluka-Sigma-Aldrich, Germany) supplemented by 10% sterile defibrinated mutton blood (Microbiol, Italy). After one week-growth, grey colonies, capable of haemolysis, were selected and re-streaked for the confirmation of the bacterial identity. AAB, LAB, SFB and *P. larvae* isolated from honeybee larvae and adults were employed for the establishment of a bacterial collection, in which the reference strain *Bacillus thuringiensis entomocidus* HD110 (Cherif et al., 2006) was also included. BT was routinely maintained on TSA at 30°C.

Identification of bacterial strains

Total genomic DNA was extracted from all the bacteria by proteinase K and sodium dodecyl sulphate treatments as previously described (Favia et al., 2007). Bacterial isolates were clustered in several groups according to internally transcribed spacer (ITS)-PCR fingerprinting with primers ITSF (5’-GCC AAG GCA TCC AAC-3’) and ITSR (5’-GTC GTA ACA AGG TAG CCG TA-3’) as previously described (Daffonchio et al., 1998). Representatives of each ITS group were then subjected to phylogenetic analyses. Partial 16S rRNA gene was amplified with universal bacterial primers 27F (5’-TCG ACA TCG TTT ACG GCG TG-3’) and 1495R (5’-CTA CGG CTA CCT TGT TAC GA-3’) following protocols and thermal conditions described by Ruiz and colleagues (2000). Finally 16S rRNA fragments (about 800 bp) were sequenced (Primm, Milano, Italy) and the sequences were aligned in GenBank using NCBI tool.

Inhibition assays of bacterial isolates

Inhibition activity assays of the isolated bacteria against the pathogen *P. larvae* 20it and *P. larvae* BMG93 were performed by the use of the “well diffusion” test as described elsewhere (Forsgren et al., 2010; Tagg and McGiven, 1971). Briefly,

all the tested bacteria (LAB, AAB, SFB) were grown in their specific media, whereas the indicator strains (*P. larvae* 20it and *P. larvae* BMG93) were grown in Columbia blood agar. Bacterial supernatants were recovered by centrifugation (10 minutes, 3000 rpm) and stored at 4°C, until inhibition tests were performed. In the meantime Petri dishes were filled with a thick layer of MYPGP (1,5 % agar) medium (following the composition indicate by Dingman and Stahly, 1983). Then, MYPGP tubes (0,7% agar) were inoculated with *P. larvae* strains with a final concentration of 10^3 cells, carefully mixed and then poured onto the MYPGP (1,5 % agar) plates. After solidification, various numbers of holes were punched out of the agar. The base of each hole was sealed with a drop (0,05 ml) of melted MYPGP (1,5 % agar), and bacterial supernatants (obtained from 48 hours culture) were then placed inside the wells (about 100 μL) and incubated at 37 °C. Inhibition of *P. larvae* strains by LAB, AAB and SFB was defined as the distance, in cm, of the hole's edges and the first line of *P. larvae* growth after 24 hours.

In vivo larval rearing and feeding tests

From the apiary sited in Caluso (Turin, Italy) one comb with 1 day-old larvae was recovered and moved to the laboratory for carrying out the experiments. Larvae were grafted from the comb and placed into 96-well plates. Two different experimental trials were carried out in order to evaluate the bacterial influence toward honeybee larvae following an infection with *P. larvae*. A total 1056 larvae was reared in 96-well plates with a sugar-based diet (NC) enriched with symbiotic (BL), reference (BT) and/or pathogen (*P. larvae* 20it, PL) bacteria. NC contained 50% of Royal Jelly (ErbaVita, San Marino Republic) and 50% of an aqueous sterile solution of yeast extract (1%), D-fructose (6%) and D-glucose (6%). Larvae in 96-well plates were placed in an incubator at 35 °C with humidity condition at 97%. Following the protocol suggested by Aupinel et al. (2005) larvae were reared in 96-well plates with NC enriched with symbiotic (BL; *Saccharibacter* sp. AM169; *Lactobacillus alvei* AM34), reference (BT) and/or pathogen (PL) bacteria. Foreseen treatments, in replications, are listed in Tab. S6. First-instar (1-day old) larvae were fed with 20 of (NC) on the first day, added with a given concentration of 1 or more bacteria (Tab. S6). Then, 20, 30, 40 and 50 μl of NC were administered to the larvae on the second, third, fourth and fifth day of rearing, respectively. For the following days no feed was added. Larval mortality was daily checked for 12 days as the larvae become "pupae". Average larval mortality was finally calculated for each treatment (Tab. S7).

Quantitative Polymerase Chain Reaction (qPCR) on RNA transcripts of innate immune related-antimicrobial peptides (AMPs)

Honeybee larvae, sampled after the different bacterial feedings, were collected 6 days after the administration of the bacteria and immediately frozen at -20°C in "RNA Protect Bacteria Reagent" (Qiagen, Milan, Italy).

Total RNA was isolated from each sample using "Total RNA Isolation Nucleospin RNA II kit" (Macherey Nagel, Milan, Italy) following the manufacturer's protocol. RNA quantification was measured using a Nanodrop 1000 Spectophotometer with samples eluted in RNase-free water. Then, 2 µg of total RNA were used for the synthesis of 1st strand cDNA using "RevertAid™ first strand cDNA Synthesis Kit" (Fermentas, Milan, Italy) with oligo-dT primers according to the manufacturer's protocol. Four genes involved in the honey bee innate immune response, abaecin, hymenoptaecin, defensin and lysozyme were amplified by qPCR by using an Icycler real-time PCR thermal cycler (Bio- Rad, Milan, Italy). Twentyfive-microliter reactions were carried out on 2 µg cDNA along with 1X Brilliant Ultra-Fast SYBR®Green QPCR Master Mix (Agilent Technologies, Milan, Italy) and 0,4 µM of each specific primer. Abaecin and Hymenoptaecin primers were designed from precursor sequences for these genes (Casteels-Josson et al. 1994; GenBank accession numbers: U15954 and U15955, respectively). Primer sequences were: abaecin.F 5'-CAG CAT TCG CAT ACG TAC CA-3'; abaecin.R 5'-GAC CAG GAA ACG TTG GAA AC-3'; hymenoptaecin.F 5'-CTC TTC TGT GCC GTT GCA TA-3'; and hymenoptaecin.R 5'-GCG TCT CCT GTC ATT CCA TT-3'. Defensin primer, selected from the literature, (Evans, 2006; Antunez et al., 2009) was the built on Defensin1 gene: defensin.F 5'-TGC GCT GCT AAC TGT CTC AG-3' and defensin.R 5'-AAT GGC ACT TAA CCG AAA CG-3'. For which concern the Lysozime, the honeybee genome contains 3 different lysozyme (Evans et al, 2006b) in this study it was selected Lys1. The primers sequences were Lys1.F 5'-GAA CAC ACG GTT GGT CAC TG-3' and Lys1.R 5'-ATT TCC AAC CAT CGT TTT CG-3'.

Transcript levels for a gene whose activity is closely tied with mRNA concentration (ribosomal protein S5, GenBank accession numbers: BG101562, Evans and Wheeler 2000) were used to normalize against variable mRNA levels. Primers for this gene were AmRPS5.F 5'-AAT TAT TTG GTC GCT GGA ATT G-3' and AmRPS5.R 5'-TAA CGT CCA GCA GAA TGT GGT A-3'. Real time PCR cycling program consisted of an initial pre-incubation at 95°C for 4 min followed by 43 cycles of denaturing at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min 30 s. Fluorescence was measured during the annealing step. Melt-curve analysis is used to confirm that the fluorescence measured was the result of amplified products of the predicted size.

Cycle threshold (Ct) values were calculated using Bio-Rad real time software (version 3.0a) as indicated in manufacturer's instructions. Threshold cycle numbers for the target genes were then subtracted from the ribosomal protein S5 (RPS5) threshold for each sample. This value was then scaled as a power of "E" to produce an estimate of relative cDNA abundance for each gene. The value "E" represents PCR efficiency and it is calculated by the equation "E=[10(-1/slope)]–1", whereas the slope is obtained by a standard curve that was constructed in order to validate the efficiency of the qPCR.

Localization of the administered bacteria by means of fluorescent in situ hybridization (FISH)

FISH was performed on 20 honeybee larvae fed with the potential probiotic following the rearing procedure listed in the Tab. S6 to observe the localization and co-localization of PL, BL and BT within the insect gut. Insect guts have been dissected and fixed in 4% paraformaldehyde before proceeding with hybridization according to a method previously described (Crotti et al., 2009). Specific fluorescent probes targeting the 16S rRNA gene have been used. Eubacterial specific probe EUB338 GCT GCC TCC CGT AGG AGT (Fuchs et al., 1998) have been used to match all the members of the Eubacteria, whereas probe 5'-CTA ACC TG GCG TCT CCC GAA-3'has been used to match PL (Yue et al., 2008). In the case of BT and BL, probes have been designed using ARB (Ludwig et al., 2004): 5'-GTT CAA AAT GTT ATC CGG-3' labeled 5' FITC for BT and 5'-GCA CTG TTT CTT CCC TAA CAA-3' for BL. Probe EUB has been labeled at its 5'end with the fluorophore Texas Red (absorption and emission at 595 nm and 613 nm, respectively). Probe PL has been labeled with the fluorophore Cy3 (absorption and emission at 548 nm and 561 nm, respectively). Probe BT has been labeled with the fluorophore FITC (absorption and emission at 495 nm and 519 nm, respectively). Probe BL has been labeled with the fluorophore JOE (absorption and emission at 520 nm and 548 nm, respectively).

Production of BL and BT spores for in field trials

After an overnight pre-inoculum in Nutrient Broth (NB) at 30°C, 5% (v/v) of BT and BL cultures were inoculated into NB and grown for 7h at 30°C. Then 4% (v/v) of BL and 1% (v/v) of BT culture were transferred into a modified Nutrient Yeast Extract Salt Medium, NYSM-BE (glucose 10 g/L, NaCl 5 g/L, beef extract 8,5 g/L, $MgCl_2$ $6H_2O$ 0,203 g/L, $CaCl_2$ 0,102 g/L, $MnCl_2$ 0,01 g/L) and incubated for 72h in the case of BL and 36h in the case of BT and followed by pasteurization. One application for one hive (hosting 20,000 larvae weekly) contained 2×10^9 BT spores and 2×10^8 spores BL.

In field trials

Eight hives were planned to be treated for 7 consecutive weekly treatments during in field trials. Sixteen bee hives placed in Caluso (Turin-Piedmont-Italy) have

been analysed in the experiment. Eight of these hives, were kept as negative control, being not treated with bacterial spores. The remaining 8 hives were subjected to the treatment with bacterial spores. Each hive has been treated with 2×10^9 BT spores and 2×10^8 spores BL, re-suspended in 100 ml of tap water. Every hive has been sprayed with the spore suspension, covering as much as possible, the working bees laying on the combs, maximizing in this way spore transmission during larval feeding. Twentyfour hours after the exposure, two combs for each treatment have been drawn from treated and non-treated hives and move to the laboratory. Forty-eight 1st-instar larvae for each treatment have been grafted and transferred to 96-well plates containing 10 µl of liquid sterile diet (NC). Free cells around the larvae have been maintained to get proper humidity of the animals. For the following 5 days larvae have been fed with NC. At the 1st rearing day 1×10^5 spores ml^{-1} of PL 20it have been administered to treated and not treated larvae. Hence, the plates have been placed at 35°C with cotton pads wet of K_2SO_4 and a Becker glass with sterile water to keep humidity at 97%. For twelve days, as larvae become pupae, larval mortality has been daily checked for any treatment. Dead larvae have been removed from the wells.

Statistical analysis

Two different analysis were performed. In the case of inhibition test and *in vivo* feeding and rearing experiments, statistics were calculated by using Microsoft Excel software (Millar, 2001). Variation between different groups was evaluated by T-test, using as parameters tail=2 and type=2. *P*-values below 0,05 were considered statistically significant. Mean and standard deviation were determined for three independent experiments and results were presented as mean ± SD. In the case of the measure of the transcript levels of innate immune related-AMPs and field rearing and feeding test, data sets were different and often without a normal distribution; thus a full factorial Permutational Analysis of Variance (PERMANOVA; Anderson, 2001) was used to test the null hypothesis of no differences. Post-hoc pairwise tests (p-hpt) were performed when appropriate. All data are expressed as means ± SE and the analyses were performed using the PERMANOVA+ routines for PRIMER 6 (Anderson et al., 2008).

Acknowledgements
The authors thank for financial support the European Union in the ambit of project BIODESERT (European Community's Seventh Framework Programme CSA-SA REGPOT-2008-2 under grant agreement no. 245746) and the Ministry for Research (MIUR), in the ambit of projects PRIN 2009 (Interazioni tra insetti vettori e microrganismi simbionti: nuove prospettive per il biocontrollo dei patogeni trasmessi alle piante, agli animali e all'uomo). C.H. was supported for a Short Term Scientific Mission to the University of Milan by Cost Action FA0701: 'Arthropod Symbiosis: From Fundamental Studies to Pest and Disease Management'.

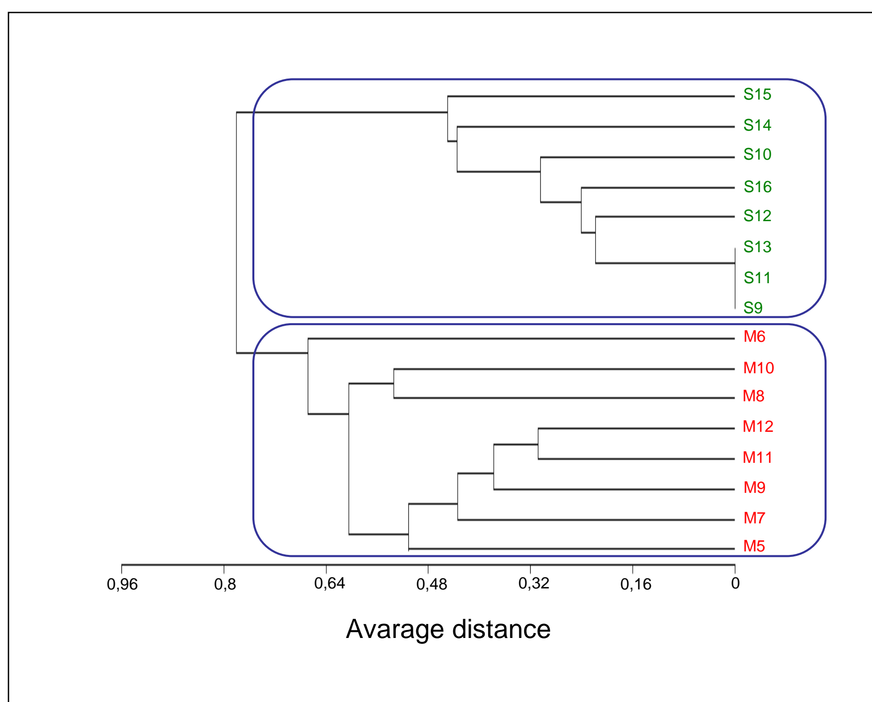
Supplementary Materials

Tab. S1. Identification of microorganisms associated to honeybee specimens according to DGGE profiles in Fig.1.

Band	Closest relative (Acc. N.)	No. of identical bp/total no. of bp (% homology)	Taxonomic group
	DGGE BANDS FROM LARVAE		
1-2-4-5	*Lactobacillus kunkeei strain* Amshot7 (HM534857)	532/535 (99%)	Firmicutes[100%]
3	*Saccharibacter floricola* strain S-877 (NR_024819)	492/510 (96%)	Alphaproteobacteria[100%]
6-7-9-10-32-36	*Bacterium NLAE*-zl-P630 (JQ607192)	461/489 (94%)	Clostridiales[100%]
8	*Candidatus Gilliamella apicola* clone pAJ206 (AY370192)	521/529(98%)	Gammaproteobacteria[100%]
11-12-13-14-16-19-37	*Lactobacillus kunkeei* strain B6-1 (JQ009353)	528/532(98%)	Firmicutes[100%]
15	*Frischella perrara* strain PEB0191(JX878306)	525/528 (99%)	Gammaproteobacteria[100%]
17	*Lactobacillus* sp. Achmto2 (HM534754)	529/529(100%)	Firmicutes[100%]
18	*Lactobacillus sp.* fhon13 (HM534758)	523/533 (98%)	Firmicutes[100%]
20-21	*Fructobacillus fructosus* NBRC 3516 (AB680098)	499/530(94%)	Firmicutes[100%]
22-23-24-25-26-27-28-29-31	*Paenibacillus larvae* strain BMG 245 (FJ649364)	525/525 (100%)	Firmicutes[100%]
30	*Leuconostoc sp.* C2 (NR_075017)	231/245(94%)	Firmicutes[100%]
33-34	*Acetobacteraceae bacterium* CS14 (JX896641)	345/350(99%)	Alphaproteobacteria[100%]

35	*Acetobacteraceae bacterium* CS14 (JX896641)	241/250(96%)	Alphaproteobacteria[100%]
	DGGE BANDS FROM ADULTS		
38	*Acetobacteraceae bacterium* EW911 (EU096231)	1263/1319(96%)	Alphaproteobacteria[100%]
39	*Weissella* sp. LMG 26217 (HE576795)	477/482 (99%)	Firmicutes[100%]
40-41-44-45-46-48-49	*Lactobacillus sp.* Amsbbr24 (HM534853)	518/521 (99%)	Firmicutes[100%]
42-43-50-51-54-57-58	*Snodgrassella alvi* strain wkB2 (JQ746651)	1336/1347(99%)	Betaproteobacteria[100%]
55	*Lactobacillus sp.*A A44(JX896496)	518/525 (99%)	Firmicutes[100%]
47-53	*Lactobacillus sp.* AmmhmR3 (HM534864)	1029/1034(99%)	Firmicutes[100%]
52	*Acetobacter tropicalis* partial (FN297837)	313/339(92%)	Alphaproteobacteria[100%]
56	*Lactobacillus sp.* F7 (EU753690)	241/250(96%)	Firmicutes[100%]
59	*Paenibacillus larvae* strain BMG 245 (FJ649364)	525/525 (100%)	Firmicutes[100%]

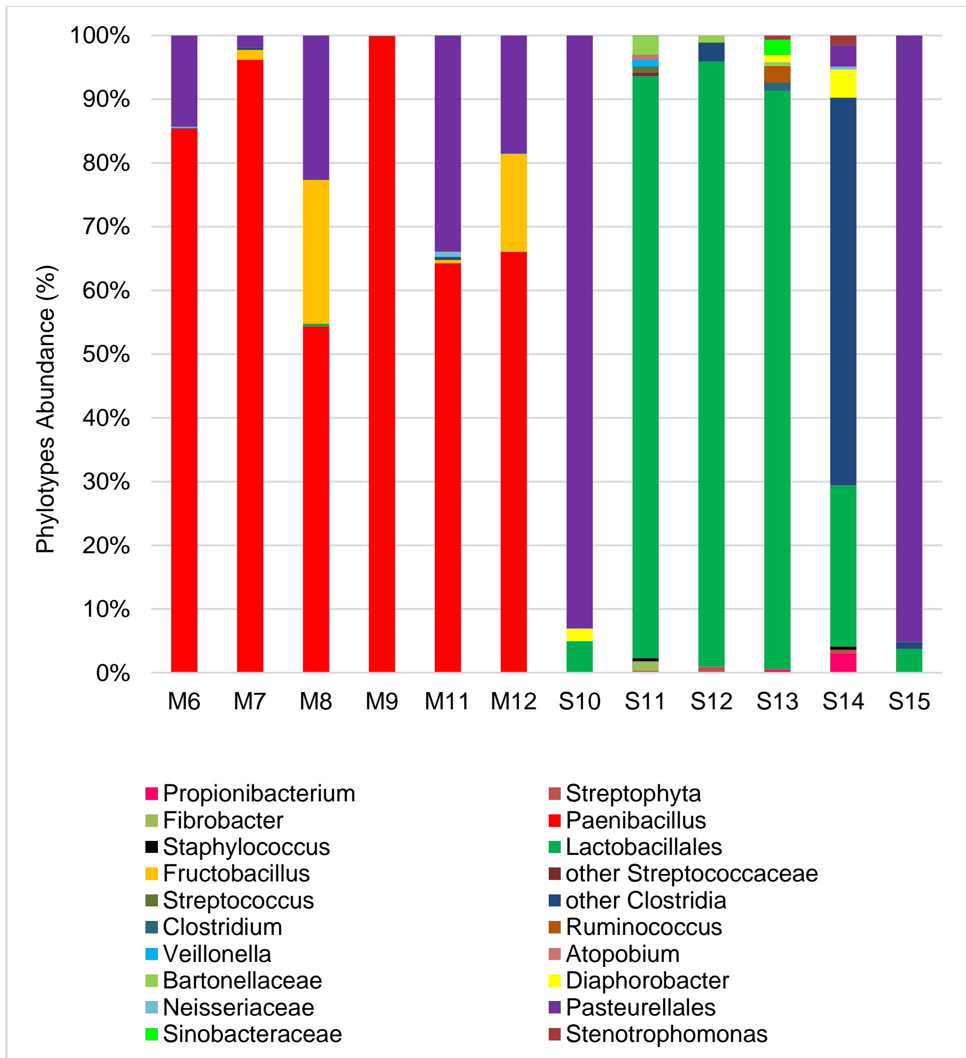
Fig. S1. Statistical analysis based on DGGE band profiles of Italian asymptomatic and symptomatic larvae. Symptomatic samples are indicated in red, while asymptomatic ones are in green.
Diversity of the bacterial population from honeybees was described through cluster analysis based on PCR-DGGE results. DGGE scanned gels were analysed with Quantity One software package version 4.6.6 (BioRad, Berkeley - California), in order to detect all the bands. The positions of the identified bands of each DGGE profile were digitized and transposed to a presence/absence matrix. The similarity among various samples was compared by cluster analysis of the digitized profile performed with the Multi Variate Statistical Package software MVSP 3.13m (Kovach Computing Services, Anglesey, Wales), using as clustering method UPGMA (Unweighted Pair Group Method with Arithmetic Mean) and adopting the average distance as linkage criteria.



Tab. S2. Number of 16S barcoding reads obtained from each sample after trimmering the chimeras and after applying a correction factor, according to which singletons and sequences with a relative abundance inferior to 0.1% have not been included in the analysis.

Samples	Without chimeras	After correction
M6	7148	6843
M7	1545	1381
M8	3736	3334
M9	6012	5791
M11	8967	8671
M12	7837	7541
S10	910	817
S11	9260	8653
S12	2263	2146
S13	6042	5735
S14	1954	1038
S15	5032	4739

Fig. S2. Bar graph showing the abundances of the different bacterial phylotypes in asymptomatic and symptomatic honeybee larvae from Italian colonies.

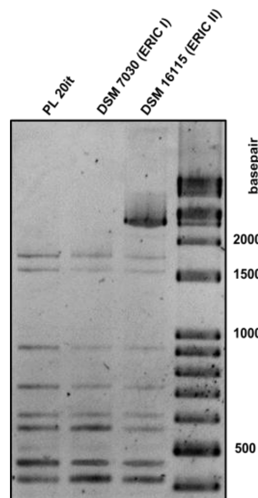


Tab. S3. Cultivable fraction associated to honeybee specimens. A) Tunisian isolates, from larvae and adult samples. B) Italian isolates, from larvae and adult samples. C) Resume of the most representative groups.

Number of isolates	Sequenced isolates	Closest relative and accession number	Homology (%)	Taxonomic groups	Origin
ISOLATES FROM TUNISIAN INDIVIDUALS					
2	L46	*Bacillus licheniformis* (GU121483)	99	Firmicutes	Larva
1	BMG180	*Bacillus amyloliquefaciens* (HQ337540)	100	Firmicutes	Larva
8	BMG68	*Bacillus thuringiensis* (HM047298)	99	Firmicutes	Larva
2	BMG188	*Sporosarcina ginsengisoli* (EU308121)	100	Firmicutes	Larva
2	BMG207	*Brevibacillus choshinensis* (FJ613127)	96	Firmicutes	Larva
5	BMG57	*Brevibacillus laterosporus* (D16271)	99	Firmicutes	Larva
4	AB13	*Bacillus pumilis* ((FJ705814)	100	Firmicutes	Larva
4	AB6	*Staphylococcus pasteuri* (HM854230)	99	Firmicutes	Larva
1	B82	*Staphylococcus sciuri* (FR687216)	100	Firmicutes	Larva
18	L33	*Lactobacillus kunkeei* (AB498042)	98	Firmicutes	Larva
9	B57	*Lactobacillus kunkeei* (AB498042)	99	Firmicutes	Larva
10	B93	*Lactobacillus kunkeei* (AB498042)	99	Firmicutes	Adult
1	B67	*Kocuria rhizophila* (FR682683)	97	Actinobacteria	Adult
1	B64	*Kocuria rosea* (HQ202874)	100	Actinobacteria	Adult
8	L27	*Micrococcus* sp. (AB576089)	99	Actinobacteria	Adult
2	L19	*Arthrobacter oxydans* (EF154243)	99	Actinobacteria	Adult
2	L37	*Paracoccus* sp. (AY278919)	98	α-Proteobacteria	Adult
3	L13	*Paracoccus* sp. (EU867311)	100	α-Proteobacteria	Adult
23	B88	*Acetobacter estunensis* (AB032349)	96	α-Proteobacteria	Adult
9	S1	*Acetobacter estunensis* (AB032349)	97	α-Proteobacteria	Adult
11	B59	*Gluconobacter cerinus* (AB436556)	97	α-Proteobacteria	Adult
2	L41	*Burkholderia* sp. (GQ468397)	98	β-Proteobacteria	Adult
4	BMG67	*Pseudomonas poae* (GU188949)	99	γ-Proteobacteria	Adult
2	BMG118	*Acinetobacter baumannii* (HM209768)	98	γ-Proteobacteria	Adult
ISOLATES FROM ITALIAN INDIVIDUALS					
67	AM40-92-55	*Lactobacillus* sp. Hma8N(JX099551)	100	Firmicutes	Adult
64	AM93	*Lactobacillus* sp. AmmhmR3 (HM534864)	100	Firmicutes	Adult
3	AM2	*Bacillus endophyticus* (EU221417)	99	Firmicutes	Adult
1	AM7	*Bacillus mojavensis* strain NS02 (JX126863)	100	Firmicutes	Adult
1	AM25	*Lactobacillus* sp. Amsbbr6 (HM534855*)*	96	Firmicutes	Adult
1	AM41	Lactobacillus sp. M4(KF543103)	99	Firmicutes	Adult

1	AM47	*Lactobacillus* sp. Mbohs2r12 (HM534800)	98	Firmicutes	Adult
1	AM51	*Lactobacillus* sp. Biut2N (JX099550)	99	Firmicutes	Adult
1	AM111	*Staphylococcus aureus* (X70648)	98	Firmicutes	Adult
1	AM143	*Staphylococcus sp.* YIM 75784 (JQ808063)	99	Firmicutes	Adult
1	L1	*Staphylococcus pasteuri* strain Z1 (GU201873)	100	Firmicutes	Larva
1	L2	*Bacillus* sp. DB166 (HM566879)	99	Firmicutes	Larva
3	L3	*Bacillus pumilus* strain S_T_TSA_70 (NR_042776)	100	Firmicutes	Larva
1	L6	*Bacillus pumilus* strain GTG-11 (JX841107)	100	Firmicutes	Larva
2	L10	*Bacillus amyloliquefaciens* strain WJ25 (JX966406)	99	Firmicutes	Larva
3	L12	*Paenibacillus barcinonensis* strain BP-23 (NR_042272)	99	Firmicutes	Larva
1	L18	*Bacillus insolitus* strain DSM 5T (NR_042709)	99	Firmicutes	Larva
1	L21	*Bacillus safensis* strain FO-036b (NR_041794)	100	Firmicutes	Larva
1	L22	*Paenibacillus turicensis* strain MOL722 (NR_037003)	99	Firmicutes	Larva
1	L23	*Staphylococcus warneri* strain AW 25 (NR_025922)	99	Firmicutes	Larva
1	L33	*Paenibacillus pabuli* strain HSCC 492T (NR_040853)	98	Firmicutes	Larva
2	L35	*Bacillus amyloliquefaciens* strain NBRC 15535 (NR_041455)	99	Firmicutes	Larva
1	L37	*Paenibacillus alvei* strain DSM 29 (NR_042091)	99	Firmicutes	Larva
4	L45	*Bacillus safensis* strain FO-036b (NR_041794)	99	Firmicutes	Larva
3	AM35	*Bifidobacterium coryneforme* strain Amshmt5(HM534861)	99	Actinobacteria	Adult
1	AM42	*Bifidobacterium* sp. Amsbbr10(HM534860)	99	Actinobacteria	Adult
1	AM52	*Bifidobacterium* sp. Bisn6 (EF187233)	99	Actinobacteria	Adult
1	AM85	*Bifidobacterium asteroides strain* Mbobb2t12 (HM534830)	99	Actinobacteria	Adult
1	AM94	*Bifidobacterium asteroides* PRL2011 (NR_10286)	99	Actinobacteria	Adult
1	AM95	*Bifidobacterium* sp. Achmro11 (HM534827)	94	Actinobacteria	Adult
6	AM90-96-97	*Bifidobacterium* sp. Achmro11 (HM534827)	97	Actinobacteria	Adult
1	L7	*Microbacterium sp.* I_GA_A_1_16 (FJ267583)	100	Actinobacteria	Larva
1	L13	*Microbacterium foliorum* strain P 333/02 (NR_025368)	99	Actinobacteria	Larva
1	L14	*Leifsonia shinshuensis* strain DB102 (NR_043663)	99	Actinobacteria	Larva
1	L15	*Streptomyces griseoaurantiacusstrain* NBRC (NR_041186)	98%	Actinobacteria	Larva
87	AM1-2-12-14-16-113-122-123-128-137-144-152-161-165-168-169-170-173-183	*Saccharibacter floricola* strain S-877 (NR_024819)	95	α-Proteobacteria	Adult
1	L48	*Neisseria subflava* strain U37 (NR_041989)	99	β-Proteobacteria	Larva
3	L39	*Neisseria subflava* strain U37 (NR_041989)	99	β-Proteobacteria	Larva
2	L29	*Pseudomonas psychrotolerans* strain C36 (NR_042191)	99	γ-Proteobacteria	Larva

Fig S3. ERIC profiles of *P. larvae* PL20it compared to *P. larvae* DSM7030 and DSM16115, as representative of ERIC I and ERIC II genotypes, respectively. Below, the sequence alignment of the sequences relative to the gene S-layer protein SplA used to discriminate ERIC I genotype from ERIC II genotype.
Genotyping was performed using ERIC1R and ERIC2 primers, as described by Versalovic et al. (1994). PCRs were performed in a total volume of 25 µl. The reaction mixture contained the diluted buffer 1 X, 1,5 mM $MgCl_2$, 5% of DMSO, 0,12 mM of a mixture of dNTPs, 0,25 µM of each primer, 1 U Taq polymerase, and 20 ng of template. If necessary, DNA was properly diluted. Cycling conditions used to amplify the gene fragment were with an initial activation step at 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min 30 seconds, with a final extension at 72°C for 10 min. All fingerprint profiles were generated from at least three independent experiments to determine their reproducibility. PCR amplified products were analyzed by electrophoresis loaded in a agarose gel 1.5% with TBE 0,5%. After the run, the gel was stained in a Ethidium Bromide solution 0,5 mM (Sigma, Milan, Italy). The gel was observed using the GEL DOC 2000 system and analysed using the software QUANTITY ONE (Bio-RadTM, Berkeley, California).



In order to confirm the genotyping analysis carried out using the REP-PCR, the sequence of *P. larvae* SplA was performed by a TBLASTN analysis, according to Poppinga et al. (2012).
At first, *P. larvae* (target strain and reference strains) DNA was amplified with primers SPL-F2 (5’-ACT ATC AGC AAA TCG TTA TTG AAG G-3’) and SPL R1 (5’-CTG TTT TTT CGT TAA GCA TGG TT-3’; Poppinga et al., 2012). PCRs were performed in a total volume of 25 µl. The reaction mixture contained the diluted buffer 1 X, 1,5 mM $MgCl_2$, 0,12 mM of a mixture of dNTPs, 0,3 µM of each primer, 1 U Taq polymerase, and 20 ng of template. If necessary, DNA was properly diluted. Cycling conditions used to amplify the gene fragment were with an initial activation step at 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. The amplicons were sequenced (Macrogen, Seoul, South Korea) and aligned by Nucleotide Blast. The homology with the sequence *P. larvae* strain 04-309 S-layer protein A (Acc. Number JQ353714) was 99%. Thus, SplA sequence of the strain PL20it was compared with SplA sequences of two reference strains: DSM 7030^T (ERIC I) and DSM 16115 (ERIC2; Genersch et al., 2006). The multi alignment performed using ClustalX evidenced that SplA sequence of the strain PL20it presented the Adenine at the position 894 (highlighted in yellow) such as the strain DSM 7030^T (ERIC I), differently from the sequence of DSM 16115 (ERIC2).

Strain	**Sequence**
DSM16115 (ERICII)	881-GAGATACTACTAT-TTCTCTGGTTGCTTATAACGGTGAAAA -920
DSM7030^T (ERICI)	881-GAGATACTACTATATTCTCTGGTTGCTTATAACGGTGAAAA -920
PL20it (Italy)	881-GAGATACTACTATATTCTCTGGTTGCTTATAACGGTGAAAA -920

Tab S4. Treatment conditions applied for the evaluation of PL dosage to be administered to the 1st instar larvae.

Treatment*	Rearing day 1st		Rearing day 2nd		Rearing day 3rd		Rearing day 4th		Rearing day 5th	
	NC (µl)	CFU ml^{-1}	NC (µl)	CFU ml^{-1}	NC (µl)	CFU ml^{-1}	NC (µl)	CFU ml^{-1}	NC (µl)	CFU ml^{-1}
NC	20	0	30	0	30	0	40	0	50	0
PL	20	**5×10^2**	20	0	30	0	40	0	50	0
PL	20	**2×10^3**	20	0	30	0	40	0	50	0
PL	20	**5×10^4**	20	0	30	0	40	0	50	0

*Abbreviation: NC, sugar-based diet; PL, *P. larvae* 20it.

Tab S5. Larval mortality with two different doses of PL 20it. Mortality rates in relation to PL dose.

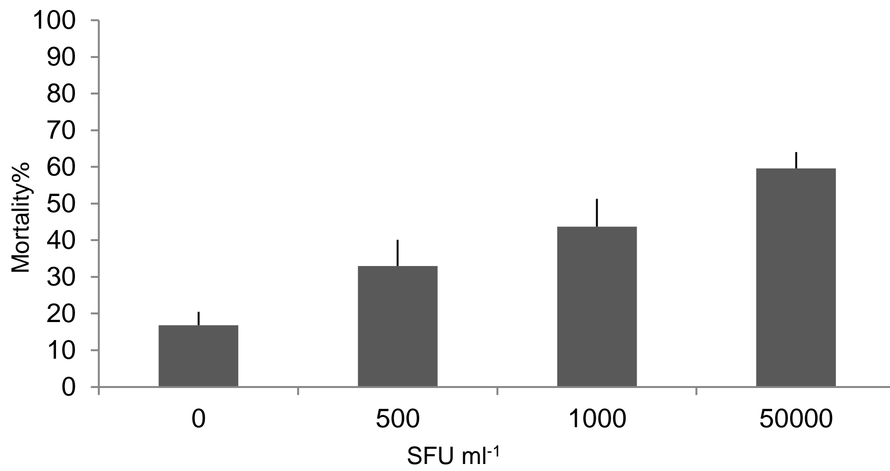
	Mortality rate (%)					Average Mortality rate (%)	SD Mortality rate (%)**	T-test value***
Treatment*	E.[1] 1	E. 2	E. 3	E. 4	E. 5			
NC	21,9	20,8	14,6	14,6	14,6	17,3	3,7	-
PL 5×10^{2} cfu mL^{-1}	30	40				35	7,1	<0,01**
PL 2×10^{3} cfu mL^{-1}	45,8	56,5			41,7	48	7,6	<0,01**
PL 5×10^{4} cfu mL^{-1}			62,5	56,5		59,4	4,4	<0,01**

*Abbreviation: NC, sugar-based diet; PL, *P. larvae* 20it.

**SD: standard deviation

***Values considered significantly different from the control ($p<0.05$).

Fig. S4. Determination of LC_{50} from PL 20it.



Abbreviation:SFU ml^{-1}: Spore Forming Units administered to larvae.

Tab. S6. Treatments applied during the *in vivo* trials.

Treatment	Rearing day 1st		Rearing day 2nd		Rearing day 3rd		Rearing day 4th		Rearing day 5th	
	NC (µl)	CFU ml^{-1}	NC (µl)	CFU ml^{-1}	NC (µl)	CFU ml^{-1}	NC (µl)	CFU ml^{-1}	NC (µl)	CFU ml^{-1}
NC	20	0	20	0	30	0	40	0	50	0
BT	20	5×10^6	20	0	30	0	40	0	50	0
BL	20	5×10^5	20	0	30	0	40	0	50	0
BT+BL	20	$5 \times 10^6/5 \times 10^5$	20	0	30	0	40	0	50	0
AM34	20	5×10^6	20	0	30	0	40	0	50	0
AM169	20	5×10^4	20	0	30	0	40	0	50	0
EC	20	5×10^6	20	0	30	0	40	0	50	0
PL	20	5×10^4	20	0	30	0	40	0	50	0
BT+PL	20	$5 \times 10^6/5 \times 10^4$	20	0	30	0	40	0	50	0
BL+PL	20	$5 \times 10^5/5 \times 10^4$	20	0	30	0	40	0	50	0
BT+BL+PL	20	$5 \times 10^6/5 \times 10^5/5 \times 10^4$	20	0	30	0	40	0	50	0
AM34+PL	20	$5 \times 10^6/5 \times 10^4$	20	0	30	0	40	0	50	0
AM169+PL	20	$5 \times 10^4/5 \times 10^4$	20	0	30	0	40	0	50	0
EC+PL	20	$5 \times 10^6/5 \times 10^4$	30	0	30	0	40	0	50	0

*Abbreviation: NC, sugar-based diet; BT, *B. thuringiensis* HD 110; BL, *B. laterosporus* BMG65; PL, *P. larvae* 20it; EC, *E. coli* SC110; AM169, *Saccharibacter* sp. AM169; AM34, *L. alvei* AM34 .

Tab. S7. Larval mortality of the *in vivo* trial.

	Mortality rate (%)									Average Mortality rate (%)	SD Mortality rate (%)	T-test value*
Treatment	E. 1	E. 2	E. 3	E. 4	E. 5	E. 6	E. 7	E. 8	E. 9			
NC	14,5	16,2	16,2	41,2	28,7	19,3	16,2	15,2	21	20,9	8,8	
EC		13,1					19,3	25,2		19,2	6,1	0,76
PL		88,7	98,1		59,9	88,7	98,1	79,4		85,5	14,3	<0,01***
BT					19,3				20	19,7	0,5	0,85
BL	16,2	19,3	19,3		9,9					16,2	4,4	0,33
AM169				34,9		16,2				25,6	13,3	0,54
AM34				24,2		20,6				22,4	2,5	0,83
BL+BT					21,1				24,4	22,8	2,3	0,79
EC+PL			39				37,2			38,1	1,3	0,03***
BT+PL					33,0				30,5	31,8	1,8	0,13
BL+PL			19,3		22,4					20,9	2,2	0,99
AM169+PL	16					14,0				15,0	1,4	0,38
AM34+PL	23					19,0				21,0	2,8	0,99
BT+BL+PL					14,3				18	16,2	2,6	0,48

*Abbreviation: E., number of experiment.

**Treatment against the control.

***T-test values considered significantly different from the control ($p<0.05$).

Tab. S8. Transcript levels for abaecin, hymenoptaecin, defensin and lysozyme.

Sample*	Number of samples	Relative quantity	RATE vs NC
Abaecin transcript levels			
NC	15	0,335	-
BT+BL	13	0,777	2,3
AM 34	14	0,207	0,6
AM 169	14	0,071	0,2
EC	13	0,014	0,04
PL	15	0,292	0,8
Hymenoptaecin transcript levels			
NC	15	0,225	-
BT+BL	13	1,989	8,8
AM 34	14	0,076	0,3
AM 169	13	0,024	0,1
EC	13	0,004	0,01
PL	15	0,016	0,05
Defensin transcript levels			
NC	13	1,429	-
BT+BL	13	1,382	0,9
AM 34	14	1,674	1,1
AM 169	12	1,313	0,9
EC	13	0,076	0,05
PL	14	0,184	0,12
Lysozyme transcript levels			
NC	15	0,816	-
BT+BL	13	0,006	0,007
AM 34	14	0,004	0,0005
AM 169	14	0,001	0,001
EC	13	0,064	0,08
PL	15	0,009	0,01

*Abbreviation: see caption of Tab. S6.

Tab. S9. Bacterial treatments in field trials. Eight hives were weekly treated with a bacterial suspension of BT+BL spores for seven consecutive applications, while 8 further hives were also kept as controls. The day after each treatment, 96 larvae from two randomly selected treated and untreated hives were collected and reared in 96-well plates. Forty-eight larvae (half of the amount of the withdrawn larvae) from each hive were challenged with the pathogen, while the other 48 were left untreated. Field trials were performed in duplicate.

Treatment and PL exposure		1st day Rearing		2nd day Rearing		3rd day Rearing		4th day Rearing		5th day Rearing	
		NC (µl)	PL CFU ml^{-1}	NC (µl)	PL CFU ml^{-1}	NC (µl)	PL CFU ml^{-1}	NC (µl)	PL CFU ml^{-1}	NC (µl)	PL CFU ml^{-1}
From 2 hives **treated** with BT+BL spore suspension											
Hive I	NC[1]	20	-	20	-	30	-	40	-	50	-
	PL[2]	20	5×10^4	20	-	30	-	40	-	50	-
Hive II	NC	20	-	20	-	30	-	40	-	50	-
	PL	20	5×10^4	20	-	30	-	40	-	50	-
From 2 hives **not treated** with BT+BL spore suspension											
Hive I	NC	20	-	20	-	30	-	40	-	50	-
	PL	20	5×10^4	20	-	30	-	40	-	50	-
Hive II	NC	20	-	20	-	30	-	40	-	50	-
	PL	20	5×10^4	20	-	30	-	40	-	50	-

Abbreviation: [1]NC, non challenged with PL, [2]PL, challenged with PL

Tab. S10. Larval mortality (%) of the field trials.

Diet		3rd treatment	4th treatment	5th treatment	6th treatment	7th treatment
Treated	NC[1]	48,9	62,5	45,8	33,3	27
	PL[2]	60,1	61,4	47,9	35,4	23,9
	NC	54,1	57,2	30,2	40,6	21,8
	PL	53,1	59,3	45,8	43,7	26
Not treated	NC	58,3	63,5	45,8	33,3	28,1
	PL	85,4	83,3	78,2	50	44,7
	NC	54,1	57,2	39,5	38,5	19,7
	PL	82,9	87,5	70,8	69,7	54,1

Larval mortality (%) of the field trials calculating the average of hive1+hive2.

Diet		3rd treatment	4th treatment	5th treatment	6th treatment	7th treatment
Treated	NC[1]	50,4	59,9	37,9	37,0	24,6
	PL	63,0	60,9	49,1	38,4	24,0
Not treated	NC	41,1	59,8	48,3	43,8	25,6
	PL	85,9	85,4	74,5	59,9	62,6

Abbreviation: [1]NC, non challenged with PL, [2]PL, challenged with PL.

Tab. S10. Larval mortality (%) of the field trials.

Diet		3rd treatment	4th treatment	5th treatment	6th treatment	7th treatment
Treated	NC[1]	48,9%	62,5%	45,8%	33,3%	27%
	PL[2]	60,1%	61,4%	47,9%	35,4%	23,9%
	NC	54,1%	57,2%	30,2%	40,6%	21,8%
	PL	53,1%	59,3%	45,8%	43,7%	26%
Not treated	NC	58,3%	63,5%	45,8%	33,3%	28,1%
	PL	85,4%	83,3%	78,2%	50%	44,7%
	NC	54,1%	57,2%	39,5%	38,5%	19,7%
	PL	82,9%	87,5%	70,8%	69,7%	54,1%

Larval mortality (%) of the field trials calculating the average of hive1+hive2.

Diet		3rd treatment	4th treatment	5th treatment	6th treatment	7th treatment
Treated	NC[1]	50,4%	59,9%	37,9%	37,0%	24,6%
	PL	63,0%	60,9%	49,1%	38,4%	24,0%
Not treated	NC	41,1%	59,8%	48,3%	43,8%	25,6%
	PL	85,9%	85,4%	74,5%	59,9%	62,6%

Abbreviation: [1]NC, non challenged with PL, [2]PL, challenged with PL.

Tab. S11. Bacterial counts of BT and BL, recovered after re-isolation trials from larvae fed with BT and BL, respectively

Strain	Number of administered spores (SFU) ml^{-1}	Number of re-isolated bacteria	
		Vegetative cells (CFU) per larva	Spores (SFU) per larva
BT	5×10^{6}	1.5×10^{3}	8.2×10^{2}
BL	5×10^{5}	1.4×10^{4}	6.5×10^{3}

*Abbreviation: see caption of Tab. S6.

Tab. S12. Relative quantity of transcript levels for abaecin and hymenoptaecin from larvae collected during field trials. Before starting with the application of treatments (treatment 0) and after treatment number 5 and 7, larvae were collected from treated and untreated. The transcript levels of these two AMP were measured considering as reference gene the honeybee 5S (Pfaffl, 2001).

Number treatment	Number samples	Untreated hive (NT)	Treated hive (T)	T/NT
Not exposed to PL 20it				
Abaecin transcript levels				
0	8	0,0257	0,008	0,34
5	8	0,142	0,119	0,83
7	8	0,214	0,457	2,13
Hymenoptaecin transcript levels				
0	8	0,0197	0,007	0,36
5	8	0,4151	0,164	0,39
7	8	0,5169	0,568	1,09
Exposed to PL 20it				
Abaecin transcript levels				
0	8	0,093	0,051	0,55
5	8	0,062	0,002	0,03
7	8	0,232	0,051	0,22
Hymenoptaecin transcript levels				
0	8	0,091	0,074	0,81
5	8	1,390	0,013	0,01
7	8	0,894	0,072	0,008

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Conclusions

Since its importance as crop pollinator, *Apis mellifera* is determinant for the food supply chain and for the survival of the wild crops too (Moran et al., 2012). In the last years, a strong honeybee decline is occurring worldwide causing a widespread concern and impacting the global economy; multiple factors co-operate as determinant causes of this decline, such as chemicals, pests and environmental stresses (Oldroyd, 2007). There is an urgent need to develop effective control strategies to prevent further mass losses.
The surveys of the bacterial diversity in different arthropods allow the determination of the microbial structure and composition, contributing to lay the foundations for the understanding of roles played by bacteria during different host life stages and in different body organs (Evans and Armstrong, 2006). Insect-bacteria associations range from facultative short-term interactions to highly co-dependent symbioses. Specifically, social insects provide unique resource of microbial symbionts, thanks to different features, such as the high density of individuals within colonies, the sharing of food and other resources, and the coexistence of colony members from multiple generations. Not surprisingly then, symbioses between social insect species and microbial species are common and often highly coevolved. There is an increased interest to develop new and effective strategies in order to improve honeybee health, considering the complexity of stressing factors that are present into the beehive. Indeed, the aim of this thesis was to get through the gut microbial diversity of honeybee collected in the Mediterranean area and its interaction with the host and the pathogen, and, finally, to develop a pathogen biocontrol strategy, based on the use of honeybee symbionts, in order to improve the host health and to counteract pathogen infection. In particular, I focused my attention on the pathogenic model *Paenibacillus larvae*, the causative agent of the American Foulbrood Disease (AFB). In particular, the main aim of my thesis was to assess the ability of different intestinal honeybee symbionts to preserve the insect health, evaluating also if synergistic activities of different classes of bacteria could occur.
The first part of the present work was dedicated to the characterization of the pathogen. Results obtained from the biochemical characterization of 75 isolates of *P. larvae* from a relatively small area of Northern Tunisia showed that *P. larvae* is not a monoclonal species like other pathogens, supporting previous observations of a phenotypic variability in the former subspecies *P. larvae subsp. larvae* and *P. larvae subsp. pulvifaciens* (Heyndrickx et al., 1994; Neuendorf et al., 2004). Indeed, 16S rRNA gene sequencing of the isolates confirmed their identity with *P. larvae*; however, sequence variability among

the isolates showed the lack of a strict clonality in the species according to the biochemical studies. A relative intraspecific diversity within the 75 Tunisian isolates was further observed by BOX-PCR typing, which firstly allowed the distinction of *P. larvae* from the related *Paenibacillus* species. Moreover, BOX profiles showed polymorphic *P. larvae*-specific bands that could be used as new markers for the discrimination of the pathogen from other pathogenic bacilli (Cherif et al., 2002). Alippi and coworkers (1998) detected only three genotypes by BOX-PCR typing of isolates that were retrieved from a geographic area (in Argentina) much larger than Northern Tunisia. Again, using a different molecular typing technique, such as the combination of BOX-PCR and REP-PCR, four genotypes were detected in isolates from Germany (Genersch and Otten, 2003; Neuendorf et al., 2004). The results carried out in this study could suggest the existence of a larger phenotypic variability in comparison to the one described until yet, in previous studies. Nevertheless the higher genotypic variability, a not clear correlation among biochemical, phylogenetic and molecular features was present among *P. larvae* isolates. This outcome could suggest an evolutionary pathway within the species.
In further experiments with the aim to correlate phenotypical and genotypical differences among the isolates with their pathogenicity, no difference in larval mortality rate was recorded for the *P. larvae* isolates tested. Thus, the selected *P. larvae* isolates, showing different patterns in the BOX-PCR analysis and belonging to different 16S rRNA gene phylotypes, presented the same virulence level against honeybee larvae.
The second part of this thesis sheds light on the microbial diversity of honeybees collected in two different climate zones, in order to survey the microbial differences of the insects from different life stage, geographic area and AFB infection stage. Different techniques were used in order to get deeply inside the topic and to compare the results. The metagenomic survey by Denaturing Gradient Gel Electrophoresis (DGGE)-PCR, 16S rRNA gene barcoding pyrosequencing and phylochip confirmed that *Proteobacteria* of the α-, β- and γ- subgroups and *Firmicutes* were the major bacterial taxa associated to *A. mellifera* larvae and adults. In particular, a special attention was directed toward several bacterial groups, namely Spore-Forming Bacteria (SFB), Lactic Acid Bacteria (LAB) and Acetic Acid Bacteria (AAB), which are recently receiving a great notice (Olofsson and Vasquez 2008; Crotti et al. 2010). Other two phylotypes, from γ-Proteobacteria (*Giliamella* sp.) and from β-Proteobacteria (*Snodgrassella* sp.), considered of a great relevance in recent studies (Moran et al., 2012), were also detected in the samples of this study. Moreover, in adults, the results obtained confirmed the presence of a "core microbiome": specific taxa are present in honeybees from different

geographical areas, as reported by other authors (Jeyaprakash et al.2003; Mohr and Tebbe 2006; Babendreier et al. 2007).
Interestingly, an increasing intestinal unbalance in the larval microbiome (dysbiosis) associated with the development of the disease was observed. This outcome allows to suppose that such a dysbiosis may reflects physiological changes accompanying the development of the disease, as it was demonstrated for other diseases, underlying that symbionts could exert a key role in the physiological homeostasis of the honeybees (Cox-Foster, 2007).
By cultivation dependent methods, a high number of Lactic Acid Bacteria (LAB), Acetic Acid Bacteria (AAB) and Spore Forming Bacteria (SFB) were obtained. The aforementioned taxa are among the main groups of bacteria present in honeybees and in the last years great attention was focused on them for their role in disease control. LAB and SFB are, in fact, known as common inhabitants of animal gut and several study have already proved that they own antagonistic effects against *P. larvae*. Moreover, AAB were considered since they were reported to be involved in the host immune and metabolic homeostasis (Ryu et al., 2008; Shin et al., 2011). They also own some peculiar and intriguing features, like the ability to change environmental pH (allowing the survival of gut inhabitants that tolerate low pH) and to prevent the pathogens' colonization of gut epithelia through a massive production of extracellular polysaccharides (Kounatidis et al. 2009; Crotti et al. 2010).
The inhibition tests performed *in vitro* showed that SFB were the most active against *P. larvae*. Interestingly, a synergistic effect could be measured when two SFB supernatants were tested together. The synergy of the two bacteria seemed to be effective in other mechanisms, in relation to the improvement of the healthy state of the host. The analysis of the transcript levels of some immune system-related genes, performed using RT Real Time qPCR, demonstrated an increase of the production of two antimicrobial peptide (AMP) transcripts when the honeybee was reared in the presence of both the two SFB, in comparison to transcripts of larvae reared with AAB and LAB. Furthermore, a significant inhibition activity was also present when measuring the pathogen inhibition using the supernatants obtained from the smashed guts of larvae fed with the probiotics. Thus, it is possible to hypothesize two mechanisms mediated by the potential probiotics: the release of bacteriocins, and the stimulation of the AMP production. According to Evans and Lopez (2004), the activation of the immune system prophylactically could be an extra cost for the honeybee. Although a slight growth cost from immunopeptide production has been found in the beetle *Tenebrio molitor*, there are still have no evidences for such a cost in bees.

Furthermore, SFB were analysed for their ability to colonize the gut environment and to hinder the pathogenesis of the *P. larvae* by competitive exclusion. The re-isolation experiments of the administered SFB, carried out using molecular typing by BOX-PCR, evidenced that SFB colonised efficiently the honeybee gut at 6 days after the administration. *Fluorescence in situ hybridization* showed that the probiotic bacteria, even in the presence of massive concentration of *P. larvae*, were able to colonise the gut, competing for food and niche with the pathogen. The competitive exclusion is another proposed mechanism mediated by the symbionts that could hinder the colonization of the pathogen and thus prevent the development of the disease.

Although, the molecular pathogenesis of AFB still remains elusive (Poppinga et al., 2012; Garcia-Gonzalez and Genersch, 2013), it is still not clear whether the bacterial proliferation takes place in the midgut itself or in the detritus of destroyed epithelial cells (Gregorc and Bowen, 1998). However, this work highlights the key role played by the microbial symbiotic community, considered in terms of richness (the number of species) and evenness (the relative abundance of individuals within a species) in preventing the occurrence of the invasion phenomena (Wittebolle et al., 2009).

Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit for the host” (Joint FAO/WHO Working Group, 2002). In this study, several mechanisms by which probiotics give benefit to its host have been suggested, i.e. the suppression of pathogen growth by the production of bacteriocins, the ability to outcompete the pathogen blocking its adherence to the host epithelium and/or competing for food, and the immunostimulation of the host. All of these factors contribute in the prevention of a subsequent PL invasion both *in vivo* and field trials.

Field trials, finally, were useful to understand if the scaling up of a process, effective in laboratory condition, was effective even on field. An advantage in the use of probiotic bacterial spores is the readily availability as veterinary and human dietary supplements (Evans and Lopez, 2004); it would be relatively easy to generate a supply of probiotic treatments for bees.

During the seven weeks of probiotic administration, a mortality test was coupled. Data clearly demonstrated a strong decrease of the mortality of honeybee larvae treated with the symbionts and after challenged with the pathogen (administered in laboratory conditions) in comparison to untreated ones. A statistically significant decrease was evident since the 3rd treatment, confirming the effectiveness of probiotics in acting against the pathogen in a very short time, confirming *in vivo* experiments’ results.

Levels of AMP transcripts (abaecin and hymenoptaecin) of larvae treated or not in field condition with the two probiotic strains and then challenged with the

pathogen, were measured by Real Time RT-PCR. A disease prevention response, measured as significant increases of abaecin and hymenoptaecin transcript levels, occurred when the larvae were treated with the probiotic bacteria in comparison to not treated larvae. Conversely, when the larvae treated with the probiotic strains were exposed to the pathogen, a decrease in the levels of the abaecin and hymenoptaecin transcripts, respect to the non treated larvae, occurred at the fifth week of treatments with the probiotic strains, despite such a treatment significantly decreased the larval mortality induced by the pathogen. Such decreases of the two transcript levels induced by the pathogen were abolished at the seventh week of treatment with the two probiotic strains, in coherence with the maintained decreased mortality. This indicates that the influence of the two probiotic strains on the AMP expression, when the larvae were continuously treated with the two strains overtime, prevailed on that driven by the pathogen and that the two probiotics support the immune response homeostasis even in presence of the pathogen challenge.
This second part of the present Ph.D. thesis confirms the importance of the role of symbionts not only in preventing the pathogen invasion, but even in improving the honeybee general healthy state. Afterwards, the efficiency of the entire family could be improved, exploiting its energies in pollination activity and production of honeybee food storage (such as honey, bee bread, royal jelly). The diversity, maintenance and dynamics of symbiont bacteria in the honeybee have a pivotal role in bee health, with major implications not only for research on bee decline but even for a sustainable pollinator management (Vàsquez et al., 2012). The importance of this research is focused not only on its scientific sense, but even on the development of a feasible application of probiotics in "field" conditions, giving a strong answer to the large use of chemicals on honeybee hives that, to date, are effective in one hand, but not environmentally friendly in the other hand.

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Acknowledgments

Three years of strong, passionate and deep research are not easy to carry on without the best support.
At first I'd would like to thank my family. They have been close to me even if there were 900 km of distance.
Then I would like to thank Professor Daffonchio, who gave me the chance to reach great professional results in a high level laboratory.
Thanks to Annalisa and Elena, my "scientific mothers", that supported me in every moment with patience and a smile.
Thanks to all DD lab and FC lab, for the (hundred) of amazing and (few) difficult moments lived togheter, because we like Bella Vita. I want remember the Lab38, a style of life.
A particular "shukran" goes to Chadlia Hamdi, that with her passion and knowledge increased my passion for this topic.
I even send my thanks to Professor Alma and his group, in particular Elena "To", for the hospitality in the laboratories of Grugliasco at the University of Turin (DISAFA), during the summer seasons.
I'd like to thank Andrea Bianco, I could consider myself an "amateur beekeeper" with his teachings.
Thanks to Don Matteo: one day I wish to be thanked for being a good "spannometric" tutor.
Thanks to Davide (Davidino), for the fantastic support during my first period in the lab.
Thanks to my best friends in Bari because they were and they will be in every moment.
Thanks to my best friends in Milan, for the unforgettable moments spent togheter and a special thank to the residence U22 also called "little corner of paradise".
Last but not the least, thanks to the honeybees, incredible creatures I am in love with!

Activities performed during the Ph.D.

-Publication: Genetic and biochemical diversity of Paenibacillus larvae isolated from Tunisian infected honey bee broods
Hamdi C, Essanaa J, Sansonno L, Crotti E, Abdi K, Barbouche K, Balloi A, Gonella E, Alma A, Daffonchio D, Boudabous A and Cherif A
Biomed Research International 2013:2013-9.

-Publication: Microbial symbionts of honeybees: a promising tool to improve honeybee health
Crotti E, Sansonno L, Prosdocimi EM, Vacchini V, Hamdi C, Cherif A, Gonella E, Marzorati M, Balloi A
New Biotechnology 2013;30(6):716-722.

-Publication: Microbial symbionts: a resource for the management of insect relative problems
Crotti E, Balloi A, Hamdi C, Sansonno L, Marzorati M, Gonella E, Favia G, Cherif A, Bandi C, Alma A, Daffonchio D
Microbial Biotechnology 2011;5(3):307-317.

-Publication: Symbiotic control strategies for managing honeybee diseases
Hamdi C, Essanaa J, Sansonno L, Crotti E, Balloi A, Abdi K, Barbouche A, Daffonchio D, Boudabous A and Cherif A
Proceedings of the BIODESERT International Conference on Microbial Resource Management for Agriculture in Arid Lands, December 16-19th, 2012, Hammamet, Tunisia.

-Publication: Acetic acid microbiome associated to the spotted wing fly *Drosophila suzukii*
Crotti E, Gonella E, Vacchini V, Prosdocimi EM, Mazzetto, Chouaia B, Mandrioli M, Sansonno L, Daffonchio D and Alma A
Proceedings of the BIODESERT International Conference on Microbial Resource Management for Agriculture in Arid Lands, December 16-19th, 2012, Hammamet, Tunisia.

-Poster: *In vitro* and *in vivo* control of the causal agent of the American Foulbrood Disease *Paenibacillus larvae* by honey bees symbionts
Hamdi C, Balloi A, Crotti E, Sansonno L, Essanaa, Gonella, Raddadi, Boudabous A, Borin S, Manino A, Bandi C, Alma A, Daffonchio D, Cherif A.

Presented during the International Conference “MICROBIAL DIVERSITY 2011, Environmental Stress and Adaptation” 1st edition, 26-28 October 2011,at the faculty of Agriculture of University of Milan.

-Poster: Microbial symbionts as a tool to improve honey bee health
Crotti E, Sansonno L, Hamdi C, Balloi A, Gonella E,Manino A,Alma A, Cherif A and Daffonchio D
Presented during the III CONVEGNO NAZIONALE of Società Italiana di Microbiologia Agraria, Alimentare e Ambientale (SIMTREA), the 26th-28th, June 2012, Bari, Italy.

-Poster: Microbial symbionts as a tool to improve honey bee health
Crotti E, Sansonno L, Hamdi C, Balloi A, Gonella E,Manino A,Alma A, Cherif A and Daffonchio D
Presented during the III CONVEGNO NAZIONALE of Società Italiana di Microbiologia Agraria, Alimentare e Ambientale (SIMTREA), the 26th-28th, June 2012, Bari, Italy.

-Poster: Symbiont Resource Management in honey bee health protection
Crotti E, Hamdi C, Sansonno L, Balloi A, Gonella E, Manino A, Alma A, Cherif A, Daffonchio D

-Poster: Acetic acid bacteria and the factors driving their roles as insect symbionts
Crotti E, Chouaia B, Vacchini V, Prosdocimi EM, Sansonno L and Daffonchio D
Presented during EU US Environmental Biotechnology Workshop, 5 - 7 Novembre 2012St. Louis, Missouri, USA.

-Poster: Bacterial probiotics to improve honey bee health
Crotti E, Sansonno L, Hamdi C, Balloi A, Gonella E, Chouaia B, Manino A, Marzorati M, Alberto Alma A, Cherif A and Daffonchio D
Presented during the International Conference: “Integrated Insect Immunology: From Basic Biology to Environmental Applications”, 23-28 September 2013, Polonia Castle Pultusk, Poland.

-Joined the International Training Course: “Biology of infectious and parasitic diseases of honeybees and traditional and innovative strategies for control”, the 25th of February 2011, Milan, Italy.

-Joined the laboratory of entomology of the group of Prof. Alberto Alma from Di.Va.P.R.A. (University of Turin) during the period of June-July 2011.

-Speech:"Microbial symbionts: a resource for pollinators health management". Presented during the VII workshop "DOCTORATE IN CHEMISTRY BIOCHEMISTRY AND ECOLOGY OF PLANT PROTECTION PRODUCTS AND XENOBIOTICS", 26th-27th January 2012; Faculty of Agriculture, Milan, Italy.

-Conference Secretariat at "1st International Conference on Microbial Diversity", 26-28 October 2011, Milan, Italy. Speech: Bees and probiotics: a new solution to contain the American Foul Brood disease.

-Speech: "Microbial symbionts: a resource for health management of insect pollinators". Presented during the meeting "CORTONA-PROCARIOTI 2012", 3rd-5th May 2012 in Cortona (Ar), Italy.

-Joined the laboratory of entomology of the group of Prof. Alberto Alma from Di.Va.P.R.A. (University of Turin) during the period of June-July 2012.

-Speech: Bees and probiotics: a new solution to contain the American foulbrood disease
Hamdi C, Sansonno L, Crotti E, Balloi A, Gonella E, Essanaa J, Marzorati M, Manino A, Alma A, Daffonchio D, Cherif A.
EurBee 5, 5th -7th September 2012, Halle en Saale, Germany.

-Joined the Seminar "Aggiornamenti sanitari in apicoltura", at the Veterinary Hospital of the University of Milan, Pavia, 21 September 2012.

-Speech:"Microbial symbionts: a new tool to preserve pollinators health". Presented during the VII workshop "DOCTORATE IN CHEMISTRY BIOCHEMISTRY AND ECOLOGY OF PLANT PROTECTION PRODUCTS AND XENOBIOTICS", 20th-21th January 2013; Faculty of Agriculture, Milan, Italy.

-Joined the course: "*Bacillus thuringiensis* an important resource for pest control: ecology, genetics, biotechnology" (Prof. Daffonchio), at the University of Milan, January 2013.

-Joined the Workshop “Nuove associazioni tra parassotoidi indigeni e insetti esotici” (Maria Luisa Dindo, Santolo Francati, Elisa Marchetti, Fabrizio Santi - DiPSA - Alma Mater Studiorum Università di Bologna), DeFENS (Department of Food, Environmental and Nutritional Sciences), Univeristy of Milan, 27 february 2013.

-Joined the Seminar “Study of microorganisms based on color” (Dr. Patricia Sanmartín Sánchez - University of Santiago de Compostela, Spain), DeFENS (Department of Food, Environmental and Nutritional Sciences), Univesity of Milan, 14 february 2013.

-Speech: “Utilizzo di probiotici per la salvaguardia delle api”, held at Istituto Tecnico Commerciale-Ragioneria L. Einaudi, 20th April 2013 in Magenta (Mi), Italy.