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GUT-BACTERIA SYMBIOSIS IN INSECT PESTS

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Cover:
Adult *Psacothea hilaris hilaris.*
Photo: Daniela Lupi

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Summary

Insects are one of the most fascinating taxa on Earth: their diversity, diffusion, colonization of different niches are unparalleled in the animal kingdom. Besides, they have a remarkable impact on human life: they are parasites for people, animals and crops, vectors of diseases, pollinators, and even breeding animals (e.g. honeybees, silkworms). This extraordinary evolutionary success and diversification is partially due to the symbiotic relationships that insects have with a wide range of bacteria. These symbionts can be divided into primary, secondary symbionts and gut bacteria. Primary symbionts are found in very specialized cells (the bacteriocytes), strictly maternally transmitted and not cultivable. They are essential for their host, and vice-versa: they can actually be considered part of a single organism called "holobiont". Secondary symbionts are not necessary for the host survival, although often beneficial, and they can inhabit various organs and tissues. In this category fall also reproductive parasites, as *Wolbachia*, which spreads in the population by maternal transmission, manipulating the reproduction of the host to favour the birth of infected daughters. Finally, gut bacteria are a more vague category, comprising organisms that live in the insect intestine because they are ingested with the diet, but also symbionts that establish a close relationship with the host, being essential for its survival and development. The roles of all these microorganisms are, to different extents, important for the insect physiology. Primary symbionts are generally essential to complement unbalanced diets and secondary ones contribute to the host fitness, while reproduction parasites deeply affect the reproduction mode of their hosts. Even commensals have been demonstrated to influence the development, mating choice and immune responses in *Drosophila* flies. For these reasons, the understanding of the biology of an insect can not do without the characterisation of its microbiota.

In the second chapter of my PhD thesis, a review on the microbial ecology techniques applied to the study of insect microbial communities gives an overview on the methods that can be applied to this purpose. On one hand, molecular analyses based on the 16S gene sequencing, such as 16S rRNA barcoding (pyrotag) and Denaturing Gradient Gel Electrophoresis (DGGE) are the most powerful methods to get a complete picture of the microbial community composition and structure. Microscopic localisation of symbionts can be also achieved by Fluorescent *In Situ* Hybridisation. On the other hand, the isolation of bacteria allows to deeply characterize the cultivable fraction, verifying through direct *in vitro* tests the activities of the strains. Taking advantage of a strain collection isolated from the target insect, the symbiotic relationship can be investigated through *in vivo* experiments. The more common ones involve i) the labeling of the strains with fluorescent proteins and the recolonization of the insects, to evaluate their localisation and colonisation ability, ii) the assessment of the detrimental effects of symbionts deprivation on the hosts, and iii) the comparison of insects monoassociated with different strains to check the effects on host fitness. To further analyse

the interaction between bacteria and their hosts from a genetic point of view, advanced techniques, such as Signature Tagged Mutagenesis or *In Vivo* Expression Technology, can be performed. Many of these techniques have been applied in the case studies here presented, in which the microbial communities associated to three insect pests have been characterised.
In the third chapter is presented a study on the spotted-wing fly *Drosophila suzukii*. Unlike its relative *D. melanogaster*, which feeds on rotten fruit, this fly feeds and lays eggs on healthy fruits. The most damaged crops are members of the Drupaceae family (e.g. cherries) and berries (strawberries, raspberries, blueberries). The bacterial community associated to this pest have been characterised with a focus on acetic acid bacteria (AAB), important symbionts of many sugar-feeding insects. According to our findings, *D. suzukii* harbours a diverse community of AAB, detected both in the isolate collection and in culture-independent screenings (pyrotag, DGGE). They are primarily localised in the gut, attached to the peritrophic matrix, as showed by FISH micrographs. The ability of three AAB species (*Gluconobacter oxydans, Acetobacter tropicalis* and *Acetobacter indonesiensis*) to colonise the gut has been proved by recolonization experiments of the insect using GFP-marked strains.
In the fourth chapter, the bacterial community of the wood-feeding beetle *Rhynchophorus ferrugineus* has been analysed. Commonly named Red Palm Weevil (RPW), this insect is an important pest for palm trees. The plants are damaged mainly by the larvae, which dig tunnels in the trunks until pupation. Bacteria associated to the red palm weevil have been studied primarily by molecular means (pyrotag). Our results outline that the bacteria hosted by *R. ferrugineus* are mainly acquired from the environment while feeding. Indeed, a sharp difference has been registered between field-caught and bred specimens. While field caught RPW harbour more bacterial taxa which are in common with their feeding plants, the animals fed on apple in the laboratory show a higher prevalence of lactic acid and acetic acid bacteria, which presumably grow on the rotten fruit. The latter result is further confirmed by the bacterial isolations performed on apple-fed specimens. Besides, the DNA sequence of a primary symbiont, *Candidatus* Nardonella, has been detected. This bacterium has been shown to inhabit a wide range of insects of the same family of the RPW, Curculionidae.
The fifth chapter is about the gut bacterial community of *Psacothea hilaris hilaris*. Native of Japan and east China, this longicorn beetle (family: Cerambicidae) arrived in Italy as a consequence of the wood trade, and settled as a stable population in a small area in Como province. Its larvae dig tunnels in the trunks of the trees of the Moraceae family, while the adults feed on leaves. The most damaged by its feeding habits are mulberry and fig trees. This beetle hosts a variegate gut microbiota, that, as shown by DGGE, greatly changes according to the diet and to the gut tract examined. The cultivable fraction of this microbiota has been tested for several activities that proved the capability of the community as a whole to exploit the food sources in the insect gut (primarily, sugars from plant cell walls) and to assist their host in carbon and nitrogen absorption. Thus, even if acquired from the environment, these bacteria seem to be adapted to a symbiotic lifestyle.

From the comparison among these three studies, some conclusions can be drawn. All three case studies outline the importance of the diet in shaping the insect microbial community. In detail, wild insects always show higher diversity and individual variability in their associated microbiota. Reared insects appear, on the contrary, dominated by the species that can rapidly grow on laboratory diets, such as Lactobacillales and Enterobacteriales. Secondly, these studies depict a more accurate image of the commensal bacteria, which are not merely acquired by chance through feeding, but are capable to actively colonize insect guts, and to efficiently exploit this niche to multiply and spread in the environment. Finally, the research data point out that the origin and the function of many of the organisms detected in insects are yet poorly understood. For this reason, these studies can be considered a basis to for future research, aimed to a more in-depth understanding of the roles of these bacteria and their interactions with the hosts.

Riassunto

Gli insetti sono uno dei più affascinanti taxa sulla terra: la loro varietà, diffusione, colonizzazione di diverse nicchie non hanno eguali nel regno animale. Inoltre, hanno un notevole impatto sulla vita umana: possono essere parassiti per le persone, gli animali e le piante coltivate, vettori di malattie, impollinatori e anche animali di allevamento (come ad esempio api da miele e bachi da seta). Questo straordinario successo evolutivo e diversificazione sono parzialmente dovuti alle relazioni simbiotiche che gli insetti intrattengono con una vasta gamma di batteri. I batteri simbionti si possono dividere tra simbionti primari e secondari, e batteri intestinali. I simbionti primari si localizano in cellule altamente specializzate (i batteriociti), sono trasmessi esclusivamente per via materna e non sono coltivabili. Sono essenziali per i loro ospiti e viceversa: i due possono essere a buon diritto considerati come parti di un singolo organismo chiamato “olobionte”. I simbionti secondari, invece, non sono necessari per la sopravvivenza dell’ospite, nonostante spesso gli conferiscano dei benefici, e possono colonizzare diversi organi e tessuti. In questa categoria ricadono anche i parassiti riproduttivi, come ad esempio *Wolbachia*, che si diffonde nelle popolazioni per trasmissione materna, manipolando la riproduzione dell’ospite per favorire la nascita di figlie infette. Infine, i batteri intestinali sono una categoria più vaga, comprendente organismi che vivono nell’intestino degli insetti perché ingeriti con il cibo, ma anche simbionti che intrattengono una stretta relazione con gli ospiti e sono essenziali per la loro sopravvivenza e il loro sviluppo. I ruoli di tutti questi microorganismi sono, importanti, in misura diversa, per la fisiologia degli insetti. I simbionti primari sono spesso essenziali per complementare diete sbilanciate e i secondari contribuiscono al benessere dell’ospite, mentre i parassiti riproduttivi ne influenzano profondamente la riproduzione. E’ stato dimostrato che anche i commensali possono condizionare lo sviluppo, la scelta del partner e le risposte immunitarie di *Drosophila melenogaster.* Per queste ragioni, la

comprensione della biologia di un insetto non può prescindere dalla caratterizzazione del suo microbiota.
Nel primo capitolo della mia tesi, una review sulle tecniche di ecologia microbica applicate allo studio delle comunità batteriche degli insetti fornisce una panoramica dei metodi che possono essere applicati per raggiungere questo obiettivo. Da una parte, le analisi molecolari basate sul sequenziamento del gene codificante il 16S rRNA, come il 16S rRNA barcoding (pyrotag) e l'elettroforesi su gel in gradiente di denaturante (DGGE) costituiscono gli strumenti più efficaci per ottenere un'immagine completa della composizione e della struttura della comunità microbica. La localizzazione dei simbionti può essere poi studiata attraverso l'ibridazione *in situ* fluorescente (FISH). Dall'altra parte, l'isolamento in coltura dei batteri permette di caratterizzare dettagliatamente la frazione coltivabile, verificando attraverso dei saggi *in vitro* le attività dei singoli ceppi. Avvalendosi di una collezione di ceppi isolati dall'insetto oggetto di studio, è possibile inoltre analizzare i meccanismi dell'interazione simbiotica attraverso esperimenti *in vivo*. I più comuni riguardano: i) la marcatura dei batteri con proteine fluorescenti e la ricolonizzazione degli insetti, per valutarne la localizzazione e la capacità di colonizzazione, ii) la misurazione degli effetti negativi causati sull'ospite dalla mancanza del simbionte, iii) il paragone tra insetti associati a singoli ceppi per studiarne gli effetti sul benessere dell'ospite. Per approfondire ulteriormente l'interazione tra i batteri e i loro ospiti da un punto di vista genetico, possono essere applicate tecniche avanzate quali la Signature Tagged Mutagenesis (mutagenesi con sequenza marcata) o la In Vivo Expression Technology (tecnologia dell'espressione *in vivo*).
Molte tra le tecniche citate sono state applicate ai casi studio presentati di seguito, nei quali si caratterizzano le comunità microbiche associate a tre insetti dannosi per l'agricoltura.
Nel secondo capitolo è presentato uno studio sul moscerino dei piccoli frutti, *Drosophila suzukii*. A differenza del suo congenerico *D. melanogaster*, che si nutre di frutta marcescente, questo moscerino si nutre e depone le uova su frutta sana: le coltivazioni maggiormente danneggiate sono le drupacee (ad esempio le ciliegie) e i piccoli frutti a bacca (fragole, lamponi, mirtilli). La comunità batterica associata a questo parassita è stata caratterizzata focalizzandosi sui batteri acetici (AAB, acetic acid bacteria), importanti simbionti di molti insetti con diete ricche di zuccheri. Dai risultati di questa ricerca emerge che *D. suzukii* ospita una variegata comunità di batteri acetici, individuati sia nella collezione di isolati che nelle analisi indipendenti dalla coltivazione, quali pyrotag e DGGE. Questi batteri sono principalmente localizzati nell'intestino, adesi alla matrice peritrofica, come dimostrato dalle microfotografie FISH. La capacità da parte di tre specie di AAB (*Gluconobacter oxydans, Acetobacter tropicalis* e *Acetobacter indonesiensis*) di colonizzare l'intestino è stata dimostrata in esperimenti di ricolonizzazione degli insetti effettuati usando ceppi marcati con GFP.
Nel terzo capitolo si analizza la comunità batterica del coleottero xilofago *Rhynchophorus ferrugineus*. Comunemente chiamato punteruolo rosso delle palme (Red Palm Weevil, RPW), questo insetto è un importante parassita delle

palme. Le piante sono danneggiate principalmente dalle sue larve, che scavano cunicoli nei tronchi fino all'impupamento. I batteri associati al punteruolo rosso sono stati studiati principalmente con tecniche molecolari (pyrotag). I risultati evidenziano che i batteri residenti in *R. ferrugineus* sono in gran parte acquisiti dall'ambiente con il cibo. Infatti, è stata registrata una netta differenza tra esemplari prelevati in campo e allevati. Mentre i punteruoli prelevati in campo ospitano più taxa in comune con le piante di cui si nutrono, gli animali nutriti in laboratorio su mela mostrano una più alta proporzione di batteri lattici e acetici, che probabilmente crescono sulla frutta marcescente. Quest'ultimo risultato è ulteriormente confermato dagli isolamenti di ceppi batterici effettuati a partire da esemplari nutriti di mela. Infine, è stata individuata la sequenza di DNA appartenente a un simbionte primario, *Candidatus* Nardonella. Questo batterio è associato a una vasta gamma di insetti appartenenti alla stessa famiglia del punteruolo rosso, quella dei Curculionidi.
Il quinto capitolo riguarda la comunità batterica intestinale di *Psacothea hilaris hilaris.* Originario del Giappone e della Cina orientale, questo cerambicide è giunto in Italia con il commercio internazionale di legname e si è stanziato in una piccola area della provincia di Como. Le sue larve scavano cunicoli nel tronco degli alberi della famiglia delle Moraceae, mentre gli adulti si nutrono di foglie: i più danneggiati dalle sue abitudini alimentari sono i gelsi e i fichi. Questo coleottero ospita un microbiota variegato, che, come illustrato dalle analisi DGGE, cambia molto a seconda della dieta e del tratto intestinale esaminato. La frazione coltivabile è stata sottoposta a diversi saggi che hanno dimostrato la capacità della comunità nel suo insieme di sfruttare le risorse alimentari nell'intestino dell'insetto (soprattutto polisaccaridi che compongono parete cellulare delle cellule vegetali) e di coadiuvare il loro ospite nell'assorbimento di carbonio e azoto. Dunque, anche se acquisiti dall'ambiente, questi batteri sembrano essere adattati a uno stile di vita simbiotico.
Dal confronto tra questi casi studio si possono trarre alcune conclusioni comuni. Tutti e tre sottolineano l'importanza della dieta nel dare forma alla comunità batterica degli insetti. In particolare, il microbiota associato ad insetti prelevati in campo mostra sempre una maggiore variabilità individuale e diversità tassonomica; gli insetti allevati appaiono, al contrario, dominati da quei microorgansmi che crescono più rapidamente sulle diete da laboratorio, come i membri delle famiglie Enterobacteriales e Lactobacillales. In secondo luogo, questi studi contribuiscono a dipingere un'immagine più accurata dei batteri commensali, che non sono semplicemente acquisiti casualmente con l'alimentazione ma sono in grado di colonizzare attivamente l'intestino degli insetti, e di sfruttare questa nicchia in modo efficiente per moltiplicarsi e diffondersi nell'ambiente. Infine, questa tesi evidenzia come l'origine e la funzione di molti degli organismi individuati negli insetti siano ancora poco conosciute. Per questa ragione, i risultati di questa ricerca possono essere considerati la base per una ricerca futura volta a una più profonda comprensione dei ruoli di questi batteri e delle loro interazioni con gli ospiti.

Chapter 1

Aim of the thesis

The interest on bacterial symbionts has greatly increased in recent years, giving birth to impressive scientific initiatives, as the human microbiome project (1). *Drosophila* flies, with their simple gut bacterial community, have been used as models to investigate the relationships between the gut microbiota and the host, and the regulation of the immune response to bacterial colonization (2–6). These studies proved the great importance of the microbial symbionts, that influence the survival, development, mating choice and immune response of the insect hosts. Besides, the body of insects emerged as an interesting and new environment to investigate with the methods of microbial ecology. The importance of this class of Arthropoda on planet Earth is overwhelming, in terms of diffusion, number of taxa and colonization of different environments. This makes the insects themselves and their diversity a fascinating topic, not only as a model to investigate the interactions between the microbial symbionts and the host. Moreover, many insects are studied for their immediate impact on human life, health and economy as pests, disease vectors, pollinators or breeding animals (e.g. silkworms, honeybees). In this light, the knowledge about composition and function of the bacterial community of insects is useful to deepen our understanding of insect physiology, and to explain the extraordinary evolutionary success of this taxon. This information will allow to develop better strategies to manage dangerous and useful insects (7).
In this thesis three case studies are presented, in which the microbiota associated to insect pests is characterized with different methods. In all the three cases, molecular techniques such as 16S rRNA barcoding (pyrotag) and Denaturant Gradient Gel Electrophoresis (DGGE) are applied, to obtain a complete image of the whole bacterial community. Bacterial isolations are then performed to deepen the knowledge about the cultivable fraction of the microbiota.
In the third chapter is presented a study on the black-spotted wing fly *Drosophila suzukii*, a pest of fruits and, in particular, berries. Unlike its relative *D. melanogaster*, that feeds on rotten fruit, the black-spotted wing fly feeds and lays eggs on healthy fruits still on the plants. The bacterial community of this insect has been investigated with a particular focus on the acetic acid bacteria. In fact, this taxon has been recognized as widespread among insects with a sugar-rich diet, and some of its members are proved to be important for *Drosophila* flies development, acting on the host insulin signaling pathway (3). In this study, the presence in *D. suzukii* of several members of the Acetobacteraceae family, such as *Gluconobacter*, *Gluconacetobacter* and *Acetobacter*, has been demonstrated by pyrotag, Denaturing Gradient Gel Electrophoresis (DGGE) and Fluorescent *in-situ* hybridization (FISH). All the three genera have been isolated from adults and larvae. Furthermore, three of the isolates (belonging to the species

Gluconacetobacter oxydans, *Acetobacter tropicalis* and *Acetobacter indonesiensis*) have been marked with the green fluorescent protein (GFP) and their ability to colonize insect gut has been shown through recolonizations.
The fourth chapter is about *Rhynchophorus ferrugineus*, the red palm weevil, a member of the family Curculionidae. This large beetle is regarded as the major pest of palm trees: its spread from the Middle East to the Mediterranean basin is threatening, among others, the ornamental *Phoenix canariensis* palms on the Italian coasts, which have a great landscape and historical value. The red palm weevil lays eggs at the base of palm leaves, and its larvae dig tunnels in the trunk, feeding on wood, until they reach the length of 3 cm. Even in this case, the bacterial community of the insect has been investigated by molecular means with pyrotag. The sequencing has been performed on field-caught specimens and on specimens reared on apple. This comparison showed that the bacterial community evenness undergoes a dramatic reduction after a month of rearing, underlining the importance of the diet in shaping the gut bacterial community. Bacterial isolations have been then performed from apple-reared specimens, confirming the results of the pyrotag.
In the fifth chapter another xylophagous beetle is presented. Indeed, *Psacothea hilaris hilaris* feeds on the trunks of mulberry and fig trees at the larval stage, and on the leaves of the same plants at the adult stage. Commonly named yellow-spotted longicorn, this member of the Cerambycidae family is endemic in eastern Asia, where its feeding habits greatly damage sericulture. In Italy, several observations of this insect have been registered, but it stably colonized only a small area of the Como province. Nevertheless, the danger of a possible spreading should not be underestimated, taking into account the importance of the fig cultivation in the Mediterranean basin. A rearing of *P. h. hilaris* has been established in our department, allowing us to investigate the changes in the gut bacterial community of specimens fed on different diets or field-caught, and to further verify in a different animal model the observations we made on *R. ferrugineus*. DGGE has been applied to investigate the bacterial community diversity of different gut tracts in wild larvae and in larvae fed with different artificial diets. Then, bacterial isolations have been performed with a focus on microorganisms capable to degrade recalcitrant plant polymers, such as cellulose, xylan and pectin. A selection of strains of the isolate collection has been subsequently tested for several activities connected to the carbon and nitrogen metabolisms. We named these activities “symbiotic potential” because they could promote a mutualistic relationship between the bacteria and the host. On an evolutionary perspective, bacterial strains which have the capability to persist in the host, to exploit the food resources in its gut and moreover contribute to its physiology should be advantaged themselves, spreading rapidly in the environment and in other hosts.
The last chapter presents the conclusions and the future perspectives of this work. This thesis shows how different microbiology techniques can be successfully applied to the investigation of microbial communities in insects. All the insects treated here are important pests, and the knowledge about their bacterial community will hopefully help to effectively address the problem of their management. Indeed, for understanding the physiology of

these pests, their symbiotic relationships can not be underestimated. Moreover, these exploratory analyses are the essential precondition to begin more in-depth studies that aim to elucidate the mechanisms of interaction between bacteria and non-model insects from different environments.

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Chapter 2

Microbial ecology methods to characterize the microbiome of non-model insects

Abstract

Insects are an extraordinary animal group, unparalleled for diffusion, diversity and number of different niches occupied. Besides this, they raise the interest of the researchers as animal models, human and agricultural pests and disease vectors, as well as breeding species (e.g. honeybee and silkworm). So far, it has been demonstrated that insects are involved in a number of different relationships with microbial symbionts, which influence their survival and fitness, development, mating habits and immune system. Thus, the research that aims to deepen the knowledge about a particular insect species can not do without a characterization of its microbial partners. In this review, we present several microbiology and microbial ecology techniques that can be applied to the taxonomical and functional analysis of the microbiota of non-model insects. Our goal is to give to the researchers that are approaching this topic an extensive overview of the basic methods, with a focus on operational details, and a concise guide to the advanced techniques that are available to extend the research beyond the simple description of microbial communities.

Introduction

The awareness of the importance of microbial symbionts for animal physiology is growing fast, together with the increasing number of studies focusing on the non-pathogenic bacteria hosted by a wide range of organisms. Although the attention of scientists has been for decades primarily focused on the pathogenic bacteria, the vast majority of the microorganisms associated to animals are likely harmless, variable and acquired from the environment (1). Besides, a number of beneficial bacteria had been found, and among these a group of essential, vertically transmitted endosymbionts that form with their host an inseparable holobiont (2). Insects have been investigated by microbiologists as models for human-microbiota interactions, as well as for their importance as agricultural pests and disease vectors. The most studied associations between bacteria and insects can be roughly divided in two groups, i.e. heritable symbionts (including primary and secondary symbionts) (2) and gut symbionts. On one hand, most heritable bacteria are obligate symbionts (2). They can be divided into primary (P) and secondary (S) symbionts. The first category comprises bacteria that are necessary for the insect survival and/or reproduction, and inhabit highly specialized cells –the bacteriocytes- that can be interspersed in the gut

epithelium or grouped within specialized organs called bacteriomes. Typically, they share a long evolutionary history with their host, as they propagate only through maternal transmission, and they cannot colonize naïve insects. This kind of symbiosis is common in insects with poor diets, such as aphids, which feed exclusively on phloem sap. Aphids need to host in their bacteriomes a □-proteobacterium of the genus *Buchnera* that is able to synthesize the amino acids that are absent in their diet (3). P-symbionts undergo to a process of "genome shrinkage", losing genes that are necessary for a free-living habit and retaining genes that are necessary for the activities involved in the symbiotic relationship (in the case of *Buchnera*, synthesis of aminoacids). Secondary symbionts are not essential for host survival, although they can improve the fitness of the host, and they colonize various cells and organs, including hemolymph. They are able to infect new hosts and establish with them stable associations through maternal transmission (4). Into this category fall also the reproductive manipulators that, rather than conferring fitness benefits to the host, spread into the population by promoting the reproduction of the infected females through daughters. Given that the transmission of the symbiont is maternal, this behaviour boosts its dissemination, and can be accomplished in various ways, such as cytoplasmic incompatibility, parthenogenesis, male feminization and son-killing (5). On the other hand, gut symbionts have been extensively reviewed in Engel and Moran (6). Most of these are commensals and they reside in the gut, being not clearly harmful nor beneficial for the host (7). With the term "commensals" we can refer to a broad range of microorganisms, varying greatly even among members of the same species. These bacteria are generally not transmitted maternally, but acquired from the environment and from the diet, and selected by the chemical and physical conditions inside the gut, such as pH, oxygen availability, and retention time of the food bolus. Furthermore, host immune system plays an active role in selecting certain bacteria, as it is elicited by specific bacterial features, for example the excretion of uracil (8). Despite the extreme variability of this type of microbial consortium, there is raising evidence that commensals can critically affect host physiology, acting on the immune system (8), on the larval development (9) and even on the mate choice (10) (reviewed in (6)). These effects, despite sometimes important, cannot lead to a sharp classification of the microorganism as "mutualistic" or "pathogen" (7). For example, in many cases the simple presence of the commensal microflora itself can prevent the colonization by pathogens (11), and the alteration of the bacterial community can lead to a "dysbiosis" that is detrimental for the host (12). Moreover, some cases are known of specialized gut symbionts whose relationship with the host resembles a primary symbiosis (genome shrinkage, strict heritability (13)). In the group of hemiptera, the vertical transmission of gut simbionts, which are smeared on the eggs or encased in symbiont capsules, is well studied (14–17).

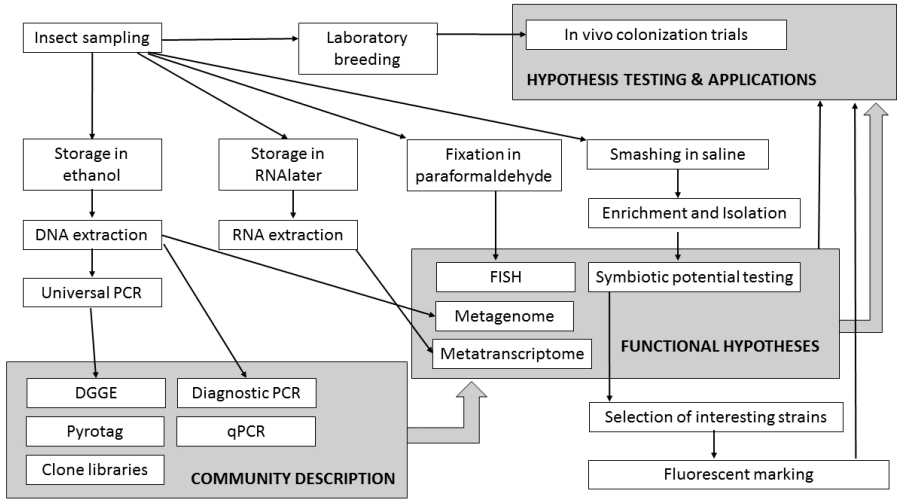

Figure 1: Schematic representation of the workflow for starting the characterization of the microbial community of an insect.

Taking into account the crucial roles of the symbionts listed above, a descriptive study of any insect can not disregard the characterization of the bacterial community associated with it. Thus, the research on emerging pests, disease vectors, or beneficial insects (e.g. honeybees and silkworms) should certainly comprise a microbiological screening. Aside from giving a more complete image of the physiology of the insect itself, this kind of survey could be the precondition to develop useful applications, such as probiotics or pest and disease control strategies (18). Looking at these potential technological developments, it is advisable not to underestimate culture-dependant assays, which, even if far less powerful than molecular methods (above all Next Generation Sequencing) in describing bacterial communities, give the promising opportunity to manipulate single cultivable strains.
The aim of this review is to give to the researchers that are approaching this kind of study an overview of the microbiological methods available (see Figure 1), together with the practical considerations that are necessary to apply them to insects and to correctly interpret the results. Addressing to the scientists that are new to this field, the focus will be mainly on the best-known and more feasible techniques. Nevertheless, also some advanced techniques of genetic analysis will be presented, to suggest the possible progress of these studies beyond the simple taxonomic characterisation of the microbial community.

Sampling

Recovering insect specimens suitable to the microbial community analysis can be challenging in many ways. Depending on the target species and its habitat,

the field season is often restricted to a short period of the year and the collection and identification of samples requires time and experience. Therefore, it is important to start with a precise plan of the number and type of individuals required.

Specimen choice

The composition of the microbiota of insects, within the same species, is largely dependent on ecological parameters such as temperature, geographical location and, above all, diet. Several papers report qualitative and quantitative differences in the gut community composition of insects reared on different diets (19–23). In some cases, insects of highly divergent taxonomical groups feeding on the same substrate can harbour very similar microbial communities, overcoming the effect of the host taxonomy in shaping the microbiota (21). This is true, for example, for some species that live on decaying wood (23). Moreover, the microbiota found in guts of wild insects is generally more diverse than the one of reared insects. Studies on *Drosophila melenogaster* show that different laboratory populations can host diverse strains that, nevertheless, can exert important and similar physiological roles (9, 24, 25). However, wild *Drosophila* gut microbiotas are much more complex, with the more diverse communities present in the flower and mushroom feeders (22). Finally, it is important to note that most standard diets used in insect stocks contain preservatives and/or antibiotics that avoid the proliferation of microorganisms on the diet itself, but necessarily have an impact on the natural microbiota of the insects. Even the distribution of secondary endosymbionts such as *Wolbachia*, *Spiroplasma*, or *Rickettsia* can depend on ecological factors such as diet, temperature and snowfall, or even the co-occurrence of other symbionts (26). The reproduction-manipulation strategy of endosymbionts like *Wolbachia* can rapidly spread the bacterium across one population, determining a patchy distribution. Moreover, this behaviour can influence the frequencies of other vertically-transmitted symbionts through a hick-hiking effect (26). On the contrary, by definition, the primary symbionts are present in all the specimens of one species, as they establish a tight interdependence with the host. For all these reasons, according to the aim of the research it may be necessary to sample insects of different populations. On one side, if describing the gut bacterial community of one insect is the primary goal, it is necessary to analyse directly wild samples rather than rely on laboratory stocks. Even feeding the insects on an artificial diet for short periods can lead to important biases (27). Moreover, to explain the possible differences in the gut bacterial composition of the specimens is advisable to monitor some ecological parameters on the collection site, at least the substrate on which the samples are feeding. Unfortunately, the dissection of the guts of wild animals can be far more challenging that the dissection of specimens reared on artificial diets. In fact, the latter usually have larger amounts of good quality food available, resulting in a bigger and more swollen gut that is clearly distinguishable from surrounding tissue. Besides, to survey the presence/infestation rate of endosymbionts in one species the sampling sites should be as geographically diverse as possible. If the infestation rate is always 100% across genetically

and spatially separate populations, the hypothesis of a primary symbiosis should be verified.

Target organs.

The choice of the organs to target largely depends on the size of the specimens and thus to the ease of the dissection. While to address single organs of smaller insects is very difficult, for larger ones this could be an obliged choice. In fact, hard cuticles and high quantities of biomass can impair DNA extraction. Moreover, the gut bacterial community usually outnumbers the bacterial cells in any other body district, thus, examining the insect as a whole can give similar results as if sampling the gut only (17). Therefore, at least in the case of "dissectable" insects it is useful to know which organs are more likely to host symbionts.

Gut. The gut of insects usually hosts a complex community of commensal symbionts. It can be divided at least in three tracts, named foregut, midgut and hindgut, which can host different bacterial communities. In foregut and hindgut, that are of ectodermal origin, a cuticular layer made of chitin separates the epithelium from the lumen, while midgut is the main site in which the nutrient absorption takes place. In the latter, a peritrophic matrix is secreted by the epithelial cells of many insects. This permeable envelope separates the bolus and the gut bacteria from the epithelium, allowing the transit of nutrient molecules, and is continuously replaced as it is shed. In the anterior hindgut the Malpighian tubules, insect excretory organs, deliver waste products such as uric acid, collected from the body cavity in which they extend. Therefore, in this area the mixture of nitrogen and food waste discriminate the gut environment of insects from the one of vertebrates, in which the nitrogen waste is separated. Depending on the species and the life stage, these three tracts can assume very different shapes and comprise diverticula or extremely specialized compartments, in which specific bacteria are hosted, as in the case of termites (28). Microsensor measurements in the beetle *Pachnoda ephippiata* show that the redox condition and the pH can vary sharply along the gut: the bacterial community composition varies accordingly (29). Therefore, the knowledge on the differences between the tracts can be critical in understanding the gut physiology. Nevertheless, dissecting the gut prior to analysis is a delicate step: a special attention is necessary to avoid the leakage or the mixing of the gut content of the different parts.

Bacteriomes. Bacteriomes, or mycetomes, are organelles composed by the bacteriocytes in which most primary symbionts reside. Often associated to the external midgut (tsetse flies (30), louse (31)), they appear as whitish round shaped bodies, and their first observation can be traced back to the seventeenth century (32). Bacteriocytes can be also located in the fat body, where they can be identified by microscopy because of their cytoplasm densely populated with bacteria (33), or intercalated in the midgut tissue (34). Separating the bacteriomes from the gut tissue without breaking the gut epithelium can be difficult in smaller animals. However, the detection of endosymbionts with molecular means, as diagnostic PCR, allows the analysis

of the whole insect, avoiding this step (see below). In the case in which target of the research are primary symbionts, bacteriomes must be investigated.
Gonads. Reproductive manipulators, as well as maternally transmitted mutualists, colonize ovaries where they infect trophocytes and oocytes, through which they are transmitted to the progeny (30). Moreover, in the arthropod *Ixodes ricinus*, an hard tick, an intra-mitochondrial endosymbiont that inhabits almost exclusively the ovaries is harboured (35). Testis also can be colonized, enabling a paternal transmission from the male to the mating female and to the offspring, as in the case of *Asaia* and *Anopheles* mosquitoes (36).
Salivary glands. The presence of endosymbionts has been investigated in the salivary glands of blood-sucking insects and ticks. *Asaia* colonizes the salivary glands of mosquitoes, co-localizing with the malaria vector *Plasmodium* spp., if present (4). The presence of *Midichloria mitochondrii* has been demonstrated in the salivary glands of *Ixodes ricinus*, by which is inoculated in parasitized humans in sufficient amounts to stimulate antibody production (37). These tiny organs have been mainly examined by Fluorescent In Situ Hybridization (FISH, a technique that it will be discussed in the following section).
Cuticles and Antennae. A peculiar type of symbiotic relationship exists between ants and bacteria of the genus *Streptomyces*. The bacterium occupies specific locations on the bacterial cuticle and produces an antifungal compound that suppresses the growth of parasitic *Escovopsis*, which threaten the fungal gardens grown by the ants (38). Another bacterium of the same genus plays a similar role in a symbiotic relationship with beewolves of the genus *Philantus* (39). Adult wasps carry the symbionts in specialized glands in the antennae and females secrete it in the brood chamber in which they lay eggs. Thanks to the antibiotic activity of the *Streptomyces* strain, with this behaviour the wasp protects the larva and the cocoon against bacterial pathogens that otherwise can easily grow on the prays that represent larval food (39).
Other locations. Wigglesworthia glossinidia, symbiont of the tse-tse flies, is transmitted to the offspring through the secretion of the milk gland. The secretion of this gland feeds the larvae that develop inside the mother's uterus until they reach the third instar (30).

Life stages.

Insects are characterized by a unique development, changing dramatically in morphology and lifestyle according to the life stage. Within the largest insect class, the Pterigota, there are two main types of postembryonic development. In the exopterigota group, the young individual hatched from the egg is similar in morphology to the adult. To reach the adult stage, it undergoes a series of moults, increasing his body mass without changing substantially in shape. Among exopterigota can be listed the cockroaches (Blattodea), the termites (Isoptera), crickets and grasshoppers (Orthoptera), stinkbugs, bedbugs, leafhoppers and aphids (Hemiptera). On the contrary, insects belonging to the endopterigota group are born as larvae, and are subject to a complete metamorphosis prior to the adult stage. Among them, can be

mentioned beetles (Coleoptera), butterflies (Lepidoptera), flies and mosquitoes (Diptera), ants and bees (Hymenoptera).

Exopterigota (heterometabolous insects). Within this group, the modifications of the gut bacterial community during development have been studied mostly in hemiptera. In the European firebug *Pyrrhocoris apterus* the gut microbiota appears to be stable along the several moulting steps, with presence of the most represented bacterial taxa already within the egg (17). However, as confirmed also in another member of the same order, *Riptortus pedestris,* the colonization of the insect by the specific bacterial community is not completed before the third larval instar (16). During the moults, an increase in the production of antimicrobial peptides causes a drop in the bacterial load of *R. pedestris* midgut (40). A possible explanation is the vulnerability of the molting insect to injury and pathogens, which leads to an up-regulation of the immune system. Regarding the primary endosymbionts, in aphids it has been demonstrated that the development of the bacteriome occurs in the first embryonic stages, even in the absence of symbiotic bacteria (aposymbiont insects) (41). This particularly intimate symbiotic relationship is not affected by the insect development in any way, as the two organisms can be regarded as a single holobiont that develops as a whole.

Endopterigota (holometabolous insects). The evolutionary success of holometabolous insects, that are widespread all over the word with an astonishing number of species, has been attributed on the differentiation of the food sources utilized by larvae and adults that avoids the competition between young and mature conspecifics. This differentiation is reflected in the composition of the gut community. Generally, the pupation phase implies a simplification and reduction of the gut microbiota, which, nevertheless, is not completely erased. After that, as the emerging adult starts to feed, bacteria start to grow again, comprising on one hand the species that survived the pupation and, on the other hand, new species that, according to the difference in diet, may or may not be similar to the ones in the larvae (27, 42). A particular case is the honeybee, *Apis mellifera*, in which, due to the cleaning behaviour of nutrices, the larvae are regarded as an almost-sterile environment and the worker bee acquires a characteristic microflora as it emerges from the brood cell, with two mechanisms: trophallaxis and contact with the hive (43). The maternal transmission of the endosymbionts that are present from the first larval stages to the reproducing adults indicates that the metamorphosis does not affect the presence of the bacteria in the bacteriomes through all the life cycle. Nevertheless, the bacterial load can change with time. The dynamic of bacteriocytes during metamorphosis has been studied in the carpenter ant *Camponotus floridanus*, hosting the mutualistic symbiont *Blochmannia floridanus*. In these ants a surprising increase of the bacterial load and of the number of bacteriocytes in the midgut epithelium takes place during metamorphosis, starting in the last larval instar (34). During the metamorphosis *Blochmannia* appears to colonize also non-bacteriocyte cells, while in the adults it gradually decreases.

In conclusion, if surveying the presence of maternally-transmitted symbionts, the life stage of the specimens could be neglected. Otherwise, if dealing with

gut bacteria, it is necessary to pay attention to this feature, especially in the case of holometabolous insects.

Sample storage and dissection

Storage. The fate of each collected specimen has to be determined since the very beginning of the sampling. In fact, different techniques require different storage of the samples. Samples for the molecular analyses are usually stored in ethanol and kept at -20°C. Especially when cooperating with unqualified personnel or volunteers during sampling campaigns, it is important to underline that denatured ethanol is not suitable for sample preservation. To ensure the permeation of the body of larger insects with ethanol, one or more legs can be removed. If dissection is required, it is better to perform it immediately and store separate organs. In fact, the dehydration of tissues caused by ethanol could hamper the dissection even after rehydration in saline. Specimens dedicated to bacterial isolations should be immediately analysed. Regarding this, it should be remarked the fact that feeding the insects on artificial diets, even for a few days, can dramatically change their gut communities, due to the fast growth of specific bacterial contaminants on the diets. For this reason, the best option when the immediate analysis is not feasible is collecting insects together with their native feeding substrate and analysing them as soon as possible.
Dissection. Prior to dissection, the insects should be killed or at least anesthetized. In fact, although the studies on nociception in *Drosophila* are still in the early stages, there is no doubt that insects show a pain response to mechanical, thermal and chemical noxious stimuli (44). A well established entomologic technique for killing insects is to saturate the atmosphere in their cage with chloroform or ether. This technique is suitable for molecular studies, as it is not likely to damage nucleic acids. However, to our knowledge it is not known the possible effect of these gases on living bacterial symbionts inside the insect; therefore some authors would rather use for specimens dedicated to bacterial isolation the anesthetisation (42) or sacrifice (17) by exposition to low temperatures (ice or refrigerator). To avoid the contamination by bacteria attached to the cuticle, which are likely influenced by the environment and by manipulation, most researchers perform a surface sterilization as a first step before the dissection or before the smashing for bacterial isolation purposes. In the washing procedure, the specimen is rinsed a few times in water or ethanol (42, 45), or detergents as SDS solution could be used (17). If they permeate the insect body, ethanol and detergents can affect the viability of the microbial symbionts. For this reason the timing of the exposure to these chemicals must be evaluated in relation to the insect's body mass: for big beetles with thick cuticles this observation is irrelevant, while it can be important, for example, for small flies and mosquitoes. In any case, the ingestion of the chemicals by the insect should be avoided as possible. The dissection of larger insects is usually performed starting with the removal of the cuticle. At this point, with some experience, it is possible to visualize the native arrangement of the gut and other organs in the abdominal cavity (46). The organs of interest can then be removed. For larvae,

the cuticle is usually cut along the side of the body, and all the fat tissue is gradually removed to uncover the gut. From small insects, like mosquitoes or *Drosophila* flies, it is still possible to obtain entire guts by gently pulling the head with pincers until the gut slides out of the body, attached to the masticatory apparatus. It is important to notice that it is impossible to surface-sterilize single organs. Therefore, each of them can be virtually contaminated by bacteria from the haemocoel and abdominal cavity.

Culture-independent methods for community characterization

As the price of new generation sequencing (NGS) lowered in the course of the last few years, the number of papers in which bacterial communities are described exclusively by means of sequencing greatly increased. Nowadays, next generation sequencing of 16S genes amplicons (pyrotag) can be considered the principal strategy for this purpose. Besides pyrotag, other molecular techniques have been traditionally applied, and among them Denaturing Gel Gradient Electrophoresis (DGGE), although more labour-intensive, is still useful for specific tasks. The basis of these methods is the amplification through PCR and the sequencing of the well-known "molecular clock" 16S rRNA gene (47). Briefly, this highly conserved gene encompasses nine variable regions, whose sequences are informative to determine the species of one bacterium on the basis of their sequence. Among them, there are as many conserved regions that are useful to design primers annealing on 16S rRNA gene of different bacterial species. On one hand, the biggest advantage of applying molecular methods is the detection of non-cultivable species, which fairly outnumber the cultivable ones in almost every environment. In particular, insect primary endosymbionts are not cultivable. Secondly but yet importantly, samples for molecular analyses can be collected through all the field season and stored in ethanol without affecting the analysis. On the other hand, the main problem connected to PCR-based methods is the reliability of the quantitative analyses, given that the relative amplification of the templates is affected by multiple factors, which have been extensively described as reported below. Hence, this analysis can be considered semi-quantitative, as it gives an indication of the relative abundance of organisms that has to be validated by more accurate methods, such as qPCR. Although a number of steps in PCR-based methods can bias the final results, there is not accordance on the best protocols to apply, which largely depend on the environment of interest: here you will find an overview.

DNA extraction.

The first critical step is the extraction of DNA. In soil, different extraction methods result in markedly different community profiles (48, 49). Although soil is considered one of the most "difficult" substrates for DNA extraction, even in insects there are some critical factors to take into account. Generally speaking, the extraction protocols starts with the lysis, for which a combination of mechanical, enzymatic and chemical treatments is used. As a first step, for entire insects, especially those with hard cuticles, liquid

nitrogen freezing and ceramic pestle smashing is the most popular option, while single organs (e.g. guts) can be smashed as well in saline using a small, tube-fitted pestle. Bead beating or sonication are also reported (21). However, unlike in the soil, bacteria in insect guts are not so tightly associated to hard substrates, and separating them should not require an aggressive beating: Santo Domingo and coworkers report that even a single brief centrifugation could be sufficient to separate most bacteria from gut cells and other debris (19). To perform the chemical lysis, proteases, lysozyme or other lytic enzymes are added. Lysozyme treatment is advisable to enhance the release of nucleic acids from gram-positives, which, due to their harder cell wall, can have lower extraction yields. The use of specific enzymes to extract DNA from particular gram-positives is also reported: Nikodinovich et al. (50) for example recommend a chromopeptidase to improve the extraction from Streptomycetes. Finally, chemical lysis disrupts cell membranes and releases DNA: this is particularly important for endosymbionts, which are enclosed in bacteriocytes. For this purpose, the sample is treated with detergents such as CTAB or SDS. Sometimes, EDTA is added during the mechanical or chemical lysis to impair the DNAses that are naturally present in the sample. The enzymatic lysis has to be performed before the addition of detergents, because the latter denaturate the proteins preventing the enzymatic activity.
After the lysis and release of nucleic acids from the cells, the DNA has to be purified. There are two main strategies to accomplish this step: i) chlorophorm-isoamilic acid extraction followed by isopropanol precipitation of DNA, ii) binding of the DNA to a silica membrane in the presence of chaotropic salts: most commercial extraction kits rely on this method (e.g. MoBio Power Soil, Qiagen Blood&tissue). The first protocol, described in (45), is usually more cost-effective although labour-intensive. However, when dealing with insects from soil, humic acids may be co-extracted with this procedure. Regarding the second method, a number of kits and protocols are available. For example, the popular Qiagen Blood&tissue kit comprises a protocol that is specific for insects. In this case, it is important to point out that the goal of this kit is extracting the DNA from the insect itself, rather than from the bacteria, and therefore at least a lysozyme pre-treatment should be added. If inhibition by humic acids is a problem, MoBio Power Soil could be a good choice. This kit has been used also for insects that live above ground (27). If using one of these kits, attention should be paid to the grinding or removal of the cuticles, which easily clog the microcolumns. Finally, DNA is eluted in water, TrisHCl or TrisEDTA buffer. A major problem in extracting DNA from insects are PCR inhibitors. A range of these compounds can be co-extracted with DNA from the insects: their type and amount vary according to the insect environment, diet and species. For example, obtaining effective PCR reactions from gut contents of soil inhabiting invertebrates seems more challenging than from above-ground livers, possibly due to the presence of humic acids in their gut or on their surface (51). Conversely, the presence of hard cuticles inhibits PCR at least with the use of direct-PCR approach (52). The presence of PCR inhibitors has been reported also for insect eyes (53) and guts (51), although both their nature and the best way to remove them are yet unclear. For this reason, it is advisable to test the presence and quality of

bacterial DNA by direct 16S rRNA gene PCR, rather than by measuring the DNA concentration in extracts photometrically or by gel electrophoresis. Moreover, in this case a high DNA yield from the extraction could not reflect a high amount of bacterial DNA, as the insect genome is always co-extracted. If the PCR fails due to inhibitors, diluting the extract could help.

Primer choice.

When amplifying the 16S rRNA gene, the critical points of a good primer choice are three: i) universality; ii) variability of the amplified region; iii) specificity for bacteria.

Universality. Although in the literature a wide range of primers on 16S rRNA gene are described, none of them is truly universal, at least without many degeneracies. This problem has been addressed in depth after the diffusion of pyrosequencing, but it affects DGGE as well. Several studies test primer pairs in silico (54, 55) for their universality, proposing new couples. Since it is probably impossible to state which is the perfect couple, it is yet important to ensure that the chosen primer pairs are suitable for each research purpose. To this extent, the primer pairs can be tested for their universality using RDP ProbeMatch tool (http://rdp.cme.msu.edu/probematch/search.jsp) or Silva TestPrime (http://www.arb-silva.de/search/testprime/). Both of them are web-based tools that compare given primers with a specific 16S database, returning a list of bacterial taxa that could or could not be amplified by the given primer pair. They also support primer degeneracies. Noteworthy, most sequences in the databases are not full-length, making this tools less suitable for testing the primers in the first and last bases of the gene. However, while the Silva tool clearly points out the percentage of sequences excluded from the analysis because too short, the user of the RDP tool has to be aware of this problem, eventually restricting the search to specific regions of the gene in order to use only the sequences that comprise the target region.

Variability of the amplified region. Given that for both pyrotag and DGGE should be generated an amplicon of no more than 500 base pairs, the analysis has to be restricted to one or two variable regions of the 16S rRNA gene. To these purpose, the most common choice is to select the V1-V3 or V5-V6 regions, as the major variability is registered in V1, V3 and V6 regions (56). Amplicons spanning one single variable region are not advisable because too short to correctly identify taxa. The deep analysis of Yarza and colleagues (57) shows that only the combination of two or more variable regions can lead to a full recovery of the species present in the sample. Cutting the high-quality sequences of the Silva Tree of Life Project Database, they simulated short amplicons spanning different variable regions and clustered the sequences to different percentages of similarity, corresponding to different levels of taxonomy. The best recovery of all ranks is obtained by combining two or more variable regions.

Specificity for bacteria. Working with mixed eukaryotic-prokaryotic DNA, the risk of unspecific amplification should not be underestimated. In the DNA solution extracted from an insect there is a mix of bacterial DNA and DNA from the insect and its food source (e.g. from the pray, or from vegetal material). Some of the priming regions of 16S rRNA are conserved in plastid

DNA or in eukaryotic 18S rDNA (58, 59). In some cases, the size of the amplicon from eukaryotic DNA is different from the size of the prokaryotic amplicon, allowing a separation of the two by means of gel electrophoresis. When the size is similar, the problem is difficult to detect. In both cases, due to the higher proportion of eukaryotic DNA in the source sample, the bacterial amplicons will be underrepresented in the PCR product. Moreover, since in the PCR reaction reagents are limiting, rare sequences in the quote of the bacterial amplicons will be lost. To check primer pairs for this problem, PrimerBlast is a useful tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) although it does not support primers with degeneracies. If is not possible to find a suitable primer pair, one option could be a nested-PCR approach. A first PCR amplification is performed with the primers 27F – 1492R, that are highly specific for bacteria and amplify the whole 16S rRNA gene; the result of this amplification is used as a template for a second PCR with the 16S primers of choice (59). To reduce the bias that comes from the double amplification (see following paragraph) the number of cycles in the first PCR should be reduced as possible. Another option is the design of a specific blocking primer (60), although this strategy could require a great effort for optimization. The universal bacterial PCR introduces a source of bias, determining the unreliability of the PCR-based methods for the quantification of bacterial community members. Two mechanisms produce this bias: PCR selection and PCR drift (61). The first implies a preferential amplification of certain templates at higher levels. There are several possible causes for such a "preference": i) the presence of a mismatch in one template, even in the 5' region (62), ii) the higher stability of the annealing of G or C variants of a degenerate primer respect to the A or T variants, (61) iii) different accessibility of the 16S rRNA genes in the bacterial genomes. The PCR drift is related to the stochastic variation in the amplification of different templates in the first cycles of PCR, and likely it has a less important effect, easily overcome by mixing replicate PCR reactions. A more important issue is the different copy number of 16S genes in bacterial genomes, recently reviewed by Vetrovsky & Baldrian (63). The authors, based on a comparative study on published bacterial genomes, point out also the presence of differences in the sequence of the copies within the same bacterium, which lead to an overestimate of the bacterial diversity in metagenomic studies.

Denaturing Gradient Gel Electrophoresis (DGGE)

Although considered by many an outdated technique, DGGE is still useful for many applications. This method is based on the separation of the different amplicons generated by a 16S rRNA PCR on a denaturing gradient gel. Briefly, a PCR reaction is performed on a mixed DNA sample using GC-clamped primers that target conserved regions of 16S rRNA gene. This generates amplicons that differ in their sequence and –as a consequence- denaturation behaviour, and reflect the diversity in the microbial community. The gradient of denaturating reagents (urea and formamide) in a polyacrylamide gel allows every amplicon subjected to electrophoresis to denaturate in a specific point of the gel. Once denaturated, the DNA molecules slow their run, creating thus a profile of bands that is viewable upon staining the gel with an intercalating

dye. It is assumed that every band corresponds to a bacterial species within the original sample, with a detection limit of 1% of the total community (64). Then, the bands can be cut and sequenced to identify the bacteria. Unlike pyrotag results, that require some bioinformatics analysis, band profiles are immediately and visually comparable, allowing the researcher to have an idea of the complexity and variability of the bacterial community within many insect samples (up to 20 in a single gel). This is useful in many cases: i) to choose the number of samples and the sequencing depth to perform a good pyrotag assay, ii) to directly compare individuals across time-scales, or different rearing condition, and to test if the factor in exam influences the bacterial community, iii) to test for the presence of recognisable patterns of bands, common to one group of samples. Compared to pyrotag, this method requires more bench work and optimization, but less bioinformatics analysis. However, it is possible to analyse the gel data both qualitatively and quantitatively with both proprietary software like Quantity One (Bio Rad) and open-source software like ImageJ. To this purpose, it is important to include markers on both sides of the gel, to make different images comparable. Laboratory-made markers can be easily obtained from a selection of DNA extracts from different isolates. If we exclude special cases, such as the highly bio-diverse gut of termites, the DGGE profile that we obtain from an insect consists of less than 20 bands. As a first trial, the denaturing gradient can be set to 40%-60% of denaturing agents. Bands originated from endosymbionts, whose genomes are usually rich in A and T, are located on the top of the gel. If looking for endosymbionts, it is advisable to choose a denaturing gradient starting from low percentages of denaturing agents (<40%) to better separate these bands. If dealing with vegetable-feeding insects, even if nested PCR has been performed to improve specificity of the amplification, bands corresponding to chloroplast can appear in the middle part of the gel. Sometimes cutting and sequencing of the bands at different heights reveals that more than one band is referable to a single species, thus, cutting and sequencing two representatives of each band height, at least in the first trials, is always advisable.

Clone library

To produce a 16S library, the product of a PCR amplification of a portion of the 16S rRNA gene is ligated into a plasmid vector and used to transform a competent *E. coli* cells. There are a number of commercial kits comprising the essential reagents for all the phases of the work (e.g. Pgem-T systems, Promega, or TOPO-TA systems, Life Technologies). Then, the 16S gene in the plasmid is PCR-amplified and sequenced separately from each *E. coli* colony, allowing the separation between different 16S genes coming from different bacterial species. This technique has an output incomparably lower than pyrotag in term of number of sequences. Nevertheless, it is useful to retrieve full-length 16S rRNA gene sequences to produce accurate phylogenies and identifications for the most representative members of a bacterial community (17), even if they are uncultivable. For this reason, to take maximum advantage of this technique the primers 27F and 1492R, or other primer pairs spanning the entire 16S rRNA gene should be used. Clones can be sequenced

by Sanger method in both directions with a high level of accuracy, and the information about the whole 16S gene can be used also for the design of specific primers and probes.

Diagnostic PCR

This technique consists in directly testing the presence of a specific symbiont within an insect individual performing a PCR reaction with specific primers targeting the symbiont. The research so far produced a long list of insect endosymbionts, many of which are restricted to certain taxa (especially P-symbionts), while others are widespread through diverse orders (reproduction manipulators). This knowledge has been exploited to design a number of specific primers that can be used to detect the symbiotic bacteria. This kind of screening is the easiest way to assess the presence of known primary symbionts in newly studied insects. Moreover, it is useful to assess the distribution of S-symbionts across species, sampling sites and populations (65). Specific primers can also be used to set up qPCR assays, which are the most reliable method to quantify the presence of uncultivable symbionts, whereas the pyrotag can give only an approximation of the contribution of a single species to the overall bacterial community (66). In literature it is possible to find a number of diagnostic primers for the best known symbionts: a list is present, for example, in Russell et al. (65). As an alternative, it is possible to directly design the specific primers from the 16S rRNA gene sequence of the target bacterium. Primer specificity has to be validated in silico with the over mentioned ProbeMatch or TestPrime tools and with PrimerBlast, and in control PCR reaction with both positive and negative control template DNA extracts.

Next Generation Sequencing of the 16S rRNA gene

Methods based on Next Generation Sequencing (NGS) have became in the last few years the main tool to investigate microbial communities and are increasingly popular. In fact, one of the principal applications the NGS platforms is the sequencing of 16S amplicons, that is possible from almost every kind of environmental samples. To apply the NGS workflow, DNA must be extracted and PCR-amplified with 16S universal primers. DNA molecules in the PCR product are directly and individually sequenced, giving as result a number of sequences that greatly outnumbers the sequence output from DGGE or cloning studies. Amplicons from different samples can be pooled on the same sequencing plate, by adding to one or both the universal primers a sequence tag.

Platform choice. For sequencing, a few different devices can be choose, among which the most used are Roche 454FLX, which was the first pyrosequencing platform available, and Illumina (HiSeq or MiSeq), which is rapidly overtaking it because of its lower cost and higher sequencing depth. The choice between the two should be driven by the aims of the study. Illumina systems are the most valuable option if we take into account the price and output in terms of number of number of sequences (in a single plate, 4000 millions with HiSeq and 25 millions with MiSeq). As a result, the coverage of the bacterial community is much deeper. However, the sequences are quite short (125 pb,

HiSeq, 300 bp, MiSeq), allowing the amplification of a single variable region of the 16S. Short sequences are not suitable for an accurate taxonomic assignment, and the description of the community is usually limited to the family level. The paired-end systems partially overcome this problem by sequencing the amplicon from both ends (2x300 bp with MiSeq). However, this requires an additional step to assemble two overlapping strands, possibly increasing the formation of chimeric sequences. 454FLX is more expensive and gives a lower number of sequences (700000 for one plate, amplicon sequencing with GS FLX Titanium XLR70) but longer (up to 700 bases in the same platform). This allows a far better taxonomic resolution, with the identification of genera and species. In summary, Illumina is designed for comparing very complex microbial communities, for which a deep sequencing is required. If we exclude some very particular cases, such as termites, this do not seem to be the situation in insects, in most of which bacterial communities are not as diverse as in environmental samples. However, the trend is towards an increasing usage of Illumina systems and there are some papers investigating insect communities with this method yet (27). In this context, Illumina technology can be successfully applied to compare bacterial communities from many different insects, and assessing the modifications of the microbiota occurring with the change of environmental parameters, populations or species. 454FLX is more suitable to study in-depth the composition of the microflora of a single insect, inferring information about its function. In fact, due to horizontal gene transfer and the rapid and complex evolution of Bacteria, members of the same genera and even the same species could have different lifestyles and metabolic features. For example, *Bacillus thuringiensis* produces the insecticidal Cry proteins, an important and biotechnologically exploited trait that is absent in *Bacillus cereus*, even if the two species are almost undistinguishable even by complete 16S sequencing. Therefore, to have some hints about the function of the bacterial consortium is necessary to reach the lowest taxonomic level possible, elongating as much as possible the sequenced region.

Number of sequences required. Pilot studies. The number of sequences per sample is largely dependent on the microbial community therein. Hence, the most accurate way to determine the minimum number of sequences is to perform a pilot study and inspect the rarefaction curve that correlates the number of taxa found to the sequencing effort. Different types of rarefaction curves can be easily obtained following the Qiime alpha-diversity pipeline (see below).A pilot study to assess the community diversity can be performed also by DGGE. This method is quite laborious to set up, but if a DGGE system is already available, it can be fruitfully used to test the diversity in each sample. In fact, in some species the bacterial composition of different individuals can vary greatly. For example, when analysing whole insects, it should be noted that sometimes primary and secondary symbionts, and particularly *Wolbachia*, can be present at very high loads compared to other bacteria, "shadowing" the remaining diversity. For the same reason, this kind of preliminary analysis is useful to decide if it is worth to pool small insects together or not. In this respect, should be considered that pooling always

implies a loss of information on individual variability that can lead to a misinterpretation of the data.

Data analysis. One of the most popular platforms for data analysis is Qiime (Quantitative Insights in Microbial Ecology, www.qiime.org) (67). It requires a Linux operating system and a basic knowledge on linux command line writing. The Qiime workflow comprises: 1) assignation of sequences to the samples based on the tag and denoising; 2) clustering of the sequences in groups (OTUs) based on similarity and picking one representative of each OTU; 3) taxonomy assignation to each OTU based on the comparison with a specialized ribosomal DNA database such as Silva (www.arb-silva.de) or Greengenes (www.greengenes.lbl.gov); 4) analysis of alpha-diversity (diversity within sample) with rarefaction curves and diversity indices; 5) analysis of beta-diversity (diversity between samples) with the Unifrac diversity matrix (www.bmf.colorado.edu/unifrac); 6) production of tables and graphs that can be exported or used for publications. The Qiime analysis pipeline can be run both in an almost totally automated way (using default parameters) or highly customized. Moreover, the software is open-source and can be modified by anyone with experience in phyton programming. Detailed tutorials are available on the website. An important note for data coming from insects regards the use of databases. In fact, Silva databases comprise eukaryotic 18S sequences, allowing the detection of possible insect sequences in the sample (originated by poor primer specificity, as seen before). On the contrary, Greengenes databases are exclusively prokaryotic: eukaryotic sequences would not be recognised and they will be shown as unassigned. An alternative to Qiime is Mothur (http://www.mothur.org). A valuable instrument to reconstruct phylogenies and identify the species from sequences is ARB (68). This software package is available on the Silva website (http://www.arb-silva.de) and requires a Linux operating system, although it is not run by command line like Qiime. ARB allows to align sequences taking into account the secondary structure of the 16S rRNA, that due to its stems and loops produces self-matching regions in the gene sequence. Moreover, it is possible to download highly curated trees of type and non-type strains, as well as constructing customized trees, and to place new sequences in the existing tree using a maximum parsimony algorithm. In this way, the taxonomy can be assigned based on evolution, and not to similarity, with the most rigorous and theoretically correct approach, and species level can be reached with a higher degree of certainty, with the method described, for example, by Franca and colleagues (69). However, compared to the Qiime pipeline, this requires much more time, experience and manual work, to inspect and evaluate the trees.

Metagenomes and Metatranscriptomes

With the term “metagenomes” and “metatranscriptomes” we refer here to the shotgun sequencing of the whole DNA or RNA within a sample. The resulting sequence data can be analysed to understand both the taxonomical composition and the metabolic potential of a given bacterial community, by comparison with sequenced genes coding known enzymes. Indeed, the first developed metatranscriptomic technique is the microarray, but we will not discuss this technique here, as it requires a good knowledge of the genomes

of target organisms, being thus more suitable for model or well-known symbiotic relationships, as for example, the one of *Buchnera aphidicola* with pea aphids (70). To our knowledge, metagenomic/metatranscriptomic sequencing has been applied to insect symbionts by a few authors so far, with a specific interest in wood degrading systems (71, 72) or in honeybee gut physiology (73, 74). More scientific literature is available on the transcriptomic profiles of insect themselves instead, sometimes connected to bacterial colonisation (75). Scully and colleagues (72, 76) published two coupled research papers describing the metagenome and metatrascriptome of the gut of the cerambycid beetle *Anoplophora glabripennis*, shading light on the contribution of both the host and its bacterial community to the digestion of recalcitrant polymers. One major decision has to be taken in planning this kind of experiment: if to sequence only the bacterial DNA (RNA) or to sequence the nucleic acids from the symbionts and the host together. The DNA and RNA content of eukaryotic cells is much higher than those of prokaryotes, thus, depending on the sequencing depth, the information from bacteria could be insufficient for a proper analysis. The gut bacteria can be separated from the surrounding insect tissue mechanically, or by centrifugation (71, 72) while in the case of endosymbionts no separation is possible. It is not advisable to describe a standardized analysis pipeline for metagenomic or metatranscriptomic experiments, because the "information mining" within large quantities of sequence data depends on the specific questions of the experimenter. For example, in the above-mentioned studies on wood feeding insects, the metagenome and metatranscriptome data are compared to the CAZy database (http://www.cazy.org/) (77) to retrieve sequences related to enzymes that metabolize carbohydrates. A nice overview of the available techniques, both for the sample preparation and the data analysis can be found in the review by Julia di Bella and coworkers (78). To have a detailed idea of the technical issues involved in transcriptomic experiments we suggest the review by Westermann and colleagues (79). Although speaking about pathogens rather than symbionts, it discusses in detail the questions arising from an experimental design that involves at the same time one eukaryotic host and one or more bacterial residents. A last interesting example on the application of these methods to the insect gut symbiosis can be found in the paper by Xie and collegues (80) which detected in the *Bemisia tabaci* microbiota a number of genes for the resistance to xenobiotics.

Bacterial isolation and cultivation

Although there is not accordance on the estimation of the proportion of uncultivable bacterial species, it is common knowledge that they greatly outnumber cultivable ones. Therefore, a culture-based survey of a bacterial community can not claim to be complete. For example, endosymbionts are not cultivable yet, therefore the culture efforts should focus on the gut microflora. Nevertheless, handling a collection of bacterial strains, even incomplete, gives the unique opportunity to test directly, *in vivo* and *in vitro*, hypotheses on their role for the insect physiology. Nowadays there are a

number of research works describing the bacterial communities within insects, lacking in many cases hypotheses on their function. Coming back to the old-fashioned bacterial isolations, this could be the first step to fill this gap in knowledge. Finally yet importantly, isolates can be used in a range of applications beyond the pure investigation of natural environments. Bacteria have been proposed as probiotics to improve the health of useful insects like honeybees (12), or to enhance the effectiveness of the release of sterile males to control a pest population (81). On the other hand, paratransgenesis is a promising technique against vector-borne diseases (82). A drawback of the culture-dependent methods is the requirement of fresh insect specimens collected from the field, that can not be fed in the lab nor preserved after sacrifice. Thus, the isolation schedule has to be carefully planned according to the field season. Bacterial isolation is typically performed by smashing insect gut (or the whole animal) with a sterile pestle in saline, and plating or inoculating dilutions of the homogenate on various culture media. The direct plating of the homogenate on a rich culture medium (e.g. Tryptic Soy Broth, Nutrient Broth) is suitable to estimate the bacterial load, by counting and averaging the colonies arising from the same dilution of the inoculum in multriple plates (usually triplicate plates). With this strategy, would be isolated mainly the bacteria which are present in higher loads and grow faster than others. In insect guts, usually fall in this category a number of Enterobacteriales. In spite of this, with this method that does not operate a selection among bacteria (as happens in enrichment, see below) is more likely to retrieve a higher proportion of the bacterial diversity within the sample. The serial dilutions should at least span a range from undiluted to 10^7. More rare bacteria, or bacteria with a slower growth, become visible only on highly diluted plates, due to the overgrowth of more common and fast species on the concentrated plates. Similarly, colonies of some bacteria can take considerably more time than others to become visible. To maximize the proportion of cultivated strains, the best practice for media preparation is mimicking as close as possible the natural environment of the bacteria. To obtain information about how the gut environment does look like, in terms of physico-chemical conditions, micro-sensors have been used. As an example, Lemke et al. (29) provide a detailed description of important parameters such as pH, redox potential, gas exchange along the entire gut tract of the *Pachnoda ephippiata* larvae (Coleoptera: Scarabeidae). These parameters can vary both spatially (in different gut tracts) and temporally (in different life stages). The pH is actively regulated by the host. Extreme levels of alkalinity are reported in specific regions in guts of some insects feeding on soils or decaying wood (scarabeid beetles (29), termites (28, 83)). This phenomenon has been explained with the enhancement in the digestion of recalcitrant wood polymers, or, particularly for humivores, the detachment of humic acids and other organic matter from clay particles. The guts of Lepidoptera are alkaline as well, this improving the nutrient availability in tannin-rich diets. Extreme pH values do not prevent microbial colonization, but provide a selective environment to which only a small group of microorganisms is adapted. Oxygen availability in the gut can vary greatly among insects, but it is usually higher in smaller insects, while in bigger ones the gut can be almost totally

anoxic (46) or host anoxic compartments. In these case, oxygen usually enters with the food in the foregut and decreases in midgut and hindgut. Anoxic conditions for the isolation of bacteria can be obtained through the use of an anaerobic chamber or, if not available, in sealed jars with oxygen subtractors such as Anaerocult A (Merck Millipore). If looking for particular activities (such as degradation of specific substrates) or for certain species, enrichment cultures can be obtained in liquid culture media prior to plate. As an example, a range of enrichment media for cellulose degrading bacteria are reported in literature (84), containing filter paper or carboxymethyl-cellulose, while the isolation of acetic acid bacteria, widespread symbionts of sugar-feeding insects, can be performed on acid media containing high proportions of sugars or ethanol (85). Several steps of enrichment can be performed by inoculating an aliquot of the enrichment culture in fresh enrichment medium. Single colonies resulting from homogenate platings or enrichment media platings are usually picked with sterile instruments and re-streaked on fresh medium. Typically, to ensure strain purity the process is repeated three times; after that, the strains can be preserved at -80° by adding glycerol to a usual final volume of 20%. The diversity in the strain collection can be assessed with fingerprinting techniques; the most used to this purpose is Internal Transcribed Spacer (ITS)-PCR. PCR amplification of the ITS produces indeed a characteristic profile of amplicons of different sizes, usually visualized on an agarose gel, thought to be specific for a strain (86). The identification of the strains is thus achieved by sequencing the 16S rRNA gene amplified with universal primers 27F and 1492R. Forward Sanger sequencing of this amplicon typically produces a 1000 bp sequence, spanning variable regions from V1 to V5 and allowing the identification by BLAST search or other online tools such as RDP classifier (87), SINA aligner (88) or EZTaxon (89). Sequences can also be used to reconstruct phylogenies together with sequences from type and non-type strains, using ARB (68). To obtain bacterial DNA for ITS and 16S amplification, a proper DNA extraction is not required, as in the large majority of the cases boiling lysis is sufficient. For some gram-positives, in which the thick layer of peptidoglycan of the cell wall can retain the DNA inside the cell, the boiling lysis can be preceded by an incubation with lysozime.

Isolate screening

Some studies infer metabolic potential of the gut microbiota from metagenomic and metatranscriptomic data (72, 74, 76). A direct screen of the isolates for specific activities is another straight and simple way to assess the possible contribution of the bacteria to the host metabolism. There are many *in vitro* tests that can be performed quite easily and others can be created or re-adapted from literature to fit every case study. For example, after the finding of pectate-lyases during a metagenomic survey on the honeybee gut microbiota, Engel et al. (74) verified the pectin degradation ability of the isolates from honeybee gut. Interestingly, only some strains within the *Gilliamella* genus possess this activity that could contribute to the digestion of pollen.

Carbon metabolism. There are a number of insects that feed on recalcitrant plant polymers. Grass and the growing parts of plants, such as leafs and stems, are mainly constituted by primary cell walls. The latter are flexible layers of cellulose and hemicellulose fibrils, embedded in a pectin matrix. In wooden parts, besides primary cell walls, there is a hard secondary wall layer, made of cellulose fibres, lignin and xylan. Although many insects secrete enzymes to digest such substances (90), in most cases the digestion of complex matrices of polysaccharides likely result from a cooperation between the insect and its bacterial and fungal microbiota. The best known wood-degrading microbial consortia in insects are found in termites (91). To assess the degrading capabilities of bacterial isolates a number of plate tests can be easily performed (Table 1). Isolates can also be grown on a mineral medium in the presence of unique carbon sources, for example sugar monomers and dimers originated by complex sugar hydrolysis: arabinose and xylose are the mayor components of xylan, while cellobiose is a cellulose dimer (two glucose molecules joined by a beta bond). To this end, a plate reader spectrophotometer can be used for O.D. (optical density) measurements, allowing multiple assays at a time. Survival of the isolates on chemically defined, minimal media needs to be tested before the experiment.

Test	References	Description
Endoglucanase	(84, 92)	Congo-red staining
Xylanase	(93)	Congo-red staining
Pectin degradation	(74, 93)	CTAB staining
Protease secretion	(94)	Degragation halo in milk agar plates
EPS production	(95)	Sucrose-rich medium
Urease	(96)	Colorimetric assay
Uricase	(97)	Degradation halo in uric acid agar plates
Nitrogen fixation	(98)	PCR assay
Ammonia production	(99)	Colorimetric assay

Table 1: Examples of screening tests to assess the symbiotic potential of the isolate collection.

Nitrogen metabolisms. Herbivorous diets are generally regarded as poor in nitrogen. For example, in wood the carbon to nitrogen ratio can be as high as 1000/1. To help their host coping with nitrogen scarcity, bacterial symbionts can directly fix atmospheric nitrogen, or recycling the nitrogen waste from the insect itself. In fact, in insects the nitrogen excretion passes through the Malpighian tubules that collect uric acid in the body cavity and confer them to the anterior hindgut. Other nitrogen sources in the gut environment are the urea produced by the gut microbiota itself and the cell-wall proteins. Easy biochemical tests can reveal the presence of urease or uricase activity, or the secretion of proteases. Although a number of nitrogen-free media have been published, the evidence of nitrogen fixing abilities through the growth on

these media can be difficult to obtain. Another option is to detect the presence of the nitrogenase gene by means of PCR (98). Ultimately, nitrogenase activity can be measured by acetylene reduction assay (100).
Screening for other nutritional activities. Bacteria can be involved in the detoxification of diet compounds. Strikingly, it has been demonstrated that they can even confer resistance to pesticides (101). Many herbivorous insects specialize to feed on plants that synthesize toxic metabolites to protect themselves. A common defence against insects is the production of tannins, which reduce the protein availability in diet. Several bacteria have been showed to produce tannases (92). The survival of isolated bacteria on toxic compounds can be tested directly in liquid or agar cultures.

Fluorescent microscopy methods

Microscopy techniques are an invaluable resource to get information about symbionts localization and function. As mentioned previously, the presence of bacteria in different body districts is indicative of their role and route of transmission. Since the bacteria often inhabit very specialized sub-niches in the gut or localize in specific positions in the bacteriocytes and in the reproductive organs, microscopy is the best way to observe the situation in detail. For example, *Candidatus* Midichloria mitochondrii, as suggested by the name, was found to inhabit the mitochondria of the ovarian cells (35, 103) of ticks. To this purpose, the most popular method to detect a bacterial species in a sample is *in situ* hybridization (ISH) based on an oligonucleotide probe complementary to a small fragment of 16S rRNA gene. The visualization of the probe can be achieved by immunohistochemistry or, more often, fluorescence microscopy. In the first case, the sample is treated with a DNA probe joined to the digoxygenin antigen, which in turn is bound by an antibody conjugated with an enzyme. Finally, the activity of the latter enzyme is detected by adding a chromogenic substrate (4, 103) that is visualized with light microscopy. This method is quite labour-intensive and it has been replaced in most cases by Fluorescence *In Situ* Hybridization (FISH), in which a DNA probe is conjugated with a fluorescent label.
FISH. Fluorescence *in situ* hybridization is based on the hybridisation of a DNA oligonucleotide conjugated with a fluorochrome to the bacterial DNA. This technique is a powerful method to detect bacteria directly in field-collected samples. Tissues can be preserved upon fixation, thus enabling the researchers to choose the most suitable timing for their experiments. The first step to perform FISH is to design or select from literature a specific probe. If the aim is detecting a particular species, the probe should hybridize on the 16S rRNA gene of the target species. The number of bacterial 16S rRNA gene sequences in the databases allows the researchers to design specific probes and, conversely, the high copy number of ribosomal rRNA genes inside bacterial cells provides a strong fluorescence signal. However, particular features of bacterial communities can be investigated designing FISH probes for functional genes. To design the probes for 16S rRNA, the PROBE_DESIGN tool within ARB software package can be used (68). The website of SILVA is another useful source of information about how to perform FISH

(http://www.arb-silva.de/fish-probes/). The probes can be verified in-silico also on the online tool "probe match" of RDP (http://rdp.cme.msu.edu/index.jsp, (104)) If possible, FISH probes should be then tested on bacterial isolates, or known samples, to assess the specificity and adjust the stringency conditions. This step is necessary also for probes from the literature, because different working conditions can lead to different specificity features, and, moreover, different substrates can include or not species closely related to the target – situation that requires a higher degree of stringency in the assay. It is thus important to verify that probes can detect the target species and are not cross-hybridizing with other bacterial DNA in the sample. To this purpose, FISH can be performed directly on bacterial cultures of the isolates or, if the target bacterium is unculturable, on a *E. coli* clone from a library, carrying the 16S of the target bacterium (105). Usually, a probe targeting all the bacteria is always included in the assay. A second preparatory step for the FISH assay consists in the observation of the target insect tissue in an epifluorescence microscope. Indeed, insect tissues can have a level of autofluorescence. Moreover, a plant-based diet usually fluoresce in the wavelengths of red in the gut sections. According to the endogenous level of fluorescence of the sample and the area in which bacteria are expected to be, the researcher can choose appropriately the fluorescent dyes conjugated to the probes. Briefly, FISH is performed after incubation in 4% paraformaldehyde to fix tissues. Samples are washed in PBS. A pepsin digestion step can be added to enhance the penetration of the probe in the tissues. The probes are hybridized ad the appropriate temperature in the presence of formamide and washed again in PBS before the observation (36). An additional DAPI staining is recommended to better localize the bacteria within tissues, using the position of eukaryotic nuclei as a reference. To the same purpose, actin filaments in the host cells can be marked using fluorescent phalloidin. For small insects, such as mosquitoes or *Drosophila* flies, the staining can be performed on whole organs in an Eppendorf tube and the glass slide mounted as a final step. According to their thickness, these thin samples could be observed in an epifluorescence microscope or (better) in a confocal fluorescence microscope, enabling the observation of multiple focal planes at different depths. For larger insects, organs (and particularly guts) may be too thick for the observation of a suitable focal plane on Z-axis, or to allow the penetration of the light to the desired plane. In this case, to get good images micro sections are necessary. One of the most feasible techniques to this extent is cryo-sectioning. This method preserves the tissue morphology, without the need of histological preparations that require more time and equipment, such as resin infiltrations. According to this method, after fixation in 4% paraformaldehyde, the sample is incubated in growing concentrations of sucrose, which acts as a cryoprotectant. Then, it is frozen on dry ice and embedded in an optimal cutting temperature resin (OCT). Thin sections (from 30 to 60 µm) are obtained in a cryostat and collected on microscope slides. In this case, the DAPI staining and hybridization with probes could be performed on the tissue slices on the glass slides. Unfortunately, gut sections are especially fragile, because the gut content is easily detached from the tissue during the washing and staining procedures.

For this reason, it is also possible to perform the DAPI staining and hybridization on whole organs before the freezing and to immediately observe the slides after the cut. The success of this second option depends on the penetration rate of the probe in the insect tissue, which in turn is linked to the size of the organ, and can be improved by the pepsin treatment. However, some preliminary experiments are usually required in order to choose the best visualization options for each case study.

Fluorescent strains

If an interesting strain is found among the isolates, the first step to characterize its interaction with the host could be transforming it with a plasmid carrying a fluorescent protein. The fluorescent strain can be subsequently administered to the host to investigate i) the colonization routes and timing, ii) the persistence in time and across different life stages, iii) the transmission routes (maternal, paternal, environmental) iv) the cross-colonization of different species (106, 107). This experimental set up requires on one hand a bacterial strain that is easily cultivated and transformed, on the other hand the management of a stable insect rearing.

Marking systems for bacteria. In literature are described several marking systems to transform bacteria with genes encoding for fluorescent proteins: among them, each researcher has to choose according to his target species and experimental constraints. Here an overview of the principal features of these systems is reported, in order to help the researchers that are new to this field to better understand the available options. Depending on the final position of the marker gene (usually encoding a fluorescent protein, e.g. GFP) in the host strain, the marking can be chromosomal, if the marker gene is located on the bacterial chromosome, or episomal, if it is on a plasmid.

The genes for fluorescent proteins are usually encoded in specific plasmids, to enable them to be transferred to the target strain, and coupled with an antibiotic resistance gene, to select cells that acquire them. If the marking is chromosomal, the plasmid lacks the replication origin that allows the plasmid to replicate in the host. Thus, it can not persist in the host cytoplasm but integrates in the host genome through molecular mechanisms derived by transposons or fagi. In some cases the insertion occurs in a specific, neutral site (such as attTn7 in tn7-based systems (108, 109)), while in others the insertion is random, raising the issue of the influence of the insertion on gene expression. Chromosomal marking is usually more stable and can allow to cultivate the bacterium without selective pressure (i.e. antibiotics). As a consequence, insects receiving the bacterial inoculum do not need to be under antibiotic treatment, thus resembling more closely the natural environment. Conversely, for the episomal marking is used a vector that carries an origin of replication recognized by the host, and thus continuously replicates in its cytosol. In this case, a continuous antibiotic selection is needed to avoid plasmid loss through generations; nevertheless, the fluorescence is much more bright, due to the multiple copies of the plasmid that can be present at the same time in a single cell. However, the level of expression of the fluorescent protein is largely dependent on the bacterial strain. Another important difference among marking systems regards the mode of delivery

of the marking gene to the bacterial cell: by transformation or by conjugation. In the first case, only two strains are involved: the target and the donor (usually *E. coli*). The plasmid is purified from the donor, usually using a commercial kit, while the target is grown in liquid culture and washed to obtain competent cells. The purified DNA is then directly introduced in the competent cells by heat-shock transformation or electroporation. The first technique works well with *E. coli*, but is generally regarded as less effective in terms of number of transformants. For this reason, electrotransformation is advisable in the case of environmental isolates. This second method requires an electroporator equipment to subject the competent cells, mixed to the plasmid DNA, to a voltage difference (111). After the transformation, the bacteria are usually grown for one hour or more in a rich liquid medium without antibiotics, and then plated on the appropriate plates added with antibiotic(s) to select the transformed cells.

Fluorophore	Transformation method	Strains/plasmids required	Target bacteria	Ref.
Chromosomal GFP (mini tn7); Insertion site attTn7	Conjugation and transposition	Delivery strain: *E. coli* XL1-Blue/miniTn7; Helper strain: *E. coli* HB101/pRK600; Helper strain: *E. coli* SM10 λpir/pUX-BF13	*Pseudomonas*, *Asaia*	(107, 108)
Chromosomal dsREd (mini tn5); Insertion site casual	Conjugation and transposition	Delivery strain: *E.coli* Mv1190λpir/TTN151; Helper strain: *E. coli* HB101/pRK600	*Pseudomonas*, *Asaia*	(36, 110)
Episomal GFP	Electroporation	*E. coli* pHM2-Gfp	Gram negatives	(4)

Table 2: Some marking systems that have been used to label bacteria in insect-related studies.

The conjugation method exploits the natural horizontal DNA transfer between bacteria of different species. To transfer the DNA by conjugation a complex molecular machinery is required; thus, unless the donor strains retains all the required features, more than two strains can be involved: the donor strain, the target bacterium and one or two helper strains. All the strains are separately grown in liquid cultures, washed, mixed together in appropriate ratios and plated or spotted on filter paper. Only after the conjugation time (usually an overnight, or more) the exconjugants are exposed to the antibiotic(s) in order to select the target bacteria that acquired the marker gene. To plan a transformation experiment, a marking system has to be choose primarily according to the target bacterial species. The various marking systems that have been developed differ in many features, such as

origin of replication of the plasmids and delivery mode, primarily to be suitable to different taxa. Many are the taxon-specific characteristics that affect the transformation efficiency: recognized replication origin, methylation of the DNA, type of cell wall (i.e. Gram positives/negatives), codon usage. Prior to start the experiment it is necessary to verify the antibiotic resistance profile of the target strain: it should be sensitive to the antibiotics used in the selection of the transformed cells. If using a conjugation-based system, the target strain should have one antibiotic resistance in the genome, or should grow in special selective conditions to allow the separation from the donor strain. To this purpose, it is sometimes possible to select naturally occurring mutants that are resistant to rifampicin. Furthermore, the fluorescent protein suitable for each study has to be selected according to the natural fluorescence of target insect organs, to optimize the visualization. The bacteria themselves can also have a natural autofluorescence that should be checked. In the Table 2 there is a short list of marking systems that have been used in experiments involving insect colonisations.

In vivo experiments

The recolonization of an insect with marked symbionts can have several purposes: i) to assess the beneficial effect of the symbionts on insect growth or fitness, ii) to verify the transmission routes of the bacterium iii) to assess the localization of the bacterium within the insect. All the three aspects are nicely discussed by Kikuchi and colleagues in a study about *Riptortus pedestris* (112). Conversely, it could be useful to deprive the insect of a symbiont to investigate if it is necessary, beneficial or neutral to the host (113).

Symbiont deprivation and monoassociated insects. The creation of "aposymbiotic" insects is the best method to assess the importance of a symbiont for the host survival and fitness. Moreover, it is the only applicable strategy when the target bacterium is an uncultivable endosymbiont, which effect can be only proved in a negative manner (by subtracting it rather than adding). Aposymbiotic insects are studied since the fifties (114) and can be obtained in various methods, reviewed by Wilkinson (115), among which the most popular and effective is the antibiotic treatment. This strategy allowed, for example, to assess the dependency of the pea aphids on the *Buchnera* symbionts for the production of some essential amino acids (116). Germ-free *Drosophila* can be produced also bleaching the embryos and rearing them on autoclaved medium, without the use of antibiotics. However, sterile conditions are very difficult to maintain, thus, the germ-free stocks are usually reared on diets supplemented by a mix of antibiotics (25). The comparison between germ-free (GF) and wild-type (WT) animals is a powerful method, but not sufficient for most scientific purposes. Indeed, aposymbionts are deprived of their entire microbiota, that is in most of cases composed by more than one species, and is thus impossible to attribute the better performance observed in WT animals to a single strain. Besides, it is difficult to prove that the antibiotic itself is harmless for the insect. For these reason,

in many studies aposymbiotic insects are used as a starting point to produce monoassociated insects, which are inoculated with a particular strain usually provided with the feeding medium. In *Drosophila,* this strategy allowed the screening of an entire library of mutated strains, to assess which exact bacterial genes were responsible for the beneficial properties of the tested bacterium (9). In this case, the monoassociated insects were compared to both WT and GF animals. In another study authored by Chouaia and collegues (113) a rifampicin-resistant *Asaia* strain is used to recolonize rifampicin-treated mosquitoes. The development of rifampicin-treated specimens was delayed, while the colonization by *Asaia* restored the normal development rate, showing that the longer growing time was due to the absence of the symbionts rather than to rifampicin itself. Many gut symbionts are commensals acquired from the environment, which are selected in the gut by the complex habitat created by the host and by the resident microbial community. Reasonably, these strains could be able to persist and to be beneficial to the insect also through the interactions with other members of the gut community. For these reasons, in some cases the colonization of insects with isolates can be performed directly on WT insects (117). One critical aspect of these experiments is the delivery time and mode of the bacterium. Since most cultivable symbionts are primarily located in the gut, the bacteria are usually mixed with the diet. To ensure the acquisition of the bacteria, insects can be subjected to a starvation time before being exposed to the inoculated food. Alternatively, the inoculated food is provided for a sufficient time span to ensure that the insects have at least one meal. The timing is chosen according to the size and feeding habits of the animal. For example, Kikuchi and colleagues (112) let the *Riptortus pedestris* nymphs without water for one night, and the animals immediately drink the inoculated water as it is given to them the following morning. Storelli and colleagues (25) allow the eggs of *Drosophila* flies to hatch on inoculated medium, while mosquitoes are fed with inoculated sugar diet for two hours (106). In some cases, symbionts are acquired in a specific temporal window during the development, that should be considered in planning experiments (16).

Fitness measurements. The quantification of the effect of a symbiont on the host fitness is not trivial. First, in many cases the beneficial effects become visible only in situations of stress or nutrient scarcity, while insects are usually reared in controlled conditions, with plenty of food and optimal temperature and light/dark cycles. For example, the influence of *Acetobacter pomorum* on *Drosophila* fly larvae development is visible only when the amount of yeast extract in the diet is reduced (9), while in a applicative study on the fruit fly *Ceratitis capitata* the symbiont *Klebsiella oxytoca* is used to restore the fitness of □-irradiated males (81). Second, the measurement of the "fitness" can be performed in different ways and at different life stages according to the expected effect of the bacterium. In most cases, the growth rate or the development time is measured at larval stages. In fact, holometabolous insects grow mainly as larvae, and different larval stages can be monitored observing the moults, thus registering the development time (9, 113). Insects can also be measured and weighted, or mated to assess the mean number of eggs laid as an indicator of reproductive success (112). To

specifically address the problem of mating success of males of *C. capitata*, Gavriel and colleagues observed the proportion of individuals that mated with WT females among inoculated, sterile males and among sterile males inoculated with dead bacteria (81).

Measurements of gene expression. In addition to the fitness measurements, the immune response of the insect to the administration of a bacterium can be evaluated by measuring the expression of specific genes, such as the insect anti-microbial peptides (AMPs), using real-time PCR assays. AMPs are important and well-characterized immune effectors of insects and invertebrates in general, which lack an adaptive immune system (118)

Localization and transmission routes. Having access to a marked strain of the target bacterium allows the researchers to localize it in colonized insects. To do that, it is possible to take advantage of the same microbiology techniques reviewed in the FISH paragraph. Fluorescent proteins are not affected by tissue fixation in paraformaldehyde or freezing. According to the colonization pattern observed, it is possible to make and test hypotheses about the bacterial transmission. For example, Favia, Crotti and Damiani (and colleagues) first localized the symbiont *Asaia* in the gut, salivary glands and gonads of *Anopheles stephensi*, and then demonstrated its maternal, paternal and environmental transmission, besides the capability to colonize different species (4, 36, 106).

Genetic manipulation techniques for advanced in-vivo studies

An advanced goal of the research on symbionts is the understanding of the genetic mechanisms that allow the interaction between an insect and its bacterial hosts. To this extent, besides metatranscriptomic studies, some authors started to apply advanced genetic techniques that have been primarily set up in the medical research. These methods provide elegant demonstrations in hypothesis-based experiments, as well as the possibility to apply wide screenings. However, the preconditions to set up this kind of experiments are severe. It is necessary to have a well-established breeding of the target insect, with appropriate and fast screening systems to evaluate the fitness of the animals colonized by different bacteria. Besides, the bacterium of interest should be easily cultivable and transformable. Finally, a great help comes from the availability of its sequenced genome.

Screenings based on insect phenotype

Site-specific mutations. Particular hypothesis on the interaction between bacteria and insects can be tested impairing a specific gene on the bacterial genome and assess the effect of the mutant strain on monoassociated insects. In the research group of Elke Genersch, this method has been used to validate the discovery of new virulence factors of the honeybee pathogen *Paenibacillus larvae* (119, 120). In their work, healthy honeybees are infected with *P. larvae* strains knocked-out in putative virulence genes, and the lack of a key symptom of the illness, the degradation of peritrphic matrix, is observed. With the constant increase of genomic and trascriptomic data on bacterial

symbionts will be possible to formulate and test an increasing number of specific hypotheses. As an alternative, a group of different mutants can be tested at the same time by transposon mutagenesis.

Transposon mutagenesis. Shin and colleagues (9) created a library of *Acetobacter pomorum* in which each strain carried an insertion of a Tn5 transposon in one random position on the genome. The library was used to create cohorts of monoassociated *Drosophila*, which were compared according to weight and growth rate. The strains that produced slow-growing flies were subsequently analysed to understand which genes had been impaired by the insertion of the transposon. They found that 11 genes involved in the periplasmic pyrroloquinolinequinone–dependent alcohol dehydrogenase (PQQ-ADH)–dependent oxidative respiratory chain were damaged in the selected strains. This strategy allowed them to understand that the production of acetic acid by *A. pomorum*, mediated by the PQQ-ADH pathway, was important to enhance larval growth on a sub-optimal diet.

Screenings based on bacterial survival in the host

IVET (In Vivo Expression Technology) and STM (Signature Tagged Mutagenesis), reviewed and compared in Chiang et al. (121), have been developed to discover In Vivo Induced (IVI) genes. Reasonably, pathogenic organisms encounter, as they enter the host, environmental conditions that they do not experience outside. Thus, they react to the mutated situation changing the gene expression profile, to enhance the transcription of the genes involved in colonization and pathogenesis. Therefore, many IVI genes are expected to be virulence factors. The same reasoning can be applied to symbionts. In the mutated conditions of the host body, they do not activate virulence factors but they should be able to modify their gene expression in order to escape the immune system, to colonize their specific niche in the body and to exploit the food resources therein without damaging the insect, but, possibly, giving it an advantage. For this reason, the application of these techniques to the field of insect symbiosis appears, in our opinion, very promising. Both these techniques are based on a genetic selection that is independent from the fitness of the host.

STM. Signature-tagged mutagenesis consists of a large-scale screening to detect the bacterial genes that are necessary for the survival of the symbionts inside the host body. Like in the method described above, a transposon is used to impair genes at random. In this case, a number of different transposon plasmids, each containing a sequence tag, is used to produce a set of libraries of randomly mutated strains of the same bacterium. Each library is originated by a transposon with a different tag. The tags are flanked by two invariant regions, to easily amplify them by PCR with the same primers. The libraries are used to create Input Pools (IP), combining one strain from each library. Every IP is administered to a different group of animals. After the experimental time, bacteria are recovered from the animals, generating the Output Pools (OP). Strains that are mutated in genes fundamental for the in-vivo survival of the bacteria will not overcome the phase inside the host, and will not be retrieved in the OP. IP and OP are compared by dot blot, after the amplification of the tags. Strains that are present in the IP but not in the OP

are thus analysed to detect their mutated genes, which are supposed to be necessary for the permanence of the bacterium inside the host. To ensure that each gene in the bacterial genome is mutated at least in one strain, it is necessary to repeat the experiment with many input pools. STM has been successfully applied to human enteric pathogens (reviewed in (122)) as well as to the plant symbiont *Sinorhizobium meliloti* (123). This screening method is suitable to select the genes that are necessary for the survival of the symbiont in the host gut, among which, reasonably, would be found critical factors for colonization and interaction with the host.

IVET. A more refined genetic manipulation is required for the In Vivo Expression Technology. The aim of this technique is to select the bacterial promoters that are active only *in vivo*, i.e. only when the bacterium is inside the host. As a first step, the genomic DNA of the target bacterium is randomly fragmented through a partial enzymatic digestion. The fragments are inserted upstream to a promoterless antibiotic resistance gene (a variant is possible using a gene to complement an auxotrophy) into a specific plasmid, that is subsequently used to transform the target bacterium. In the latter, these plasmids are not able to replicate, but can integrate in its genome through a homologous recombination event between the cloned genomic sequence and the corresponding region on the bacterial chromosome. Thus, a library of mutants is produced. As a result of the homologous recombination process, each of the mutants carries two tandem copies of a specific fragment of the genome; downstream of one of the copies lays the promoterless antibiotic-resistance gene. This library is subjected to a first screening step on antibiotic medium, to eliminate the clones that are resistant to the antibiotic in-vitro (with a replica-plating-like process). Indeed, only the bacteria in which a promoter present in the cloned genomic fragments drives the expression of the antibiotic resistance gene will survive on the antibiotic plates. These clones harbour a cloned copy of a promoter that is probably constitutive, as it is active in the optimal laboratory conditions, without any stress stimulus. These clones have to be excluded from further screenings. The remaining clones of the library of mutants are introduced in the eukaryotic host as a whole, and, as in STM, retrieved after some time. Differently from STM, for the whole duration of the experiment the animal host is subjected to an antibiotic therapy. In these conditions, only the bacteria that are able to express the antibiotic resistance gene, due to the presence of a cloned, *in vivo* activated promoter upstream, will survive. The bacteria in which the cloned portion of the genome coincides with inactive promoters or regions with other functions, will die or at least remain quiescent inside the host. To further refine the selection, the retrieved pool of clones can be used for a second in vivo inoculation. In the end, the output pool will contain only bacteria in which a second copy of an in vivo activated promoter is present. Further analyses are needed to sequence it and determine his native function in the genome, in order to understand which are the in vivo transcribed genes in its operon. To this extent, the knowledge of the genome of the target bacterium is very useful. Many variants of this general idea have been developed. The screening can be based on the complementation of an auxotrophy rather than on an antibiotic resistance, avoiding the need to treat the hosts with an antibiotic

(124). Finally, different methods have been developed to select against the constitutive promoters. An advancement of the IVET is called RIVET – Recombinase-based IVET – and involves a recombinase that, when expressed due to promoter activation, permanently cleaves a marker gene elsewhere in the genome. RIVET is much more sensitive and can detect promoters activated at low levels and transiently. Both IVET and RIVET are reviewed by Merrell and Camilli (125). IVET has been applied also to the research on *Bacillus cereus* as a pathogen of insects, using the honeycomb moth *Galleria mellonella* as a model (126). IVET screening is more refined than STM, giving as output only the genes that are activated selectively *in vivo*, and therefore are responsible of the "switch" between the free and the symbiotic lifestyle.

Conclusion

In recent years, the literature data on insect bacterial symbionts greatly increased, together with the fast development of sequencing techniques. However, a large number of works do not go beyond the description of taxa associated to insects of various genera and orders. In our opinion, this great amount of data is an important resource that, so far, have not been deeply exploited. Indeed, in most cases the function of insect symbionts is unknown. Conversely, metagenomic studies show that RNA transcripts from symbionts can exert a variety of functions, but they give only a rough idea of which bacterium is actually producing them. Connecting taxonomy to function and, possibly, to isolates constitutes a great advance for science and technology. The extraordinary evolutionary success of insects, which represent 5/6 of all the Metazoa and inhabit any type of ecological niche all over the world, is at least partially due to their symbiotic relationships. For example, insect guts are complex ecosystems in which a variety of food sources are efficiently exploited, including poor, unbalanced, toxic and difficult to digest substrates. Assessing the mechanisms through which this happens would be precious for the research on the management of wastes, including their possible reuse and exploitation, as well as for the detoxification of contaminated substrates. In this context, to manage isolated strains would be a high added value, both for deepening the knowledge and for developing applications. On the other hand, knowing the processes that underlie the survival and resistance to treatments of many agricultural pests and disease vectors could give a relevant contribution to the research on animal and plant illnesses. In insect vectors, symbionts and pathogens coexist, but their interactions are far to be fully elucidated. Besides, it is well-documented the interaction of gut symbionts with the host immune-system, which can be elicited or down-regulated by the resident microbiota possibly affecting the survival and transmission of the pathogens. Several management strategies based on symbionts (reviewed by Crotti and colleagues(18)) are already applied to control insect pests. As an example, the Sterile Insect Technique (SIT), based on the release in the environment of sterilized males to control a pest population, can be greatly improved by the use of microbial symbionts to improve the fitness of irradiated males (81).

For these reasons, insect symbionts are a promising field, to be investigated using all the techniques available, some of which are summarized in this review, not only to describe microbial diversity but also to link it to function and to enable applications.

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Chapter 3

The Acetic Acid Bacterial Microbiome of the Spotted Wing Fly, *Drosophila suzukii*

Abstract

Drosophila flies are mainly considered as secondary parasites, due to their behaviour to attack rotten fruits. They harbour an inconsistent microbiome composed of several bacterial taxa, among which the acetic acid bacteria (AAB) are found to be important modulators of insect development through insulin signalling. Conversely, the spotted wing fly *Drosophila suzukii* (Diptera: Drosophilidae) is a highly invasive pest, native of Eastern and South-eastern Asia, rapidly spreading in the many countries, laying eggs in healthy fruits, with a consequent economic damage. With the aim to unravel the microbiome associated to *D. suzukii*, reared on fruits or on artificial diet, cultivation-independent and -dependent techniques have been used, giving a particular attention to AAB symbionts. By DGGE-PCR on 16S rRNA gene, AAB of the genera *Acetobacter* and *Gluconobacter* have been frequently detected. According to 16S rRNA barcoding, the two groups of insects (reared on fruits or on artificial diet) showed to cluster separately, but in both cases sequences related to Rhodospirillales order, to whom AAB belong, were a predominant group. Isolation data evaluated the extensive presence of cultivable AAB (*Acetobacter, Gluconacetobacter* and *Gluconobacter*) in the fly body, investigating different life stages (larvae, pupae, adults). Recolonization experiments by the use of *green fluorescent protein* (Gfp)-labelled strains and fluorescent *in situ* hybridization indicated the dispersal of AAB in the insect gut. In *D. suzukii* larvae and adults, AAB are mainly localized on the midgut epithelium.

Introduction

The vinegar fly *Drosophila suzukii* Matsumura (Diptera: Drosophilidae), also known as spotted wing *Drosophila* in the USA, is an exotic pest, endemic to South Asia and recently introduced in the continental USA, Canada and Europe, most probably by the international trade (1–3). Characterized by a rapid spreading behaviour, this fly is an economically damaging pest, due to the ability, unlike its vinegar fly relatives that attack rotten fruits, to feed on healthy soft summer fruits, laying eggs on them, thanks to the females' large serrated ovipositor (4). Once hatched, larvae grow in the fruit, destroying it (4, 5). *D. suzukii* is able to develop on many host plants either in its native and introduced habitats, with berries being the preferred hosts (3, 4, 6, 7). In most attacked countries *D. suzukii* causes severe economic damage to soft fruits

every year (8, 9). In particular, in Italy the most significant growers' associations reported extensive crop losses (3).
A recent increased attention has received the study of the bacterial microbiome associated to *Drosophila* flies. Drosophilid flies belonging to different species and with various feeding habits, reared in laboratory conditions or field-captured, have been investigated through deep sampling analysis by 16S barcoding or 16S clonal libraries (10–12). A bacterial community dominated by four families, Lactobacillales, Acetobacteraceae, Enterobacteriaceae and Enterococcaceae is commonly associated to these flies, with variations of the bacterial members at the genus level (11, 12). In lab reared and field-sampled flies, acetic acid and lactic bacteria (AAB and LAB, respectively) are dominant symbiotic taxa harboured in the intestinal tract (10, 13). In particular, AAB establish a delicate balance with the insect innate immune system, being involved in the suppression of the growth of pathogenic bacteria in healthy individuals (i.e. colonization resistance), hence contributing to the host health (13, 14). An interesting experiment conducted on the experimental model *Drosophila melenogaster* by the same research team (15) demonstrated AAB promotion ability of the insulin pathway, with consequent enhancement of the larval developmental rate, body size, intestinal stem cells activity and energy metabolism. AAB positive role has been also demonstrated in a different insect model, represented by anopheline mosquitoes: the acetic acid bacterium *Asaia* plays a beneficial role in the development of the mosquitoes and in fact, a delay in the development in *Anopheles stephensi* larvae was observed after antibiotic treatment; the larval development rate compared to the control one could be restored after administration of an antibiotic-resistant *Asaia* strain (16). Another work confirmed that *Asaia* administration boosted the developmental rate of *An. gambiae* larvae, affecting genes involved in cuticle formation (17).
AAB are symbionts of insects mainly localized in the insect gastrointestinal tract (GIT) (18). Particularly, the midgut is a sugar and ethanol rich environment, and represents a specific and beneficial habitat for these bacteria (19). They own different ways of transmission, with the horizontal as the favorable one (18, 20, 21). The recent comparison of AAB genomes showed several symbiotic traits that could favor the adaptation of AAB as insect symbionts (22). In particular, cytochrome bo3 ubiquinol oxidase might be involved in AAB adaptation to the diverse oxygen levels in the arthropod gut. In fact, AAB cannot be only considered as insect symbionts; they are generally found on sugar- and ethanol-rich substrates, spread in the environment on fruits, vegetables and fermented matrices, niches that they share with insects and from which insects can re-acquire them (18). Given that strong associations are common between acetic acid bacteria and selected insect orders, in the present study we assessed the presence of this important microbial group in *D. suzukii* individuals, reared on fruits or artificial diet, by means of cultivation-independent and -dependent techniques. We also provided information on tissue localization of these endosymbionts. The knowledge of the bacteria associated to this insect pest could be applied in future biocontrol approaches, as discussed.

Materials and Methods

Insects.

Wild specimens of *D. suzukii* were field collected as adults/larvae in Trentino Alto Adige region (Italy) and reared in laboratory condition both on fruits and on a sugar-based artificial diet (composed with 71 g of corn flour, 10 g of soy flour, 5.6 g of agar, 15 g of sucrose, 17 g of brewer's yeast, 4.7 ml of propionic acid, 2.5 g of vitamins mix for each Kg of the preparation) at the Dipartimento di Scienze Agrarie, Forestali e Alimentari (DISAFA), University of Turin. Insects were kept in cages at 25 °C with a 14:10 h light-dark photoperiod.

DNA extraction.

Larval, pupal and adult individuals of *D. suzukii* were killed, washed once in ethanol 70% and twice in saline and immediately stored at -20°C in ethanol until molecular analysis. Total DNA was individually extracted from larvae, pupae and adults of the laboratory strains by sodium dodecyl sulfate-proteinase K-acethyltrimethyl ammonium bromide treatment, as described in (23).

Characterization of the bacterial community associated to *D. suzukii* by Denaturing Gradient Gel Electrophoresis (DGGE).

A 550 bp fragment of the 16S rRNA gene was amplified from the total DNA extracted from *D. suzukii* individuals, using the forward primer GC357f, containing a 40-bp GC clamp, and the reverse primer 907r, as previously described (23). Gels with a denaturant gradient of 40–60% were prepared with a gradient maker (Bio-Rad, Milan, Italy) following the manufacturer's instructions. Bands were excised and used as template in PCR re-amplification reactions with primers 357f and 907r, as described previously (23). PCR products were sequenced (Macrogen, South Korea), and the resulting sequences were compared, using BLAST (http://www.ncbi.nlm.nih.gov/blast), with deposited sequences in the National Center for Biotechnology Information (NCBI) sequence database (24).

Characterization of the bacterial community associated to *D. suzukii* by 16S barcoding.

DNA extracted from fly individuals (namely DS54, DSM, DS41, DS55, Ds159, Ds164, Ds165, Ds167, FP1, FP3, LP1, LP3, MP3 and PP2, Tab. 2) were used in 454 Pyrotag sequencing. The variable regions V1–V3 of the bacterial 16S rRNA gene was amplified by MR DNA (Molecular Research LP, Texas, USA) using the universal bacterial primers 27Fmod (5'-AGR GTT TGA TCM TGG CTC AG-3') and 519Rmodbio (5'-GTN TTA CNG CGG CKG CTG-3') as described in Montagna *et al.,* 2013 (25). In total, 178,856 raw reads were obtained. Pyrosequencing adaptors, low quality base calls (<30 Phred score) and size-selected (between 350 and 500 bp) were performed by using the QIIME pipeline filtering scripts (26). The resulted reads were clustered into operational taxonomic units (OTUs), applying a sequence identity threshold of 97%, using *Uclust* (27). A representative sequence of each OTU was, then,

aligned to Greengenes (http://greengenes.lbl.gov/) using PyNast (28). Chimeras were removed using *Chimeraslayer* (29). The results of OTUs assignment were then used in the diversity analyses using the various scripts of the QIIME pipeline.

Prevalence of AAB in *D. suzukii* specimens.

A total of 50 *D. suzukii* adults (25 males and 25 females), reared on fruits, were used for assessing the prevalence of different AAB. After DNA extraction from single flies following Raddadi *et al.* (2011) (23), samples were submitted to AAB-specific amplification of the 16SrRNA gene, followed by digestion with the restriction endonuclease TaqI (Promega, Madison, USA) (30). Pure cultures of the most representative strains isolated from *D. suzukii* were employed for restriction profile reference.

Localization of *D. suzukii* symbionts by fluorescent in situ hybridization (FISH).

FISH was carried out on tissues and organs dissected from field-collected *D. suzukii* adults in a sterile saline solution. The dissected organs were fixed for 2 min at 4°C in 4% paraformaldehyde and washed in PBS. All hybridization experiment steps were performed as previously described (21, 31), using fluorescent probes, specifically designed for the acetic acid bacterial group (AAB455, sequence TGCACGTATTAAATGCAGCT) and for *Gluconobacter* (Go15, sequence AATGCGTCTCAAATGCAGTT and Go18, sequence GTCACGTATCAAATGCAGTTCCC). Moreover, the universal eubacterial probe, Eub338 (sequence GCGGGTACCGTCATCATCGTCCCCGCT), was used to detect the localization of the overall bacterial abundance and presence in the organs analysed (32). Probes for AAB and Eubacteria were targeted at the 5' end with the fluorochrome Texas Red (TR; absorption and emission at 595 nm and 620 nm, respectively), whereas probes Go15 and Go18 were labelled with indodicarbocyanine (Cy5; absorption and emission at 650 nm and 670 nm, respectively).

Isolation of AAB.

Insects (5 males, 6 females, a pool of 3 males and a pool of 3 females), reared on fruits, were surface sterilized by rinsing once with ethanol 70% and twice with 0.9% NaCl under sterile conditions, before being homogenized by grinding in 200 µl of 0.9% NaCl. Forty µl of each insect homogenate were inoculated in different enrichment liquid and solid media, selected for AAB growth: enrichment medium I (hereafter indicated as TA1, (33, 34)), enrichment medium II (hereafter indicated as TA2, (35)), a basal medium (hereafter indicated as TA4, (36)), Hoyer-Frateur medium (37), acid YE medium (yeast extract 2%, ethanol 2%, acetic acid 1%, pH 6). One hundred µl of serial dilutions of the insect homogenate were spread on plates containing mannitol agar medium (mannitol 2.5%, peptone 0.3%, yeast extract 0.5%, pH 7, agar 15 g/L) or R2A agar (38), both supplemented with 0.7% $CaCO_3$ and 0.01% cycloheximide. Other 6 insect adults reared on the artificial diet, 6 adults, 3 pupae and 3 larvae reared on fruit diet, were washed three times with deionized water and the washing water of the last step was plated on MA solid

medium. Pupae and larvae were smashed, as previously described, and inoculated in TA1 and TA2 enrichment media. All the enrichment liquid media were incubated at 30°C, in aerobic condition with shaking, until turbidity of the liquid media was reached. Serially dilutions were plated on MA medium, supplemented with $CaCO_3$ (1% D-glucose, 1% glycerol, 1% bactopeptone, 0.5% yeast extract, 0.7% $CaCO_3$, 1% ethanol, 1.5% agar, pH 6.8) and incubated at 30 °C, in aerobic conditions. For the solid media, colonies were picked up and streaked on MA solid medium, with $CaCO_3$. Colonies capable of clearing the calcium carbonate were purified on agarized MA medium, and pure strains were conserved in 15% glycerol at -80 °C. Total DNA was extracted from the isolates by boiling protocol and stored at – 20 °C.

16S rRNA gene-based identification and fingerprinting analysis of the isolates.

Internally transcribed spacer (ITS)-PCR fingerprinting was performed with primers ITSF (5'-GCC AAG GCA TCC AAC-3') and ITSR (5'–GTC GTA ACA AGG TAG CCG TA-3') as previously described (39). ITS-PCR amplification patterns of all the isolates were visually compared to cluster the isolates into ITS groups or profiles. At least 2 candidates for each ITS profile were selected and 16S rRNA gene was amplified and sequenced for identification by Macrogen (South Korea). 16S rRNA gene was amplified with universal bacteria 16S rRNA gene primers 27F (5'-TCG ACA TCG TTT ACG GCG TG-3') and 1495R (5'-CTA CGG CTA CCT TGT TAC GA-3'). Reaction mixture for 16S amplification was carried out in a final volume of 50 µL , using 1 unit of Taq DNA polymerase, 1X PCR Buffer, 0.12 mM of each dNTP, 0.3 µM of each primer, 1.5 mM $MgCl_2$ and 2 µL of DNA. Reaction was run for 4 min at 94°C, followed by 35 cycles of 1 min at 90°C, 1 min at 55°C, 2 min at 72°C and then a final extension of 10 min at 72°. 16S rRNA gene sequences were compared to the databases at the National Centre for Biotechnology Information (NCBI) using BLASTn (24) and aligned with their closest type strain relatives using Clustal W (http://align.genome.jp/).

Transformation of *Gluconobacter oxydans* DSF1C.9A, *Acetobacter tropicalis* BYea.1.23 and *Acetobacter indonesiensis* BTa1.1.44 with the plasmid pHM2-Gfp.

G. oxydans strain DSF1C.9A, *A. tropicalis* BYea.1.23 and *A. indonesiensis* BTa1.1.44 were transformed through electroporation introducing the plasmid pHM2-Gfp (40). Electrocompetent cells were prepared according to this procedure: exponential phase cells (OD 0.5) grown in GLY medium (2.5% glycerol, 1.0% yeast extract, pH 5) were washed twice with cold 1 mM Hepes, pH 7, and once with cold 10% glycerol. Then, cells were resuspended in cold 10% glycerol to obtain 160-fold concentrated competent cells. Aliquots were stored at -80°C. Sixty µl of competent cells were gently mixed with about 0.2 µg of plasmidic DNA, put in a cold 0.1-cm-diameter cuvette, and pulsed at 2000 V with the Electroporator 2510 (Eppendorf, Milan, Italy). After the pulse, 1 ml of GLY medium was added to the cells, which were subsequently incubated at 30°C in aerobic condition with shaking for 4 h. Transformed cells

were selected by plating serial dilutions on GLY agarized medium, supplemented with 100 μg ml^{-1} kanamycin, 40 μg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (XGal), and 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for Lac^+ phenotype detection. When growth occurred, transformant colonies were chosen and the Gfp expression was checked by fluorescence microscopy. ITS amplification of wild type and transformant strains was performed and compared to ensure the identity of the transformants.

Evaluation of plasmid stability.

To verify plasmid stability in the absence of selection, *G. oxydans* DSF1C.9A(Gfp), *A. tropicalis* BYea.1.23(Gfp) and *A. indonesiensis* BTa1.1.44(Gfp) were grown overnight in GLY medium with 100 μg ml^{-1} kanamycin, with shaking. When growth was visible, suitable dilutions were plated on non-selective GLY agar an incubated at 30°C till the growth of well-separated colonies. Four colonies were then chosen, resuspended in 1.0 ml of GLY medium and vortexed intensely to obtain free cells. Suitable dilutions were plated on selective and non-selective GLY agar. The proportion of kanamycin-resistant bacterial cells was determined through the ratio between the kanamycin-resistant bacterial cells and the total number of cells grown.

Colonization experiments of *D. suzukii* with *G. oxydans* DSF1C.9A(Gfp), *A. tropicalis* BYea.1.23(Gfp) and *A. indonesiensis* BTa1.1.44(Gfp).

G. oxydans DSF1C.9A(Gfp), *A. tropicalis* BYea.1.23(Gfp) and *A. indonesiensis* BTa1.1.44(Gfp) were grown in GLY medium containing 100 μg ml^{-1} kanamycin up to a concentration of 10^8 cells ml^{-1}. Cells were harvested, then washed and resuspended in sterile water to a final concentration of 2×10^8 cells ml^{-1} or 1×10^9 cells ml^{-1} for colonization experiments of *D. suzukii* adults. Colonization experiments of adults were performed by placing the adults in a small cage. A bacterial suspension (10^7 or 10^8 cells) was added to 0.5 g of adult sterile food and small drops of the obtained mixture were placed inside the cage on parafilm-covered glass slides. Appropriate controls without the addition of bacteria were done. The insects were fed *ad libitum* for 48 h with a sugar solution containing the Gfp-labelled strain, and then they were allowed to feed for 20 h with honey. Organs were then dissected in Ringer solution (0.65% NaCl, 0.014% KCl, 0.02% $NaHCO_3$, 0.012% $CaCl_2$ $2H_2O$, 0.001% NaH_2PO_4 $2H_2O$, pH 6.8) and fixed in 4% paraformaldehyde at 4°C for 10 min, mounted in glycerol and analyzed by fluorescence (Leica Microsystems, Germany) and confocal laser scanning microscopy, Confocal Laser Scanning Microscopy (CLSM, Leica Microsystems, Germany).

Results

Characterization of the bacterial diversity in *D. suzukii* by DNA-based analysis.

The bacterial community associated to 32 *D. suzukii* specimens was analysed by 16S rRNA gene DGGE-PCR, using as template the DNA isolated from whole single individuals. In particular, 5 larvae (n. 1-5; Fig 1A), one pupa (n. 6; Fig 1A) and ten adults (n. 7-16; Fig 1B) reared on fruits have been analysed, as well as 4 larvae (n. 29-32), 4 pupae (n. 25-28) and 8 adults (n. 17-24) reared on the artificial diet (Fig.1C). Generally, *Drosophila* flies host low complexity bacterial community, with the presence of few abundant bacterial taxa (11, 12). Here, a lower variability in the community profiles was observed among larvae reared on fruits and among the specimens reared on the artificial diet (Fig. 1A-C): many bands were rather conserved among the samples respectfully to the group to which each specimen belonged. Conversely, only few conserved bands were detected among the adults reared on fruits, which showed to have more complex profiles than larvae reared on fruits or specimens from the artificial diet (Fig. 1A-C). The majority of the bands from specimens reared on fruits showed similarity with species belonging to the Alphaproteobacteria class which comprises AAB, whereas the others indicated closeness with Betaproteobacteria, Gammaproteobacteria, Firmicutes and Bacteroidetes classes (Tab.1). Among larvae reared on fruits, bands A6 and A7 were the most remarkable ones, observed in a frequency of 50% (3 larvae out of 6) and 33% (2 larvae out of 6) of the tested individuals, and with 99% similar to the 16S rRNA gene of *Acetobacter tropicalis* and *Acetobacter persicus*, respectively. In all the larvae and in the sole pupa, a sequence with 98% identity with *Bacillus* sp., corresponding to A3 band in the PCR-DGGE gel, was detected, while band A1, corresponding to a sequence strictly related to *Paracoccus* sp. of the Alphaproteobacteria, was identified only for one individual. For 33% (2 out of 6) of the larvae analysed, sequences with 99% of similarity with the genus *Stenotrophomonas* and with the species *Enterococcus casseliflavus*, which are related to bands A4 and A2 respectively, were found not give clear results (lanes 22, 23, 24; Fig. 1C).

Adults reared on fruits showed a massive presence of AAB-related sequences; in particular, they showed a high sequence similarity with a *Gluconobacter* and several *Acetobacter* species (Fig. 1B and Tab. 1). Bands B1 and B5 indicate both 100% sequence identity with *Gluconobacter albidus*, whereas bands, such as B4, B6, B10, B11, and B12, showed identity with the genus *Acetobacter*. Band B4, observed for only 10% of the tested adults, showed 99% similarity with *A. cibinongensis*; band B6, detected in few (20%) of the tested individuals, showed 100% identity with *A. tropicalis*, whereas band B11, which was repeatedly found (5 out of 10) among the samples had *A. aceti* as the closest relative, with the 98% sequence similarity.

Band	Most related species	GenBank Accession no.	% nt similarity (identical bp/total bp[a]	Classification	No. of positive indiv./ total[b]
A1	*Paracoccus sp.*	JX515659	98%(551/561)	Alphaproteobacteria; Rhodobacterales	1/6
A2	*Enterococcus casseliflavus*	KC150018	99%(576/580	Firmicutes; Lactobacillales	2/6
A3	*Bacillus sp.*	AM888231	98%(550/562)	Firmicutes; Bacillales	6/6
A4	*Stenotrophomonas sp.*	KC153268	99%(583/587)	Gammaproteobacteria; Xanthomonadales	2/6
A5	*Wolbachia sp.*	NR_074437	99%(537/542)	Alphaproteobacteria; Rickettsiales	5/6
A6	*Acetobacter tropicalis*	AB681066	99%(542/544)	Alphaproteobacteria; Rhodospirillales	3/6
A7	*Acetobacter persicus*	AB665071	99%(511/512)	Alphaproteobacteria; Rhodospirillales	2/6
B1	*Gluconobacter albidus*	AB178412	100%(507/507)	Alphaproteobacteria; Rhodospirillales	3/10
B2	*Acinetobacter sp.*	HM045831	100%(531/531)	Gammaproteobacteria; Pseudomonadales	4/10
B3	*Chitinophaga sp.*	GQ369124	92%(487/532)	Bacteroidetes; Sphingobacteriales	7/10
B4	*Acetobacter cibinongensis*	JN004206	99%(514/516)	Alphaproteobacteria; Rhodospirillales	1/10
B5	*Gluconobacter albidus*	AB178412	100%(507/507)	Alphaproteobacteria; Rhodospirillales	3/10
B6	*Acetobacter tropicalis*	JF930137	100%(516/516)	Alphaproteobacteria; Rhodospirillales	2/10
B7	*Lampropedia hyalina*	AY291121	98%(526/536)	Betaproteobacteria; Burkholderiales	6/10
B8	*Wolbachia pipientis*	AJ306307	514/519 (99%)	Alphaproteobacteria; Rickettsiales	7/10
B9	*Ochrobactrum sp.*	FJ233847	517/517(100%)	Alphaproteobacteria; Rhizobiales	5/10
B10	*Acetobacter pasteurianus*	AB608081	520/531 (98%)	Alphaproteobacteria; Rhodospirillales	7/10
B11	*Acetobacter aceti*	AJ419840	508/509 (99%)	Alphaproteobacteria; Rhodospirillales	5/10
B12	*Acetobacter senegalensis*	HQ711345	524/535 (98%)	Alphaproteobacteria; Rhodospirillales	6/10
C1	*Ochrobactrum sp.*	JN571744	99%(509/510)	Alphaproteobacteria; Rhizobiales	4/16
C2	*Ochrobactrum sp.*	KF737384	99%(499/505)	Alphaproteobacteria; Rhizobiales	4/16
C3	*Acetobacter sp.*	AB665071	99%(476/480)	Alphaproteobacteria; Rhodospirillales	4/16
C4	*Acetobacter sp.*	AB665071	99%(503/505)	Alphaproteobacteria; Rhodospirillales	5/16

Band	Most related species	GenBank Accession no.	% nt similarity (identical bp/total bp[a]	Classification	No. of positive indiv./ total[b]
C5	*Lactobacillus sp.*	JX826566	99%(512/518)	Firmicutes; Lactobacillales	1/16
C6	*Lactobacillus plantarum*	KF225698	98%(487/496)	Firmicutes; Lactobacillales	1/16
C7	*Comamonas sp.*	KC853135	99%(526/529)	Proteobacteria; Burkholderiales	5/16
C8	*Comamonas sp.*	KC853135	98%(515/528)	Proteobacteria; Burkholderiales	5/16
C9	*Acetobacter sp.*	AB665071	99%(491/493)	Alphaproteobacteria; Rhodospirillales	4/16
C10	*Acetobacter sp.*	AB665071	99%(486/489)	Alphaproteobacteria; Rhodospirillales	4/16
C11	*Lactobacillus plantarum*	HE646352	96%(493/512)	Firmicutes; Lactobacillales	2/16
C12	*Lactobacillus plantarum*	KF225698	99%(509/516)	Firmicutes; Lactobacillales	3/16
C13	*Lactococcus lactis*	KC293821	100%(464/464)	Firmicutes; Lactobacillales	2/16
C14	*Comamonas sp.*	KC853135	100%(517/517)	Proteobacteria; Burkholderiales	9/16
C15	*Ochrobactrum sp.*	JN853243	93%(350/376)	Proteobacteria; Rhizobiales	1/16
C16	*Acetobacter sp.*	AB680014	99%(452/454)	Alphaproteobacteria; Rhodospirillales	15/16
C17	*Acetobacter sp*	AB680014	99%(508/510)	Alphaproteobacteria; Rhodospirillales	13/16
C18	*Acetobacter sp.*	AB665082	99%(470/477)	Alphaproteobacteria; Rhodospirillales	2/16
C19	*Tsukamurella sp.*	KF499506	100%(438/438)	Actinobacteria; Actinomycetales	1/16
C20	*Streptomyces sp.*	KF889277	100%(429/429)	Actinobacteria; Actinomycetales	1/16
C21	*Propionibacterium sp.*	KF479576	99%(432/433)	Actinobacteria; Actinomycetales	1/16
C22	*Streptomyces sp.*	EU551673	99%(507/509)	Actinobacteria; Actinomycetales	1/16
C23	*Tsukamurella tyrosinosolvens*	AB478957	97%(528/544)	Actinobacteria; Actinomycetales	1/16
C24	*Streptomyces sp.*	HM153793	99%(506/507)	Actinobacteria; Actinomycetales	1/16

Table 1: Identification of microorganisms associated to D. suzukii according to DGGE profiles in Fig. 1.
a nt, nucleotide.
b Number of individuals positive for the presence of the specific band in the DGGE analysis compared to the total number of individuals analyzed.

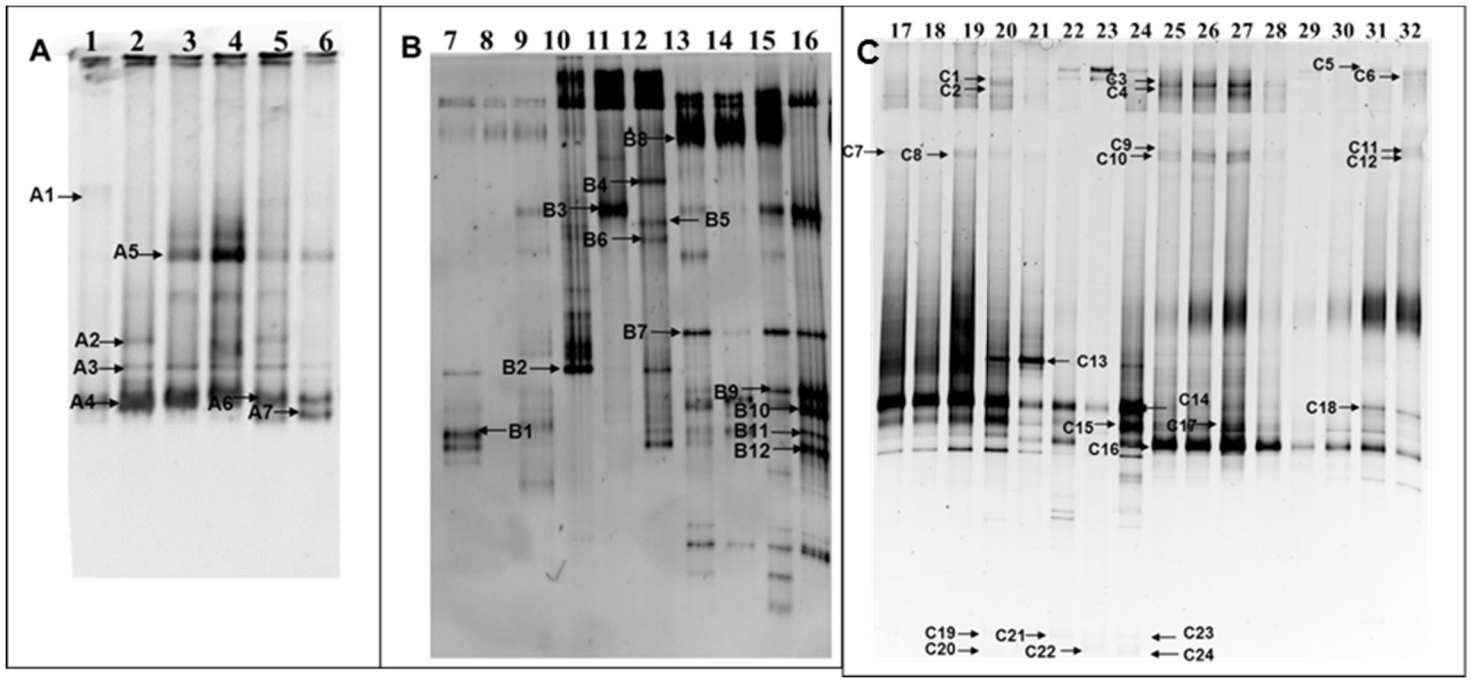


Figure 1: Bacterial diversity associated with D. suzukii by DGGEITS. DGGE profiles, in 7% polyacrylamide gels with 40 to 60% denaturation gradient, of partial 16S rRNA bacterial genes amplified from DNA extracted from whole insects reared on fru (pane(panels A and B) or artificial diet (panel C). Numbers above the lanes refer to tested individuas. Specimens on fruit: 1-5 larvae; 6, pupa; 7-16 adults; specimens from artificial diet: 17-24 adults; 25-28 pupae; 29-32 larvae. Bands marked with arrow were sequenced; data referred to s equences are given in Table 1.

Finally, bands B10 and B12 were 98% similar to the 16S rRNA gene of *A. pasteurianus* and *A. senegalensis*, respectively, with a detection frequency of 70 and 60%, respectively. Other sequences matched with *Lampropedia hyaline* (98%), *Acinetobacter* sp. (100%), *Chitinophaga* sp. (92%) and *Ochrobactrum* sp. (100%).

In both larval and adult specimens reared on fruits, a remarkable presence of *Wolbachia pipientis* was documented, particularly in 5 out of 6 larvae and 7 out of 10 adults (bands A5 and B8 for larvae and adults, respectively, Fig. 1 and Tab. 1). Sequences had a 99% identity with *Wolbachia* spp.

Larvae and pupae reared on the artificial diet showed a huge presence of AAB sequences clustering to *Acetobacter* genus (bands C3, C4, C9, C10, C16, C17 and C18 with values of 99% identity), together with sequences related to *Lactobacillus* genus (bands C5, C6, C11 and C12; 96-100% identity). In the case of the adults reared on artificial diet, sequences related to *Ochrobactrum*, *Comomonas*, *Lactobacillus*, *Tsukamurella*, *Streptomyces* and *Propionibacterium* were retrieved (Fig. 1C). No sequences were found to cluster with *Wolbachia*; however, few bands in the upper part of Fig. 1C did.

To have a wide view of the bacterial community associated to the samples, 16S rRNA barcoding, by amplifying the variable regions V1-V3 of the bacterial 16S rRNA gene, was performed on 14 specimens, including both individuals reared on fruits or artificial diet and specimens from different developmental stages (larvae, pupa and adults). Intra-specimen variability among the samples was reported (Tab. 2; Fig. 2).

Sample ID	Age	Rearing environ.	Barcode sequence	N	OTUs	Chao1	H'	J
Ds159	larva	fruit	ACACGACT	12851	68	92.43	1.148	0.272
Ds164	larva	fruit	ACACGAGA	15587	132	148.24	2.151	0.440
Ds165	larva	fruit	ACACGTCA	9835	170	186.73	3.137	0.611
Ds167	pupa	fruit	ACAGAGAC	6638	153	200.57	3.290	0.654
DS41	adult	fruit	AGACGACA	9324	96	109.57	2.651	0.581
DS54	adult	fruit	AAGGTACG	19831	154	166.55	2.848	0.565
DS55	adult	fruit	AGACGAGT	6537	117	136.09	3.014	0.633
DSM	adult	fruit	AAGGCGTA	6189	53	59.0	1.761	0.444
LP1	larva	diet	ACACGTGT	15338	71	122.0	1.975	0.463
LP3	larva	diet	ACACTCTC	20032	84	85.25	1.609	0.363
PP2	pupa	diet	ACAGAGTG	17180	69	74.60	0.907	0.214
FP1	adult	diet	ACACTGTG	7298	89	104.83	2.103	0.468
FP3	adult	diet	ACACTGAC	10321	113	120.58	2.738	0.579
MP3	adult	diet	ACAGACAG	21895	40	41.0	1.162	0.315

Table 2: Alpha diversity metrics of 16S barcoding of 14 samples, at 97% identity level. N: number of reads for each sample; OTUs: number of OTUs for each sample; Chao-1: Chao-1 values for each sample, H': Shannon H diversity for each sample; J: Pielou's evenness indices for each sample.

Using the Shannon index to measure α-diversity, it was possible to visualize that all samples reached a plateau; rarefaction curves showed the saturation of the microbial diversity associated to the samples (data not shown). We obtained in total 178,856 reads after quality evaluation and chimera removal. Singletons and less significative sequences (below the 0.1% threshold) were also deleted from the analysis. Besides the number of OTUs detected for each sample, in Table 2 are reported the alpha diversity metrics of 16S barcoding of the 14 samples, at 97% identity level, i.e. Chao-1, Shannon H diversity and Pielou's J evenness indices. On the other hand, the β-diversity related to the samples was evaluated through principal coordinates analysis (PCoA) on the phylogenetic β-diversity matrix obtained by UniFrac (Fig. 2a). The components explain 49.67% of the variation (Fig. 2a). The analysis showed that three clusters could be obtained; the first one, quite separated from the other two, contained the two larvae and the sole pupa reared on the artificial diet, the second one included the adults reared on the artificial diet and the other one was constituted by the specimens reared on fruits (Fig. 2a). By the use of this analysis, adults reared on the artificial diet clustered closer to the specimens reared on fruits than the larvae or pupa reared on the artificial diet. Looking to the sample composition, results showed a high frequency of sequences belonging to the Rhodospirillales order (average percentage of reads was 24.8 per sample), to which AAB belong. Interestingly reads clustering to Rickettsiales, to which *Wolbachia* belongs, were detected only in flies reared on fruits, with an average of 27.5% among the individuals, confirming results obtained by DGGE-PCR (Fig. 2b).

In particular, DSM and DS54 showed massive presence of Rickettsiales sequences with percentages of 95.3% and 59.4% out of the total number of reads, respectively. It is noteworthy to underline that all the reads, clustering to the Rickettsiales order, clustered at the genus level with *Wolbachia*. Reads clustering within Rhodospirillales order were present in all the specimens with different loads: the major abundant presences of Rhodospirillales reads were detected in DS41, a specimen reared on fruits, and PP2, a specimen reared on the artificial diet, with percentages of 85.2% and 85.4% out of the total number of sequences for each sample, respectively. Moreover, members of other orders such as Enterobacteriales, Xanthomonadales, Lactobacillales, Rhizobiales, Burkholderiales and Sphingobacteriales constituted the most significative fractions of reads out of the total ones (Fig. 2b).

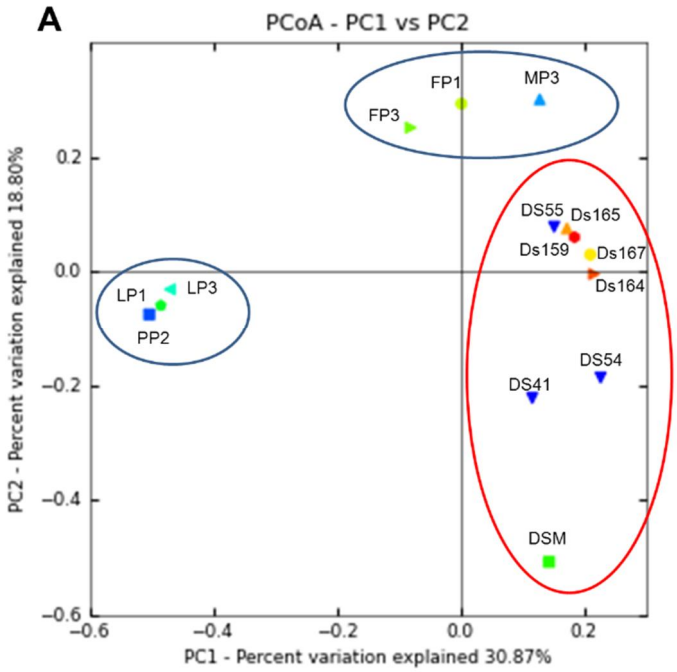
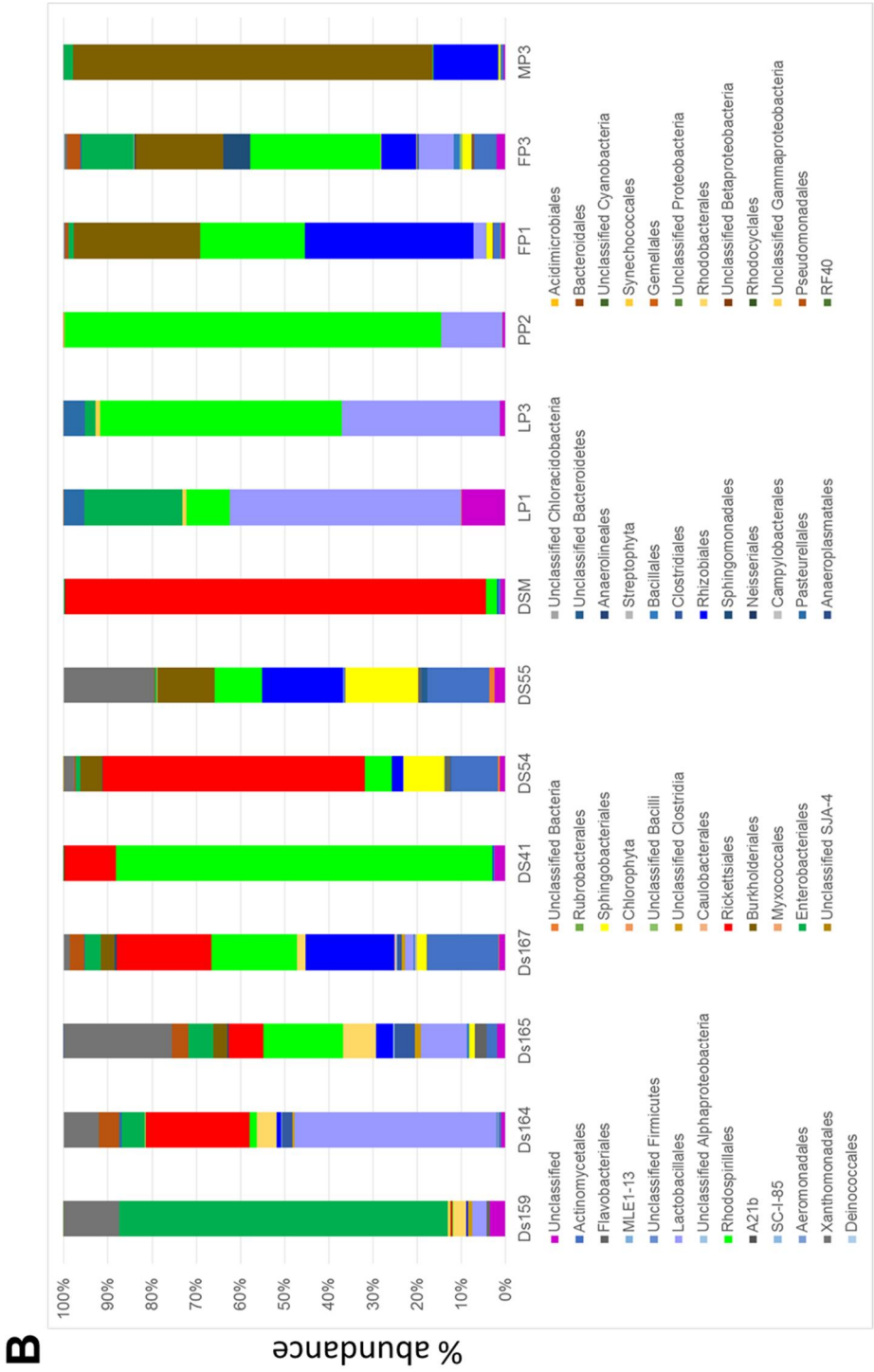


Figure 2: Bacterial diversity associated with *D. suzukii* by 16S rRNA barcoding. (A) Principal coordinate analysis (PCoA) on the phylogenetic β-diversity matrix on *D. suzukii* samples. With red circle are clustered fruit-fed individuals, while with blue circles specimens on the artificial diet. (B) (next page) 16S RNA barcoding describing microorganisms, at order level, associated with *D. suzukii*. Names, under histograms, refers to fly specimens submitted to DNA extraction and partial 16S rRNA bacterial genes amplification. In columns, the relative abundances in percentages of the orders identified.


B
% abundance
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Ds159
Ds164
Ds165
Ds167
DS41
DS54
DS55
DSM
LP1
LP3
PP2
FP1
FP3
MP3
Unclassified
Actinomycetales
Flavobacteriales
MLE1-13
Unclassified Firmicutes
Lactobacillales
Unclassified Alphaproteobacteria
Rhodospirillales
A21b
SC-I-85
Aeromonadales
Xanthomonadales
Deinococcales
Unclassified Bacteria
Rubrobacterales
Sphingobacteriales
Chlorophyta
Unclassified Bacilli
Unclassified Clostridia
Caulobacterales
Rickettsiales
Burkholderiales
Myxococcales
Enterobacteriales
Unclassified SJA-4
Unclassified Chloracidobacteria
Unclassified Bacteroidetes
Anaerolineales
Streptophyta
Bacillales
Clostridiales
Rhizobiales
Sphingomonadales
Neisseriales
Campylobacterales
Pasteurellales
Anaeroplasmatales
Acidimicrobiales
Bacteroidales
Unclassified Cyanobacteria
Synechococcales
Gemellales
Unclassified Proteobacteria
Rhodobacterales
Unclassified Betaproteobacteria
Rhodocyclales
Unclassified Gammaproteobacteria
Pseudomonadales
RF40

Prevalence and localization of AAB.

To investigate the prevalence of AAB, at the genus level, in the analysed insects reared on fruits, infection rates of the genera *Gluconobacter*, *Gluconacetobacter* and *Acetobacter* were evaluated in adult flies (Fig. 3). The frequency detected for the genera *Gluconobacter* and *Acetobacter* did not show significant differences, likewise *Gluconobacter* and *Gluconacetobacter* genera that did not present significantly different values ($p<0.05$). Prevalence indicated *Gluconobacter* and *Gluconacetobacter* as the most prevalent genera among the samples with values of 21 and 31%, respectively.

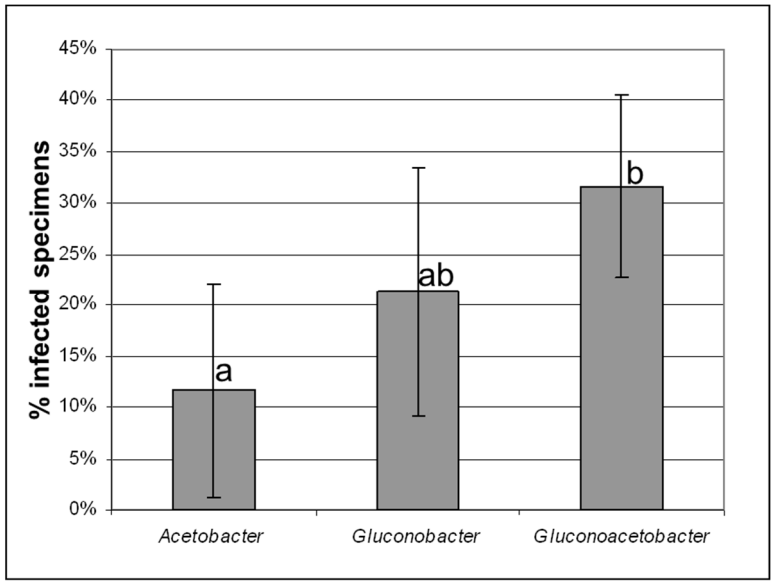


Figure 1: AAB infection rates in adult D. suzukii. columns indicate the percentage of infected individuals within th most common genera found in mass reared flies, i. e. Acetobacter, Gluconbacter and Gluconacetobacter. Bars represent the standard error. Different letters indicate significantly different values. (ANOVA, P<0,05).

With the aim to localize AAB, fluorescent *in situ* hybridization (FISH) experiments were carried out using the AAB-specific probe, AAB455, on the insect dissected organs, showing positive signals for proventriculus and gut (Fig. 4). In particular, a strong signal was detected at the level of the proventriculus epithelium, as observable by merging the interferential contrast picture (Fig. 4c) with the FISH micrograph (Fig. 4b) of a midgut section near to the proventriculus. Magnification in fig. 4d allowed to visualise fluorescent AAB microcolonies adhering to the peritrophic membrane. Since *Gluconobacter* was one of the main genera in prevalence assays, the distribution of this genus was observed in the midgut of *D. suzukii*. *Gluconobacter* specific signal was detected in the gut (Fig. 4g) providing the evidence of the distribution of this genus in the inner side of the intestinal

lumen. Fig. 4f showed the Texas red-signal for *Eubacteria*, allowing to observe the distribution of *Gluconobacter* in relation to the dispersal of the *Eubacteria* in the same portion of the organ (Fig. 4e-h). *Gluconobacter* is localized in the intestinal tract probably surrounded by other acetic acid bacteria. Attempts to design probes specific for *Gluconacetobacter* or *Acetobacter* genera failed. AAB isolation.

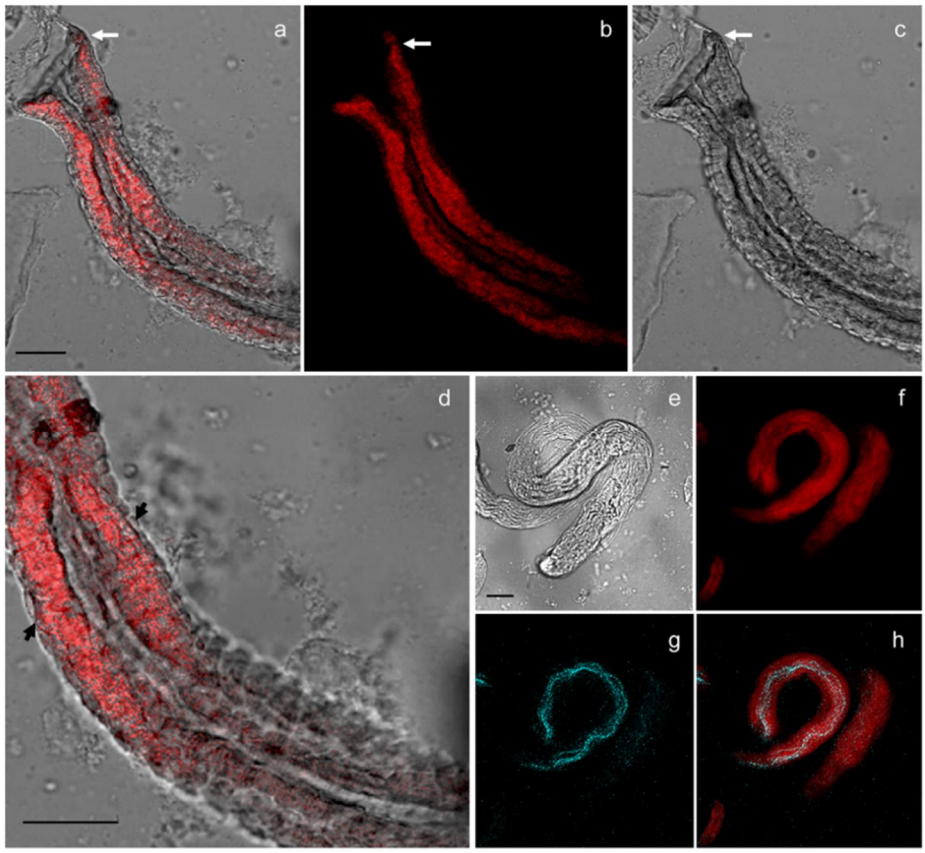


Figure 2: AAB localization in the gut of D. suzukii. (a-d) FISH of the insect gut after hybridization with the Texas red-labelled probe AAB455, matching AAB. (a) Superposition of the interferential contrast picture (c) and the FISH image (b) of a midgut part close to the proventriculus (indicated by white arrows). (d) Magnification of the image in b. The massive presence of AAB adherent to the peritrophic membrane (indicated by black arrows) is observed. (e-h) FISH of D. suzukii midgut with the Texas red-labelled universal eubacterial probe Eub338 (f) and the Cy5-labelled probe specific for Gluconobacter, Go615 and Go618 (g). (e) Intestine portion pictured by interferential contrast. (h) Superposition of hybridization signals of Eubacteria (red) and Gluconobacter (blue). Bars = 50 μm.

Isolation trials of AAB were performed with different kinds of enrichment and selective media (De Ley and Frateur, 1974, Reasoner *et al.,* 1979, Yamada *et al.*, 1999; Yamada *et al.*, 2000; Kadere, 2008). Since the condition of fruit-rearing was the most close one to the natural habit of the insect, we directed our attention manly on isolation trials from specimens reared on fruits; specimens reared on artificial diet were also included in the analysis in a lower extent. After purification, 234 isolates were obtained and subjected to de-replication analysis, clustering them in ITS fingerprinting profiles. 16S rRNA gene sequencing of the candidates chosen for each ITS fingerprinting profile showed a prevalence of bacteria belonging to *Acetobacter*, *Gluconobacter* and *Gluconacetobacter* genera, which constitute the 16.67%, 7.7% and 52.99%, respectively, of the total number of bacteria in the collection (Tab. 3). 22.7% of isolates did not belong to Acetobacteraceae family. Seven *Acetobacter* species were isolated, i.e. *Acetobacter tropicalis*, *A. cibinongensis*, *A. persicus*, *A. peroxydans*, *A. indonesiensis*, *A. orientalis*, *A. orleanensis*, with *A. persicus* being the most abundant (Tab. 3). *Gluconobacter* genus was found to be present with three species, i.e. *G. kondonii*, *G. oxydans*, and *G. kanchanaburiensis*. The single isolate of *G. kondonii* was collected from an adult fly fed on fruits, while *G. kanchanaburiensis* species were isolated from specimens reared on artificial diet.

Twelve isolates, collected from the fruit-fed adults, showed high sequence similarity with *G. oxydans* as closest described species, with isolates belonging to different ITS profiles (Tab. 3). One hundred and twenty-three isolates were sequenced and assigned to the genus *Gluconacetobacter*. In particular, 66 *Gluconacetobacter hansenii* (41 coming from females and 25 from fruit–fed males), 14 *Ga. saccharivorans*, and 8 *Ga. intermedius* isolates were isolated from fruit–fed *Drosophila*. The 8 isolates of *Ga. intermedius* derived from the same male, DSM1, but from different media, specifically the enrichment medium I and the basal medium. Three pupae and one larva revealed to harbor *Ga. liquefaciens,* when smashed and plated on enrichment medium I. Twenty-six *Gluconacetobacter* sp. could not be discriminated with the performed analysis, due to the phylogenetic proximity of the species analyzed.

Isolates	No. of isolates	ITS	Larval fly	Pupal fly	AP fly	AF fly
Micrococcus sp.	5	34, 35, 38	0	0	0	5
Microbacterium foliorum	2	39	0	0	0	2
Corynebacterium sp.	1		0	0	0	1
Sphingobacterium multivorum	1		0	0	1	0
Streptococcus salivarius	1	48	0	0	1	0

Isolates	No. of isolates	ITS	Larval fly	Pupal fly	AP fly	AF fly
Staphylococcus sp.	13	37	0	0	1	12
Paenibacillus sp.	2		0	0	0	2
Lactococcus lactis	1	60	0	0	0	1
Lactobacillus plantarum	1		0	1	0	0
Lactobacillus brevis	2		0	1	1	0
Acetobacter tropicalis	1	46	0	0	0	1
Acetobacter orleanensis/malorum/cerevisiae	5	47, 58	0	1	0	4
Acetobacter peroxydans	1		0	0	0	1
Acetobacter indonesiensis	10	49, 50, 55, 59	0	1	1	8
Acetobacter persicus	20	51	2	2	6	10
Acetobacter orientalis	1	54	0	0	0	1
Acetobacter cibinongensis	2	53	0	0	0	2
Gluconacetobacter sp.	26	8, 15, 16, 18, 23, 24, 32, 33, 40, 44	0	0	0	26
Gluconacetobacter hansenii	66	1, 2, 6, 10, 11, 12, 42, 43	0	0	0	66
Gluconacetobacter liquefaciens	4		1	3	0	0
Gluconacetobacter europaeus	3	22	0	0	0	3
Gluconacetobacter saccharivorans	14	4, 19, 41	0	0	0	14
Gluconacetobacter intermedius	8	14, 16, 17, 20, 21, 25	0	0	0	8
Gluconacetobacter nataicola	2	7, 31	0	0	0	2
Gluconobacter kondonii	1	52	0	0	0	1
Gluconobacter oxydans	12	5, 9, 26, 27, 28, 29, 45	0	0	0	12
Gluconobacter kanchanaburiensis	5		3	1	1	0

Isolates	No. of isolates	ITS	Larval fly	Pupal fly	AP fly	AF fly
Rhodobacter sp.	1		0	0	0	1
Pseudomonas geniculata	4		0	0	1	3
Serratia sp.	12		4	7	0	1
Enterobacter sp.	7		1	0	3	3
Total	234		11	17	16	190

Table 3: Identification of cultivable microorganisms associated to *D. suzukii*. AP: Adults fed with artificial diet; AF: Adults fed with fruit diet.

During the isolation procedure, few isolates belonging to the phylum Firmicutes were obtained, i.e. *Lactobacillus plantarum, Lactobacillus brevis*, *Lactococcus* sp., *Paenibacillus* sp., *Streptococcus salivarius*, and *Staphylococcus* sp. Several isolates of *Sphingobacterium multivorum*, *Corynebacterium* sp., *Micrococcus* sp. and *Microbacterium foliorum* were also found, as well as some representatives of Proteobacteria phylum, i.e. *Rhodobacter* sp., *Pseudomonas geniculata*, *Enterobacter* sp., and *Serratia* sp.

Colonization of *D. suzukii* with G. oxydans DSF1C.9A(Gfp), A. tropicalis BYea.1.23(Gfp) and A. indonesiensis BTa1.1.44(Gfp).

Isolates *G. oxydans* DSF1C.9A, *A. tropicalis* BYea.1.23 and *A. indonesiensis* BTa1.1.44 were selected for inserting a plasmid carrying the Gfp cassette in order to label the bacteria with a fluorescent protein. Plasmid stability into the three different AAB strains was evaluated and data showed that *G. oxydans* DSF1C.9A showed that it was able to retain the plasmid with an high percentage (73.125%, data not shown), while plasmids inserted in *A. tropicalis* BYea.1.23 and *A. indonesiensis* BTa1.1.44 were not stably inherited (data not shown). Colonization trials of adult flies were thus performed. Because of the quite rapid loss of the plasmid, the colonization experiments were performed under antibiotic selection by administering 100 μg ml^{-1} kanamycin in the insect food. After the administration of the Gfp-labelled strains, *Drosophila* specimens were dissected and the gut analyzed by CLSM. Gfp-labelled strains were able to massively recolonize the fly foregut and midgut (Fig. 5-6). In the case of Gfp-labelled *Gluconobacter*, the crop, proventriculus and first part of midgut were successfully colonized by the labelled bacteria (see the magnification views of the crop and the proventriculus in Fig. 5b and 5c). It is noteworthy that the Gfp-labelled cells are clearly restricted to the epithelium side of the proventriculus, embedded in a matrix, probably of polysaccharidic nature close to the peritrophic membrane (Fig. 5c). Likely, the first tract of the intestine, also the central part represented by midgut showed massive colonization pattern (Fig. 5d-e).

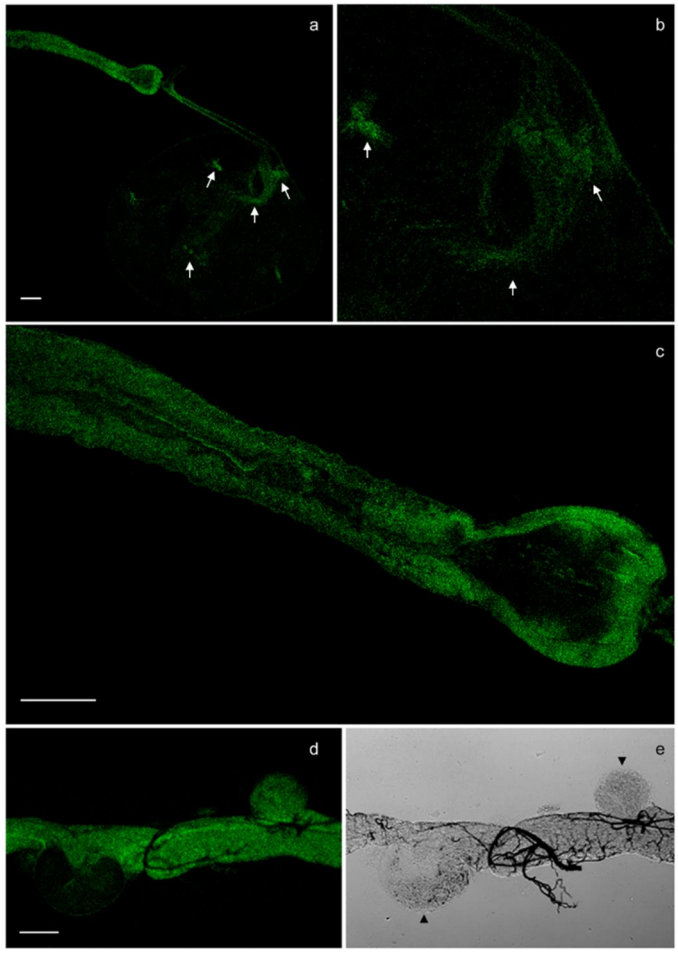


Figure 3**:** Colonization of *D. suzukii* foregut and midgut by *G. oxidans* DSF1C.9A1 documented by confocal laser scanning microscopy. (a-c) Intestine portion including the crop, the proventriculus and the first midgut part. (b, c) Magnified views of the crop (b) and the proventriculus (c) showed in a. Masses of fluorescent cells are observed in the crop (arrows); when the marked strain reaches the proventriculus it colonizes the gut part close to peritrophic membrane. (d-e) Interferential contrast (d) and confocal laser scanning (e) pictures of the midgut of *D. suzukii* massively colonized by the *G. oxidans* strain labelled with Gfp. Small hernias (arrowhead) are shown. In some cases, the flow of the gelatinous matrix entering the hernia is composed by fluorescent cells. Bars = 50 μm.

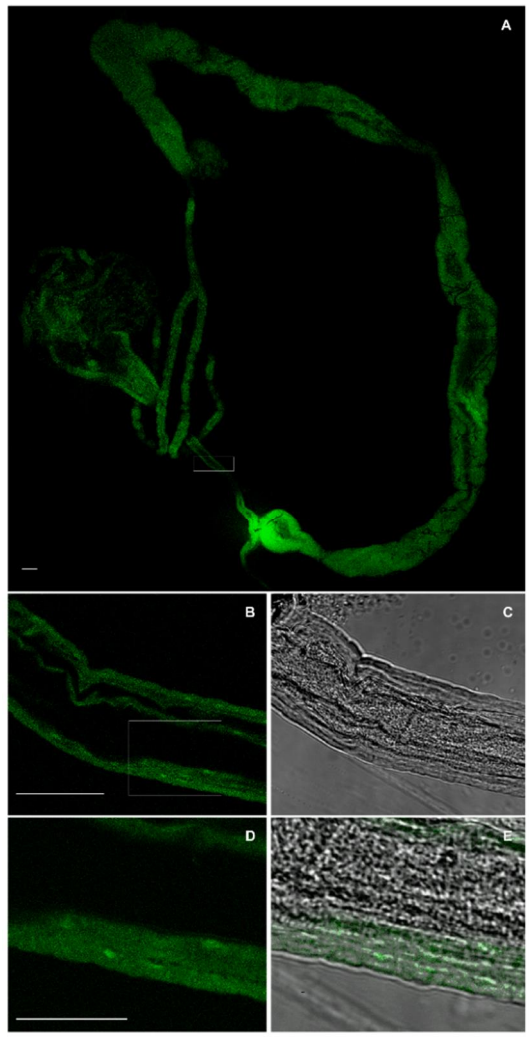


Figure 4: Confocal laser scanning micrographs showing the colonization of D. suzukii foregut and midgut by A. tropicalis BYea.1.23 (A) Reconstructed image of an intestine obtained by overlapping successive sections. The gut portion includes crop, proventriculus, midgut, and Malpighian tubules. Fluorescent A. tropicalis BYea.1.23 cells are visible in the whole tract; the symbiont is especially located close to the gut walls and in the peritrophic membrane. Bar = 50 μm. (B, C) Magnification of the framed crop part in A pictured by CLSM (B) and interferential contrast (C). Masses of fluorescent bacteria are evident in the crop. Bar = 50 μm. (D-E) Interferential contrast (D) and confocal laser scanning (E) magnifications of the framed crop part in B, showing Gfp-marked *A. tropicalis* adhering to the crop wall. Bar = 25 μm.

Since small hernias are visible by interferential contrast (indicated by black arrowheads in Fig. 5e) and since they result Gfp-positive with CLSM, the gelatinous matrix forming the hernias appears like a gel in which the bacterial cells are completely sunk. Black filaments around the organ are the Malpighian tubules, more evident in the confocal laser scanning microscopy picture (Fig. 5d). Also in the case of *A. tropicalis* BYea.1.23, the colonization of the foregut and midgut was successfully performed (Fig. 6A). The labelled bacteria are present in the whole tract and especially they are located close to the gut walls and in the peritrophic membrane (Fig. 6B-E). Images related to *A. indonesiensis* BTa1.1.44(Gfp) were similar and thus not included here.

Discussion

The highly invasive vinegar fly *Drosophila suzukii* is a poliphagous species endemic to the South East Asia and now it is emerging as a dangerous pest in many Mediterranean and North America's countries . The study of its biology, ecology and distribution is in progress, but to develop future management solutions many gaps should be filled. One of these is the characterization of insect microbiome, with particular attention to the analysis of the acetic acid bacteria, important symbionts living in association with this pest. Indeed, in other insect models, these alphaproteobacteria have been described to play important biological roles (15–17). In this work, culture-independent techniques, DGGE-PCR and 16S rRNA pyrotag in particular, gave insights on the overall bacterial community composition of *D. suzukii* and, notably, provided robust evidence of the stable association of AAB, underlining their constant presence in the samples under investigation. Results showed the presence of AAB belonging to the *Acetobacter*, *Gluconacetobacter* and *Gluconobacter* genera (Fig. 1, Fig. 2).

AAB are a diverse class of organisms, widespread in nature, as a large number of AAB strains have been isolated from a variety of sources (41). AAB are recognized by their unique ability to oxidize ethanol to acetic acid in neutral and acidic (pH 4±5) media and to produce polysaccharides that are exploited at industrial level (42). Besides *Drosophila* flies and mosquitoes, AAB have been reported in association with bees, olive fruit flies, parasitic wasps and mealybugs (34, 43, 44). For example, *Acetobacter tropicalis*, whose presence was also recorded in *D. suzukii* by the present study, was previously described in association with the olive fruit fly *Bactrocera olae*, with which it establishes a strict association (34). The appearance and behaviour of this bacterium were similar to the ones showed by the Gfp-labelled strains used in the actual work: it was indeed observed in contact with the gut epithelium of the insect, entrapped in the polisaccharidic matrix. This peculiar localization in the insect body was also documented in *Asaia* (40) and it may suggest the importance of the AAB for the insect metabolism and gut functions' maintenance.

16S rRNA barcoding allowed to discriminate three clusters among the samples (Fig. 2a); the first principal component (which explains 30.87% of the variance) segregates the microbiota of two groups of *D. suzukii*, the adults reared on the fruit and the ones on the artificial diet from the larvae and pupa

reared on the artificial diet, while the second component allows to discriminate the adults reared on the artificial diet from the fruit-fed ones (Fig 2a).

In both results of cultivation-independent techniques, DGGE-PCR and pyrotag, *Wolbachia* presence was massively recorded in insects reared on fruit. *Wolbachia* is an intracellular reproductive manipulator already described for several insect models, including different *Drosophila* species (45–48). Its finding only in samples reared on fruit and not in samples reared on the artificial diet could be explained by the presence of inhibitory compounds against *Wolbachia* in the artificial diet (Fig. 1, Fig. 2).

However, even if little is known about *Drosophila suzukii* microbiota (49), numerous studies have now been conducted in order to assess the microbial community residing in *Drosophila melenogaster* (10, 11, 13, 19, 50–55), both in the gut and in the whole body. These studies underline the simple bacterial communities in association with *Drosophila,* predominantly made up of Firmicutes phylum, represented by the families Lactobacillaceae and Enterococcaceae, and alpha and gamma classes of Proteobacteria, represented by Acetobacteraceae and Enterobacteriaceae families. Cox and Gilmore (19), who performed the analysis of the bacterial community of wild and laboratory-reared *D. melanogaster* specimens, revealing the predominant presence of *Acetobacter* genus, and consequent identification of *A. aceti*, *A. cerevisiae*, *A. pasteurianus*, *A. pomorum*, *Gluconobacter* and *Gluconacetobacter* species. A recent work showed that the differences in the diversity and dominance of bacterial species associated to several *Drosophila* species have a relationship with food source (10). Moreover, Chandler and coworkers (11) observed that all individuals of different *Drosophila* species reared on different food sources obtained a similar microbiome when moved to the same medium. Furthermore, Wong *et al.* focused on the microbial composition at different life cycle stages (10). Chandler and colleagues (49) characterized the microbiota of adult and larval *D. suzukii* collected from cherries, showing a high prevalence of the gamma-proteobacterium *Tatumella*. *Gluconobacter* and *Acetobacter* were found at lower frequency than *Tatumella*. In our case, high prevalence of Rhodospirillales reads was reported with an average of 24.8%, abundance percentages varying from 0.02% to 85.42% (Fig 2). No *Tatumella* sequences were detected among gamma-proteobacteria.

AAB have been shown to be involved in the relationship between the gut microbiota and host health, underlining the importance of the correct microbial balance for the host well-being (14). The normal flora in the fly gut is sufficient to suppress the growth of pathogenic bacteria, and to regulate host immune response (13), but also to promote the insulin pathway (15). Consequently, to gain knowledge of the detailed localization of this key group, FISH was performed with AAB-specific probe (Fig.4). The localization of the AAB probe in the wall side of the midgut portion near to the proventriculus showed their distribution, not in the lumen, but in the peripheral side of the organ suggested a role of protective layer between the lumen and the surface epithelium, able to prevent the passage of bacteria. This was already proposed by Kounatidis and colleagues (34), when observing

a similar behaviour in *Bactrocera oleae* gut, colonized by *A. tropicalis*. Several studies reported that another AAB, *Asaia,* is able to colonize the gut and the reproductive organs of different insects, such as the leafhopper *Scaphoideus titanus*, and the mosquitoes *Anopheles stephensi*, *An. gambiae* and *Aedes aegypti* (21, 31, 40, 56). Together with the hypothesis above reported, this supports the evidence that the insect digestive system is a favourable habitat for AAB, in which they establish a strict connection with the epithelial cells (18).

Recolonization data strongly supported FISH analysis: several isolates belonging to *Gluconacetobacter*, *Gluconobacter* and *Acetobacter* genera were targeted with a plasmid carrying the GFP (Fig.5-6). Several efforts were also made to achieve the transformation of *Gluconacetobacter* isolates, but no successful results were obtained. Further experiments will be planned to improve the transformation protocol. Strains *G. oxydans* DSF1C.9A(GFP), *A. tropicalis* BYea.1.23(GFP) and *A. indonesiensis* BTa1.1.44(GFP) were obtained and their dispersal in the fly body was followed by fluorescent microscopy on re-colonized specimens.

The actual control of *D. suzukii* is based on insecticides that however are not very effective (4) and the promising control strategies based on interferences with communication still need more research (57). Thus a forward-looking concept like the symbiotic control approach, under investigation in the last years (58), should be taken into account. In the light of the development of future control strategies exploiting the remarkable importance of the Acetobacteraceae family for *D. suzukii*, further experiments have to be performed to assess their distribution pattern in the host compartments, their role and involvement in the host homeostasis and possible exploitations of their properties for the host control.

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Chapter 4

Effects of the diet on the microbiota of the Red Palm Weevil (Coleoptera: Dryophthoridae)

Abstract

Rhynchophorus ferrugineus, also known as the red palm weevil, is regarded as the major pest of palm trees. Although studies of the microbiota associated with this species have been performed in recent years, little attention has been dedicated to the influence of the diet in shaping the host bacterial community. Here, we investigated the influence of food sources (i.e. palm tissues *vs* apple based substrate) on the microbial diversity associated with RPW, which was compared with the microbiota associated with wild individuals of the sister species *Rhynchophorus vulneratus*. The bacterial characterization was performed using a culture independent approach, i.e. the 16S rRNA pyrotag, and a culture dependent approach for a subset of the samples, in order to obtain bacterial isolates from RPW tissues. The bacterial community appeared significantly influenced by diet. Proteobacteria resulted to be the most abundant clade and was present in all the specimens of the three examined weevil groups. Within Proteobacteria, Enterobacteriaceae were identified in all the organs analysed, including hemolymph and reproductive organs. The apple-fed RPWs and the wild *R. vulneratus* showed a second dominant taxon within Firmicutes that was scarcely present in the microbiota associated with palm-fed RPWs. A comparative analysis on the bacteria associated with the palm tissues highlighted that 12 bacterial genera out of the 13 identified in the plant tissues were also present in weevils, thus indicating that palm tissues may present a source for bacterial acquisition.

Introduction

The Red Palm Weevil (hereafter RPW), *Rhynchophorus ferrugineus* Olivier (Coleoptera; Dryophthoridae), is regarded today as the major pest of palm, attacking over 20 palm species belonging to 16 different genera worldwide (1). RPW is native to South Eastern Asia, but due to the international exchange of infected plant material, during the last two decades it has spread to the Middle East, Africa and the Mediterranean. In 1992 RPW was first detected in Egypt (2) then spread through the Northern Mediterranean Basin (3–5), where it attacked the highly sensitive ornamental palm *Phoenix canariensis* (6). More recently RPW has been detected in Australia, China, Japan and the Caribbean (7–12). Globally, the pest has a wide geographical distribution in diverse agro-climates and an extensive host range in Oceania, Asia, Africa and Europe (1). The RPW life cycle, from egg to new-born adult, occurs in the palm tree trunks

(13) in which the weevil feeds on tissues and sap. Larvae develop inside palm trees resulting in the destruction of palm tissue leading, eventually, to the death and collapse of the tree. The palm tree trunk tissues consist of more than 80% (wt. %, dry basis) of cellulose, hemicellulose and lignin (14). For this reason, they represent a non-easily digestive substrate for most eukaryotes.
The role played by mutualistic bacterial consortia supporting their insect host with essential compounds missing from the diet (i.e. amino acids, vitamins and cofactors), or by contributing to the digestion of the ingested material, is well documented (15–20). In the cases of aphids and cicadellids, these contributions are provided by intracellular bacteria (respectively, *Buchnera aphidicola* and '*Candidatus* Sulcia muelleri'(21–24), while in termites and other insects, essential compounds are provided by complex microbial communities, in some cases including both intracellular bacteria and gut microbiota, e.g. coackroaches (15, 25, 26).
Considering the economic and social impact of RPW, the interest in this pest has significantly increased in recent decades. Most studies have focused on the efficacy of different chemical and bio-control strategies (27, 28). Conversely, little attention was paid to the microbial community associated with RPW, although an intracellular primary endosymbiont has been described in weevils and classified as '*Candidatus* Nardonella' (29–32). Regarding the gut bacterial community associated to RPW, only preliminary studies have been conducted (33–36), in some cases addressed to cultivable bacteria in order to identify potential insect pathogens useful in bio-control strategies (34, 37). Aerobic and facultative anaerobic bacteria (*Bacillus* sp., *Salmonella* sp., *Enterococcus* sp. and *Xanthomonas* sp.) and bacteria able to degrade polysaccharides and sucrose through hydrolase activity (e.g., *Klebsiella pneumoniae* and *Lactococcus lactis*) have been discovered and isolated from the RPW's gut (33, 35). To date, no studies have investigated the existence of stable core-microbiota that may be useful for the development of efficient bio-control strategies (38, 39).
The main aim of the present study is to investigate the influence of the environment (mainly food sources) in shaping the microbial diversity of RPW by *i*) comparing the microbiota of RPW individuals collected on palm tissues *vs* laboratory individuals reared on apple-based substrate; *ii*) comparing the endophytes of palm tissues with the weevil's microbiota and *iii*) evaluating the metabolic potential of the identified microbial consortia from individuals collected on palm tissues *vs* laboratory ones. Moreover, the bacterial cultivable fraction associated to laboratory-reared individuals was estimated by the use of several isolation media.

Materials and Methods

Ethics statement

Rhynchophorus ferrugineus and its sister species *R. vulneratus*, the most damaging insect pest of palms in the world, are not listed in any national or regional law as protected or endangered species. The collection of specimens in Malaysia was made in the private properties of cooperating landowners. The collection of specimens in Italy was not subjected to any restriction,

moreover the specimens sampling occurred in not protected areas and under the surveillance of Servizio Fitosanitario della Regione Sicilia.

Specimens sampling, dissection and DNA extraction

Male and female RPW adults, and host plant tissues were collected on *Phoenix canariensis* in Catania, Italy (Table 1). Among the insects, three individuals (one male and two females) were preserved in absolute ethanol, whereas six additional specimens (three males and three females) were transported to laboratory and maintained alive on diets of apple for four weeks with a natural light-dark cycle (14:10), RH = 65% and temperature = 28°C. Three adults of *Rhynchophorus* sp., firstly identified as *R. ferrugineus*, were collected using specific traps baited with the aggregation pheromone Rhyfer 220 (Intrachem Bio Italia S.p.A.) in Genting Sempah, Selangor, Malaysia (Table 1). All the collected specimens were immediately stored in absolute ethanol, furthermore, considering the size of the specimens 1 ml of absolute ethanol was injected with sterile syringes in each specimen. Before dissection, samples were surface sterilized following the protocol reported in Montagna *et al.* (40). Specimens, after anaesthetisation at -20° C, were dissected aseptically using sterilized scalpels and forceps under a Leica MS5 stereomicroscope. The insect content, including the whole gut, the fat body and reproductive system, was removed and homogenised for the DNA extraction. Palm tissues were dried at room temperature before DNA extraction. DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen), for the animal tissues, and the DNeasy Plant Kit (Qiagen), for the palm tissues, following the manufacturer's instructions in both cases. The final elution was realised in 300 µl of AE buffer and DNA was quantified by spectrophotometry. In 2013 Rugman-Jones *et al.* (41) reported the presence of two *Rhynchophorus* species in Malaysia: *R. ferrugineus* and *R. vulneratus*, which have been in synonymy until the publication of the cited work. Since the identification at species level starting from morphological features is sometimes impossible, we performed molecular identification of the three specimens collected in Malaysia. The following strategy has been adopted: total genomic DNA was extracted from three individuals sampled at Genting Sempah (Malaysia) and used as template to amplify a 5' upstream region of the cytochrome c oxidase subunit I gene (*coxI*) from the mitochondrial genome. The PCR cycle was performed using the Folmer's primers LC01490 and HC02198 (42); complete details of the laboratory procedures are reported in Rector *et al.* (43). The acquired sequences, deposited at the European Nucleotide Archive with accession numbers LN612634-LN612636, were screened for identification by a blast search over the National Center for Biotechnology Information (NCBI) GenBank nucleotide collection using the Mega BLAST procedure (44) available at its website (http://www.ncbi.nlm.nih.gov/blast). Our query sequences were unequivocally assigned to *R. vulneratus* with high sequence identity in all cases (Identity 99-100%, 0 gaps).

Pyrosequencing and data analysis

Pyrotag assays were carried out using bacterial universal primers (27 F mod 5' – AGR GTT TGA TCM TGG CTC AG – 3'; 519 R mod bio 5' – GTN TTA CNG

CGG CKG CTG – 3') targeting the variable regions of 16S rRNA V1-V3 and amplifying a fragment of approximately 400 bp. The amplified 16S rRNA regions contain enough nucleotide variability to be useful in identification of bacterial species (45, 46). Primers were modified by the addition of a GS FLX Titanium Key-Primer AGR GTT TGA TCM TGG CTC AG and a multiplex identifier (MID) sequence specific to each sample. The MID sequences (forward) were reported for the respective weevil specimen in Table 1. PCR reactions and next generation 454 pyrosequencing were performed commercially (MR DNA, Shallowater, TX – U.S.) as described in a previous work (40).

A total of 345973 raw, barcoded amplicons of the V1–V3 region of the 16S rRNA gene, were obtained. The reads were trimmed to remove pyrosequencing adaptors, low quality base calls (<30 Phred score) and size-selected (between 350 and 500 bp) using the QIIME(47) pipeline filtering scripts. The total of 138738 high quality sequence reads that were not flagged as chimeras after screening with *Chimeraslayer* were clustered into operational taxonomic units (OTUs), based on a sequence identity threshold of 97%, using *Uclust* (48); drawing one sequence for each OTU, as representative, and then aligned to Greengenes (http://greengenes.lbl.gov/) using PyNast(49). Sequences representative of each OTU were taxonomically classified by BLASTn-based comparisons to the *Greengenes* and *Silva* databases within QIIME. The resulting set of OTUs was used in diversity analyses (see below). The analyses were carried out using the various scripts of the QIIME pipeline.

The 16S rRNA gene sequences obtained by 454 pyrosequencing assays were deposited in European Nucleotide Archive with accession numbers PRJEB6918.

Diversity and statistical analyses

The diversity indices and the following analysis (exceptions are specified) were estimated using the vegan-package "Community Ecology Package: Ordination, Diversity and Dissimilarities" (50) in the R software package (R Project 3.0.2; http://cran.r-project.org/). The Shannon *H* index(51), Pielou's eveness (52) and total species richness index Chao 1 (53–55) were estimated. The significance of the differences between the analysed statistics (i.e. the number of identified OTUs within each specimen, the Shannon *H* diversity and Pielou's evenness indices) were tested with the non parametric Kruskal-Wallis tests (56) after the assessment of the equality of variances adopting Levene's test (57). In case of comparison between to two groups the Mann-Whitney test was adopted (58). These tests were performed using lawstat-package (59) in the R software package.

The β-diversity matrix was computed using the script *beta_diversity.py* implemented in QIIME (47) and UniFrac (60), which uses as input the OTU table with the amount of the observed 16S rRNA sequences for each OTU for each weevil and the phylogenetic tree constructed using FastTree (61). Since the purpose of our ordination analysis was to reveal significant pattern of variation in the microbiota composition between the different specimens, the

unweighted unifrac metric was adopted (62). The obtained matrix was used as input for the Principal Coordinates Analysis (PCoA (63).
The OTU table, containing the abundance of sequences clustered within each identified OTU for all of the processed insect specimens, was transformed into a presence-absence matrix in order to be processed for further analyses. The similarity between the microbial communities associated with the three groups of specimens (i.e. RPW from wild population, RPW from wild population and reared on apple for four weeks and the sister species *R. vulneratus*) was analysed through a hierarchical cluster analysis. This analysis was conducted using the function *hclust* in R stats-package (R Project 3.0.2; http://cran.r-project.org/). The dissimilarity matrix, used as input for the hierarchical cluster analysis, was estimated by *vegdist* using the Bray and Curtis dissimilarity index (64). In addition, to test the reliance of the obtained results the same analysis was also performed on the dissimilarity matrix obtained adopting Jaccard (65) and Kulczynski indices. In order to test the significant dissimilarity between the microbiotas associated to the groups identified by the clustering analysis (corresponding to RPW_{PALM}, RPW_{APPLE} and R_{VULN}), the dissimilarity matrices were subjected to a nonparametric one-way analysis of similarity (ANOSIM (66)) as in a previous work (67). In order to estimate the change in species composition between the bacterial communities harbored by RPW_{PALM} and by RPW_{APPLE}, two components of β-diversity (i.e. species turnover and nestedness) were estimated with the R package *betapart* (68) using Simpson's dissimilarity index as in Montagna *et al.*(40). To investigate the common OTUs present in the three groups of weevils, an analysis of commonality was performed and visualized through a Venn diagram using the *gplots* package in R.
The impact of ecological traits (i.e., the food source and the temperature at which the samples live) on the bacterial communities associated with insects has been evaluated by correlation with results of the OTU-table Non-Metric Multi-Dimensional Scaling (NMDS(69)). The adopted procedure has been described in a previous study(40). Concerning the diet, two substrates were considered: the palm tissues and apple; while, regarding the temperature two classes have been adopted based on the monthly average temperature at the time of the sampling: 20°C for specimens collected in late October, 2012 in Catania and, > 25°C for specimens maintained in lab and for specimens collected in Malaysia in January, 2012.

Predictive functional profiling

To explore the functional profiles of our bacterial community data set, we used PiCRUST (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States http://picrust.github.com, 3 July 2013 (70)). For the analysis, OTUs were closed-reference picked against the 18 May 2012 Greengenes database using QIIME v 1.6 according to the online protocol. We predicted the bacterial metagenome for each of our samples. The accuracy of metagenome predictions was measured by the Nearest Sequenced Taxon Index (NSTI), with lower values indicating a closer mean relationship (70). Our samples had NSTI values of 0.06 ± 0.02. For comparison, Langille *et al.* (70) found that human-associated samples had the lowest (best) NSTI values (0.03

± 0.2), while communities such as soil had a much higher NSTI value (0.17 ± 0.02). The table with the predicted gene family counts per-samples according to Cluster of Orthologous Groups (71) and identifiers adopted by KEGG Orthology (72) was cleaned removing: *i*) all categories not related to the bacterial physiology/metabolism in a symbiotic perspective; and *ii*) categories with count equal to 0. Statistical analyses (i.e. Levene's and the non parametric Kruskal-Wallis tests) were performed among the weevil groups in order to account for the differences in the amount of counts.

Bacterial isolation

Since most of the strategies adopted in biocontrol programs (e.g., the sterile insect technique or the use of bacteria as biocontrol agents) are based on insects' rearing, isolation trials were carried out in order to investigate the bacterial cultivable fraction associated to $RPW_{APPLE,}$ which may be the target of such strategies. Two adults (one male and one female) sampled in Catania, Italy, and reared under lab condition on apple for 30 days (see previous paragraph for details on the rearing condition), were dissected to obtain gut, ovaries, testes, and the female hemolymph. Each organ was smashed in 500 µl 0.9% NaCl and serial dilutions were plated on agarized LB, R2A, TSB and PDB media, supplemented with 100 µg/ml cycloheximide, and incubated in aerobic conditions or in microaerophilic Gaspak at 30°C. After isolate purification, the bacterial collection was de-replicated by the analysis of internal transcribed spacer (ITS)-PCR and one or two representatives of each ITS group were identified by partial sequencing of 16S rRNA gene after DNA extraction and amplification with the primers 27F and 1492R (73). Partial 16S rRNA gene sequences were deposited in the European Nucleotide Archive under the accession numbers LN623577-LN623640.

Results

Bacterial diversity associated with weevil (α-diversity)

A total of 138,738 bacterial 16S rRNA sequences have been obtained from the 12 RPW samples (median = 7599.5). The coverage of microbial α-diversity associated with each specimen was investigated through visual analysis of the rarefaction curves (α-diversity indices and observed species plotted *vs* simulated sequencing effort; Figures S1, S2). Using the Shannon index as a metric to measure α-diversity, all samples reached a plateau at value of ~1600 sequences per samples (except for a female specimen reared in lab for which only 1670 high-quality 16S rRNA gene sequences have been obtained), indicating that the microbial α-diversity associated with each specimen was well covered. Table 2 reports the values of the estimated diversity indices (i.e. Chao-1, Shannon *H* diversity and Pielou's *J* evenness indices) for the bacterial communities associated with the RPW and *R. vulneratus* specimens. The bacterial communities associated with the specimens belonging to the three groups were found to differ significantly in terms of diversity indices (H' χ^2 = 8.69, P = 0.013; J' χ^2 = 6.85, P = 0.032).

Identifier	Gender	Collecting locality	Lat/Long	TAG
I_palm_F1	♀	Catania, Italy	37°31.518'N/15°05.147' E	ACATCCAT
I_palm_F2	♀	Catania, Italy	37°31.518'N/15°05.147' E	ACCAACAT
I_palm_M3	♂	Catania, Italy	37°31.518'N/15°05.147' E	ACAGTGAA
I_apple_F1	♀	Catania, Italy	37°31.518'N/15°05.147' E	ACGCAACG
I_apple_F2	♀	Catania, Italy	37°31.518'N/15°05.147' E	ACGCATCG
I_apple_F3	♀	Catania, Italy	37°31.518'N/15°05.147' E	AAGGTAGC
I_apple_M3	♂	Catania, Italy	37°31.518'N/15°05.147' E	ACGAGATC
I_apple_M4	♂	Catania, Italy	37°31.518'N/15°05.147' E	ACGATCTG
I_apple_M5	♂	Catania, Italy	37°31.518'N/15°05.147' E	ACGCAACC
MYS_field_F1	♀	Genting Sempah, Selangor, Malaysia	3°21.368' N/101°47.684' E	ACGGGTAA
MYS_field_F2	♀	Genting Sempah, Selangor, Malaysia	3°21.368' N/101°47.684' E	ACGGTAAG
MYS_field_M3	♂	Genting Sempah, Selangor, Malaysia	3°21.368' N/101°47.684' E	ACGCGAAC

Table 1: *Rhynchophorus* specimens analysed in the present study.

Identifier	Group	OTUs	*H́*	*J́*	Chao-1
I_palm_F1	RPW_{PALM}	1060	5.61	0.56	1605
I_palm_F2	RPW_{PALM}	1028	5.74	0.57	1652
I_palm_M3	RPW_{PALM}	1142	5.69	0.56	1667
	RPW_{PALM}^{*}	1076.7±58.8	5.68±0.07	0.56±0.01	1641.3±32.3
I_apple_F1	RPW_{APPLE}	722	4.4	0.46	1082
I_apple_F2	RPW_{APPLE}	296	3.03	0.37	485
I_apple_F3	RPW_{APPLE}	209	3.98	0.52	345
I_apple_M3	RPW_{APPLE}	404	3.62	0.42	606
I_apple_M4	RPW_{APPLE}	420	3.67	0.42	617
I_apple_M5	RPW_{APPLE}	520	4.25	0.47	707
	RPW_{APPLE}^{*}	428.5±179.4	3.83±0.5	0.44±0.05	640.3±250
MYS_field_F1	R_{VULN}	134	2.98	0.42	189
MYS_field_F2	R_{VULN}	342	3.54	0.42	473
MYS_field_M3	R_{VULN}	633	2.56	0.27	880
	R_{VULN}^{*}	369.7±250.6	3.03±0.49	0.37±0.08	588±347.5

Table 2: Diversity indices estimated for the bacterial communities associated with the analyzed Rhynchophorus specimens.
a The mean and standard deviation of the estimated diversity indices are reported for each analyzed group of weevils; b Number of sequences obtained for each specimens after chimeric and contaminants removal.

Based on the Chao-1 index, approximately 66% of the -diversity was recovered by our analysis. The microbiotas associated with RPW_{PALM}, with J'= 0.56 ± 0.01, resulted more balanced than the bacterial communities associated both with lab-maintained RPWs (J'_{APPLE}= 0.44 ± 0.05) and with the sister species *R. vulneratus* (J'_{VULN}= 0.37 ± 0.08). The communities associated In particular, the bacterial communities associated with 3 specimens of RPW

directly collected from palm are significantly more diverse than that associated with the 6 specimens maintained in laboratory and fed with apple for four weeks (H' U = 18, P = 0.024; J' U = 18, P = 0.024). Differences were also observed in the total amount of bacterial OTUs associated to the three groups of samples (OTUs χ^2 = 6.38, P = 0.0413). This trend was also observed using the Chao-1 richness estimator (data not shown).
with the latter two groups were dominated by few taxa. In the high-evenness community associated with RPW_{PALM} the dominant OTU accounts for 8.4% ± 2.4 of the insect's microbiota respect to those explained by the dominant OTUs in RPW_{APPLE} and R_{VULN}, in which they made up respectively 15.4% ± 8.1 and 33% ± 12.1 of the total microbial diversity. These results were also visually confirmed by the rank-frequency curves plot (Figure S3), in which, for each of the 12 RPW and *R. vulneratus* samples, the number of high-quality 16S rRNA sequences clustered into each OTUs is reported. This method allows a visual evaluation of OTU richness and evenness (74, 75).

β-diversity and ecological traits

The β-diversity of bacterial communities associated with the weevil specimens was investigated through a principal coordinates analysis (PCoA) carried on the phylogenetic β-diversity matrix, obtained by UniFrac. The first two components explain a total of 42.6% of the variation (1st component, 28.3%; 2nd component, 14.3%). The analysis revealed an evident clustering of the samples according to each membership group; the first principal component segregates the microbiota of the two groups of *R. ferrugineus*, while the second component isolates the specimens of *R. vulneratus* (Figure 1A). The clustering analysis performed on the OTUs' presence-absence matrix confirms the results obtained by PCoA showing that the pattern of association of the different bacterial community was congruent with the different weevil groups (i.e. the microbiota associated to each specimen clustered together; Figure 1B). The same results were also obtained by analysing the presence-absence OTUs matrix using Jaccard index (Figure S4) and the abundance OTUs matrix adopting the Kulczynski distance (Figure S5). These results support the fact that the observed pattern in microbiota composition (i.e. the presence-absence of the different OTUs) is congruent with the grouping factor independently from the bacterial evenness associated with each community. Interestingly, microbiota from specimens of *R. ferrugineus* reared in laboratory clustered as the sister group of microbiota from *R. vulneratus* specimens.
The estimated β-diversity over the two groups of RPWs, measured as Sørensen's dissimilarity, resulted in a value of β_{SOR} = 0.982. The two components of the β-diversity, the turnover and the nestedness, resulted in β_{SIM} = 0.973 and in β_{NES} = 0.009, respectively. These values indicate that high OTU turnover and a low nested component have been recovered between the two groups of RPWs, which means that ~97% of the OTUs are different in the two communities. It is interesting to note that after only 30 days of feeding on apple, the bacterial community associated with RPW_{APPLE}, derived from RPW_{PALM}, dramatically changed compared to the microbiota of the original population, maintaining only a few shared OTUs.

The analyses on the OTUs common to the three groups of weevils showed (Figure 2) that over a total of 2386 OTUs associated with *R. ferrugineus*, 1369 are exclusive to RPW_{PALM} and 702 to RPW_{APPLE}. Interestingly only 34 OTUs (19 unique OTUs shared between RPW_{PALM} and RPW_{APPLE} plus 15 unique OTUs common to all) are shared between these two groups of weevils that descend from the same population. Considering all the three groups, only a total of 15 OTUs are shared among them (taxonomic assignment of these OTUs with a comparative analysis with bacterial OTUs isolated from palm tissues are reported below).

Figure 3 reports the results of the NMDS analysis performed on the bacterial OTU table fitted with both ecological traits: *i*) diet consisting in apple for the lab-reared RPW and palm tissues for field collected RPW and *R. vulneratus* and *ii*) the temperatures at which the specimens have been maintained (lab) or are assumed to have developed (wild collected). Both traits significantly explain the dissimilarities among the bacterial communities associated with the three groups of weevils (diet: $R^2 = 0.87$, $P = 0.003$; temperature: $R^2 = 0.94$, $P = 0.006$). These results also suggest that temperature may be a confounding factor to explain the impact of different diets on the bacterial diversity and richness associated with the different weevil groups.

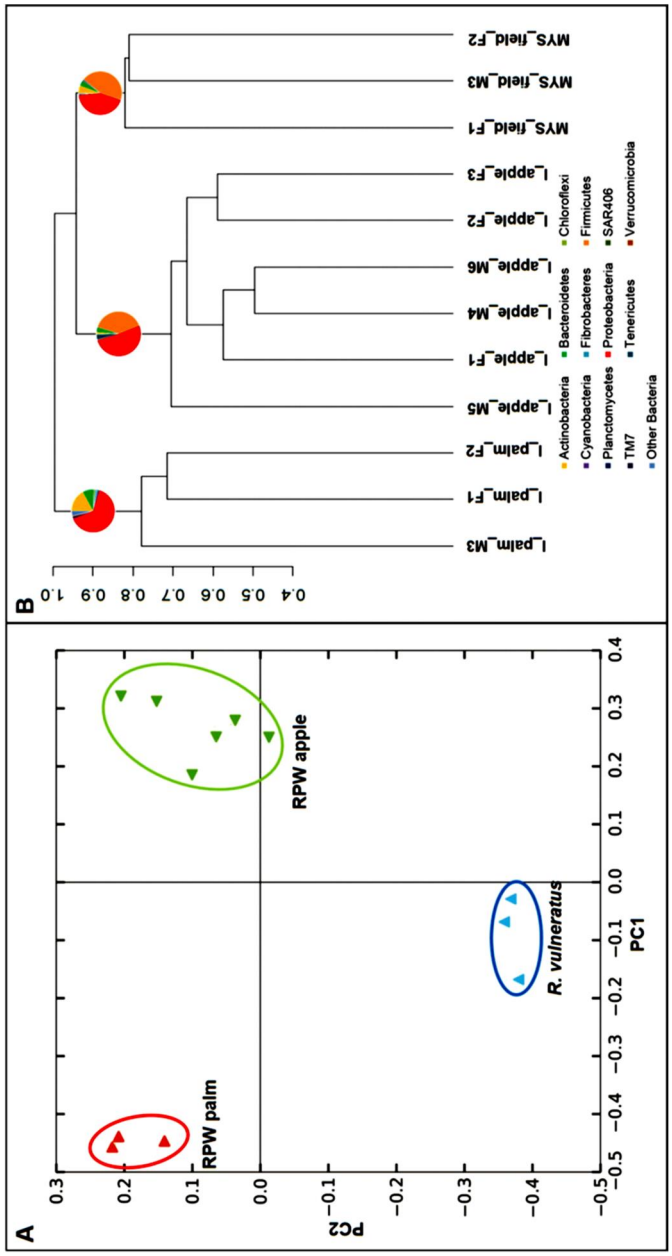

Figure 1 A: principal-coordinate analysis on the phylogenetic β-diversity matrix obtained starting from the OTU table. The explained variance is as follows: 28.3% 1st component, 14.3% 2nd component. B: hierarchical clustering dendrogram representing the OTU table pairwise dissimilarities between the different analyzed weevils; the pie charts represent the relative abundance of bacterial communities at phylum level.

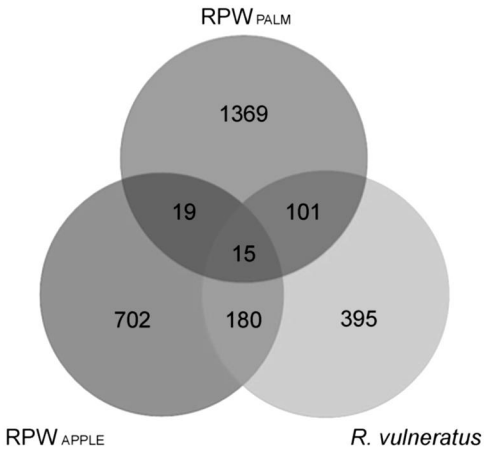

Figure 2: Venn diagram showing the shared bacterial OTUs (at 97% similarity) between all studied weevils groups.

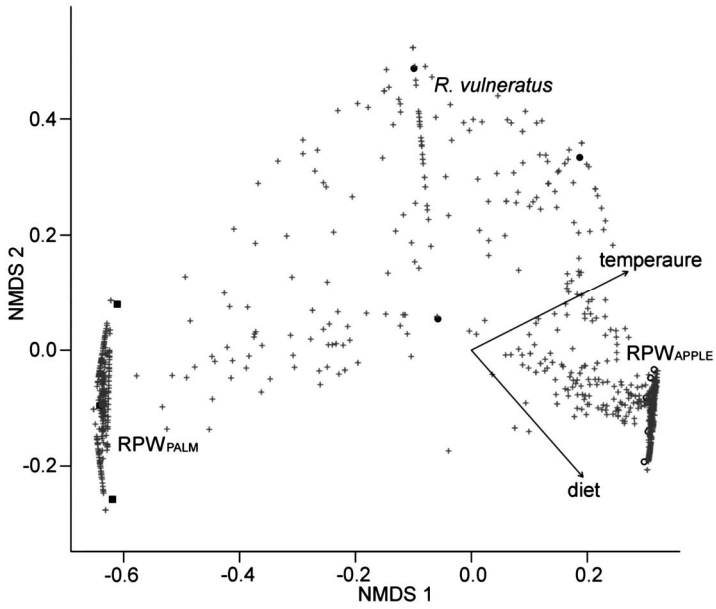

Figure 3: Biplot of the first 2 axes for the Non-Metric Multi-Dimensional Scaling representing correlations between the OTUs Chao dissimilarity index and ecological factors (i.e. diet and temperature). The black squares, the black and open circles represent respectively: RPW_{PALM}, *R. vulneratus* and RPW_{APPLE}; while black crosses represent the identified OTUs. The vectors represent the

mean direction and strength of correlation of diet and temperature (p-value < 0.05).

Taxonomic classification of OTUs

The results of the taxonomic assignment analysis at the phylum and family levels are reported in Figure 4 (see also table S1-S2). The analysis revealed that the most abundant taxa shared by all the members of the three insect groups belong to Proteobacteria (on average c.a. 64.6% of the sequences in the specimens of RPW_{PALM} group, 51.6% in RPW_{LAB} and 43.3% in R_{VULN}; Figure 4A). The specimens of the groups RPW_{APPLE} and R_{VULN} harbour a second dominant taxon represented by Firmicutes (on average 39.1% and 44.1%), while members of this taxon are scarcely represented in the microbiotas associated to RPW_{PALM} specimens (0.3%). The members of the three groups of insects harbour, with different abundance, also members of Actinobacteria (RPW_{PALM} = 17.1%, RPW_{APPLE} = 1.2% and R_{VULN} = 5.6%) and Bacteroidetes (RPW_{PALM} = 8.2%, RPW_{APPLE} = 3.6% and R_{VULN} = 4.3%). In Table 3 are reported the relative abundances of the bacterial genera (with abundance > 1%) associated with the three groups of weevils. Within the *R. ferrugineus* feeding on palm the most abundant taxa belong to Xanthomonadaceae (mean 13.2% ± 4.03) and Rhodobacteraceae (mean 11.9% ± 11), to which belong the genera *Luteimonas* (mean 6.6% ± 2) and *Paracoccus* (mean 7% ± 7.8), respectively. In the microbiota associated with RPW_{PALM} specimens, but not with those reared in laboratory, members of the genus *Demequina* (Cellulomonadaceae) and members of Rhodobacteraceae, Phyllobacteraceae and Rhizobiales were recovered. In contrast, the most abundant genus in the bacterial community associated to RPW_{APPLE} and R_{VULN} specimens was *Leuconostoc*, which represents respectively 17.8% (s.d. 14.1) and 37.2% (s.d. 34.1) (Table 3). This genus was not observed in the microbiota of RPW_{PALM} specimens. Other dominant components of the RPW_{APPLE} microbiota are the bacteria of the genera *Acetobacter* (12.9% ± 9.2) and *Lactococcus* (9.3% ± 6.2). Bacteria of the genera *Lactobacillus* and *Entomoplasma* are present in all specimens of *R. ferrugineus* reared under lab conditions and in that of the sister species *R. vulneratus*, but are not associated with RPW_{PALM}. Besides *Leuconostoc*, the dominant bacteria in the *R. vulneratus* microbiota belong to the family Comamonadaceae (11.2% ± 7.3) and to the genus *Ralstonia* (Burkholderiaceae). Table S2 reports the relative abundances of the bacterial families associated with the different weevil samples. In agreement with the results obtained by the biodiversity analysis, the higher number of bacterial families are associated with RPW_{PALM}, while samples of *R. ferrugineus* reared under lab conditions and those of the sister species *R. vulneratus* harbour a lower number of bacterial taxa.

	Rhyncophorus ferrugineus		*Rhyncophorus ferrugineus*						*Rhyncophorus vulneratus*			
	palm		apple						palm			
	f[a]	*f*[a]	*m*[a]	*f*[a]	*f*[a]	*f*[a]	*m*[a]	*m*[a]	*m*[a]	*f*[a]	*f*[a]	*m*[a]
Demequina	2.96	4.13	1.13	-	-	-	-	-	-	-	-	-
Gordonia	0.21	0.55	1.19	-	-	-	-	-	-	-	0.30	0.03
Aeromicrobium	0.74	2.99	1.50	-	-	-	-	-	-	-	-	-
Pimelobacter	0.27	0.94	3.20	-	-	-	-	-	-	-	-	-
Propionibacterium	-	-	-	0.04	0.09	0.24	-	-	-	0.17	2.92	0.03
Dysgonomonas	-	-	-	1.64	0.67	0.06	6.64	6.76	1.92	-	0.50	-
Flavobacterium	-	-	0.09	-	-	-	-	-	-	-	1.55	-
Wautersiella	0.15	1.27	0.96	-	-	-	-	-	-	-	0.17	-
Enterococcus	-	-	0.04	0.24	0.01	-	0.66	11.48	8.97	-	-	-
Lactobacillus	-	-	-	2.60	0.60	1.56	0.99	0.38	0.79	0.04	0.07	0.10
Leuconostoc	-	-	-	5.07	2.17	22.16	40.88	22.15	14.54	41.21	1.37	69.17
Lactococcus	-	-	-	11.05	2.92	18.62	4.49	13.61	4.94	0.25	-	0.35
Clostridium	-	-	-	-	-	-	-	-	-	-	2.30	0.04
Erysipelothrix	-	-	-	1.29	-	-	0.07	0.01	0.41	1.53	0.14	-
Planctomyces	1.01	0.59	0.22	-	-	-	-	-	-	-	0.27	0.04
Asticcacaulis	1.01	0.54	0.33	-	-	-	-	-	-	-	-	-
Devosia	3.68	1.09	1.48	-	-	-	-	-	-	0.17	0.95	0.07
Hyphomicrobium	0.82	1.37	0.69	-	-	-	-	-	-	0.17	-	-
Defluvibacter	0.02	0.06	0.06	-	-	-	-	-	-	-	1.29	0.06
Kaistia	1.11	0.07	0.06	-	-	-	-	-	-	-	-	0.02
Paracoccus	1.03	15.79	4.22	0.04	0.02	-	-	-	-	0.30	1.79	0.25
Acetobacter	0.07	-	-	22.16	23.91	14.79	4.22	1.22	10.95	0.04	-	0.20
Swaminathania	-	-	-	1.07	0.42	0.30	0.19	0.04	0.16	-	-	0.17
Ralstonia	-	-	-	-	-	-	-	-	-	8.37	38.90	2.38
Escherichia	0.03	0.02	0.28	0.06	0.10	-	7.25	-	1.21	0.30	-	-
Gluconacetobacter	-	-	-	0.49	0.16	1.80	0.01	0.41	0.90	-	-	-
Serratia	-	0.02	0.50	1.28	0.19	-	1.19	1.10	2.03	-	1.65	1.39
Trabulsiella	0.02	-	0.31	4.31	0.06	-	0.02	0.52	0.55	1.57	-	0.01
Acinetobacter	0.27	0.46	5.46	0.01	0.01	0.12	-	-	-	0.08	2.07	0.35
Pseudomonas	1.82	1.07	4.41	0.30	-	0.12	-	-	-	0.04	0.26	-
Luteimonas	8.88	4.91	6.00	-	-	-	-	-	-	-	-	-
Stenotrophomonas	-	0.04	0.70	-	0.01	-	-	-	-	0.13	1.64	0.05
Thermomonas	1.68	0.44	1.76	-	-	-	-	-	-	-	-	-

Table 3: (previous page) Genera of bacteria identified in the weevil microbiotas with their relative average abundance expressed as percentage. In this table are reported the bacterial genera present with abundance > 1% in at least one specimens. The main differences in in the bacterial genera associated with apple and palm are highlighted in bold.
a The gender of the specimens is reported; f: female, m: male

Interestingly, no OTUs of the *R. ferrugineus* primary endosymbiont "*Candidatus* Nardonella" were recovered in the first analysis of the data using the well-curated RDP database to taxonomically identify OTUs. A more detailed analysis of the OTUs, which were previously identified as unknown Gammaproteobacteria, performed by BLAST against known sequences of "*Ca.* Nardonella" allowed the identification of different OTUs as belonging to this taxon (Table S3). The identity of these OTUs was later confirmed as the primary symbiont by bidirectional BLAST with values of sequence similarity > 97%. Noteworthy, all the identified OTUs matched with the sequence FJ626262, endosymbiont of *Sphenophorus levis.* The prevalence of "*Ca.* Nardonella" was 100% in all the RPWs and *R. vulneratus* specimens. This result confirms the presence of "*Ca.* Nardonella" also in this *Rhynchophorus* species. The fact that the titer of the primary symbiont within the analysed samples was low can be attributed to the fact that the bacteriome and mesenteric caeca, colonized by the primary symbiont (76), represent a small fraction of the total sampled tissues colonisable by bacteria. In addition, the titer of endosymbionts has been demonstrated to vary along the life cycle of their arthropod host (e.g.,(77)).

Bacteria associated with palm tissues

The majority of the 16S rRNA sequences, 60755 out of the obtained 61189 via 454 sequencing from the palm tissues were removed from the analysis, since they matched with the chloroplast. From the three palm samples, a total of 434 16S rRNA sequences were of bacterial origin, and were clustered in 193 ± 78.5 OTUs. The taxonomic composition of the bacterial communities associated with the palm tissues is dominated by members of Firmicutes and Proteobacteria, representing on average the 62% and the 34% of the microbiota (Figure 5A). The composition of family-level microbiota associated with palm tissues is represented in the pie chart reported in Figure 5B. The genus *Brevibacillus* (Paenibacillaceae), detected in all the processed palm samples, is the dominant one (average 60 ± 37.7% of the obtained 16S rRNA sequences).
A comparative analysis on the bacteria associated with weevil and palm samples were performed on the genus-level identified OTUs in order to detect any patterns of commonalities. Interestingly, 12 out of the 13 bacterial genera identified in the palm tissues, are recovered, with different degree of prevalence and abundance, also in the weevil microbiota (only *Klebsiella* is exclusive of these tissues). *Brevibacillus*, the dominant bacterium in the palm microbiota, is found to be associated also with two samples of RPW although with a low abundance.

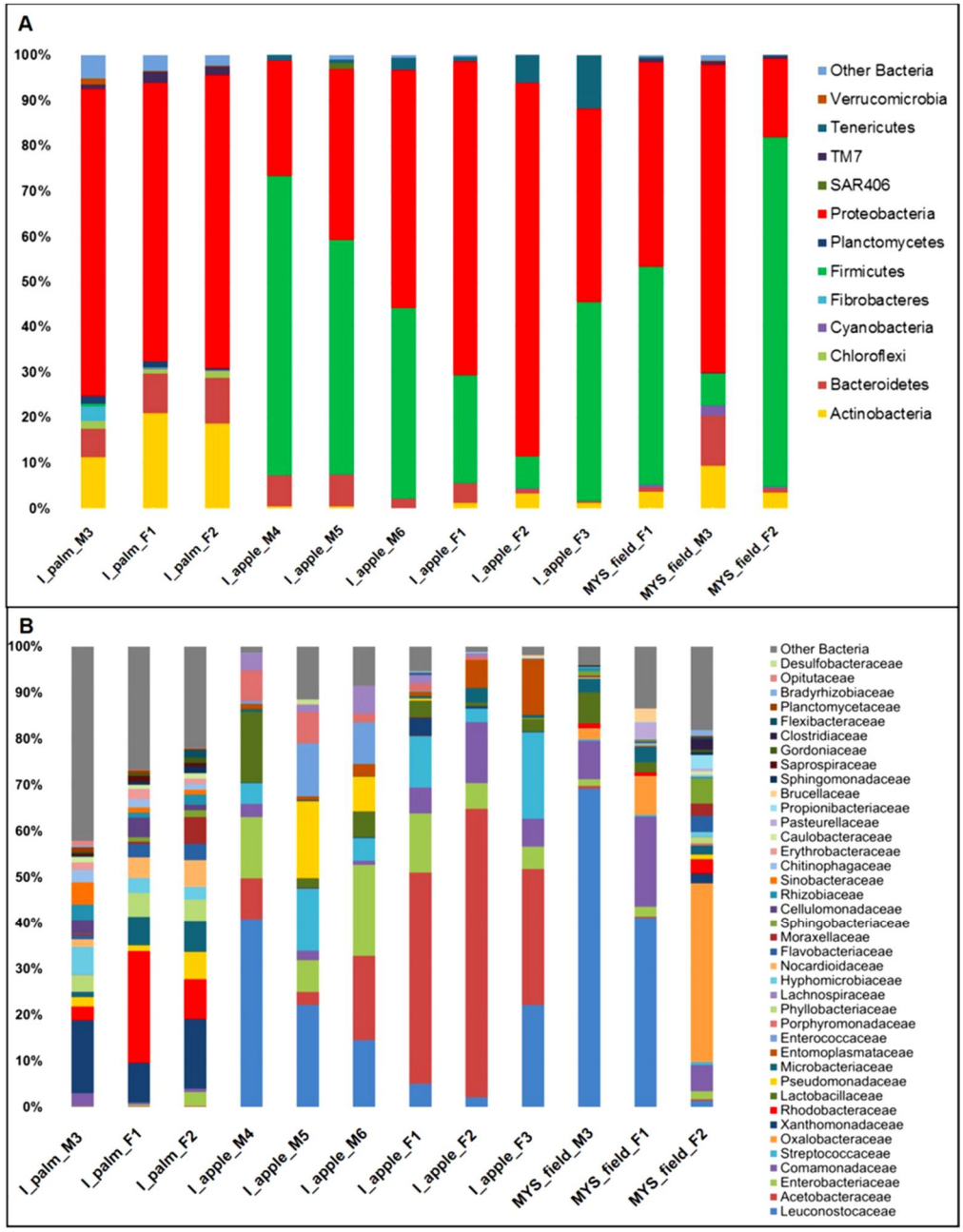

Figure 4: Histogram representing the taxonomic assignment of bacterial 16S rRNA gene sequences associated with the analyzed weevils; A: phylum level, B: family level.

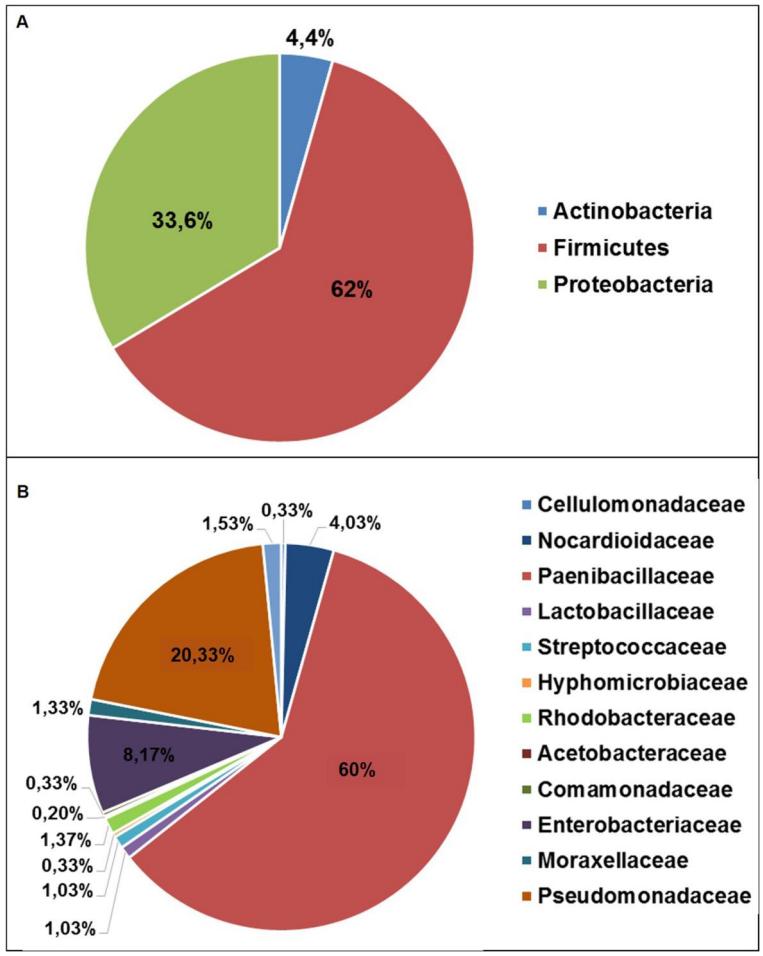

Figure 5: Pie charts representing the taxonomic assignment of bacterial 16S rRNA gene sequences associated with the analyzed the palm tissues; A: phylum level, B: family level.

Metabolic potential

In order to investigate and compare the metagenomic functional potential associated with the different bacterial communities harboured by the three groups of weevils, the 16S rRNA sequences obtained from each specimen were analysed with a dedicated bioinformatics tool (PiCRUSt, (70)). In Table S5 are reported the results of the analysis containing the predicted gene family counts per sample for all the categories that have been related to the bacterial physiology/metabolism, in light of a symbiotic relationship, such as the amino acid metabolism, the biosynthesis of secondary metabolites and the metabolism of cofactors and vitamins.

The full results of the analysis are reported in Table S4. Within the cellular processes category, significant differences between all the three insect groups

were recovered in bacterial chemotaxis and in bacterial motility proteins (respectively Kruskal-Wallis $\chi^2 = 6.54$, $df = 2$, $P = 0.038$ and Kruskal-Wallis $\chi^2 = 7.27$, $df = 2$, $P = 0.026$). In the amino acid metabolism category, significant differences ($P < 0.05$) between the three groups of weevil were reported for Lys, Val, Leu and Ile degradation and for Phe, Trp and β-Ala metabolism.

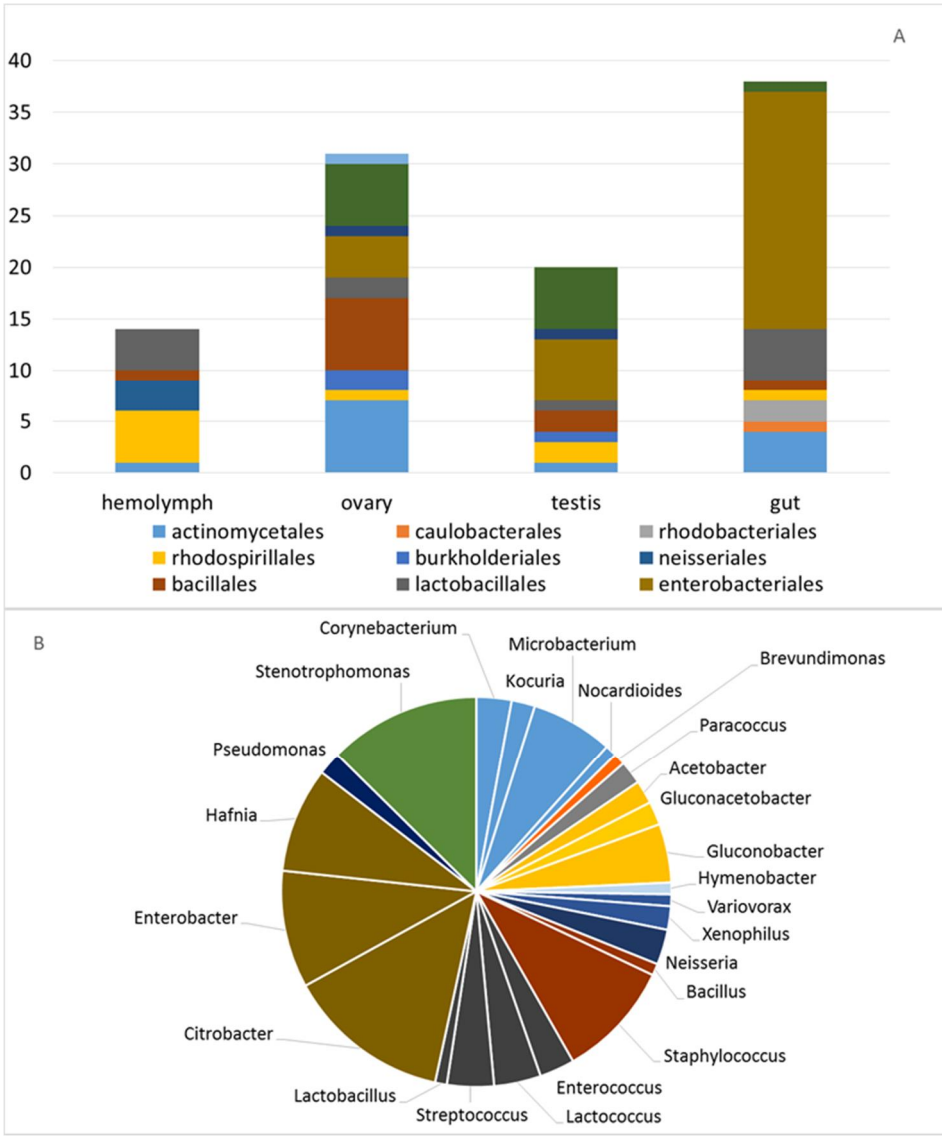

Figure 6: Relative abundance of the strains isolated from the different organs of RPW. A: histogram representing the relative abundance of strains at order level isolated by different organs; B: pie chart representing the cumulative abundance of the different strains at the genus level.

None of the predictions obtained for the category of biosynthesis of other secondary metabolites resulted different based on the Kruskal-Wallis test.Interestingly, other statistically significant differences between-groups were observed in the categories of carbohydrate/energy metabolism (pentose phosphate pathway and nitrogen metabolism) and in the metabolism of cofactor and vitamins (thiamine metabolism). Pairwise comparison carried on the subsets of differentially predicted metabolic pathways, using the Mann-Whitney test, indicated that most of the differences result to be between the *R. ferrugineus* that were fed on palms and those fed on apples ($p<0.05$; Table S5). From the PICRUSt prediction, these pathways result to be present at a higher percentage in the microbiota associated with RPW_{PALM} than in the one associated with RPW_{APPLE}.

Bacterial isolation from laboratory reared weevils

The bacterial cultivable fraction was investigated on $RPW_{APPLE.}$. This work allowed the isolation of a total of 103 isolates from different insect organs dissected from laboratory weevils, with the aim to investigate the bacterial diversity associated to the different body districts (Table S6). Partial 16S rRNA sequencing results showed that the majority of the isolates belonged to Proteobacteria (64%) and Firmicutes (22%, Figure 6A). Among Proteobacteria, Gamma-subdivision was the most abundant (46% in comparison to Alpha and Beta ones which accounted with 12% and 6%, respectively) with members of Enterobacteriales and Xanthomonadales. Among Firmicutes bacteria belonging to the orders Lactobacillales and Bacillales were isolated. Acetic Acid Bacteria (AAB) accounted for 9% of the total isolates (Figure 6).
We did not appreciate major differences in the bacterial composition of the different organs and tissues analysed, even though an important fraction of the isolates were obtained from the intestine (no. 38) (Figure 6B; Table S6).
The isolated strains belong mainly to the taxa Enterobacteraceae, Lactobacillales, Actinobacterales and Acetobacteraceae. These taxa were also observed in the metabarcoding analysis.

Discussion

The bacterial communities harboured by the three groups of weevils are dominated by members of Proteobacteria; in addition, RPW_{APPLE} and R_{VULN} specimens harbour a second dominant taxon represented by Firmicutes (see Fig. 4). The unbalanced composition of the bacterial communities associated with these insect groups is reflected also in the Pielou's evenness index, where RPW_{APPLE} and R_{VULN} show significant lower values that those of RPW_{PALM}. In fact, the microbiota associated with RPW_{PALM} is characterized by the absence of dominant bacteria. Most of the RPW_{PALM} exclusive taxa are present with low abundance (<1%); conversely, the microbiota associated with RPW_{APPLE} and R_{VULN} are dominated, with an average abundance of 17.85% and 37.27%, by bacteria of the genus *Leuconostoc*, which is absent in the microbiota of RPW_{PALM}. The microbiota of RPW_{APPLE} specimens is characterized also by bacteria of the genera *Acetobacter* (14.47% ±13.6) and *Lactococcus* (9.25% ± 6.2). Interestingly the three groups of weevils shared 15 common OTUs (Table S7). Among these

OTUs, taxa such as *Serratia* and *Ochrobactrum* were observed. Both taxa have been described in association with several arthropod taxa (40, 78, 79)(*Amblyomma rotundatum*, data not shown). While *Serratia* plays several roles ranging from host protection against parasitoids (80) to the enhancement of host fitness (81), the role of *Ochrobactrum* is yet to be investigated. Noteworthy, three of these shared OTUs belonged to taxa that were present also in palm tree tissues (i.e., one belonging to Comamonadaceae and two to *Stenotrophomonas*).

Culture-dependent methods on organs and tissues of RPW_{APPLE} have allowed obtaining 103 isolates, which were identified by sequencing of partial 16S rRNA gene. Interestingly, a large part of the isolates was constituted by Proteobacteria and Firmicutes, which were present in all the different dissected organs and in the hemolymph. This is in accordance with the data obtained with DNA-based method. In fact, using both cultivation dependent and independent technique, Proteobacteria and Firmicutes members resulted abundant in the laboratory population. Particularly, members of Gammaproteobacteria, with Enterobacteriales and Xanthomonadales representatives, and Firmicutes, with *Lactococcus*, *Streptococcus*, *Enterococcus* and *Lactobacillus*, were retrieved. Bacteria belonging to the genus *Lactococcus* were also previously isolated from wild specimens of RPW (34). It is interesting to note that members of AAB were isolated from the hemolymph. In insects, these bacteria are generally described as gut associated, but they have been shown to colonize different organs even after administration with food; this indicates that AAB are able to cross the gut barrier and reach other organs, likely through the hemolymph (81–83). Moreover, in accordance with pyrotag data, laboratory insects harbour also Actinobacteria and Betaproteobacteria, with respectively 13% and 6% of abundance.

The comparative analysis performed on the bacterial consortia associated with the palm tissues with those associated with weevils highlight some patterns of commonalities: 12 bacterial genera out of the 13 identified in palm tissues were also recovered in weevils from the original palm population. Based on this finding, we can hypothesise that these bacteria are ingested by weevils along with palm tissues. Even if we cannot completely exclude that these bacteria are transient components of the weevil microbiota and do not represent stable consortia, the fact that most of them (nine out of 13) are present also in specimens reared on apple for 30 days or in *R. vulneratus* suggests that these are stable component of the weevil microbiota. The alternative hypothesis is that palm tissues are contaminated by weevil faeces; the shared insect-palm bacteria would thus represent contaminants from the insects. However, palm tissues examined in this study were healthy and not colonized by the weevil.

Interestingly, even if the microbiota associated with weevils clearly differ among groups in terms of diversity and composition, the predicted metagenome functional potentials related to the bacterial physiology/metabolism in light of a symbiotic relationship were maintained in most aspects. Statistically significant differences among the three groups of weevils were reported for the metabolism of a few amino acids (degradation of Lys, Val, Leu and Ile; metabolism of Phe, Trp and □-Ala), in

carbohydrate/energy metabolism (pentose phosphate pathway and nitrogen metabolism) and in thiamine metabolism. These biochemical pathways are linked with the recycling of nitrogen and are thus expected to be highly represented in the microbiota of organisms feeding on nitrogen-poor food sources (e.g., palm tissues, apple). The differences in the carbohydrate/energy metabolism could be explained by the high-sugar content in the food resources dispensed to RPWs in laboratory respect to those feeding on the palm tissues. The apple-based diet provided in the laboratory enriched and selected for *Lactococcus* and *Acetobacter*. These bacteria have been observed in association with several other insects that have a sugar-rich diet(73, 83).
The taxonomic composition of the microbiota associated with *R. ferrugineus* specimens collected in Catania (Italy) clearly differ from those described in a previous study, where a comparable approach was adopted on *R. ferrugineus* collected in Al-Hassa Oasis (Saudi Arabia) (35). In this study, even if a seasonal variability in the gut microbiota was observed, the dominant bacteria in adult specimens belonged to the genera *Lactococcus* and *Acinetobacter*, whereas *Klebsiella* and *Lactococcus* were detected in larvae. In our study, bacteria of the genus *Lactococcus* were recovered in specimens reared in laboratory on apple, while members of the genus *Klebsiella* were only recovered in the palm tissues. The last findings lead to the hypothesis that the pattern found by Jia and colleagues (35) could result from the effect of the bacteria transmitted to the weevil by the palm tissues.
The bacterial communities associated with the three groups of weevil under study (*R. ferrugineus* from wild population, *R. ferrugineus* from wild population reared in laboratory for four weeks feeding on apple, and *R. vulneratus* from Malaysia) were significantly different. The specimens belonging to *R. ferrugineus* of the invasive population collected in Catania harboured the highest number of bacterial OTUs (OTUs = 1077), while a significant decrease in the number of harboured OTUs has been observed in the specimens reared in laboratory (OTUs = 429). This reduction has been observed after a relatively short time of maintenance (30 days) under stable environmental conditions (temperature, humidity, light-dark cycle and food resources) feeding on a sugar-rich resource as the apple. The number of OTUs identified in the sister species *R. vulneratus*, collected in the *Rhynchophorus* native area, was lower (OTUs = 370) respect to those of the two groups of *R. ferrugineus*. Interestingly, the number of OTUs associated with adult specimens from Saudi Arabia and to larvae reared under hot condition (i.e. 32°C) resulted of ~ 400 (35), a value comparable to those obtained for the RPW_{APPLE} and of *R. vulneratus* from Malaysia, but not with the recovered value for RPW_{PALM} and larvae reared at 20°C (1077 and 1049, respectively). We cannot exclude that the differences in OTU number between our specimens and those from Saudi Arabia (35) are due to differences in the used 16S rRNA regions. The number of OTUs detected in the analysed weevils was higher in respect to those observed in other Coleoptera (40, 84–86). Moreover, the composition of the bacterial community associated with the three groups of weevils, which have been analysed through PCoA and ANOSIM, resulted statistically different. These results have also been confirmed by the analysis on the two components of the □-diversity, which was performed on the bacterial

community associated with the two groups of RPW. The hierarchical clustering grouped the bacterial community associated with RPW_{APPLE} together with those associated with the sister species *R. vulneratus* instead of with those harboured by the co-specific specimens from wild population.
Based on the achieved results on the bacterial OTU diversity, integrated with those obtained by Jia and colleagues (35), we can hypothesize that high temperatures (as those of rearing facilities, of Saudi Arabia and of Malaysia) have caused a decrease in the level of bacterial diversity associated with weevil. Conversely, low temperatures (as those experienced by adults collected in Catania and larvae reared at 20°C) increase the bacterial OTU diversity associated with the insect. Both studies confirmed the high plasticity, in terms of turnover, of the microbiota associated with RPW. Based on our results, environmental abiotic factors, such as the temperature, could play an effect in shaping the diversity of weevil's microbiota. Similar results have been obtained for another group of phytophagous beetles, in which the altitude is related with the structure of the insect's microbiota (40). The interpretation of these findings by biological and evolutionary perspectives can be done in the light of the hologenome hypothesis (87), which argues that the real unit under natural selection is the eukaryotic host together with its associated microorganisms. Harbouring a more diverse and evenly represented microbiota, in addition to the capability to acquire new bacterial taxa from the environment, may confer selective advantages to the host in changing environments (e.g., food resources exploitation, capability to survive in polluted environments).
In conclusion, our study shows that: *i*) the bacterial diversity and evenness decrease in a short time when RPW specimens are reared in laboratory under controlled conditions (temperature, humidity, light-dark cycle and food resources); *ii*) the composition of the bacterial community associated with the three weevil groups clearly differs both within the same population (influenced by the diet) and between the considered species; *iii*) most of the members of the bacterial community associated with palm tissues, from which the specimens of *R. ferrugineus* were collected, are also present in the insects microbiota; *iv*) bacterial isolation performed on laboratory reared weevils confirmed pyrotag data; *v*) both the present study and the previous one by Jia *et al.* (35) do not identify a fixed microbiota in RPW, suggesting the importance of the environment in shaping it. The knowledge of the bacterial community associated to this important pest, the metabolic potentials exerted by its bacterial partners, the bacterial dynamics in relation to the environment/diet and their distribution and localization, both in palm and insect organs, together with the possibility to cultivate the bacterial symbionts of this insect, could open new interesting perspectives towards the development of novel strategies for the symbiotic control of weevils.

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Chapter 5

Characterization of the gut microbiota of the cerambycid beetle *Psacothea hilaris hilaris* and of its "symbiotic potential".

Abstract

Psacothea hilaris hilaris is a xylophagous beetle belonging to the Cerambycidae family. Its larvae grow inside mulberry tree and fig trunks, reaching the length of up to three centimetres and damaging the plants to death. In Japan, this is a serious pest for sericulture, while its recent establishment in northern Italy could threaten the fig cultivation in the Mediterranean basin. In this study, we characterized by DGGE the bacterial communities in the guts of wild and reared *P. h. hilaris* larvae, assessing the significant effect of the diet and the gut tract in modifying their taxonomical composition. Moreover, we established a collection of bacterial isolates from the guts of wild larvae and we evaluated the possible contribution of the isolates to the host physiology (in terms of contribution to carbon or nitrogen absorption) through *in vitro* tests. From our observations, the cultivable gut bacterial community of *P. h. hilaris* appears to include many different commensals, mainly acquired from the environment, which are suitable to exploit the food sources in the gut and to give a contribution to the host metabolism.

Introduction

Psacothea hilaris hilaris, (Figure 1) commonly known as the yellow spotted longicorn, is a cerambicyd beetle widely spread in east Asia. The subspecies *hilaris* is native to China and Japan, where it is an economically relevant pest for the trees belonging to the Moraceae family, above all the fig (*Ficus carica*) and the mulberry (*Morus* spp.). In Japan, the wood-feeding larvae of *P. h. hilaris* represent a serious threat for sericulture, as they damage the mulberry trees that are the food source for silkworms, by boring deep tunnels in the trunk (1). Due to the international commerce of wood and trees, in the last years the insect was observed outside of the native area, in North America, Canada and United Kingdom. In northern Italy, the presence of this pest is recorded from 2005 but, according to the last observations, a stable population is present only in a small area in Como province. However, this observation should not be underestimated, taking into account the potential danger of the diffusion of the parasite for the fig culture in the Mediterranean basin (1)

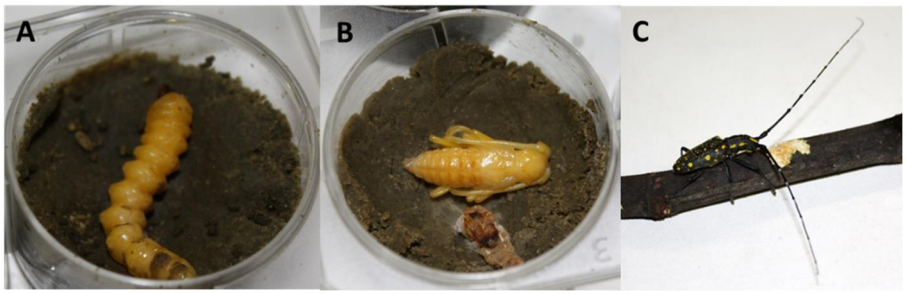

Figure 1: *Psacothea hilaris hilaris* larva (A) pupa (B) and adult (C) from the laboratory breeding. (Photo: Daniela Lupi)

One aspect of insect physiology that proved to be very important is the symbiosis with gut bacteria. Recent studies demonstrated how the bacterial communities in the gut of insects deeply affect the physiology of the hosts, influencing not only the nutritional capabilities but also the immune system (2), the larval development (3) and the mate choice (4). Even the commensal bacteria, which do not establish a permanent or essential symbiotic relationship with the host, can have relevant effects on host physiology (5). These bacteria are mainly acquired from the environment; thus, they vary respective to the diet and sampling site. However, they do not simply reflect the bacterial community in the environment in which the insect lives, because they undergo a selective process inside the gut. Commensal strains must be capable to efficiently exploit the food sources within the insect gut, to find a suitable niche in which they can persist, irrespective of the continuous flow of food bolus, and survive the particular pH conditions of the gut. Finally yet importantly, they must be able to positively interact with the host immune system (5). Indeed, they are retained inside the gut and live within the insect without causing illness and death (which turns to be disadvantageous to the bacterium itself, which loses a favourable niche). To do that, they should not strongly elicit the immune system as a pathogen (a situation that that leads to bacterial elimination or insect death), nor behave as a pathobiont, leading to a chronic production of reactive oxygen species (2).
The diet of the xylophagous *P. h. hilaris* larvae suggests that gut commensal microbiota could have a role in the nutritional balance of the insect. In fact, a wood-based diet is generally considered to be poor in nitrogen (present mainly in cell-wall proteins) and rich in recalcitrant sugar polymers, such as cellulose and hemicellulose (6, 7). High level of activity against the principal dietary carbohydrates (including cellulose, hemicellulose and pectin) has been detected in *P. h. hilaris* gut extracts, although is not clear if these enzymes are secreted by the host itself or by its gut microflora (7). Indeed, at least one cellulolytic enzyme, characterized by Sugimura et al. (8), is produced and secreted by the insect. A recent study by Scully et al. (6) on a closely related cerambycid beetle (*Anoplophora glabripennis*) revealed the presence of a complex set of enzymes in the microbial gut community, that are hypothesized to help the host in the digestion of recalcitrant carbohydrates,

as well as in nitrogen and other nutrients acquisition. Moreover, a culture-dependent study on the microbiota of eight cerambycid species, including *P. hilaris*, shows that these insects harbour xylanase- and pectinase-secreting bacteria (9).
In this study, we aimed to characterize the gut bacterial community of *P. h. hilaris* larvae combining molecular and culture-dependant methods, and to describe its change in composition after feeding the larvae on different artificial diets. Moreover, we assessed in the isolated bacterial strains the presence of several metabolic capabilities, which likely allow the persistence of these microorganisms in the gut of the host as commensals and, possibly, the establishment of a mutualistic (though not essential) symbiotic relationship.

Methods

Psacothea hilaris hilaris sampling and rearing

Wild specimens of *P. h. hilaris* have been collected from Ponte Lambro, in northern Italy (45°50'N 9°14'E). Trunks and bigger branches of *Ficus carica* trees have been cut and brought to the laboratory, where the larvae were extracted. Adult specimens have been collected in the same area from the tree canopy. The reared beetles were fed during all the larval stages with a commercially available diet (10) developed for silkworms, made of mulberry leaves (increased to 40%), soy flour, wheat flour, corn starch, citrate, agar, and a vitamin mix. As preservatives, the diet includes β-sitosterol (0,5% w/w), sorbic acid (0,2%) and propionic acid (0,7%), together with the antibiotic chloramphenicol (0,01%) (10). The larvae for groups 3 and 4 (see the paragraph about DGGE) were collected in field as described above, and then reared for ten days on the same diet, but without chloramphenicol (diet without antibiotic, group 4) or without β-sitosterol, sorbic acid, propionic acid and chloramphenicol (diet without preservatives and antibiotic, group 3). The larvae were reared individually in the wells of 6-wells titer plates, and were transferred in a new well with fresh diet every three days. The climatic chamber was set to 25±0.5°C; 70% R.H. with a 16:8 light-dark photoperiod. After pupation, adults were transferred on cut fig tree branches and trunks, to allow them to mate and lay eggs.

Denaturing Gradient Gel Electrophoresis (DGGE)

P. h. hilaris larvae of the third or fourth larval instar (determined by cephalic capsule measurement) were killed by putting them in a hermetic vial saturated with ether, and dissected with sterile instruments to extract the gut, after being surface sterilized by one washing in 70% ethanol and two washings in sterile water. The three parts of the gut (foregut, midgut and hindgut, Figure 2) were separated and preserved in 100% ethanol at -20°C.

Figure 2: *P. h. hilaris* larval gut, divided in its three sections. (Photo: Davide Venegoni)

DNA extraction was performed with the chloroform-phenol method (11). A first step of PCR amplification was performed using bacterial primers 27F and 1492R, which amplify almost the whole 16S rRNA gene. The 20 µl reaction mixture contained the diluted buffer 1X, 1.5 mM $MgCl_2$, 0.2 mM of a mixture of dNTPs, 0.3 µM of each primer, 0.8 U of Taq polymerase and 1 to 3 µl template DNA. Thermal cycle was 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, with a final elongation step of 10 min at 72°C. PCR products were conveniently diluted 10, 100 or 1000 folds and used as a template for the nested amplification of a smaller fragment of the 16S rRNA gene comprising the variable regions from 3 to 5. For this second PCR step, primers 907R and 357F with a GC-clamp were used (12). The nested PCR approach is necessary to overcome the aspecific amplification of the 18S rRNA gene of the insect host (13). PCR was performed in 0.2 ml tubes with a 50 µl reaction volume. The reaction mixture contained the diluted buffer 1X, 1.5 mM $MgCl_2$, 5% DMSO, 0.12 mM of a mixture of dNTPs, 0.3 µM of each primer, 1 U Taq polymerase and 2µl of diluted template. Cycling conditions were: 94°C for 4 min, followed by 10 cycles of 94°C for 30 sec, 61°C for 1 min and 72°C for 1 min; followed by a further 20 cycles of 94°C for 0.5 min, 56°C for 1 min and 72°C for 1 min; and a final extension at 72°C for 7 min. Three microliters of a 1:5 dilution of the PCR product were visualized and quantified by electrophoresis in 1.5% agarose gel. About 80 ng of the PCR product from each sample was loaded on a polyacrylamide gel (8% of a 37:1 acrylamide–bisacrylamide mixture in a Tris acetate EDTA 1X buffer -TAE-, 0.75 mm thick, 16 × 10 cm, with a 40–60% or 35-55% denaturant gradient). The gels were run for 17 hours at 90 V in TAE 1X buffer at 60°C in a DCode apparatus (Bio-Rad, Milan, Italy) and subsequently stained with 1X Sybr Green

(Life Technologies) in TAE buffer and visualized in a Gel-Doc system (BioRad). The DGGE bands were excised from the gel using a sterile scalpel and eluted in 50 µl water at 37°C for 6h. The DNA eluted from DGGE bands was PCR amplified using 907R and 357F primers without the GC-clamp, with the following protocol: 95°C for 5 min, 30 cycles of 95°C for 1 min, 56°C for 1 min, 72°C for 1 min and a final extension at 72°C for 10 min. Fragment sequencing was performed by Macrogen (South Korea), and the sequences were identified as described for isolates.

Statistical analysis of DGGE results

According to the band profiles and identifications, we compiled a presence-absence matrix describing the bacterial genera retrieved in each gut section of each specimen analysed (Figure 3). This matrix was used to calculate a resemblance matrix based on the Sorensen distance, describing the dissimilarity between each possible pair of samples. Thus, the data were analysed using the PERMANOVA method, implemented in the PRIMER6 software package (14). PERMANOVA is a permutational, multivariate analysis of variance. In this case, the variables were represented by the genera identified in each gut fraction. On one hand, we assessed the effect of the diet in shaping the bacterial community of the gut fractions (main test) and the diversity between pairs of dietary groups (pairwise test). On the other hand, we assessed the effect of the gut tract (foregut, midgut or hindgut) in influencing the composition of the gut community (main test) comparing each possible combination of pairs of gut tracts (pairwise test).

Isolation of bacterial strains

The isolation of cultivable bacteria was performed using *P. h. hilaris* larvae of the third and fourth larval instar, collected from the environment. Insects were anesthetized by incubation in ice, surface-sterilized with one washing in 70% ethanol and two washings in sterile water, and dissected with sterile instruments near a Bunsen burner. Guts were collected in Eppendorf tubes and smashed using a sterile pestle, in saline.

Serial dilutions of the homogenates were plated directly on selective agar media or inoculated in liquid enrichment media, and incubated at 30°C. An aliquot of the enrichment media was serially diluted and plated as soon as bacterial growth could be observed by checking the turbidity of the medium. Enrichment media with cellulose, paper or pectin (15) contained: $NaNO_3$ 2.5 g/l, KH_2PO_4 2 g/l, $MgSO_4$ 0.2 g/l, NaCl 0.2 g/l $CaCl_2$ ($6H_2O$) 0.1 g/l and, respectively, 2 g carboxy methyl cellulose (CMC, Fluka), or 1/8 Whatman filter paper n°1, or 2 g polygalacturonic acid (Sigma-Aldrich). A different type of pectin enrichment was made according to Compant et al. (16) ($(NH_4)SO_4$ 4 g/l, NaCl 0.1 g/l, $MgSO_4$ 0.1 g/l, $CaCl_2$ 0.1 g/l, yeast extract 0.5 g/l, Fe(III)EDTA 0.33 g/l, potassium phosphate buffer pH 7 0.05 M, polygalacturonic acid 5 g/l). Turbid cultures from CMC and paper enrichments were plated on KH_2PO_4 0.5 g/l, $MgSO_4$ 0.25 g/l, gelatin 2g/l, CMC 2 g/l, agar 15 g/l (15). Turbid cultures from pectin enrichments were plated on the same medium by Compant et al. (16) by adding agar 15 g/l.

For a second isolation trial a pool of three intestines from field-caught *P. h. hilaris* larvae was used. In this case the whole procedure was performed identically both in aerobic and anaerobic condition using an anaerobic chamber with a 10% H_2, 5% CO_2, 85% N_2 atmosphere. The intestines were smashed in a reducing solution (gelatin 2 g/l, cysteine 0.5 g/l, $CaCl_2$ 2.6 g/l, $MgSo_4$ 2 g/l, K_2HPO_4 10 g/l, KH_2PO_4 10 g/l, NaCl 20 g/l), serially diluted in the same solution and plated on BHI medium (Fluka). An aliquot of the gut homogenate was inoculated in two 30 ml vials of enrichment medium (EM) (bacto-tryptone 1 g/l, yeast extract 0.5 g/l, cysteine 1 g/l, K_2HPO_4 1.2 g/l, Na_2HPO_4 2 g/l, soil extract 100 ml/l) containing 1 g of *Ficus carica* sawdust and 2 cm^2 of Whatman filter paper n°1. The cultures were incubated at 30°C. The enrichment vials were kept at 30°C with shaking until we observed the degradation of paper. Hence, 3 ml of the culture were diluted in 30 ml of medium in a new vial. This procedure was repeated three times; after that, the culture kept in aerobic conditions, which still showed paper degradation, was plated on three different media: EM plus agar, EM plus agar without sawdust or EM plus agar with glucose 10 g/l instead of CMC and sawdust. The anaerobic colture did not show paper degradation and therefore was not plated. All the isolation and enrichment media were supplemented with cicloexymide 0,01%. For the purification of the isolated strains we picked single colonies from the plates and we streaked them on a fresh plate of the same medium using a sterile inoculating loop. The process was repeated three times. Each strain was preserved by adding glycerol to a saturated liquid culture to the ratio of 20% and keeping it at -80°C.

Identification of isolates

DNA was isolated from bacteria by boiling lysis. A loopful of bacterial cells was suspended in 50 µl of sterile TE (10 mMTris/HCl, pH 8, 1 mM EDTA), incubated at 100°C for 8 min and centrifuged at 13000g for 10 min in a benchtop centrifuge. The supernatant was stored at –20°C and used as template for PCR amplification. The strain collection was analysed by fingerprinting of the rRNA 16S-23S Intergenic Transcribed Spacer (ITS) region. PCR protocol is the same as described by Cardinale et al (17). The fingerprinting profiles were visualized by performing gel electrophoresis of the PCR products on a 1.5% agarose gel. Isolates which showed the same banding pattern were grouped in ITS haplotypes. One or two isolates of each ITS profile had been identified, forward sequencing the 16S rRNA gene after amplification with universal primers 27F and 1492R. The sequencing was performed by Macrogen Inc. (Korea) with the Sanger method. The sequences were classified by comparison of three different methods: BLAST search in the GenBank database, RDP classifier (18) and SINA aligner and classifier (19).

Screening of the activities of isolates

102 strains encompassing all the haplotypes identified by ITS-PCR were selected for further analysis.

Urease test. The strains were grown overnight in Tryptic Soy Broth (TSB, Difco) medium. Cells were harvested by centrifugation from 500 µl of culture and washed twice in sterile saline. The cell pellet was suspended in 470 µl of B

solution (KH_2PO_4 0.1 g, K_2HPO_4 0.1 g, NaCl 0.5g, phenol red 0.2% solution 1ml, distilled water 100 ml, $NiCl_2$ 5µM) and 30 µl A solution (urea 2 g, ethanol 95% 2 ml, distilled water 4 ml). The suspension was incubated at 30°C overnight and then colour was checked. Positive strains showed a colour change from yellow to bright pink (20).

Uric acid degradation test. Five microliters of an overnight TSB culture of each strain were spotted on a Nutrient Broth (DIFCO) agar plate with the addition of 0.5 % uric acid (modified from Morales-Jimènez et al. (21)). The medium has a milky appearance because uric acid is not soluble in this pH condition (pH about 6.8). After 24 hours incubation at 30°C, positive colonies showed a transparent halo due to the degradation of the uric acid.

EPS (Exopolysaccharides) production test. The strains were streaked on the sucrose-rich agar medium from Santaella et al. (22) and incubated at 30°C for five days. The EPS-producing bacteria showed a glossy appearance due to the extracellular polysaccharides.

Nitrogen fixation. We tested the presence of the nitrogenase gene with the PolF – PolR primer pair (23). The PCR reaction was performed in 25 µl with 1 µl of DNA solution, Taq polymerase (Invitrogen) 0.6 U/reaction, DNTPs 0.2 mM, primers 0.3 µM, Taq buffer 1X and DMSO 5%. The thermal cycle was 94° for 4', 30 cycles of 94°, 54° and 72° for 1'each, 70° for 7'. Genomic DNA extract from *Azospirillum brasiliensis* was used as a positive control. Bands of the expected length (400 bp) were cut from the gel and sequenced to verify the presence of nitrogenase gene.

Protease production. Five microliters of overnight culture in TSB of each strain were spotted on milk agar (casein 5 g/l, glucose 1 g/l, yeast extract 2.5 g/l, dehydrated skimmed milk 35 g/l). The plates were incubated at 30° overnight. Positive strains showed a clear transparent halo around the colony.

Cellulose and xylan degradation. Cellulose and pectin degradation were assayed as described in Compant et al. (16). Briefly, strains were streaked on CMC or xylan–rich media. After five days, bacteria were removed and the plates were stained with Congo Red. Positive result was detected as a discolouration halo on Congo red plates.

Pectin degradation. According the method used by Park and co-workers (9) the strains were grown on R2A agar medium supplemented with polyglacturonic acid (Sigma-Aldrich). The pH of the medium was adjusted to 7. After the growth, the plates were flooded with a 1% n-hexadecyltrimethylammonium bromide solution (CTAB) to observe the clear halos around the colonies that are indicative of pectin degradation.

Ammonia production. The release of ammonia was measured as described in (24). Bacteria were grown for 72 hours on peptone water, then Nessler's reagent was added (500 µl in 5 ml of culture). The color change was then evaluated: if the bacterium produces ammonia the culture turns orange-brown, while in the other cases it is bright yellow.

Inhibition by Streptomyces isolate. Streptomyces strain 44 was inoculated on a TSB agar plate in order to trace the diameter of the plate, and incubated at 30° for 36 or 48h. Then, another strain was inoculated perpendicularly and let for additional 48 h at 30°. Strains inhibited by 44 should not grow in the vicinity of *Streptomyces*' colonies on the diameter of the plate.

Utilization of sugars. Thirty strains able to grow on Yeast Nitrogen Base (YNB, Fluka) medium were selected for this test. Each strain was grown overnight in Tryptic soy broth (TSB) medium at 30°C. Five hundred microliters of culture were harvested by centrifugation (4°C, 4000g) and washed twice in saline. Then, the cells were suspended and diluted 1:10 in saline. YNB medium was prepared without carbon sources or with different sugars: glucose (1%), xylose (1%), arabinose (1%), cellobiose (1%) and CMC (0.5%). The pH of the medium was adjusted to 7. The test was performed in 384-wells plates using a microplate spectrophotometer (Tecan). Two microliters of cell suspension were diluted in 100 µl of medium in each well. Each combination of bacterial strain/carbon source was repeated in four separate wells. Four wells for each medium remained without any inoculum as a negative control. The optical density (O.D.) at the wavelength of 600 nm of each well was measured every 15 minutes for 48 h. Growth curves were obtained by averaging the O.D. values of the four replicates and subtracting the value of the negative control for each measurement. For every strain, the growth curves with sugars were compared to absorbance profile in the YNB medium without inoculum. The plate filling was performed by the EpMotion liquid handler (Eppendorf).

Results

DGGE analyses

To assess the effect of the diet on the microbiota of the three parts of the gut, we run three separate DGGE gels for the foregut, midgut and hindgut of the same 12 larvae (Figure 3). The larvae have been divided in four groups according to the diet:

1) Field-caught larvae
2) Larvae from the laboratory breeding, fed on an artificial diet for generations. The diet includes chloramphenicol and, as preservatives, sorbic acid, propionic acid and β-sitosterol.
3) Larvae fed on artificial diet without chloramphenicol and preservatives for 10 days after field collection.
4) Larvae fed on artificial diet without chloramphenicol, but with preservatives, for 10 days after field collection.

According to the band profiles, the larvae of the first two groups showed a richer bacterial community, particularly in the foregut and midgut (Figure 3). The bands were cut to amplify and sequence the DNA and identify bacterial genera. Respectively 10, 10, 5 and 6 genera had been identified in the four groups (Figure 3).The PERMANOVA test identified the diet as an important factor to shape the microbial community in the gut tracts (Pseudo-F= 2,6701, df=3,35, p=0.0074) (14). More in detail, pairwise tests revealed that there was no significant difference between groups 1-2 and 3-4 (PERMANOVA p-hpt test), while in all the other group comparisons the differences were significant (Table 1). A significant difference can be also detected among the three gut tracts (PERMANOVA, Pseudo-F=2,3213, df=2,35, p=0,0325). In this case, the pairwise tests showed that the hindgut differed from the midgut, while all the other comparisons were not significant (Table 2).

Phylum	Genus	group 1								group 2						group 3								group 4						Bands		
		20			23			26			40			42			46			17			31			102			29			36			101			gel F	gel M	gel H
		f	m	h	f	m	h	f	m	h	f	m	h	f	m	h	f	m	h	f	m	h	f	m	h	f	m	h	f	m	h	f	m	h			
Proteobacteria	*Erythrobacteriaceae bacterium*				x	x	x		x	x	x	x	x	x	x	x																			8	3, 8, 14	6
Proteobacteria	Acetobacter							x	x																										31	22	
Proteobacteria	Gluconobacter								x																											35	
Proteobacteria	Methylobacterium				x						x			x																					2, 15	
Proteobacteria	Ralstonia	x	x	x							x	x		x																x					3	32	
Proteobacteria	*Oxalobacteriaceae bacterium*										x			x																						19	
Proteobacteria	*Comamonadaceae bacterium*													x																						17	
Proteobacteria	Enterobacter																x				x	x					x	x							24	22, 19	
Proteobacteria	Klebsiella		x	x				x																											5	1, 2, 3, 8, 9, 10, 32, 33	
Proteobacteria	Pseudomonas																						x						x						28, 30		
Firmicutes	Staphylococcus				x			x															x													28	
Firmicutes	Bacillus					x								x			x																		10, 11, 12, 13, 14		
Firmicutes	Leuconostoc																			x				x			x	x							21	26, 27	
Firmicutes	Enterococcus	x	x	x	x						x						x	x	x		x		x			x			x	x	x				22, 34, 40	33, 34, 40	15, 16, 23, 24, 30, 39
Firmicutes	Lactobacillus																									x									20		
Bacteroidetes	Flavobacterium				x																															25	
Acidobacteria	*Acidobacteria Gp6 bacterium*													x																						18	
Actinobacteria	Propionibacterium										x						x												x						16		21
	Chloroplast																																		5		29

Figure 3: (previous page). DGGE gels for the three larval gut tracts (foregut, midgut and hindgut). In the bigger squares, the ID of the larval specimens (white) and the diet group (black). Small squares and numbers identify the bands that have been cut and sequenced. In the table, a presence/absence matrix describing the distribution of the identified genera in the gut tracts. In the last column of the table, the numbers refer to the band numbers reported in the gel image. "f" refers to foregut, "m" to midgut and "h" to hindgut.

Groups	t	P(MonteCarlo)
1, 2	0,7905	> 0,05
1, 3	2,1113	0,009
1, 4	1,6632	0,0462
2, 3	2,3686	0,0035
2, 4	1,7502	0,0326
3, 4	< 0,01	> 0,05

Table 1: PERMANOVA post-hoc pairwise test to evaluate the contribution of the diet to explain the overall bacterial diversity in the gut tracts.

Groups	t	P(MonteCarlo)
foregut, midgut	1,1092	0,2927
foregut, hindgut	1,6463	0,0611
midgut, hindgut	1,8324	0,0181

Table 2:. PERMANOVA post-hoc pairwise test to evaluate the contribution of the gut section (foregut, hindgut, midgut) to explain the overall bacterial diversity in the gut tracts.

The most represented genera in the larvae of 3 and 4 group were *Enterococcus* and *Leuconostoc*, belonging to the phylum Firmicutes, while in the groups 1 and 2 Proteobacteria was the more abundant phylum, represented by the genus *Ralstonia* and by a bacterium of the Erythrobacteraceae family.

Isolation and screening of bacterial strains

In total, 210 bacterial strains have been isolated from 6 *P. h. hilaris* larval guts. Bacteria had been isolated from three individual specimens (ID codes 41, 43, 45) on selective media to enrich the culture with cellulose, lignin and pectin degraders. Three of the guts (88, 89, 90) were pooled together, smashed in an anaerobic chamber and used to perform bacterial isolations in aerobic and anaerobic conditions, following the same protocol, on the nutrient-rich medium BHI. We counted approximately $1{,}3 \times 10^6$ CFU/gut for the anaerobic trial and $2{,}3 \times 10^6$ CFU/gut for the aerobic trial. However, none of the isolates is strictly anaerobic, as they are able to grow even in the presence of oxygen. Among the isolates we identified 31 genera, belonging to the phyla Proteobacteria (52% out of the total isolates), Actinobacteria (32%) Firmicutes (13%) and Bacterioidetes (3%).The most abundant orders in the collection are represented by Enterobacteriales (32%) and Actinomycetales (21%). Together

with Bacillales (9%), the Actinomycetales are present in all the four samples (Figure 4).

Screening of the isolates. From the total bacterial collection, two representatives for each ITS profile (one in the case of unique strains, 102 strains overall) had been selected for the screening of the "symbiotic potential" activities, i.e urea degradation, uric acid degradation, protease secretion, nitrogen fixation, ammonia production, EPS production, cellulose degradation, pectin degradation, xylan degradation. The results are summarized Table 3. All the isolates belonging to the genus *Streptomyces* showed a clear halo upon Congo-red staining, both for the xylan and the CMC degradation tests. Furthermore, they were able to degrade pectin. To better identify these isolates, forward sequencing of the 16S rRNA gene was combined with reverse sequencing (1492R) to obtain the nearly full length of the 16S rRNA gene. All the sequences can be aligned obtaining 99,5% identity, thus being referable to a single species that was identified by BLAST search as *Streptomyces thermocarboxydus* (GenBank accession of the closest relative: KJ571048.1). *Streptomyces* isolate 44 has been tested for inhibitory effects towards all the 102 members of the strain selection, giving negative results in all cases.

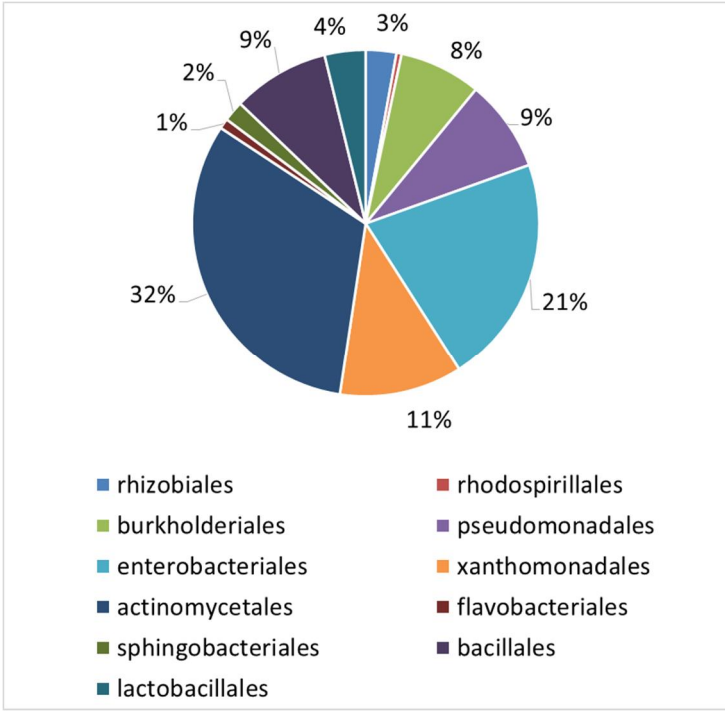

Figure 4: Distribution of the isolates in the collection according to the order.

Utilization of different carbon sources. A selection of 31 strains has been tested for growth with the following sugars as the only carbon source:

glucose, arabinose, xylose, cellobiose and CMC. The strains have been selected according to the species (determined by the closest relative found by BLAST search) and to their capability to grow on the YNB medium. Controls had been performed using the same growth medium without any sugar. We considered as a positive result all the growth curves above the threshold determined by the negative control. Results are summarized in Table 4.

Isolate ID	genus	urea degradation	uric acid degradation	protease secretion	nitrogen fixation	ammonia production	EPS production	cellulose degradation	pectin degradation	xylan degradation
70	*Corynebacterium*	+	-	-	-	-	-	-	-	-
75	*Corynebacterium*	+	-	-	-	-	-	-	-	-
154	*Corynebacterium*	+	-	-	-	-	-	-	-	-
156	*Corynebacterium*	+	-	-	-	-	-	-	-	-
173	*Corynebacterium*	+	-	-	-	++	-	-	-	-
174	*Corynebacterium*	+	-	-	-	-	-	-	-	+
175	*Corynebacterium*	+	-	-	-	-	-	-	-	-
208	*Gordonia*	-	-	-	-	-	-	-	-	-
106	*Tsukamurella*	-	-	-	-	-	-	-	-	-
138	*Tsukamurella*	+	-	-	-	-	-	-	-	-
223	*Tsukamurella*	-	-	-	-	++	-	-	-	-
105	*Rhodococcus*	-	-	-	-	-	-	-	-	-
181	*Rhodococcus*	+	-	-	-	-	+	-	-	-
77	*Brevibacterium*	-	-	++	-	++	-	-	-	-
94	*Brevibacterium*	-	+	-	-	++	-	-	-	-
97	*Brevibacterium*	-	-	-	-	++	-	-	-	-
99	*Brevibacterium*	-	+	-	-	++	-	-	-	-
160	*Brevibacterium*	-	-	-	-	++	-	-	-	-
162	*Brevibacterium*	-	-	-	-	++	-	-	-	-
184	*Brevibacterium*	-	-	-	-	++	+	-	-	-
192	*Brevibacterium*	-	-	-	-	++	-	-	-	-
200	*Brevibacterium*	-	-	-	-	++	-	-	-	-
207	*Brevibacterium*	-	-	-	-	++	-	-	-	-
25	*Microbacterium*	-	+	-	-	++	-	-	-	-
32	*Microbacterium*	-	-	-	-	++	-	-	-	-
198	*Microbacterium*	-	-	-	-	++	-	-	-	-
60	*Micrococcus*	-	-	++	-	++	-	-	-	-
61	*Micrococcus*	-	-	-	-	++	-	-	-	-
44	*Streptomyces*	-	-	-	-	-	-	+	+	+
51	*Streptomyces*	-	-	-	-	++	-	+	+	+
68	*Streptomyces*	-	++	-	-	+	-	+	-	+
197	*Streptomyces*	-	-	-	-	++	-	+	-	+
276	*Ochrobactrum*	+	-	-	-	++	-	-	-	-
121	*Rhizobium*	+	-	-	-	++	+	-	-	+
122	*Rhizobium*	-	-	-	-	++	+	-	-	-

Isolate ID	genus	urea degradation	uric acid degradation	protease secretion	nitrogen fixation	ammonia production	EPS production	cellulose degradation	pectin degradation	xylan degradation
158	*Rhizobium*	-	-	-	-	++	-	-	-	-
125	*Agrobacterium*	+	-	-	-	++	+	-	-	-
193	*Roseomonas*	+	-	-	-	+	-	-	-	-
10	*Comamonadaceae Bacterium*	+	-	-	-	-	-	-	-	-
17	*Comamonadaceae Bacterium*	+	-	-	-	-	-	-	-	-
40	*Comamonadaceae Bacterium*	+	-	-	-	-	-	-	-	-
55	*Comamonadaceae Bacterium*	+	-	-	-	-	-	-	-	-
258	*Comamonadaceae Bacterium*	-	-	-	-	+	-	-	-	-
259	*Comamonadaceae Bacterium*	-	++	-	-	+	-	-	-	-
261	*Comamonadaceae Bacterium*	-	-	-	-	-	-	-	-	-
277	*Bacterium*	-	++	-	-	+	-	-	-	-
54	*Comamonas*	+	-	-	-	-	-	-	-	-
111	*Acidovorax*	-	+	-	-	+	-	-	-	-
112	*Acidovorax*	-	+	-	-	+	-	-	-	-
229	*Enterobacteriaceae Bacterium*	-	-	-	-	++	-	-	-	-
210	*Enterobacter*	-	-	-	-	+	+	-	-	-
204	*Enterobacter*	-	-	-	-	++	+	-	-	-
224	*Enterobacter*	-	-	-	-	+	+	-	-	
225	*Enterobacter*	-	-	-	-	++	+	-	-	-
251	*Enterobacter*	-	-	-	-	++	+	-	-	-
269	*Enterobacter*	-	-	-	-	++	+	-	-	-
246	*Klebsiella*	+	-	-	+	++	+	-	+	-
256	*Klebsiella*	+	-	-	+	+	+	-	+	-
90	*Rahnella*	-	-		-	+	+	-	-	-
186	*Rahnella*	-	-	-	-	++	+	-	-	-
187	*Rahnella*	+	-	-	-	+	+	-	-	-
189	*Rahnella*	-	-	-	-	++	+	-	-	-
190	*Rahnella*	-	-	-	-	+	+	-	-	+
191	*Rahnella*	-	-	-	-	+	+	-	-	+
2	*Enhydrobacter*	-	-	-	-	++	-	-	-	-
5	*Enhydrobacter*	-	-	++	-	-	-	-	-	-
15	*Acinetobacter*	+	-	-	-	-	-	-	-	-
58	*Acinetobacter*	+	-	-	-	-	-	-	-	-
116	*Acinetobacter*	+	-	-	-	-	-	-	-	-
52	*Pseudomonas*	-	+	++	-	++	+	-	+	-
120	*Pseudomonas*	-	-	++	-	++	+	-	+	+
238	*Pseudomonas*	-	-	++	-	++	+	-	-	+
249	*Pseudomonas*	-	-	++	-	++	+	-	-	+

Isolate ID	genus	urea degradation	uric acid degradation	protease secretion	nitrogen fixation	ammonia production	EPS production	cellulose degradation	pectin degradation	xylan degradation
252	*Pseudomonas*	-	++	-	-	++	-	-	-	-
273	*Pseudomonas*	-	++	++	-	++	+	-	-	+
12	*Stenotrophomonas*	-	-	++	-	++	-	-	-	-
16	*Stenotrophomonas*	-	-	++	-	++	-	-	-	-
56	*Stenotrophomonas*	-	-	++	-	++	-	-	-	-
57	*Stenotrophomonas*	-	-	++	-	++	-	-	-	-
218	*Luteimonas*	-	-	-	-	++	-	-	+	-
151	*Olivibacter*	-	-	-	-	++	+	-	-	-
152	*Olivibacter*	-	-	-	-	++	+	-	-	-
255	*Sphingobacterium*	-	-	-	-	++	-	-	-	-
134	*Epilithonimonas*	-	-	-	-	++	-	-	+	-
137	*Epilithonimonas*	-	-	-	-	++	-	-	-	-
76	*Bacillus*	-	++	-	-	++	-	-	-	-
4	*Staphylococcus*	+	-	-	-	-	-	-	-	-
41	*Staphylococcus*	-	-	-	-	++	-	-	-	-
67	*Staphylococcus*	+	-	-	-	++	-	-	-	-
69	*Staphylococcus*	+	-	-	-	+	+	-	-	-
203	*Staphylococcus*	+	-	-	-	+	-	-	-	-
219	*Staphylococcus*	-	-	-	-	+	-	-	-	-
220	*Staphylococcus*	-	-	++	-	++	-	-	-	-
222	*Staphylococcus*	-	-	++	-	++	-	-	-	-
236	*Staphylococcus*	+	-	-	-	+	-	-	-	-
232	*Enterococcus*	-	-	-	-	-	-	-	-	-
209	*Lactococcus*	-	-	++	-	+	-	-	-	-
228	*Lactococcus*	-	-	+	-	+	-	-	-	-
233	*Lactococcus*	-	-	+	-	+	-	-	-	-
237	*Lactococcus*	-	-	+	-	+	-	-	-	-
243	*Lactococcus*	-	-	+	-	+	-	-	-	-
248	*Lactococcus*	-	-	-	-	+	-	-	-	-

Table 3: Results of the screening of 102 isolates for the "symbiotic potential" activities.

Discussion

DGGE

The bacterial communities of larvae reared for ten days on a diet without antibiotics (groups 3 and 4) seem to be very poor and dominated by *Enterococcus* and *Leuconostoc*. These species probably overgrew on the diet without antibiotics, and entered the insect gut in large quantities together with the ingested food, replacing the native microflora. An identical situation

has been observed in a paper by Montagna et al. (submitted and presented in Chapter 4) in which it is shown that in *Rhinchophorus ferrugineus* specimens fed on apple for a month the community evenness dramatically decreases, with the growth of bacteria that are typical of rotting fruit (as for example Acetobacteraceae and Lactobacillaceae members). Surprisingly, the *P. h. hilaris* larvae from the laboratory breeding, grown for generations on an artificial diet complemented with chloramphenicol and preservative compounds, seem to host a bacterial community that resembles much better the microflora from the field-caught larvae.

isolate ID	genus	glucose	arabinose	xilose	cellobiose	CMC
70	*Corynebacterium*	+	+	-	-	-
223	*Tsukamurella*	+	-	-	-	-
99	*Brevibacterium*	+	-	-	-	-
184	*Brevibacterium*	+	+	+	+	-
200	*Brevibacterium*	+	-	-	-	-
25	*Microbacterium*	+	+	+	+	-
61	*Micrococcus*	+	+	+	+	-
44	*Streptomyces*	+	+	+	+	+
51	*Streptomyces*	+	+	+	+	+
276	*Ochrobactrum*	+	-	-	-	-
121	*Rhizobium*	+	+	+	+	-
125	*Agrobacterium*	+	+	+	+	-
10	*Comamonadaceae bacterium*	+	-	-	-	-
40	*Comamonadaceae bacterium*	+	-	-	-	-
54	*Comamonas*	+	-	-	-	-
186	*Rahnella*	+	+	+	+	-
191	*Rahnella*	+	+	+	+	-
204	*Enterobacter*	+	+	+	-	-
229	*Enterobacteriaceae bacterium*	+	+	+	-	-
246	*Klebsiella*	+	+	+	+	-
251	*Enterobacter*	+	+	+	+	-
15	*Acinetobacter*	+	+	-	-	-
52	*Pseudomonas*	+	+	+	-	-
238	*Pseudomonas*	+	+	-	-	-
151	*Olivibacter*	+	+	+	+	-
134	*Epilithonimonas*	+	+	-	-	-
255	*Sphingobacterium*	+	+	-	+	-
76	*Bacillus*	+	-	-	-	-
219	*Staphylococcus*	+	+	+	+	-
220	*Staphylococcus*	+	-	-	+	-

Table 4. Results for the screening of 30 isolates for the growth on different carbon sources.

This could be explained with the low amount of antibiotic in the diet and the insurgence of resistance phenomena. In fact, the preparation of the diet requires cooking, a step that possibly lowers the effective concentration of chloramphenicol. Thus, the latter seems to be enough to prevent the bacterial proliferation on the diet itself, but could allow the appearance of resistant cells among the native microbiota in the gut. The bacterial community in the larvae from the rearings and in the ones collected directly from field is richer, comprising mainly Alpha- and Beta-Proteobacteria.
Regarding the gut tracts, a significant difference has been shown between the midgut and the hindgut. Indeed, in midguts and, to a lesser extent, in foreguts there was a higher diversity of Proteobacteria, while hindguts were dominated by *Enterococcus*, belonging to the Firmicutes phylum. There are many possible explanations to this difference: in the hindgut of *P. h. hilaris* a higher pH has been measured than in the foregut and midgut (8). Moreover, the Malpighian tubules deliver the nitrogen waste collected in the hemocoel at the cephalic border of the hindgut. Thus, the environment in this tract is characterized by a mix of nitrogen and food waste.
The group "Erythrobacteriaceae" is largely represented in foreguts and midguts of both wild and reared larvae (groups 1 and 2). This family, comprised in the Sphingomonadales order, has been recently separated from Sphingomonadaceae (25): it comprises rod-shaped bacteria that have been mainly isolated from freshwater and that contain pigments as bacterio-chlorophyll *a* and carotenoids. Sphingomonadaceae are known for their catabolic flexibility, as they are able to degrade a broad range of recalcitrant compounds (26). These catabolic competences are largely encoded in "megaplasmids", which are also transferred by conjugation. All the sequences have been loaded on the pre-constructed tree of the Non Redundant 16S database downloaded from the Silva website (SSURef_NR99_119_SILVA_14_07_14) using the maximum parsimony tool of the ARB software. Interestingly, one of the sequences identified as *Klebsiella* in the hindgut is closely related to two sequences previously identified as *Candidatus* Nardonella by Rinke and colleagues (27) (accessions FJ626254 and FJ626262). *Nardonella* is an endosymbiont of coleoptera of the family Curculionidae, which comprises many wood-feeding insects. Similarly, other two *Klebsiella* sequences seem to be close to *Candidatus* Annandia pinicola, an endosymbiont of the pine bark adelgid *Pineus strobi* (28) (accession KC64418). In both cases, the bands of interest were not present in all the individuals in analysis. During the dissection, attention was paid to detect possible bacteriomes, but it we did not find any. In spite of this, the abovementioned bacterial endosymbionts (*Ca.* Nardonella and Annandia pinicola) can be hosted in mycetocytes intersperse in the fat body or attached to the gut external epithelium. Thus, it is possible that mycetocytes have been effectively collected in some samples and not in others. For these reason, further analyses are required to verify any hypothesis about endosymbionts in *P. h. hilaris*.
In contrast to the bacterial isolation, that lead to the identification of nine genera of Actinobacteria, only one representative of this phylum (*Propionibacterium*) has been detected by the DGGE. There are two possible

explanations to this observation. On one side, the extraction of DNA from some Actinobacteria, in particular Actinomycetales, could be problematic(29). On the other side, these bacteria could survive inside the gut only as spores.

Isolation and screening of bacterial strains

Most of the isolates in our collection belong to Gamma-Proteobacteria. This class is widespread in the guts of insects. However, none of these genera was present in all the samples analysed. Actinomycetales were present in all the *P. h. hilaris* samples with 9 identified genera. Among them, *Brevibacterium* was present in all the four samples, while *Corynebacterium* and *Streptomyces* were present in three of them. The absence of *Streptomyces* in the 88-89-90 sample could be due to the different isolation method used for this sample. Notably, the Actinobacteria bacteria could persist in the gut as spores. In this case, they do not participate in a symbiotic relationship with the host but they could exploit the insect as a vector to spread over the plant tissues.

Despite the isolation trial made in the anaerobic chamber, no strictly anaerobic bacterium was found, but some strains able to grow both in aerobic and anaerobic conditions identified as *Enterobacter, Enterobacteriaceae bacterium* and *Lactococcus*. This suggests that the gut environment in *P. h. hilaris* could be aerobic (in contrast with the observations made by Ceja-Navarro and colleagues on another large wood feeding beetle, *Odontotaenius disjunctus* (30)). Alternatively, we may think that the resident bacteria are all acquired randomly from the environment, in which they can survive in aerobic conditions, but some of them can as well survive in anaerobic niches in the gut.

One or two representatives for each strain in the collection had been tested for several activities that could favour the persistence in the gut of the host and the insurgence of a mutualistic relationship. In fact, though insect gut communities largely consist of environmental bacteria ingested with the food, only a small portion of these bacteria can persist, due to their colonization abilities and to the selection operated by the host immune system and physical-chemical conditions in the gut tract. Moreover, commensal bacteria must be able to exploit food sources present in the insect diet, which in this case is based on wood (5).

The xylem-based diet is regarded as poor in nitrogen, as the plant cell-wall proteins are generally not considered to be a sufficient nitrogen source, and the C:N ratio can be as high as 1000/1(6). Therefore, both larvae and commensals should have effective recycle systems or the ability to exploit atmospheric nitrogen. We tested the isolates for the presence of NifH gene for the fixation of nitrogen using PolF-PolR primer set (23). Only *Klebsiella oxytoca* (isolates tested 246 and 256) gave a positive result. All the total 11 isolates of *Klebsiella* belong to the 88-89-90 samples: this is possibly due to the different isolation medium used for these samples. *Klebsiella* has been identified also by DGGE in two individuals from the laboratory breeding. Regarding the nitrogen recycling, we tested our isolates for the ability to degrade urea and uric acid, that are, respectively, waste products of the bacterial and host metabolism. In insects, uric acid is collected from the tissues by the Malpighian tubules, that flow into the first tract of hindgut, near the midgut-

hindgut border. Uric acid can be metabolized by bacteria to urea, and subsequently to ammonia and carbon dioxide. Urea is also produced by bacteria as a by-product of purine and aminoacids metabolism. Notably, 29 of the 102 isolates tested (including the previously mentioned *Klebsiella oxytoca*) are able to degrade urea, and 12 are able to degrade uric acid. Furthermore, most of the isolates (78 out of 102) are able to excrete ammonia derived from the peptides catabolism during the growth on peptone-water. The secretion of proteases may further improve the availability of amino acids and peptides in the gut, allowing the exploitation of the proteins released from the plant cell walls after cellulose degradation (6) or from the microorganisms themselves. Nineteen isolates showed, to a different extent, the release of proteases in the extracellular medium.

About 73% of the dry weight of the mulberry tree wood on which larval *P. hilaris* feed is constituted by cellulose and hemicellulose (7). The major components of hemicellulose polysaccharides are xylan and xyloglucans. *P. h. hilaris* produces at least one cellulase that has been isolated by Sugimura et al. (8). Another study demonstrated the presence in *P. hilaris* gut extracts of degradative activities against several polysaccharides, comprising pectin, carboxymethyl-cellulose (CMC) and xylan (7). However, it is not clear whether these activities are due to the production of degradative enzymes by the insect itself or by its gut microflora. The isolates in our collection had been tested for CMC, pectin and xylan degradation, finding respectively 4, 8 and 12 degraders. We also tested the ability of the strains to grow on sugars derived from the degradation of plant cell walls as the only carbon source. In detail, xylose and arabinose result from the degradation of xylan, a major constituent of hemicellulose, while cellobiose is the dimeric unit of cellulose. Of the 30 strains tested, only two isolates of the same species (*Streptomyces thermocarboxydus)* grew on cellulose (CMC), while 15 grew on cellobiose, exploiting the degradative activity of the cellulases secreted by the host and by the *Streptomyces thermocarboxydus* strain. Regarding the xylan by-products, 21 strains grew on arabinose and 16 grew on both arabinose and xylose. Many strains grew on cellobiose at higher rates than on glucose (isolates 51, 151, 186, 193, 204, 219, 220, 225, 251, for identification see Table 4) or showed a well-defined preference for arabinose (15, 191 204, 219, 276) xylose (121, 125, 184, 251) or both (186, 229). In conclusion, only a few of these microorganisms can effectively degrade complex plant cell walls, but many of them can take advantage from the degradative processes carried out by other components of the microflora or by the insect itself.

Finally, we tested the capability of our strains to produce an exopolysaccharides (EPS). The secretion of EPS is an important step in the formation of biofilms, and could enhance the adhesion of bacteria and their persistence on the gut epithelium. Twenty-seven strains, mainly Gamma-Proteobacteria, showed a visible enhancement in the production of extracellular matrix upon growth on sucrose, proving their ability to produce EPS.

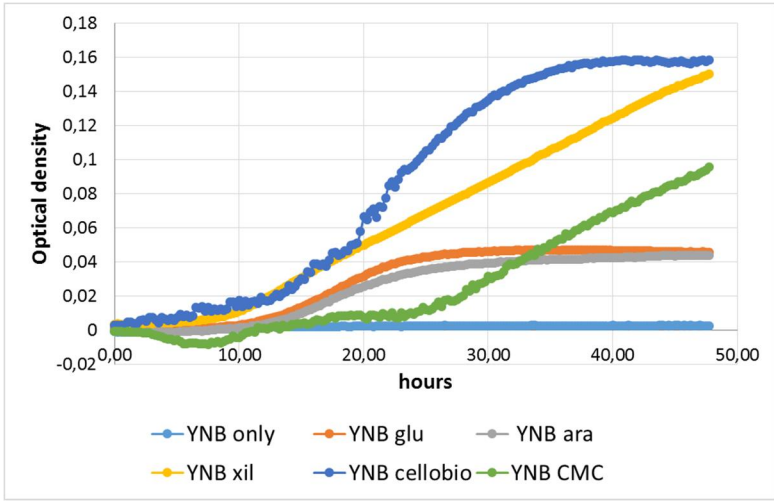

Figure 5: Growth curves of the isolate 44 (*Streptomyces thermocarboxydus*) on different carbon sources.

All the isolates of *Streptomyces* showed a wide range of degradative activities through plant sugar polymers (cellulose, pectin, xylan). This bacterium has been isolated in the first three specimens 41, 43 and 45, but not in the 88-89-90 pool, probably due to the different isolation method. On one side the nutrient-rich medium BHI favours the isolation of fast-growing gamma Proteobacteria and Staphylococci against the slow-growing *Streptomyces*. On the other side, from the enrichment medium in which we observed the degradation of the filter paper we did not isolate any cellulolytic bacterium, thus suggesting some bias in the isolation, possibly due to the overgrowth of other bacteria. *Streptomyces* can exploit as carbon sources all the tested substrates, showing a rapid growth especially on xylose and cellobiose. It is very efficient also in utilizing cellulose, though the growth starts lately, reflecting the time needed to degrade the long-chain polymer (Figure 5).
Given that many *Streptomyces* are able to secrete antibiotics, we tested our strain for inhibitory effects against the other strains in the collection, obtaining only negative results. On the contrary, the presence of this strain could enhance the survival of other commensal bacteria that grow on by-products of cellulose degradation. However, preliminary *in vivo* studies demonstrated that our *Streptomyces* strain doesn't have the ability to grow into larval gut and to give a selective advantage to colonized larvae (data not shown). These data suggest that it could persist in the gut as spores, exploiting the larvae as a reservoir to easily colonize the inside of trunks, in which it can easily grow on the wood matter due to its degradative abilities.

Conclusion

Both DGGE and isolation results depict *P. h. hilaris* bacterial gut community as a highly variable assemblage of environmental bacteria that are not shared

among different individuals. They can be regarded as commensals, which are able to persist in the insect gut and to efficiently exploit the food sources therein, as well as to use the insect as a reservoir to propagate in the environment. The latter mechanism can be hypothesized primarily for sporulating bacteria, as for example the isolate 44 (*Streptomyces sp.*). The high variability of this bacterial community is remarked by the analysis on the larvae fed with different diets, which show great changes in their bacterial community. Interestingly, the bacteria that overgrow on the diet, like *Leuconostoc*, seem to replace completely the resident community. Paradoxically, the addition of a certain quantity of antimicrobial compounds to the artificial diet, rather than erasing the native gut community, better preserves it. These results help to shed light on the biology of bacterial commensals, a group of symbionts that, up to now, has been poorly investigated (5). Indeed, there are a number of research papers that describe and compare variegate communities associated to insects, but do not formulate precise hypotheses about their function (e.g. (31, 32)). Alternatively, the activities of the microflora are investigated by metagenomics (6, 33), a very powerful method that, nevertheless, makes it difficult to associate taxonomy and function. Here, we propose to verify the "symbiotic potential" of a collection of bacterial isolates through direct *in vitro* tests, drawing inspiration to the well-established approach that is used to characterize PGP (plant-growth promoting) symbionts of plants (34). Although it is important to consider that the response of the bacteria to the gut environment could be very different from the *in vitro* behaviour and that these tests exclude the large fraction of uncultivable strains, our results show that this approach can give important information about the function of commensals. In *P. h. hilaris*, gut bacterial symbionts can directly contribute to the cellulose digestion or exploit the by-products of the degradation of cell wall compounds, possibly becoming themselves a valuable food source for the insect. Moreover, they help their host to absorb nitrogen, converting waste molecules (uric acid and urea) or proteins to ammonia and smaller peptides, or even fixing atmospheric nitrogen. From an evolutionary point of view, the ability to live into an insect host and to help its fitness and survival greatly advantages these environmental bacteria, which can multiply in the insect gut and rapidly spread through faeces in very distant and non-colonized habitats with similar ecological features.of the same type.

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Chapter 6

General conclusions

This thesis, with the three case-studies presented, gives a contribution to the knowledge of the structure and function of the bacterial communities hosted in insects. All the species presented are relevant pests, belonging to very different taxa and habitats. In spite of this, some commonalities can be found and general conclusions can be drawn by comparing the results of the three studies.
The most striking evidence that regards all the three examples is the effect of the different diets on the microbial colonization of the insects. In the *Drosophila suzukii* work, we did not analyse wild specimens, but flies reared on fruit and on an artificial diet were compared. Both pyrotag and Denaturing Gradient Gel Electrophoresis (DGGE) analyses showed a higher individual variability in insects reared on fruit, as well as a higher bacterial diversity. In the PCoA graph based on the Unifrac analysis of the pyrosequences, fruit-fed specimens clustered together, and the effect of the diet seemed to overcome the effect of the life stage in explaining the variance of the bacterial communities. A higher mean load of Lactobacillales was registered in flies fed on the diet, while in the others was immediately apparent the presence of *Wolbachia* and Flavobacteriales. In addition, red palm weevils, field-collected and reared on apple for one month, were analysed by pyrotag. In this case, the evenness and diversity of the bacterial communities hosted by wild specimens were remarkably higher. Fruit fed beetles hosted *Acetobacter* and members of the Lactobacillales order (*Lactobacillus*, *Lactococcus* and *Enterococcus*), which were absent in wild ones. On the contrary, they lacked bacterial taxa which are typical of soil and plants and that can be found in wild specimens (e.g. *Gordonia*, *Devosia*). Finally, the effect of the diet on the yellow-spotted longicorn gut microbiota was assessed by means of DGGE. The insects were fed with three diets, which exerted very different effects, allowing us to elucidate how the diet could shape the microbial community. In fact, the comparative DGGE analysis on larvae fed on a diet supplemented or not with antimicrobial compounds clearly suggested that some bacteria rapidly proliferated on the diet if the conditions were favourable (absence of antimicrobials) and they quickly replaced the resident microbial community as they were ingested by the insect. The overgrowing species were, in this case, *Leuconostoc* and *Enterococcus*. This kind of replacement presumably happened not only in *Psacothea hilaris hilaris*, but also in *Rhynchophorus ferrugineus*: *Acetobacter* and Lactobacillales colonized the apple, in which they could find an appropriate habitat. In *D. suzukii* study, although all the flies were reared in laboratory, the specimens fed on fruit retained a more variegate microbial community. This was probably because this kind of diet is very close to the natural one, thus, these flies could preserve a bigger proportion of the original microbiota. On the contrary, the microbiota of the flies fed on the artificial diet was replaced by the bacteria that better grew on

that medium. All these observation seem to support the hypothesis that the insect microbiota –particularly in the gut- is mainly acquired from the food.
In a natural situation, insects ingest many different bacteria, most of which can colonize the gut, creating a biodiverse, ever-changing community. According to the uneven distribution of the bacteria in the environment, these insects always show a higher individual variability compared to bred ones. Microorganisms exploit the insects to spread in the environments in which they feed and defecate, or they find a favourable niche in the insect itself. The "symbiotic potential" screening of the cultivable fraction of *P. h. hilaris* community allowed us to verify the hypothesis that most of these bacteria are adapted to exploit the food sources in the insect gut, and to give a contribution to the host fitness. Colonization of *Drosophila* flies with GFP-tagged acetic acid bacteria proved the bacterial ability to adhere to the intestinal walls and to the peritrophic matrix, initiating a stable association. All these features can further advantage environmental bacteria which use them to multiply and spread through insects. Thus, "commensals" are not only microorganisms that enter insects by chance, but they possibly evolve strategies to survive in the insect gut, getting selective advantages over the others.
An interesting paper by Stefanini and colleagues (1) illustrates the role of social wasps for the preservation, overwintering and diffusion of vineyard yeasts. The same mechanisms seem to happen, to a lesser extent, in guts of other insects, which preserve and spread a number of environmental bacteria. *D. suzukii* seems to have a role in inoculating fruits with acetic acid bacteria, which are responsible of the rotting. *Psacothea* carries a number of Actinomycetales, at least one of which is capable to degrade wood matter, and other bacteria that live on the by-products of wood degradation.
However, while we can make hypotheses regarding the presence of some of these bacteria, for many others the situation is more vague. Considering as a whole all the species identified by the isolations and by molecular methods, it is evident that for many we are not able to understand nor the function nor the reason why we found them in such a special niche. Speaking only about isolates, for which it is possible to assign a taxonomic classification with a higher degree of certainty, we found a number of species whose closest relatives are described as coming from very different environments. As an example, *Enhydrobacter sp.*, isolated from *P. h. hilaris,* has been first described in 1987 as an aquatic organism with a gas vacuole (*Enhydrobacter aerosaccus*, (2)). Originally affiliated to the Vibrionaceae, today it is considered part of the Moraxellaceae (even if a revision has been proposed (3)). From that moment, according to the papers recorded in the PubMed database, it has been identified twice in cheese (4, 5), once in human eyes (6), in the air of Louvre museum (7) and, finally, in the salivary glands of a mosquito (8). This is not an uncommon situation, showing how the biodiversity of bacteria that can be hosted and diffused by insects is higher than expected and yet poorly understood.
The composition of the bacterial communities within the three analysed insects can be compared also from a taxonomic point of view. Despite the collection of isolates have been obtained from different enrichment media,

the cultivable fraction is always dominated by gamma-proteobacteria, and, in particular, *Enterobacter* and other members of Enterobacteriales. Another common feature of the three collections is the presence of Lactobacillales. These two orders show a fast growth on culture media and are the first that colonize laboratory diets. Nevertheless, they have been detected also in wild specimens and in molecular studies, showing that their association with insects is not due exclusively to the laboratory rearing, but, probably, the insects themselves inoculate them in the diet, in which they find an optimal habitat. Insects fed on fruits (flies and reared weevils) share also a variegate acetic acid microbiota, for which the same considerations can be done.
The high difference between bacterial collections and the data coming from molecular studies outlines that a large fraction of the bacteria resident in insects is hardly cultivable. A detailed analysis of the phylogenetic placement of DGGE sequences from *P. h. hilaris* in the tree of SILVA non-redundant database revealed that in many cases they are placed in branches populated almost exclusively by uncultured bacteria. Thus, there are a number of poorly studied organisms: the knowledge of some of these does not go beyond the 16S sequence. Knowing the great importance of uncultivable primary and secondary symbionts for insects would be of great interest to deepen the knowledge about these bacteria.
In conclusion, the study of the microbiota of three different, non model insects with a wide range of techniques allowed us to draw a more precise picture of the cultivable and uncultivable microbial communities that live in wild insects and of some factors that influence their composition. Three important pests have been chosen for these studies, giving a contribution to the knowledge of economically relevant and invasive species. Although the aim of this thesis is not to solve the problem raising from the spreading of these insects, this contribution will hopefully help to effectively address it. Indeed, the study of the symbiotic microbiota and of its roles is an essential step for the understanding of the insect biology. Furthermore, a number of control strategies exist based on symbionts (9). While this work was mainly focused on the taxonomic characterisation of bacterial communities, the next step in the research on insect symbionts will be the in-depth investigation of the roles of these bacteria, particularly of commensals. In literature, there are a number of works describing insect gut microbiotas, but only a few succeeded in identifying and verifying hypotheses about the origin of the microorganisms, their functions and interactions with the host, or the reasons why they can be found in this niche. The scientific papers addressing these problems mainly focus on simplified consortia present in laboratory model organisms, such as *D. melanogaster* flies. These works shed light on the importance of commensal bacteria, which are not only casual “passengers”, but rather they can deeply modify the host physiology. However, despite their extraordinary importance for the discovery of general mechanisms of interaction and communication between gut bacteria and their eukaryotic hosts, these findings are only partially generalizable to wild insects. Indeed, microbial communities in insects from natural populations are dramatically more complex, and even in flies reared in different laboratories different

mechanisms of interaction with bacteria have been observed (compare (10) and (11)).
Thus, the relationship between commensal bacteria and insects in natural environments is still an unexplored and fascinating field, and this thesis represents only a starting point for its investigation.

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Supplementary materials

Chapter 4 – *Rhynchophorus ferrugineus*

	RPW palm_m1	RPW palm_f1	RPW palm_f2	RPW apple_m1	RPW apple_m2	RPW apple_m3	RPW apple_f1	RPW apple_f2	RPW apple_f3	R_vuln f1	R_vuln m1	R_vuln f2
Unclassified non-Bacteria	0,000	0,000	0,000	0,000	0,000	0,023	0,000	0,000	0,000	0,000	0,000	0,000
Unclassified Bacteria	4,096	2,749	1,853	0,096	0,637	0,525	0,537	0,069	0,000	0,000	0,611	0,080
Acidobacteria	0,446	0,111	0,241	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,636	0,020
Actinobacteria	11,397	21,033	18,766	0,567	0,434	0,194	1,257	3,312	1,317	3,781	9,508	3,633
Armatimonadetes	0,171	0,166	0,019	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
Bacteroidetes	6,135	8,690	9,893	6,693	7,109	1,940	4,395	1,019	0,299	0,850	11,003	0,939
Chloroflexi	1,662	0,996	1,649	0,000	0,014	0,000	0,000	0,000	0,000	0,000	0,000	0,000
Cyanobacteria	0,069	0,074	0,056	0,000	0,000	0,000	0,037	0,127	0,000	0,637	2,139	0,248
Fibrobacteres	3,342	0,185	0,037	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
Firmicutes	0,497	0,129	0,130	66,100	51,788	42,264	23,616	7,053	43,952	48,258	7,005	77,064
Fusobacteria	0,000	0,000	0,000	0,000	0,000	0,011	0,007	0,035	0,000	0,467	0,000	0,000
GN02	0,137	0,000	0,000	0,000	0,116	0,000	0,000	0,000	0,000	0,000	0,000	0,000
Gemmatimonadetes	0,051	0,055	0,056	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,003
Lentisphaerae	0,017	0,000	0,000	0,000	0,043	0,000	0,000	0,000	0,000	0,000	0,000	0,000
OD1	0,034	0,055	0,204	0,000	0,029	0,000	0,000	0,000	0,000	0,000	0,000	0,000
Planctomycetes	1,748	1,328	0,371	0,000	0,014	0,000	0,007	0,000	0,000	0,000	0,289	0,107
Proteobacteria	67,678	61,402	64,635	25,410	37,484	52,396	69,276	82,328	42,515	44,817	67,975	17,178
SAR406	0,000	0,000	0,000	0,000	1,303	0,000	0,000	0,000	0,000	0,000	0,000	0,000
SR1	0,034	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
Spirochaetes	0,034	0,000	0,000	0,000	0,217	0,000	0,000	0,000	0,000	0,000	0,000	0,000
TM6	0,000	0,018	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
TM7	0,874	2,546	1,871	0,000	0,000	0,000	0,015	0,000	0,000	0,765	0,636	0,661
Tenericutes	0,034	0,074	0,000	1,134	0,782	2,647	0,853	6,057	11,916	0,425	0,000	0,067
Thermi	0,017	0,000	0,037	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
Verrucomicrobia	1,422	0,166	0,167	0,000	0,029	0,000	0,000	0,000	0,000	0,000	0,198	0,000
WPS-2	0,051	0,037	0,019	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
WS2	0,034	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
WYO	0,017	0,185	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000

Table S1: Identification at Phylum level of the bacterial communities associated with the 12 weevil samples.

	RPW Palm m1	RPW Palm f1	RPW Palm f2	RPW Apple m1	RPW Apple m2	RPW Apple m3	RPW Apple f1	RPW Apple f2	RPW Apple f3	R_vuln m1	R_vuln f1	R_vuln f2
Unclassified	0,00	0,00	0,00	0,00	0,00	0,02	0,00	0,00	0,00	0,00	0,00	0,00
Other Bacteria	4,30	3,06	1,93	0,10	0,64	0,52	0,57	0,20	0,00	0,33	0,64	2,75
Acidobacteria												
Acidobacteriaceae	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,02	0,00	0,00
RB40	0,14	0,00	0,04	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
mb2424	0,03	0,02	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Solibacteraceae	0,07	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Other Acidobacteria	0,21	0,09	0,19	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,64
Actinobacteria												
C111	0,03	0,00	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Microthrixaceae	0,19	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
ZA3409c	0,00	0,00	0,00	0,00	0,13	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Actinomycetaceae	0,17	0,22	0,04	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,04	0,00
Beutenbergiaceae	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Brevibacteriaceae	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,03	0,00	0,00
Cellulomonadaceae	3,14	4,21	1,15	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,08	0,00
Corynebacteriaceae	0,00	0,06	0,00	0,00	0,00	0,05	0,29	0,02	0,18	0,21	0,00	0,46
Dermabacteraceae	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,10	0,00	0,00
Frankiaceae	0,00	0,00	0,04	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Gordoniaceae	0,21	0,55	1,19	0,00	0,00	0,00	0,00	0,00	0,00	0,03	0,00	0,52
Intrasporangiaceae	0,02	0,02	0,07	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Microbacteriaceae	1,15	6,37	6,84	0,57	0,29	0,10	0,62	3,15	0,72	2,94	3,31	1,97
Micrococcaceae	0,05	0,00	0,07	0,00	0,00	0,00	0,01	0,00	0,12	0,00	0,00	0,59
Micromonosporaceae	0,48	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Mycobacteriaceae	0,00	0,11	0,07	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,12
Nakamurellaceae	0,07	0,00	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Nocardioidaceae	1,59	4,41	5,74	0,00	0,00	0,00	0,00	0,00	0,00	0,02	0,08	0,00
Promicromonosporaceae	0,00	0,26	0,39	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Propionibacteriaceae	0,00	0,00	0,00	0,00	0,00	0,00	0,04	0,09	0,24	0,03	0,17	2,92
Pseudonocardiaceae	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01
Streptomycetaceae	0,02	0,07	0,89	0,00	0,00	0,00	0,00	0,00	0,00	0,05	0,00	0,00
Streptosporangiaceae	0,00	0,00	0,04	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Tsukamurellaceae	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,90
Bifidobacteriaceae	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00
AK1AB1_02E	0,09	0,22	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Gaiellaceae	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,58
Patulibacteraceae	0,00	0,06	0,13	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Solirubrobacteraceae	0,00	0,00	0,06	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Other Actinobacteria	4,20	4,48	2,02	0,00	0,01	0,05	0,28	0,05	0,06	0,23	0,08	1,45
Armatimonadetes												
Armatimonadaceae	0,07	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Fimbriimonadaceae	0,03	0,17	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Other Armatimonadetes	0,07	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Bacteroidetes												
Bacteroidaceae	0,00	0,00	0,00	0,01	0,10	0,00	0,16	0,00	0,24	0,00	0,00	0,00
Porphyromonadaceae	0,05	0,00	0,00	6,64	6,76	1,92	1,64	0,73	0,06	0,00	0,17	0,50

	RPW Palm m1	RPW Palm F1	RPW Palm f2	RPW Apple m1	RPW Apple m2	RPW Apple m3	RPW Apple f1	RPW Apple f2	RPW Apple f3	R_vuln m1	R_vuln f1	R_vuln f2
Prevotellaceae	0,02	0,07	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,04	0,00
Rikenellaceae	0,00	0,00	0,00	0,00	0,06	0,00	0,00	0,00	0,00	0,00	0,00	0,00
S24-7	0,05	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
VC21_Bac22	0,00	0,00	0,00	0,00	0,13	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Paraprevotellaceae	0,00	0,00	0,00	0,00	0,00	0,00	0,02	0,00	0,00	0,00	0,00	0,00
Cryomorphaceae	0,09	0,00	0,20	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Flavobacteriaceae	0,86	2,93	3,52	0,00	0,00	0,00	0,54	0,01	0,00	0,00	0,34	3,54
Chitinophagaceae	2,72	1,81	1,37	0,04	0,00	0,00	0,14	0,28	0,00	0,03	0,00	0,45
Flammeovirgaceae	0,75	0,54	0,09	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01
Flexibacteraceae	0,09	0,00	1,65	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,42
Saprospiraceae	0,51	1,25	0,76	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Sphingobacteriaceae	0,07	1,00	1,54	0,00	0,00	0,00	0,01	0,00	0,00	0,91	0,25	5,46
Other Bacteroidetes	0,93	1,09	0,76	0,00	0,06	0,01	1,89	0,00	0,00	0,00	0,04	0,63
Chloroflexi												
Anaerolinaceae	0,03	0,00	0,83	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Caldilineaceae	0,12	0,55	0,43	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
A4b	1,27	0,04	0,06	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Other Chloroflexi	0,24	0,41	0,33	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Fibrobacteres												
Fibrobacteraceae	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Other Fibrobacteres	3,32	0,18	0,04	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Firmicutes												
Bacillaceae	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,07	0,04	0,13
Paenibacillaceae	0,14	0,07	0,00	0,00	0,00	0,00	0,03	0,42	0,00	0,00	0,00	0,66
Planococcaceae	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00
Staphylococcaceae	0,00	0,00	0,06	0,00	0,00	0,00	0,01	0,00	0,00	0,16	0,81	0,29
Aerococcaceae	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,74
Enterococcaceae	0,00	0,00	0,04	0,68	11,52	9,03	0,24	0,01	0,00	0,00	0,00	0,00
Lactobacillaceae	0,00	0,00	0,00	15,33	2,10	5,65	3,67	0,74	2,75	6,78	2,17	0,18
Leuconostocaceae	0,00	0,00	0,00	40,88	22,15	14,54	5,07	2,17	22,16	69,19	41,21	1,37
Streptococcaceae	0,00	0,00	0,00	4,49	13,62	4,95	11,05	2,93	18,74	0,35	0,38	0,62
Catabacteriaceae	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Clostridiaceae	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,02	0,00	0,04	0,00	2,30
Lachnospiraceae	0,09	0,02	0,00	3,73	1,65	6,00	1,74	0,60	0,00	0,00	0,00	0,17
Peptococcaceae	0,03	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Peptostreptococcaceae	0,00	0,00	0,00	0,00	0,09	0,00	0,00	0,01	0,00	0,00	0,00	0,00
Ruminococcaceae	0,09	0,00	0,00	0,00	0,00	0,01	0,02	0,00	0,24	0,02	0,04	0,00
Veillonellaceae	0,00	0,00	0,00	0,00	0,04	0,00	0,01	0,03	0,00	0,34	0,59	0,40
Coriobacteriaceae	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00
Erysipelotrichaceae	0,05	0,00	0,00	0,07	0,01	0,43	1,34	0,00	0,00	0,00	1,53	0,14
Other Firmicutes	0,09	0,04	0,04	0,92	0,59	1,65	0,42	0,12	0,06	0,10	1,49	0,00
Fusobacteria												
Fusobacteriaceae	0,00	0,00	0,00	0,00	0,00	0,01	0,01	0,03	0,00	0,00	0,47	0,00
Other Bacteria	0,24	0,11	0,26	0,00	0,19	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Planctomycetes												
Isosphaeraceae	0,09	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,06	0,00	0,00

	RPW Palm m1	RPW Palm f1	RPW Palm f2	RPW Apple m1	RPW Apple m2	RPW Apple m3	RPW Apple f1	RPW Apple f2	RPW Apple f3	R_vuln m1	R_vuln f1	R_vuln f2
Pirellulaceae	0,51	0,37	0,07	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,02
Planctomycetaceae	1,01	0,59	0,22	0,00	0,00	0,00	0,00	0,00	0,00	0,04	0,00	0,27
Other Planctomycetes	0,14	0,37	0,07	0,00	0,01	0,00	0,00	0,00	0,00	0,01	0,00	0,00
Proteobacteria												
Caulobacteraceae	1,20	0,94	1,19	0,00	0,00	0,00	0,04	0,00	0,00	0,03	0,13	0,77
Kiloniellaceae	0,05	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Aurantimonadaceae	0,03	0,07	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Bradyrhizobiaceae	0,36	0,00	0,04	0,00	0,00	0,00	0,00	0,00	0,00	0,07	0,04	1,27
Brucellaceae	0,00	0,00	0,00	0,00	0,03	0,00	0,00	0,00	0,48	0,00	2,80	0,00
Hyphomicrobiaceae	6,05	3,28	2,82	0,00	0,00	0,00	0,00	0,00	0,00	0,07	0,34	0,97
Methylocystaceae	0,17	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,08	0,69
Phyllobacteriaceae	3,60	5,18	4,65	0,00	0,00	0,00	0,00	0,00	0,00	0,20	0,00	1,29
Rhizobiaceae	3,41	1,16	2,26	0,00	0,00	0,00	0,00	0,00	0,06	0,87	0,08	0,36
Xanthobacteraceae	0,00	0,00	0,04	0,00	0,00	0,00	0,00	0,00	0,00	0,12	0,00	0,37
Hyphomonadaceae	0,10	0,09	0,07	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Rhodobacteraceae	2,88	24,17	8,58	0,00	0,07	0,00	0,07	0,02	0,00	0,87	0,68	2,92
Acetobacteraceae	0,17	0,22	0,20	8,94	2,81	18,28	45,98	62,71	29,64	0,62	0,25	0,34
Rhodospirillaceae	0,55	0,11	0,07	0,01	0,03	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Pelagibacteraceae	0,00	0,00	0,00	0,00	0,04	0,00	0,00	0,00	0,00	0,00	0,00	0,00
mitochondria	0,05	0,04	0,00	0,00	0,03	0,00	0,02	0,01	0,00	0,02	0,21	0,03
Erythrobacteraceae	1,59	2,18	1,07	0,00	0,00	0,00	0,00	0,00	0,00	0,03	0,00	0,00
Sphingomonadaceae	0,24	0,66	1,46	0,00	0,00	0,00	0,00	0,01	0,00	0,29	0,00	0,62
Alcaligenaceae	0,07	0,00	0,07	0,00	0,00	0,00	0,00	0,00	0,00	0,03	0,00	0,23
Comamonadaceae	2,83	0,46	0,72	2,89	2,08	0,86	5,63	13,21	6,05	8,31	19,54	5,69
Oxalobacteraceae	0,00	0,00	0,00	0,00	0,01	0,00	0,01	0,00	0,00	2,39	8,41	38,94
Methylophilaceae	0,00	0,00	0,11	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Neisseriaceae	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,06	0,00	0,00	0,11
Nitrosomonadaceae	0,29	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Rhodocyclaceae	0,14	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,02
Bacteriovoracaceae	0,00	0,26	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Bdellovibrionaceae	0,00	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Nitrospinaceae	0,00	0,00	0,00	0,00	0,03	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Geobacteraceae	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,30	0,00	0,00	0,00
0319-6G20	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Haliangiaceae	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Polyangiaceae	0,07	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
JTB36	0,00	0,00	0,00	0,00	0,06	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Desulfobacteraceae	0,00	0,00	0,00	0,00	1,06	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Syntrophobacteraceae	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Campylobacteraceae	0,00	0,00	0,00	0,00	0,03	0,00	0,00	0,00	0,00	0,00	0,08	0,23
Aeromonadaceae	0,03	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,15
Succinivibrionaceae	0,07	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Alteromonadaceae	0,24	0,42	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Idiomarinaceae	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Enterobacteriaceae	0,05	0,35	3,06	13,22	6,91	19,92	12,80	5,56	4,85	1,44	2,17	1,74
FCPT525	0,07	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00

	RPW Palm m1	RPW Palm f1	RPW Palm f2	RPW Apple m1	RPW Apple m2	RPW Apple m3	RPW Apple f1	RPW Apple f2	RPW Apple f3	R_vuln m1	R_vuln f1	R_vuln f2
211ds20	0,45	0,15	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Coxiellaceae	0,07	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00
Legionellaceae	0,00	0,07	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,11	0,04	0,09
Halomonadaceae	0,00	0,00	0,00	0,00	0,07	0,00	0,00	0,00	0,00	0,00	0,00	0,00
SUP05	0,00	0,00	0,00	0,00	0,16	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Pasteurellaceae	0,00	0,00	0,00	0,00	0,00	0,01	0,04	0,06	0,00	0,00	3,57	0,59
Moraxellaceae	0,27	0,46	5,78	0,00	0,00	0,00	0,04	0,02	0,12	0,38	0,08	2,62
Pseudomonadaceae	2,07	1,25	5,95	0,01	16,61	7,47	0,40	0,00	0,12	0,01	0,04	0,93
Salinisphaeraceae	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Thiohalorhabdaceae	0,00	0,00	0,00	0,00	0,03	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Piscirickettsiaceae	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Sinobacteraceae	4,82	1,07	0,96	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Xanthomonadaceae	15,85	8,60	15,15	0,10	0,09	0,15	4,02	0,52	0,18	0,13	0,13	2,18
Other Proteobacteria	19,78	10,17	10,30	0,24	7,30	5,69	0,21	0,19	0,66	1,19	6,12	4,82
SAR406												
A714017	0,00	0,00	0,00	0,00	1,29	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Other SAR 406	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Spirochaetes												
Spirochaetaceae	0,03	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
ST-3K10	0,00	0,00	0,00	0,00	0,16	0,00	0,00	0,00	0,00	0,00	0,00	0,00
MSBL8	0,00	0,00	0,00	0,00	0,06	0,00	0,00	0,00	0,00	0,00	0,00	0,00
TM7												
Rs-045	0,00	0,06	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Other TM7	0,87	2,49	1,87	0,00	0,00	0,00	0,01	0,00	0,00	0,66	0,76	0,64
Tenericutes												
Acholeplasmataceae	0,03	0,07	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Entomoplasmataceae	0,00	0,00	0,00	1,13	0,78	2,64	0,85	6,06	11,92	0,00	0,13	0,00
Mycoplasmataceae	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,02	0,00	0,00
Other Tenericutes	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,05	0,30	0,00
Thermi												
Deinococcaceae	0,02	0,00	0,04	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Verrucomicrobia	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Opitutaceae	1,06	0,15	0,09	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Verrucomicrobiaceae	0,26	0,02	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,20
Chthoniobacteraceae	0,03	0,00	0,07	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Other Verrucomicrobia	0,07	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00

Table S2: Identification at Family level of the bacterial communities associated with the 12 weevil samples.

OTUs	RPW palm_m1	RPW palm_f1	RPW palm_f2	RPW apple_f1	RPW apple_f2	RPW apple_f3	RPW apple_m1	RPW apple_m2	RPW apple_m3	R_vuln f1	R_vuln f2	R_vul _m1
1	0	0	12	31	9	0	38	5	46	0	17	50
2	0	0	0	10	4	0	0	0	0	0	1	0
3	0	0	2	0	0	0	0	0	0	0	0	0
4	0	0	0	2	5	0	5	0	17	0	0	0
5	0	0	1	0	0	0	0	0	0	0	0	0
6	0	0	1	146	0	0	1	1	3	0	0	0
7	1	0	0	148	2	0	0	25	1	0	0	5
8	0	0	0	10	0	0	1	1	0	0	0	0
9	0	0	0	2	0	0	0	0	0	0	0	0
10	0	0	0	1	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	1	0	0	1
12	0	0	7	0	0	0	1	0	0	0	0	0
13	0	0	0	1	0	0	0	0	1	0	0	2
14	0	1	15	139	6	0	59	57	110	0	173	721
15	0	0	1	6	4	0	23	1	15	0	0	0
16	0	0	0	0	0	0	0	0	0	0	0	1
17	0	0	0	1	0	0	0	0	0	0	7	9
18	0	0	0	3	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0	3	0	0	0
20	0	0	0	561	391	42	4	231	453	0	0	0
21	2	1	15	8	9	0	601	0	106	7	0	0
22	0	0	0	1	0	0	0	1	4	0	1	27
23	0	0	1	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	2	0	0	0	0	0
25	0	0	15	142	2	1	34	46	183	6	0	5
26	0	0	0	0	0	0	0	0	1	0	0	3
27	0	0	0	9	0	0	1	0	3	0	0	0
28	0	0	0	0	0	0	1	0	2	0	0	0
29	0	1	0	0	0	0	0	0	2	0	0	0
30	0	0	0	1	0	0	0	0	0	0	0	0
31	0	0	0	0	0	0	0	0	4	0	0	0
Tot	3	3	70	1222	432	43	771	368	955	13	199	824

Table S3: Bacterial OTUs identified as "*Candidatus* Nardonella"

Predicted gene family percentage / weevil sample	RPW_palm_m1	RPW_palm_f1	RPW_palm_f2	RPW_apple_m1	RPW_apple_m2	RPW_apple_m3	RPW_apple_f1	RPW_apple_f2	RPW_apple_f3	R_vulneratus_m1	R_vulneratus_f1	R_vulneratus_f2
Cellular Processes;Cell Growth and Death;												
Apoptosis	0,00	0,10	0,00	0,00	0,00	0,10	0,10	0,10	0,00	0,00	0,00	0,00
Cell cycle - Caulobacter	0,50	0,50	0,50	0,40	0,60	0,50	0,60	0,50	0,90	0,60	0,80	0,40
Meiosis - yeast	0,10	0,00	0,10	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,10
p53 signaling pathway	0,00	0,10	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Cellular Processes;Cell Motility;												
Bacterial chemotaxis	0,60	0,70	0,60	0,10	0,40	0,20	0,10	0,20	0,40	0,30	0,10	0,50
Bacterial motility proteins	1,60	1,30	1,60	0,10	0,90	0,50	0,10	0,40	1,00	0,70	0,70	1,20
Cytoskeleton proteins	0,20	0,20	0,20	0,20	0,20	0,30	0,40	0,30	0,20	0,30	0,20	0,20
Flagellar assembly	0,70	0,70	0,70	0,00	0,40	0,20	0,00	0,20	0,80	0,30	0,50	0,50
Cellular Processes;Transport and Catabolism;												
Lysosome	0,00	0,00	0,00	0,00	0,10	0,00	0,00	0,00	0,00	0,00	0,00	0,10
Peroxisome	0,20	0,20	0,20	0,10	0,20	0,10	0,10	0,20	0,10	0,20	0,10	0,30
Environmental Information Processing;												
Membrane Transport;ABC transporters	3,80	5,60	4,70	3,80	3,40	2,90	2,30	2,60	5,50	5,00	4,60	4,00
Membrane Transport;Bacterial secretion system	0,90	0,60	0,90	0,40	0,80	0,60	0,70	0,70	1,50	0,80	1,20	0,70
Membrane Transport;Phosphotransferase system (PTS)	0,10	0,10	0,20	1,90	0,40	0,60	0,00	0,10	0,10	0,10	0,20	0,10
Membrane Transport;Secretion system	1,90	1,20	1,70	0,90	1,30	1,20	1,10	1,20	1,70	1,30	1,70	1,30
Membrane Transport;Transporters	6,00	8,20	7,60	9,50	6,10	5,60	3,90	4,60	8,80	8,30	7,50	6,30
Signal Transduction;MAPK signaling pathway - yeast	0,00	0,00	0,00	0,10	0,10	0,10	0,10	0,10	0,10	0,00	0,10	0,00
Signal Transduction;Phosphatidylinositol signaling system	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10
Signal Transduction;Two-component system	2,10	1,90	2,00	1,20	1,50	1,50	1,40	1,40	1,40	1,20	1,20	1,90
Signaling Molecules and Interaction;Bacterial toxins	0,10	0,00	0,00	0,20	0,10	0,10	0,10	0,10	0,00	0,00	0,00	0,10
Signaling Molecules and Interaction;Cellular antigens	0,10	0,00	0,10	0,00	0,10	0,10	0,10	0,10	0,00	0,00	0,00	0,10
Genetic Information Processing;												
Folding, Sorting and Degradation;Chaperones and folding catalysts	0,90	0,70	0,90	0,90	1,00	1,00	1,10	1,00	0,90	0,80	0,90	0,70
Folding, Sorting and Degradation;Protein export	0,50	0,40	0,50	0,50	0,60	0,60	0,60	0,60	0,60	0,80	0,80	0,40
Folding, Sorting and Degradation;RNA degradation	0,40	0,30	0,40	0,40	0,50	0,50	0,50	0,50	0,50	0,50	0,60	0,40
Folding, Sorting and Degradation;Sulfur relay system	0,30	0,30	0,30	0,20	0,30	0,30	0,40	0,30	0,20	0,30	0,20	0,30
Replication and Repair;Base excision repair	0,40	0,30	0,40	0,40	0,40	0,40	0,40	0,40	0,40	0,60	0,60	0,40
Replication and Repair;Chromosome	1,20	1,10	1,20	1,40	1,50	1,50	1,60	1,50	1,30	1,20	1,30	1,00

Replication and Repair;DNA repair and recombination proteins	2,10	1,80	2,10	2,60	2,70	2,30	2,30	2,40	2,50	3,00	3,10	1,80
Replication and Repair;DNA replication	0,50	0,50	0,50	0,60	0,70	0,50	0,50	0,50	0,70	0,70	0,80	0,40
Replication and Repair;DNA replication proteins	0,80	0,80	0,80	1,10	1,20	0,90	0,90	0,90	1,10	1,10	1,30	0,70
Replication and Repair;Homologous recombination	0,60	0,60	0,70	0,80	0,90	0,70	0,70	0,80	0,90	1,00	1,00	0,50
Replication and Repair;Mismatch repair	0,60	0,60	0,60	0,70	0,80	0,60	0,50	0,60	0,80	0,80	0,90	0,50
Replication and Repair;Non-homologous end-joining	0,10	0,00	0,10	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,10
Replication and Repair;Nucleotide excision repair	0,30	0,20	0,30	0,30	0,40	0,30	0,30	0,30	0,30	0,50	0,40	0,30
Transcription;RNA polymerase	0,10	0,10	0,10	0,20	0,20	0,10	0,10	0,20	0,20	0,20	0,20	0,10
Transcription;Transcription factors	1,30	1,50	1,30	1,90	1,20	1,40	1,00	1,20	1,10	1,20	1,20	1,30
Transcription;Transcription machinery	0,70	0,50	0,60	0,50	0,70	0,50	0,50	0,60	0,60	0,70	0,70	0,70
Translation;Aminoacyl-tRNA biosynthesis	0,90	0,80	0,90	1,20	1,20	1,10	1,20	1,20	1,30	1,50	1,60	0,80
Translation;RNA transport	0,10	0,00	0,10	0,20	0,10	0,10	0,10	0,10	0,00	0,00	0,00	0,00
Translation;Ribosome	1,60	1,40	1,60	2,20	2,40	1,90	1,90	2,00	2,30	2,80	2,90	1,40
Translation;Ribosome Biogenesis	1,10	0,90	1,00	1,30	1,30	1,10	1,10	1,10	1,40	1,00	1,30	0,80
Translation;Ribosome biogenesis in eukaryotes	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,10	0,10	0,00
Translation;Translation factors	0,40	0,30	0,40	0,50	0,50	0,40	0,40	0,40	0,50	0,50	0,60	0,30
Metabolism;Amino Acid Metabolism;												
Alanine, aspartate and glutamate metabolism	0,80	0,90	0,90	0,80	1,10	0,90	1,00	1,00	0,80	1,10	1,00	0,80
Amino acid related enzymes	1,20	1,20	1,20	1,30	1,50	1,50	1,60	1,60	1,10	1,60	1,50	1,10
Arginine and proline metabolism	1,30	1,30	1,30	1,20	1,40	1,30	1,40	1,30	1,40	1,10	1,20	1,40
Cysteine and methionine metabolism	0,70	0,70	0,70	0,90	0,90	1,00	1,00	1,00	0,40	0,70	0,60	0,70
Glycine, serine and threonine metabolism	0,90	1,00	1,00	0,70	0,90	0,90	0,90	0,90	0,90	1,10	0,90	1,00
Histidine metabolism	0,50	0,60	0,50	0,40	0,70	0,60	0,70	0,60	0,30	0,40	0,30	0,60
Lysine biosynthesis	0,60	0,60	0,60	0,70	0,80	0,60	0,60	0,60	0,80	0,60	0,70	0,50
Lysine degradation	0,70	0,80	0,60	0,10	0,30	0,20	0,20	0,30	0,50	0,50	0,40	0,80
Phenylalanine metabolism	0,40	0,40	0,40	0,10	0,30	0,30	0,30	0,30	0,30	0,30	0,30	0,50
Phenylalanine, tyrosine and tryptophan biosynthesis	0,60	0,60	0,60	0,50	0,80	0,70	0,80	0,80	0,50	0,80	0,70	0,60
Tryptophan metabolism	0,80	0,80	0,70	0,20	0,30	0,30	0,30	0,30	0,50	0,50	0,40	0,90
Tyrosine metabolism	0,50	0,60	0,50	0,60	0,60	0,80	0,90	0,80	0,60	0,60	0,60	0,60
Valine, leucine and isoleucine biosynthesis	0,60	0,60	0,70	0,80	0,70	0,80	0,80	0,80	0,60	0,80	0,70	0,70
Valine, leucine and isoleucine degradation	1,10	1,20	1,00	0,30	0,50	0,30	0,40	0,40	0,70	0,90	0,70	1,30
Metabolism;Biosynthesis of Other Secondary Metabolites;												
Butirosin and neomycin biosynthesis	0,00	0,00	0,10	0,00	0,00	0,00	0,00	0,00	0,00	0,10	0,00	0,00
Isoquinoline alkaloid biosynthesis	0,10	0,10	0,10	0,00	0,10	0,10	0,10	0,10	0,00	0,10	0,10	0,10
Novobiocin biosynthesis	0,10	0,10	0,10	0,10	0,20	0,20	0,20	0,20	0,10	0,20	0,20	0,10
Penicillin and cephalosporin biosynthesis	0,10	0,00	0,10	0,00	0,00	0,00	0,00	0,00	0,00	0,10	0,00	0,00
Phenylpropanoid biosynthesis	0,10	0,10	0,10	0,30	0,10	0,10	0,00	0,00	0,00	0,10	0,10	0,10
Stilbenoid, diarylheptanoid and gingerol biosynthesis	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,10
Streptomycin biosynthesis	0,30	0,30	0,30	0,30	0,30	0,30	0,40	0,40	0,20	0,30	0,30	0,30

Tropane, piperidine and pyridine alkaloid biosynthesis	0,10	0,10	0,10	0,00	0,20	0,10	0,20	0,20	0,10	0,10	0,10	0,20
beta-Lactam resistance	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,10	0,00	0,00
Metabolism;Carbohydrate Metabolism;												
Amino sugar and nucleotide sugar metabolism	0,90	0,80	0,90	1,80	1,10	1,10	0,90	0,90	0,70	1,00	0,90	0,80
Ascorbate and aldarate metabolism	0,20	0,20	0,20	0,20	0,10	0,20	0,10	0,20	0,10	0,20	0,20	0,20
Butanoate metabolism	1,20	1,40	1,10	1,00	0,90	1,40	1,70	1,50	0,90	1,00	0,80	1,50
C5-Branched dibasic acid metabolism	0,30	0,30	0,30	0,30	0,20	0,30	0,30	0,30	0,20	0,30	0,30	0,30
Citrate cycle (TCA cycle)	0,80	0,70	0,80	0,70	0,80	0,70	0,70	0,80	0,90	0,80	0,90	0,90
Fructose and mannose metabolism	0,50	0,50	0,60	1,50	0,90	1,60	1,80	1,60	0,50	0,60	0,60	0,70
Galactose metabolism	0,30	0,30	0,40	1,00	0,40	0,40	0,10	0,20	0,20	0,30	0,30	0,30
Glycolysis / Gluconeogenesis	1,00	1,10	1,10	1,70	1,10	1,40	1,30	1,30	1,00	1,30	1,20	1,20
Glyoxylate and dicarboxylate metabolism	0,90	1,20	0,90	0,40	0,50	0,60	0,60	0,60	0,60	0,50	0,40	1,00
Inositol phosphate metabolism	0,20	0,10	0,20	0,10	0,10	0,20	0,30	0,30	0,20	0,10	0,10	0,20
Pentose and glucuronate interconversions	0,40	0,40	0,40	0,50	0,40	0,40	0,30	0,30	0,30	0,40	0,30	0,40
Pentose phosphate pathway	0,60	0,60	0,60	0,90	0,80	0,80	0,70	0,70	0,70	0,80	0,80	0,70
Propanoate metabolism	1,20	1,30	1,10	0,50	0,70	0,70	0,80	0,80	0,80	0,80	0,70	1,30
Pyruvate metabolism	1,10	1,40	1,20	1,30	1,20	1,30	1,40	1,30	1,30	1,10	1,10	1,40
Starch and sucrose metabolism	0,50	0,40	0,50	1,30	0,60	0,70	0,50	0,50	0,20	0,40	0,30	0,50
Metabolism;Energy Metabolism;												
Carbon fixation in photosynthetic organisms	0,50	0,60	0,50	0,50	0,60	0,50	0,50	0,50	0,60	0,60	0,60	0,50
Carbon fixation pathways in prokaryotes	1,10	1,20	1,00	0,70	1,00	0,90	0,90	0,90	1,00	0,70	0,80	1,10
Methane metabolism	0,90	0,90	0,90	1,00	1,00	0,90	0,90	0,90	0,50	0,80	0,60	1,00
Nitrogen metabolism	0,80	1,00	0,80	0,60	0,70	0,60	0,60	0,60	0,50	0,60	0,50	0,80
Oxidative phosphorylation	1,40	1,20	1,40	1,10	1,30	1,50	1,70	1,60	1,80	1,30	1,60	1,20
Photosynthesis	0,30	0,20	0,20	0,30	0,30	0,30	0,30	0,30	0,40	0,40	0,50	0,20
Photosynthesis - antenna proteins	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Photosynthesis proteins	0,30	0,40	0,30	0,30	0,30	0,30	0,40	0,40	0,40	0,50	0,50	0,20
Sulfur metabolism	0,30	0,30	0,30	0,30	0,30	0,40	0,50	0,40	0,10	0,20	0,10	0,30
Metabolism;Enzyme Families;												
Peptidases	1,30	1,20	1,30	1,40	1,60	1,40	1,40	1,40	1,30	1,50	1,50	1,20
Protein kinases	0,30	0,30	0,30	0,20	0,20	0,30	0,30	0,30	0,20	0,30	0,30	0,40
Metabolism;Glycan Biosynthesis and Metabolism;												
Glycosaminoglycan degradation	0,00	0,00	0,00	0,00	0,10	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Glycosphingolipid biosynthesis - ganglio series	0,00	0,00	0,00	0,00	0,10	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Glycosphingolipid biosynthesis - globo series	0,00	0,00	0,00	0,10	0,10	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Glycosyltransferases	0,30	0,30	0,30	0,30	0,40	0,40	0,40	0,40	0,40	0,30	0,40	0,40
Lipopolysaccharide biosynthesis	0,30	0,20	0,30	0,10	0,40	0,40	0,40	0,40	0,40	0,10	0,30	0,20
Lipopolysaccharide biosynthesis proteins	0,40	0,30	0,40	0,10	0,50	0,50	0,50	0,50	0,40	0,30	0,40	0,30
N-Glycan biosynthesis	0,00	0,00	0,00	0,00	0,00	0,10	0,10	0,10	0,00	0,00	0,00	0,00

Other glycan degradation	0,00	0,00	0,00	0,20	0,20	0,10	0,00	0,00	0,00	0,00	0,00	0,00
Peptidoglycan biosynthesis	0,60	0,50	0,60	0,90	0,80	0,60	0,60	0,60	0,80	0,80	0,90	0,50
Metabolism;Lipid Metabolism;												
Arachidonic acid metabolism	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,00	0,00	0,00	0,10
Biosynthesis of unsaturated fatty acids	0,30	0,20	0,20	0,10	0,20	0,10	0,10	0,10	0,20	0,20	0,20	0,40
Fatty acid biosynthesis	0,60	0,50	0,60	0,60	0,60	0,50	0,60	0,50	0,80	0,50	0,60	0,60
Fatty acid metabolism	1,00	1,00	0,80	0,30	0,40	0,40	0,40	0,40	0,60	0,70	0,60	1,20
Glycerolipid metabolism	0,30	0,40	0,40	0,50	0,30	0,40	0,40	0,40	0,30	0,30	0,30	0,40
Glycerophospholipid metabolism	0,50	0,40	0,50	0,50	0,60	0,60	0,60	0,60	0,50	0,50	0,60	0,50
Linoleic acid metabolism	0,10	0,10	0,10	0,20	0,20	0,70	1,00	0,80	0,10	0,10	0,10	0,20
Lipid biosynthesis proteins	0,80	0,70	0,70	0,50	0,60	0,60	0,60	0,60	0,80	0,60	0,70	0,90
Sphingolipid metabolism	0,00	0,00	0,00	0,10	0,10	0,10	0,10	0,10	0,00	0,00	0,00	0,10
Steroid biosynthesis	0,00	0,00	0,00	0,00	0,00	0,10	0,10	0,10	0,00	0,00	0,00	0,00
Steroid hormone biosynthesis	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,10
Synthesis and degradation of ketone bodies	0,20	0,20	0,10	0,10	0,10	0,00	0,00	0,00	0,10	0,10	0,10	0,20
alpha-Linolenic acid metabolism	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,10	0,00	0,10
Metabolism;Metabolism of Cofactors and Vitamins;												
Biotin metabolism	0,10	0,10	0,10	0,10	0,20	0,20	0,20	0,20	0,10	0,10	0,10	0,10
Folate biosynthesis	0,40	0,30	0,40	0,20	0,40	0,40	0,50	0,50	0,20	0,50	0,30	0,40
Lipoic acid metabolism	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10
Nicotinate and nicotinamide metabolism	0,40	0,40	0,40	0,30	0,40	0,40	0,40	0,40	0,40	0,50	0,40	0,40
One carbon pool by folate	0,50	0,50	0,50	0,50	0,70	0,40	0,50	0,50	0,60	0,60	0,60	0,40
Pantothenate and CoA biosynthesis	0,50	0,50	0,50	0,60	0,60	0,60	0,70	0,70	0,50	0,70	0,70	0,50
Porphyrin and chlorophyll metabolism	0,90	1,30	0,90	0,40	0,90	1,00	1,30	1,20	0,70	0,80	0,70	0,80
Retinol metabolism	0,10	0,20	0,20	0,10	0,10	0,20	0,20	0,20	0,20	0,10	0,10	0,20
Riboflavin metabolism	0,30	0,20	0,30	0,20	0,30	0,40	0,50	0,50	0,30	0,50	0,50	0,30
Thiamine metabolism	0,40	0,30	0,30	0,50	0,60	0,50	0,50	0,50	0,60	0,30	0,50	0,30
Ubiquinone and other terpenoid-quinone biosynthesis	0,30	0,20	0,30	0,30	0,30	0,20	0,20	0,20	0,40	0,40	0,40	0,20
Vitamin B6 metabolism	0,20	0,10	0,20	0,20	0,20	0,20	0,20	0,20	0,20	0,20	0,20	0,20
Metabolism;Metabolism of Other Amino Acids;												
Cyanoamino acid metabolism	0,20	0,20	0,20	0,40	0,30	0,20	0,20	0,20	0,10	0,20	0,20	0,30
D-Alanine metabolism	0,10	0,10	0,10	0,20	0,10	0,10	0,10	0,10	0,10	0,20	0,10	0,10
D-Glutamine and D-glutamate metabolism	0,10	0,10	0,10	0,10	0,20	0,10	0,10	0,10	0,10	0,10	0,10	0,10
Glutathione metabolism	0,60	0,50	0,50	0,30	0,40	0,60	0,70	0,60	0,50	0,40	0,40	0,40
Phosphonate and phosphinate metabolism	0,10	0,10	0,00	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10
Selenocompound metabolism	0,30	0,30	0,30	0,40	0,30	0,40	0,40	0,40	0,20	0,30	0,20	0,30
Taurine and hypotaurine metabolism	0,10	0,20	0,10	0,10	0,10	0,20	0,20	0,20	0,10	0,10	0,00	0,10
beta-Alanine metabolism	0,60	0,70	0,50	0,10	0,30	0,30	0,30	0,30	0,40	0,50	0,40	0,70
Metabolism;Metabolism of Terpenoids and Polyketides;												

Biosynthesis of ansamycins	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,00	0,10	0,10	0,10
Biosynthesis of siderophore group nonribosomal peptides	0,00	0,10	0,10	0,10	0,00	0,00	0,00	0,00	0,00	0,10	0,10	0,10
Biosynthesis of vancomycin group antibiotics	0,00	0,00	0,00	0,00	0,10	0,10	0,10	0,10	0,00	0,10	0,10	0,00
Carotenoid biosynthesis	0,00	0,10	0,10	0,00	0,00	0,00	0,10	0,10	0,00	0,00	0,00	0,10
Geraniol degradation	0,50	0,50	0,40	0,00	0,20	0,10	0,10	0,20	0,30	0,30	0,20	0,60
Limonene and pinene degradation	0,60	0,60	0,50	0,20	0,30	0,30	0,30	0,30	0,50	0,40	0,30	0,80
Polyketide sugar unit biosynthesis	0,10	0,10	0,10	0,20	0,20	0,20	0,20	0,20	0,10	0,20	0,20	0,10
Prenyltransferases	0,30	0,30	0,30	0,30	0,30	0,30	0,40	0,40	0,30	0,60	0,50	0,30
Terpenoid backbone biosynthesis	0,50	0,50	0,50	0,50	0,50	0,40	0,50	0,50	0,40	0,80	0,70	0,50
Tetracycline biosynthesis	0,20	0,10	0,20	0,20	0,20	0,30	0,30	0,20	0,20	0,10	0,10	0,10
Zeatin biosynthesis	0,00	0,00	0,00	0,00	0,10	0,00	0,00	0,00	0,00	0,10	0,10	0,00
Metabolism;Nucleotide Metabolism;												
Purine metabolism	1,80	1,80	1,90	2,30	2,30	2,10	2,10	2,20	2,20	2,60	2,50	1,70
Pyrimidine metabolism	1,20	1,20	1,20	1,80	1,70	1,40	1,40	1,40	1,40	1,80	1,80	1,10
Metabolism;Xenobiotics Biodegradation and Metabolism;												
Aminobenzoate degradation	0,50	0,40	0,40	0,30	0,30	0,40	0,40	0,40	0,40	0,30	0,30	0,70
Atrazine degradation	0,10	0,10	0,10	0,00	0,10	0,10	0,20	0,20	0,10	0,10	0,10	0,10
Benzoate degradation	0,70	0,70	0,60	0,30	0,30	0,20	0,20	0,20	0,40	0,40	0,30	0,90
Bisphenol degradation	0,20	0,20	0,20	0,30	0,30	0,80	1,10	0,90	0,20	0,20	0,10	0,40
Caprolactam degradation	0,40	0,30	0,30	0,00	0,10	0,10	0,10	0,10	0,20	0,20	0,20	0,50
Chloroalkane and chloroalkene degradation	0,40	0,60	0,40	0,60	0,50	1,10	1,40	1,20	0,40	0,40	0,30	0,60
Chlorocyclohexane and chlorobenzene degradation	0,10	0,10	0,10	0,00	0,00	0,00	0,00	0,00	0,10	0,10	0,10	0,20
Dioxin degradation	0,10	0,00	0,10	0,10	0,00	0,10	0,00	0,00	0,00	0,00	0,00	0,10
Drug metabolism - cytochrome P450	0,30	0,30	0,30	0,20	0,20	0,30	0,40	0,30	0,40	0,20	0,20	0,30
Drug metabolism - other enzymes	0,20	0,30	0,20	0,20	0,20	0,20	0,10	0,20	0,10	0,30	0,20	0,20
Ethylbenzene degradation	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,20
Fluorobenzoate degradation	0,10	0,10	0,10	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,10
Metabolism of xenobiotics by cytochrome P450	0,30	0,30	0,30	0,20	0,20	0,30	0,40	0,30	0,40	0,20	0,20	0,30
Naphthalene degradation	0,40	0,40	0,40	0,40	0,40	0,50	0,60	0,50	0,40	0,20	0,30	0,50
Nitrotoluene degradation	0,10	0,10	0,10	0,00	0,10	0,10	0,10	0,10	0,00	0,00	0,00	0,10
Polycyclic aromatic hydrocarbon degradation	0,20	0,20	0,20	0,20	0,20	0,20	0,30	0,30	0,20	0,20	0,20	0,30
Styrene degradation	0,10	0,10	0,10	0,00	0,10	0,10	0,10	0,10	0,00	0,10	0,10	0,10
Toluene degradation	0,20	0,20	0,20	0,10	0,10	0,20	0,20	0,20	0,20	0,10	0,10	0,30
Xylene degradation	0,10	0,00	0,00	0,10	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,10
Organismal Systems;Environmental Adaptation;Plant-pathogen interaction	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,20	0,10	0,10	0,10
Other gene families	2,40	2,00	1,70	2,50	1,30	2,30	2,30	2,30	2,50	1,60	1,90	2,00
Unclassified;Cellular Processes and Signaling;												
Cell division	0,10	0,10	0,10	0,00	0,10	0,10	0,10	0,10	0,20	0,00	0,10	0,10
Cell motility and secretion	0,30	0,20	0,20	0,20	0,30	0,40	0,50	0,50	0,40	0,20	0,30	0,20

Electron transfer carriers	0,00	0,10	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,10	0,10	0,00
Germination	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Inorganic ion transport and metabolism	0,40	0,60	0,40	0,20	0,20	0,40	0,40	0,40	0,10	0,30	0,20	0,40
Membrane and intracellular structural molecules	0,80	0,80	0,80	0,40	0,70	1,00	1,20	1,10	0,90	0,30	0,60	0,60
Other ion-coupled transporters	1,00	0,80	0,90	0,90	1,00	1,00	0,90	0,90	0,60	0,60	0,70	0,90
Other transporters	0,20	0,20	0,20	0,20	0,20	0,30	0,30	0,30	0,20	0,10	0,20	0,20
Pores ion channels	0,50	0,50	0,50	0,30	0,50	0,70	0,90	0,80	0,40	0,20	0,30	0,40
Signal transduction mechanisms	0,30	0,30	0,30	0,50	0,40	0,40	0,40	0,40	0,20	0,30	0,20	0,40
Sporulation	0,00	0,00	0,00	0,00	0,10	0,00	0,00	0,00	0,00	0,10	0,00	0,10
Unclassified;Genetic Information Processing;												
Protein folding and associated processing	0,70	0,80	0,70	0,50	0,70	0,80	0,90	0,80	0,70	0,60	0,70	0,70
Replication, recombination and repair proteins	0,70	0,60	0,70	0,90	0,70	0,90	1,00	0,90	0,80	0,60	0,70	0,70
Restriction enzyme	0,10	0,10	0,10	0,10	0,10	0,20	0,20	0,20	0,00	0,00	0,00	0,10
Transcription related proteins	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,10	0,00	0,10	0,00
Translation proteins	0,70	0,50	0,70	0,80	0,80	0,70	0,70	0,70	0,80	0,70	0,80	0,60
Unclassified;Metabolism;												
Amino acid metabolism	0,30	0,20	0,20	0,20	0,30	0,20	0,30	0,20	0,30	0,20	0,30	0,30
Biosynthesis and biodegradation of secondary metabolites	0,10	0,10	0,10	0,00	0,00	0,00	0,00	0,00	0,10	0,00	0,00	0,10
Carbohydrate metabolism	0,10	0,10	0,10	0,20	0,10	0,10	0,10	0,10	0,00	0,10	0,10	0,10
Energy metabolism	0,70	0,60	0,60	0,70	0,80	0,80	0,90	0,80	0,40	0,50	0,40	0,80
Glycan biosynthesis and metabolism	0,10	0,00	0,10	0,00	0,10	0,10	0,10	0,10	0,10	0,00	0,10	0,00
Lipid metabolism	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10
Metabolism of cofactors and vitamins	0,20	0,20	0,20	0,10	0,10	0,20	0,20	0,20	0,10	0,10	0,10	0,20
Nucleotide metabolism	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,10	0,00
Others	1,00	0,90	1,00	1,80	1,20	1,40	1,30	1,30	1,00	0,90	0,90	1,30
Unclassified;Poorly Characterized;												
Function unknown	1,50	1,40	1,50	1,40	1,30	1,70	1,60	1,60	1,40	1,20	1,40	1,30
General function prediction only	3,20	2,90	3,10	3,40	3,10	2,90	2,70	2,70	2,70	2,40	2,50	3,10

Table S4: Predicted gene families infereed on the base of the bacterial 16S rRNA.

Predicted gene family percentage / weevil sample	RPW palm_m1	RPW palm_f1	RPW palm_f2	RPW apple_m1	RPW apple_m2	RPW apple_m3	RPW apple_f1	RPW apple_f2	RPW apple_f3	R_vuln m1	R_vuln f1	R_vuln f2	χ2	p-value	p-value <0.05	Mann-Whitney U	p-value	Mann-Whitney U	p-value	Mann-Whitney U	p-value
Cellular Processes;Cell Motility;																					
Bacterial chemotaxis	0,60	0,70	0,60	0,10	0,40	0,20	0,10	0,20	0,40	0,30	0,1	0,5	6,545	0,038	**	18	0	9	0,1	7	0,7
Bacterial motility proteins	1,60	1,30	1,60	0,10	0,90	0,50	0,10	0,40	1,00	0,70	0,7	1,2	7,269	0,026	**	18	0	9	0,1	14	0,2
Flagellar assembly	0,70	0,70	0,70	0,00	0,40	0,20	0,00	0,20	0,80	0,30	0,5	0,5	4,495	0,106							
Genetic Information Processing; Folding, Sorting and Degradation;Protein export	0,50	0,40	0,50	0,50	0,60	0,60	0,60	0,60	0,60	0,80	0,8	0,4	3,878	0,144							
Metabolism;Amino Acid Metabolism; Alanine, aspartate and glutamate metabolism	0,80	0,90	0,90	0,80	1,10	0,90	1,00	1,00	0,80	1,10	1	0,8	1,054	0,590							
Amino acid related enzymes	1,20	1,20	1,20	1,30	1,50	1,50	1,60	1,60	1,10	1,60	1,5	1,1	2,105	0,349							
Arginine and proline metabolism	1,30	1,30	1,30	1,20	1,40	1,30	1,40	1,30	1,40	1,10	1,2	1,4	1,639	0,441							
Cysteine and methionine metabolism	0,70	0,70	0,70	0,90	0,90	1,00	1,00	1,00	0,40	0,70	0,6	0,7	4,172	0,124							
Glycine, serine and threonine metabolism	0,90	1,00	1,00	0,70	0,90	0,90	0,90	0,90	0,90	1,10	0,9	1	5,549	0,062							
Histidine metabolism	0,50	0,60	0,50	0,40	0,70	0,60	0,70	0,60	0,30	0,40	0,3	0,6	1,456	0,483							
Lysine biosynthesis	0,60	0,60	0,60	0,70	0,80	0,60	0,60	0,60	0,80	0,60	0,7	0,5	2,328	0,312							
Lysine degradation	0,70	0,80	0,60	0,10	0,30	0,20	0,20	0,30	0,50	0,50	0,4	0,8	7,473	0,024	**	18	0	6,5	0,5	1,5	0,1
Phenylalanine metabolism	0,40	0,40	0,40	0,10	0,30	0,30	0,30	0,30	0,30	0,30	0,3	0,5	6,522	0,038	**	18	0	6	0,6	5	0,2
Phenylalanine, tyrosine and tryptophan biosynthesis	0,60	0,60	0,60	0,50	0,80	0,70	0,80	0,80	0,50	0,80	0,7	0,6	1,375	0,503							
Tryptophan metabolism	0,80	0,80	0,70	0,20	0,30	0,30	0,30	0,30	0,50	0,50	0,4	0,9	7,558	0,023	**	18	0	6	0,7	1,5	0,1
Tyrosine metabolism	0,50	0,60	0,50	0,60	0,60	0,80	0,90	0,80	0,60	0,60	0,6	0,6	Not passed Levene Test								
Valine, leucine and isoleucine biosynthesis	0,60	0,60	0,70	0,80	0,70	0,80	0,80	0,80	0,60	0,80	0,7	0,7	4,027	0,134							
Valine, leucine and isoleucine degradation	1,10	1,20	1,00	0,30	0,50	0,30	0,40	0,40	0,70	0,90	0,7	1,3	8,095	0,017	**	18	0	6	0,7	0,5	0
Metabolism;Biosynthesis of Other Secondary Metabolites;																					
Isoquinoline alkaloid biosynthesis	0,10	0,10	0,10	0,00	0,10	0,10	0,10	0,10	0,00	0,10	0,1	0,1	2,200	0,333							
Novobiocin biosynthesis	0,10	0,10	0,10	0,10	0,20	0,20	0,20	0,20	0,10	0,20	0,2	0,1	3,667	0,160							

Predicted gene family percentage / weevil sample	RPW palm_m1	RPW palm_f1	RPW palm_f2	RPW apple_m1	RPW apple_m2	RPW apple_m3	RPW apple_f1	RPW apple_f2	RPW apple_f3	R_vuln m1	R_vuln f1	R_vuln f2	χ2	p-value	p-value <0.05	Mann-Whitney U	p-value	Mann-Whitney U	p-value	Mann-Whitney U	p-value
Penicillin and cephalosporin biosynthesis	0,10	0,00	0,10	0,00	0,00	0,00	0,00	0,00	0,00	0,10	0	0	4,482	0,106							
Phenylpropanoid biosynthesis	0,10	0,10	0,10	0,30	0,10	0,10	0,00	0,00	0,00	0,10	0,1	0,1	1,952	0,377							
Streptomycin biosynthesis	0,30	0,30	0,30	0,30	0,30	0,30	0,40	0,40	0,20	0,30	0,3	0,3	0,400	0,819							
Tropane, piperidine and pyridine alkaloid biosynthesis	0,10	0,10	0,10	0,00	0,20	0,10	0,20	0,20	0,10	0,10	0,1	0,2	0,913	0,634							
Metabolism;Carbohydrate Metabolism;																					
Amino sugar and nucleotide sugar metabolism	0,90	0,80	0,90	1,80	1,10	1,10	0,90	0,90	0,70	1,00	0,9	0,8	1,486	0,476							
Ascorbate and aldarate metabolism	0,20	0,20	0,20	0,20	0,10	0,20	0,10	0,20	0,10	0,20	0,2	0,2	Not passed Levene Test								
Butanoate metabolism	1,20	1,40	1,10	1,00	0,90	1,40	1,70	1,50	0,90	1,00	0,8	1,5	0,452	0,798							
C5-Branched dibasic acid metabolism	0,30	0,30	0,30	0,30	0,20	0,30	0,30	0,30	0,20	0,30	0,3	0,3	2,200	0,333							
Citrate cycle (TCA cycle)	0,80	0,70	0,80	0,70	0,80	0,70	0,70	0,80	0,90	0,80	0,9	0,9	3,503	0,174							
Fructose and mannose metabolism	0,50	0,50	0,60	1,50	0,90	1,60	1,80	1,60	0,50	0,60	0,6	0,7	5,321	0,070							
Galactose metabolism	0,30	0,30	0,40	1,00	0,40	0,40	0,10	0,20	0,20	0,30	0,3	0,3	0,253	0,881							
Glycolysis / Gluconeogenesis	1,00	1,10	1,10	1,70	1,10	1,40	1,30	1,30	1,00	1,30	1,2	1,2	3,570	0,168							
Glyoxylate and dicarboxylate metabolism	0,90	1,20	0,90	0,40	0,50	0,60	0,60	0,60	0,60	0,50	0,4	1	4,741	0,093							
Inositol phosphate metabolism	0,20	0,10	0,20	0,10	0,10	0,20	0,30	0,30	0,20	0,10	0,1	0,2	1,452	0,484							
Pentose and glucuronate interconversions	0,40	0,40	0,40	0,50	0,40	0,40	0,30	0,30	0,30	0,40	0,3	0,4	0,913	0,634							
Pentose phosphate pathway	0,60	0,60	0,60	0,90	0,80	0,80	0,70	0,70	0,70	0,80	0,8	0,7	6,812	0,033	**	0	0	0,1	8,5	1	
Propanoate metabolism	1,20	1,30	1,10	0,50	0,70	0,70	0,80	0,80	0,80	0,80	0,7	1,3	5,449	0,066							
Pyruvate metabolism	1,10	1,40	1,20	1,30	1,20	1,30	1,40	1,30	1,30	1,10	1,1	1,4	1,246	0,536							
Starch and sucrose metabolism	0,50	0,40	0,50	1,30	0,60	0,70	0,50	0,50	0,20	0,40	0,3	0,5	2,587	0,274							
Metabolism;Energy Metabolism;																					
Nitrogen metabolism	0,80	1,00	0,80	0,60	0,70	0,60	0,60	0,60	0,50	0,60	0,5	0,8	5,911	0,052	*	0	8	0,2	8,5	1	
Oxidative phosphorylation	1,40	1,20	1,40	1,10	1,30	1,50	1,70	1,60	1,80	1,30	1,6	1,2	1,287	0,525							
Metabolism;Glycan Biosynthesis and Metabolism;																					
Peptidoglycan biosynthesis	0,60	0,50	0,60	0,90	0,80	0,60	0,60	0,60	0,80	0,80	0,9	0,5	2,408	0,300							

Predicted gene family percentage / weevil sample	RPW palm_m1	RPW palm_f1	RPW palm_f2	RPW apple_m1	RPW apple_m2	RPW apple_m3	RPW apple_f1	RPW apple_f2	RPW apple_f3	R_vuln m1	R_vuln f1	R_vuln f2	χ2	p-value	p-value <0.05	Mann-Whitney U	p-value	Mann-Whitney U	p-value	Mann-Whitney U	p-value
Metabolism;Metabolism of Cofactors and Vitamins;																					
Biotin metabolism	0,10	0,10	0,10	0,10	0,20	0,20	0,20	0,20	0,10	0,10	0,1	0,1	5,500	0,064							
Folate biosynthesis	0,40	0,30	0,40	0,20	0,40	0,40	0,50	0,50	0,20	0,50	0,3	0,4	0,226	0,893							
Lipoic acid metabolism	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,1	0,1	Not passed Levene Test								
Nicotinate and nicotinamide metabolism	0,40	0,40	0,40	0,30	0,40	0,40	0,40	0,40	0,40	0,50	0,4	0,4	2,750	0,253							
One carbon pool by folate	0,50	0,50	0,50	0,50	0,70	0,40	0,50	0,50	0,60	0,60	0,6	0,4	0,402	0,818							
Pantothenate and CoA biosynthesis	0,50	0,50	0,50	0,60	0,60	0,60	0,70	0,70	0,50	0,70	0,7	0,5	4,321	0,115							
Porphyrin and chlorophyll metabolism	0,90	1,30	0,90	0,40	0,90	1,00	1,30	1,20	0,70	0,80	0,7	0,8	2,681	0,262							
Retinol metabolism	0,10	0,20	0,20	0,10	0,10	0,20	0,20	0,20	0,20	0,10	0,1	0,2	0,943	0,624							
Riboflavin metabolism	0,30	0,20	0,30	0,20	0,30	0,40	0,50	0,50	0,30	0,50	0,5	0,3	3,088	0,214							
Thiamine metabolism	0,40	0,30	0,30	0,50	0,60	0,50	0,50	0,50	0,60	0,30	0,5	0,3	7,492	0,024	**	4	0,1	4	1	21	0,2
Ubiquinone and other terpenoid-quinone biosynthesis	0,30	0,20	0,30	0,30	0,30	0,20	0,20	0,20	0,40	0,40	0,4	0,2	1,179	0,555							
Vitamin B6 metabolism	0,20	0,10	0,20	0,20	0,20	0,20	0,20	0,20	0,20	0,20	0,2	0,2	3,000	0,223							
Metabolism;Metabolism of Other Amino Acids;																					
Cyanoamino acid metabolism	0,20	0,20	0,20	0,40	0,30	0,20	0,20	0,20	0,10	0,20	0,2	0,3	0,493	0,782							
D-Alanine metabolism	0,10	0,10	0,10	0,20	0,10	0,10	0,10	0,10	0,10	0,20	0,1	0,1	1,100	0,577							
D-Glutamine and D-glutamate metabolism	0,10	0,10	0,10	0,10	0,20	0,10	0,10	0,10	0,10	0,10	0,1	0,1	1,000	0,607							
Glutathione metabolism	0,60	0,50	0,50	0,30	0,40	0,60	0,70	0,60	0,50	0,40	0,4	0,4	3,048	0,218							
Phosphonate and phosphinate metabolism	0,10	0,10	0,00	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,1	0,1	3,000	0,223							
Selenocompound metabolism	0,30	0,30	0,30	0,40	0,30	0,40	0,40	0,40	0,20	0,30	0,2	0,3	3,300	0,192							
Taurine and hypotaurine metabolism	0,10	0,20	0,10	0,10	0,10	0,20	0,20	0,20	0,10	0,10	0	0,1	3,379	0,185							
beta-Alanine metabolism	0,60	0,70	0,50	0,10	0,30	0,30	0,30	0,30	0,40	0,50	0,4	0,7	8,391	0,015	**	18	0	6	0,07	0,5	0
Metabolism;Nucleotide Metabolism;																					
Purine metabolism	1,80	1,80	1,90	2,30	2,30	2,10	2,10	2,20	2,20	2,60	2,5	1,7	3,862	0,145							
Pyrimidine metabolism	1,20	1,20	1,20	1,80	1,70	1,40	1,40	1,40	1,40	1,80	1,8	1,1	4,022	0,134							

Predicted gene family percentage / weevil sample	RPW palm_m1	RPW palm_f1	RPW palm_f2	RPW apple_m1	RPW apple_m2	RPW apple_m3	RPW apple_f1	RPW apple_f2	RPW apple_f3	R_vuln m1	R_vuln f1	R_vuln f2	χ2	p-value	p-value <0.05	Mann-Whitney U	p-value	Mann-Whitney U	p-value	Mann-Whitney U	p-value
Unclassified;Metabolism;																					
Amino acid metabolism	0,30	0,20	0,20	0,20	0,30	0,20	0,30	0,20	0,30	0,20	0,3	0,3	0,611	0,737							
Biosynthesis and biodegradation of secondary metabolites	0,10	0,10	0,10	0,00	0,00	0,00	0,00	0,00	0,10	0,00	0	0,1	5,343	0,069							
Carbohydrate metabolism	0,10	0,10	0,10	0,20	0,10	0,10	0,10	0,10	0,00	0,10	0,1	0,1	0,000	1,000							
;Energy metabolism	0,70	0,60	0,60	0,70	0,80	0,80	0,90	0,80	0,40	0,50	0,4	0,8	2,455	0,293							
Glycan biosynthesis and metabolism	0,10	0,00	0,10	0,00	0,10	0,10	0,10	0,10	0,10	0,00	0,1	0	2,063	0,357							
Metabolism of cofactors and vitamins	0,2	0,2	0,2	0,1	0,1	0,2	0,2	0,2	0,1	0,1	0,1	0,2	2,829	0,243							

Table S5: Predicted gene families infereed on the base of the bacterial 16S rRNA and related to the bacterial physiology/metabolism, in light of a symbiotic relationship. Kruskal-Wallis, Mann-Whitney tests and p-values are reported.

Taxa/number of isolates and source organ	Hemolymph	Ovary	Testis	Gut	Total
Actinobacteria					
Corynebacterium sp.	1	1	0	0	2
Kocuria sp.	0	2	0	0	2
Microbacterium sp.	0	1	0	4	5
Rothia sp.	0	0	1	0	1
Proteobacteria					
Acetobacter sp.	2	0	0	0	2
Asaia sp.	0	0	1	1	2
Brevundimonas sp.	0	0	0	1	1
Citrobacter sp.	0	0	1	13	14
Enterobacter sp.	1	4	0	7	12
Gluconacetobacter sp.	1	0	0	0	1
Gluconobacter sp.	2	0	2	2	6
Hafnia sp.	0	0	5	6	11
Kluyvera sp.	0	0	0	0	0
Neisseria sp.	4	0	0	0	4
Pantoea sp.	0	1	0	0	1
Paracoccus sp.	0	0	0	1	1
Pseudomonas sp.	0	0	1	0	1
Serratia sp.	0	0	0	0	0
Stenotrophomonas sp.	0	5	5	2	12
Variovorax sp.	0	0	1	0	1
Xenophilus sp.	0	2	0	0	2
Bacteroidetes					
Hymenobacter sp.	0	1	0	0	1
Firmicutes					
Bacillus sp.	0	1	0	0	1
Enterococcus sp.	0	0	0	3	3
Lactobacillus sp.	0	1	0	0	1
Lactococcus sp.	0	1	1	2	4
Paenibacillus sp.	1	0	0	0	1
Staphylococcus sp.	1	3	2	1	7
Streptococcus sp.	4	0	0	0	4

Table S6: Bacterial isolates from different insect organs dissected from laboratory weevils.

Bacterial taxa	palm tissues
Serratia	absent
Comamonadaceae	present
Stenotrophomonas	present
Enterobacter	absent
Rhodobacter	absent
Ochrobactrum	absent
Microbacterium	absent
Serratia	absent
Stenotrophomonas	present
Enterobacteriaceae	absent
Ochrobactrum	absent
Kluyvera	absent
Corynebacterium	absent
Brevundimonas	absent
Corynebacterium	absent

Table S7: Bacterial OTUs shared by the three groups of weevils, the presence of these OTUs in palm tissues is reported.

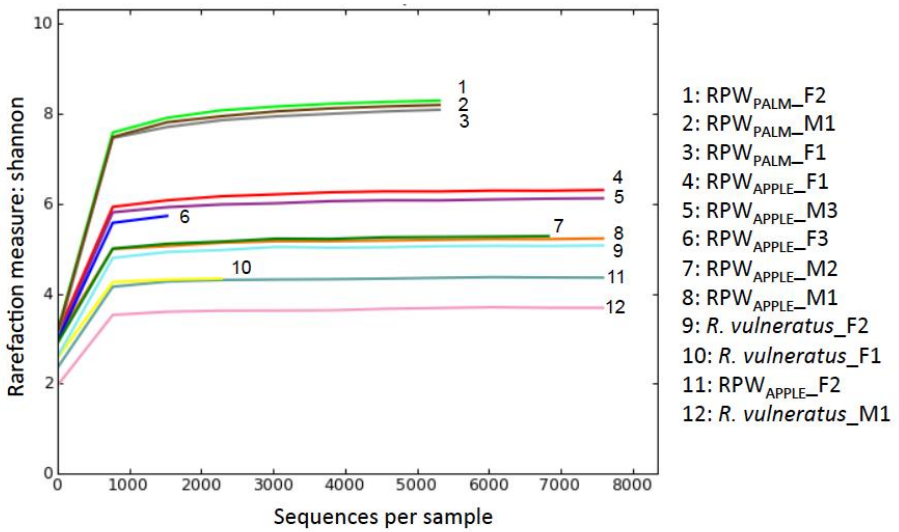

Figure S1: Rarefaction curves for total bacterial communities from the different weevil samples at 3% identity cut-off. Here are reported the curves of the Shannon index.

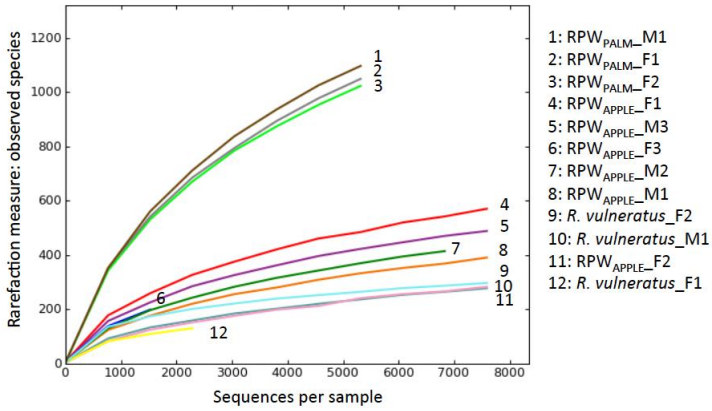

Figure S2: Rarefaction curves for total bacterial communities from the different weevil samples at 3% identity cut-off. Here are reported the curves of the cumulative number of observed species.

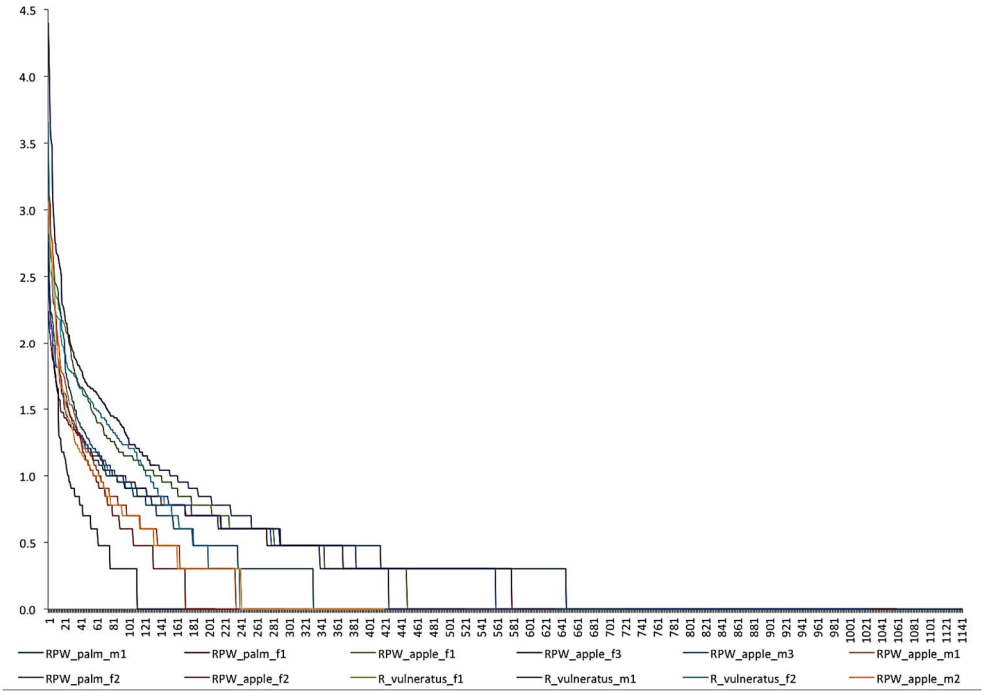

Figure S3: Log10 transformed relative abundance–rank curves for bacterial OTUs detected in the weevil specimens.

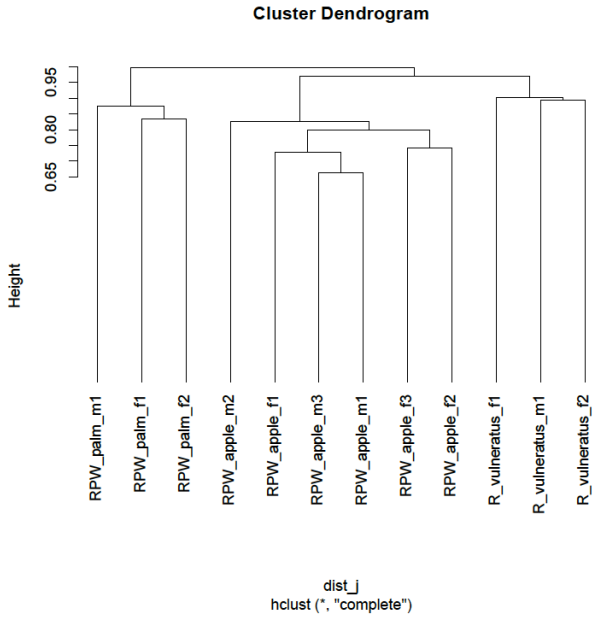

Figure S4: Hierarchical clustering dendrogram representing the OTU table pairwise dissimilarities between the different analyzed weevils. Distance matrix was estimated starting from the presence-absence OTU table adopting the Jaccard index.

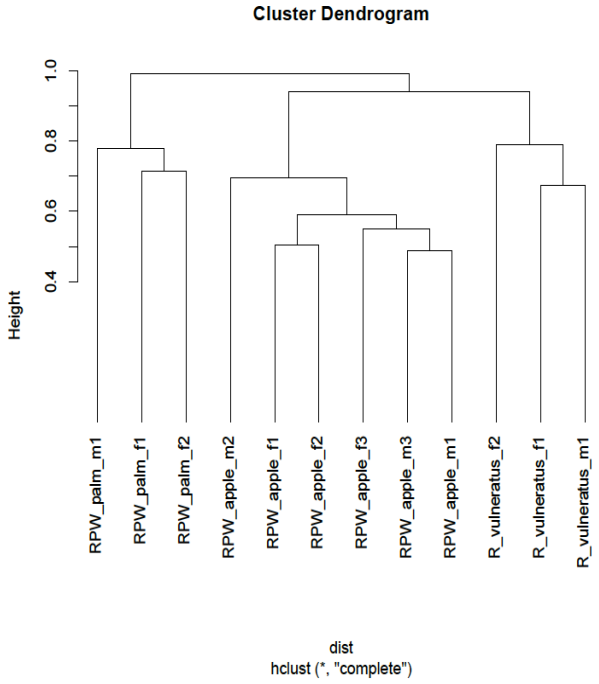

Figure S5: Hierarchical clustering dendrogram representing the OTU table pairwise dissimilarities between the different analyzed weevils. Distance matrix was estimated starting from the OTU table, with abundances, adopting the Kulczynski index.

Supplementary materials

Chapter 5 - *Psacothea hilaris hilaris*

strain ID	Sequence length	identification	RDP classifier lca_tax_greengenes lca_tax_rdp lca_tax_slv	BLAST- first described hit description	BLAST- first described hit accession	query coverage	identity
2	1098	Enhydrobacter	Enhydrobacter100% Enhydrobacter Enhydrobacter Enhydrobacter	Moraxella sp. BQEN3-02	FJ380954.1	100%	99%
4	1165	Staphylococcus	Staphylococcus100% Staphylococcus epidermidis Staphylococcus Staphylococcus	Staphylococcus epidermidis strain CIFRI D-TSB-21-ZMA	JF799891.1	100%	100%
5	1000	Enhydrobacter	Enhydrobacter100% Enhydrobacter Enhydrobacter Enhydrobacter	Moraxella sp. BQEN3-02	FJ380954.1	100%	99%
10	969	Comamonadacea e bacterium	Burkholderiales100% Comamonadaceae Xenophilus Comamonadaceae	Uncultured beta proteobacteriu m clone 120	GU202941.1	98%	95%
12	1015	Stenotrophomon as	Stenotrophomonas100% Xanthomonadaceae Stenotrophomonas Stenotrophomonas	Stenotrophomo nas sp. Ea21	JQ977692.1	100%	99%
15	1169	Acinetobacter	Acinetobacter100% Acinetobacter Acinetobacter Acinetobacter	Acinetobacter sp. NHI3-2	KC193569.1	100%	99%
16	1000	Stenotrophomon as	Stenotrophomonas99% Xanthomonadaceae Stenotrophomonas Stenotrophomonas	Stenotrophomo nas sp. Ea21	JQ977692.1	100%	98%
17	887	Comamonadacea e bacterium	Burkholderiales100% Comamonadaceae Comamonadaceae Comamonadaceae	Uncultured beta proteobacteriu m clone 120	GU202941.1	100%	97%
18	539	Stenotrophomon as	Xanthomonadaceae100% Xanthomonadaceae Stenotrophomonas Stenotrophomonas	Stenotrophomo nas sp. Ea21	JQ977692.1	100%	100%
25	1125	Microbacterium	Microbacterium99% Microbacterium Microbacterium Microbacterium	Microbacterium sp. YIM 100951	KC959569.1	100%	99%
26	840	Microbacterium	Microbacterium100% Microbacterium barkeri Microbacterium Microbacterium	Microbacterium sp. YIM 100951	KC959569.1	100%	99%
32	1133	Microbacterium	Microbacterium100% Microbacterium Microbacterium Microbacterium	Microbacterium sp. YIM 100951	KC959569.1	100%	99%
36	1350	Streptomyces	Streptomyces100% Streptomyces Streptomyces Streptomyces	Streptomyces thermocarboxy dus gene for strain: C42	AB907696.1	100%	99%
40	1007	Comamonadacea e bacterium	Burkholderiales100% Comamonadaceae Comamonadaceae Comamonadaceae	Xenophilus sp. P16	JQ928371.1	100%	95%
41	1086	Staphylococcus	Staphylococcus100% Staphylococcus epidermidis Staphylococcus Staphylococcus	Staphylococcus sp. U1368-101106-SW104	JQ082128.1	100%	99%

strain ID	Sequence length	identification	RDP classifier lca_tax_greengenes lca_tax_rdp lca_tax_slv	BLAST- first described hit description	BLAST- first described hit accession	query coverage	identity
44	1349	Streptomyces	Streptomyces100% Streptomyces Streptomyces Streptomyces	Streptomyces thermocarboxydus gene for strain: C42	AB907696.1	100%	100%
51	1030	Streptomyces	Streptomyces100% Streptomyces Streptomyces Streptomyces	Streptomyces thermocarboxydus gene for strain: C42	AB907696.1	100%	99%
52	1072	Pseudomonas	Pseudomonas100% Pseudomonas veronii Pseudomonas Pseudomonas	Pseudomonas fluorescens strain JJ8-3	KF727589.1	100%	100%
54	1377	Comamonas	Comamonadaceae100% Comamonas Comamonas Comamonas	Uncultured Comamonas sp. clone F1-7	JQ885562.1	99%	97%
55	790	Comamonadaceae bacterium	Burkholderiales100% Comamonadaceae Comamonadaceae Comamonadaceae	Uncultured beta proteobacterium clone 120	GU202941.1	100%	96%
56	1019	Stenotrophomonas	Stenotrophomonas98% Xanthomonadaceae Stenotrophomonas Stenotrophomonas	Stenotrophomonas sp.Cza49 strain Cza49	JQ977646.1	99%	99%
57	1009	Stenotrophomonas	Stenotrophomonas99% Xanthomonadaceae Stenotrophomonas Stenotrophomonas	Stenotrophomonas sp. Cza24	JQ977638.1	99%	100%
58	1130	Acinetobacter	Acinetobacter100% Acinetobacter Acinetobacter Acinetobacter	Acinetobacter sp. NHI3-2	KC193569.1	100%	99%
60	1071	Micrococcus	Micrococcus100% Micrococcus luteus Micrococcus Micrococcus	Micrococcus sp. 64A3a	KJ743999.1	99%	99%
61	1001	Micrococcus	Micrococcus100% Micrococcus luteus Micrococcus Micrococcus	Micrococcus sp. OS5	EF491955.1	100%	98%
67	1169	Staphylococcus	Staphylococcus100% Staphylococcus epidermidis Staphylococcus Staphylococcus	Staphylococcus epidermidis strain GG10H17	JN846922.1	100%	100%
68	927	Streptomyces	Streptomyces100% Streptomyces Streptomyces Streptomyces	Streptomyces thermocarboxydus gene for strain: C42	AB907696.1	100%	100%
69	831	Staphylococcus	Staphylococcus100% Staphylococcus Staphylococcus Staphylococcus	Staphylococcus hominis subsp. novobiosepticus strain ALK519	KC456583.1	100%	100%
70	939	Corynebacterium	Corynebacterium99% Corynebacterium Corynebacterium Corynebacterium	Corynebacterium glycinophilum	NR_121782.1	100%	98%
75	920	Corynebacterium	Corynebacterium100% Corynebacterium Corynebacterium Corynebacterium	Corynebacterium glycinophilum	NR_121782.1	100%	98%
76	1026	Bacillus	Bacillus100% Bacillaceae Bacillus Bacillus	Bacillus cereus strain B4	KM391942.1	99%	100%
77	925	Brevibacterium	Brevibacterium100% Brevibacterium aureum Brevibacterium Brevibacterium	Brevibacterium avium strain SS-T4	KF876886.1	100%	98%

strain ID	Sequence length	identification	RDP classifier lca_tax_greengenes lca_tax_rdp lca_tax_slv	BLAST- first described hit description	BLAST- first described hit accession	query coverage	identity
90	893	Rahnella	Enterobacteriaceae100% Enterobacteriaceae Enterobacteriaceae Rahnella	Rahnella aquatilis strain AT-113	KF817725.1	99%	99%
94	1058	Brevibacterium	Brevibacterium100% Brevibacterium aureum Brevibacterium Brevibacterium	Brevibacterium avium strain SS-T4	KF876886.1	100%	98%
97	947	Brevibacterium	Brevibacterium100% Brevibacterium aureum Brevibacterium Brevibacterium	Brevibacterium avium strain SS-T4	KF876886.1	98%	99%
99	689	Brevibacterium	Brevibacterium100% Brevibacterium aureum Brevibacterium Brevibacterium	Brevibacterium sp. SQ84A	KC921002.1	97%	99%
102	869	Luteimonas	Luteimonas99% Luteimonas Luteimonas Xanthomonadaceae	Luteimonas sp. FCS-9	KM025196.1	99%	99%
105	782	Rhodococcus	Corynebacterineae98% Rhodococcus Rhodococcus Rhodococcus	Uncultured Tsukamurella sp. clone UIL008	KC865711.1	91%	99%
106	1378	Tsukamurella	Corynebacterineae100% Tsukamurella Tsukamurella Tsukamurella	Tsukamurella paurometabola strain DSM 20162	NR_074458.1	100%	96%
111	1009	Acidovorax	Acidovorax100% Comamonadaceae Acidovorax Acidovorax	Acidovorax sp. 38a partial r gene strain 38a	HG937596.1	100%	99%
112	877	Acidovorax	Acidovorax100% Comamonadaceae Acidovorax Acidovorax	Uncultured Acidovorax sp. clone Set 2-35	JQ684146.1	92%	99%
116	1018	Acinetobacter	Acinetobacter100% Acinetobacter Acinetobacter Acinetobacter	Acinetobacter sp. NHI3-2	KC193569.1	99%	98%
120	1076	Pseudomonas	Pseudomonas100% Pseudomonas veronii Pseudomonas Pseudomonas	Pseudomonas fluorescens strain JJ8-3	KF727589.1	100%	99%
121	954	Rhizobium	Rhizobium98% Agrobacterium Rhizobium Rhizobium	Agrobacterium sp. QW10	KF737364.1	100%	98%
122	880	Rhizobium	Rhizobium100% Agrobacterium Rhizobium Rhizobium	Rhizobium sp. KT34	KJ734019.1	100%	100%
124	859	Stenotrophomonas	Stenotrophomonas99% Xanthomonadaceae Stenotrophomonas Stenotrophomonas	Stenotrophomonas sp. Cza24	JQ977638.1	100%	99%
125	847	Agrobacterium	Rhizobium100% Agrobacterium Rhizobium Rhizobium	Agrobacterium sp. ADU1	KF933534.1	100%	100%
134	1080	Epilithonimonas	Epilithonimonas99% Chryseobacterium Epilithonimonas Epilithonimonas	Epilithonimonas sp. NSG16	KC884003.1	100%	99%
135	611	Agrobacterium	Rhizobium99% Agrobacterium Rhizobium Rhizobium	Agrobacterium sp. SCAUS156	KF836041.1	100%	99%

strain ID	Sequence length	identification	RDP classifier lca_tax_greengenes lca_tax_rdp lca_tax_slv	BLAST- first described hit description	BLAST- first described hit accession	query coverage	identity
137	1007	Epilithonimonas	Epilithonimonas100% Chryseobacterium Epilithonimonas Epilithonimonas	Epilithonimonas lactis strain H1	NR_115989.1	100%	98%
138	1372	Tsukamurella	Corynebacterineae100% Tsukamurella Tsukamurella Tsukamurella	Tsukamurella paurometabola strain DSM 20162	NR_074458.1	100%	95%
148	1024	Tsukamurella	Corynebacterineae100% Tsukamurella Tsukamurella Tsukamurella	Uncultured Tsukamurella sp. clone UIL008	KC865711.1	93%	99%
151	1377	Olivibacter	Olivibacter100% Olivibacter Olivibacter Olivibacter	Olivibacter ginsengisoli strain Gsoil 060	NR_041504.1	99%	97%
152	1078	Olivibacter	Olivibacter100% Olivibacter Olivibacter Olivibacter	Olivibacter ginsengisoli strain Gsoil 060	NR_041504.1	100%	94%
153	1116	Corynebacterium	Corynebacterium100% Corynebacterium Corynebacterium Corynebacterium	Corynebacterium glycinophilum	NR_121782.1	100%	99%
154	997	Corynebacterium	Corynebacterium100% Corynebacterium Corynebacterium Corynebacterium	Corynebacterium glycinophilum	NR_121782.1	100%	98%
156	749	Corynebacterium	Corynebacterium100% Corynebacterium Corynebacterium Corynebacterium	Corynebacterium glycinophilum	NR_121782.1	100%	100%
158	883	Rhizobium	Rhizobium100% Agrobacterium Rhizobium Rhizobium	Rhizobium sp. KT34	KJ734019.1	100%	100%
160	1144	Brevibacterium	Brevibacterium100% Brevibacterium aureum Brevibacterium Brevibacterium	Brevibacterium avium strain SS-T4	KF876886.1	100%	97%
162	1130	Brevibacterium	Brevibacterium100% Brevibacterium aureum Brevibacterium Brevibacterium	Brevibacterium avium strain SS-T4	KF876886.1	99%	99%
173	999	Corynebacterium	Corynebacterium100% Corynebacterium Corynebacterium Corynebacterium	Corynebacterium glycinophilum	NR_121782.1	100%	99%
174	963	Corynebacterium	Corynebacteriaceae99% Corynebacterium Corynebacterium Corynebacterium	Corynebacterium glycinophilum	NR_121782.1	99%	97%
175	888	Corynebacterium	Corynebacterium100% Corynebacterium Corynebacterium Corynebacterium	Corynebacterium glycinophilum	NR_121782.1	100%	99%
181	885	Rhodococcus	Corynebacterineae99% Rhodococcus Rhodococcus Rhodococcus	Uncultured Tsukamurella sp. clone UIL008	KC865711.1	92%	99%
184	1000	Brevibacterium	Brevibacterium100% Brevibacterium aureum Brevibacterium Brevibacterium	Brevibacterium avium strain SS-T4	KF876886.1	100%	99%
186	1165	Rahnella	Enterobacteriaceae100% Enterobacteriaceae Enterobacteriaceae Rahnella	Rahnella aquatilis HX2 strain HX2	NR_074921.1	100%	99%

strain ID	Sequence length	identification	RDP classifier lca_tax_greengenes lca_tax_rdp lca_tax_slv	BLAST- first described hit description	BLAST- first described hit accession	query coverage	identity
187	770	Rahnella	Enterobacteriaceae100% Enterobacteriaceae Enterobacteriaceae Rahnella	Rahnella aquatilis strain TB-150	KF843720.1	100%	100%
188	865	Rahnella	Enterobacteriaceae100% Enterobacteriaceae Enterobacteriaceae Rahnella	Rahnella aquatilis strain TB-144	KF817755.1	99%	100%
189	896	Rahnella	Enterobacteriaceae100% Enterobacteriaceae Enterobacteriaceae Rahnella	Rahnella aquatilis strain AT-113	KF817725.1	99%	99%
190	870	Rahnella	Enterobacteriaceae100% Enterobacteriaceae Enterobacteriaceae Rahnella	Rahnella aquatilis strain AT-117	KF817729.1	99%	99%
191	951	Rahnella	Rahnella98% Enterobacteriaceae Enterobacteriaceae Rahnella	Rahnella aquatilis strain AT-113	KF817725.1	100%	97%
192	1039	Brevibacterium	Brevibacterium100% Brevibacterium aureum Brevibacterium Brevibacterium	Brevibacterium avium strain SS-T4	KF876886.1	99%	98%
193	1084	Roseomonas	Roseomonas100% Roseomonas mucosa Roseomonas Roseomonas	Roseomonas mucosa strain ES_21con	EU934085.1	99%	99%
197	888	Streptomyces	Streptomyces100% Streptomyces Streptomyces Streptomyces	Streptomyces thermocarboxydus strain 6-1	KJ571048.1	100%	99%
198	1135	Microbacterium	Microbacterium100% Microbacterium Microbacterium Microbacterium	Microbacterium sp. 3408bBRRJ	FJ200413.2	100%	98%
199	987	Microbacterium	Microbacterium100% Microbacterium Microbacterium Microbacterium	Microbacterium sp. YIM 100951	KC959569.1	100%	99%
200	1034	Brevibacterium	Brevibacterium100% Brevibacterium aureum Brevibacterium Brevibacterium	Brevibacterium avium strain SS-T4	KF876886.1	99%	99%
202	769	Rahnella	Enterobacteriaceae100% Enterobacteriaceae Enterobacteriaceae Rahnella	Rahnella aquatilis strain TB-144	KF817755.1	99%	99%
203	1005	Staphylococcus	Staphylococcus100% Staphylococcus epidermidis Staphylococcus Staphylococcus	Staphylococcus epidermidis strain GG10H17	JN846922.1	100%	99%
204	723	Enterobacter	Enterobacteriaceae100% Enterobacteriaceae Enterobacteriaceae Enterobacter	Uncultured Enterobacter sp. clone F7may1.10	GQ416399.1	100%	99%
206	815	Corynebacterium	Corynebacterium100% Corynebacterium variabile Corynebacterium Corynebacterium	Uncultured Corynebacterium sp. clone Z1136	EU029393.1	100%	99%
207	1374	Brevibacterium	Brevibacterium100% Brevibacterium aureum Brevibacterium Brevibacterium	Brevibacterium avium strain SS-T4	KF876886.1	100%	98%
208	905	Gordonia	Gordonia100% Gordonia Gordonia Gordonia	Gordonia bronchialis strain DSM 43247	NR_027594.1	100%	99%

strain ID	Sequence length	identification	RDP classifier lca_tax_greengenes lca_tax_rdp lca_tax_slv	BLAST- first described hit description	BLAST- first described hit accession	query coverage	identity
209	1050	Lactococcus	Lactococcus100% Lactococcus garvieae Lactococcus Lactococcus	Lactococcus garvieae strain TUB/2013/1-2	KJ909796.1	100%	100%
210	1019	Enterobacter	Enterobacteriaceae100% Enterobacteriaceae Enterobacteriaceae Enterobacter	Uncultured Enterobacter sp. clone F7may1.10	GQ416399.1	100%	100%
215	799	Enterobacter	Enterobacteriaceae100% Enterobacteriaceae Enterobacter Enterobacteriaceae	Uncultured Enterobacter sp. clone F7may1.10	GQ416399.1	99%	99%
217	846	Brachybacterium	Brachybacterium100% Brachybacterium conglomeratum Brachybacterium Brachybacterium	Brachybacterium sp. TMT4-14	JX949864.1	99%	99%
218	859	Luteimonas	Luteimonas98% Luteimonas Luteimonas Xanthomonadaceae	Luteimonas sp. FCS-9	KM025196.1	99%	99%
219	1037	Staphylococcus	Staphylococcus100% Staphylococcus epidermidis Staphylococcus Staphylococcus	Staphylococcus sp. JPR7	KM083802.1	100%	100%
220	971	Staphylococcus	Staphylococcus100% Staphylococcus sciuri Staphylococcus Staphylococcus	Staphylococcus sciuri strain APSAU153	KM192140.1	99%	99%
221	1099	Staphylococcus	Staphylococcus100% Staphylococcus sciuri Staphylococcus Staphylococcus	Staphylococcus sciuri strain Zagazig 9	KJ000305.1	100%	99%
222	1059	Staphylococcus	Staphylococcus100% Staphylococcus sciuri Staphylococcus Staphylococcus	Staphylococcus sciuri strain CtST8.1	JF935120.1	99%	99%
223	1042	Tsukamurella	Tsukamurella100% Tsukamurella Tsukamurella Tsukamurella	Tsukamurella tyrosinosolvens strain YJR102	JX154557.1	100%	99%
224	869	Enterobacter	Enterobacteriaceae100% Enterobacteriaceae Enterobacteriaceae Enterobacter	Uncultured Enterobacter sp. clone F7may1.10	GQ416399.1	100%	100%
225	839	Enterobacter	Enterobacteriaceae100% Enterobacteriaceae Enterobacteriaceae Enterobacter	Uncultured Enterobacter sp. clone F7may1.10	GQ416399.1	100%	100%
227	882	Enterobacter	Enterobacteriaceae100% Enterobacteriaceae Enterobacter Enterobacter	Uncultured Enterobacter sp. clone F7may1.10	GQ416399.1	100%	99%
228	1049	Lactococcus	Lactococcus100% Lactococcus garvieae Lactococcus Lactococcus	Lactococcus garvieae strain 29	JF831158.1	99%	99%
229	1055	Enterobacteriaceae bacterium	Enterobacteriaceae100% Enterobacteriaceae Enterobacteriaceae Enterobacteriaceae	Kluyvera sp. AaMG9	GQ915084.1	100%	99%
230	882	Enterobacter	Enterobacteriaceae100% Enterobacteriaceae Enterobacter Enterobacter	Uncultured Enterobacter sp. clone F7may1.10	GQ416399.1	100%	99%
232	1089	Enterococcus	Enterococcus100% Enterococcus Enterococcus Enterococcus	Enterococcus casseliflavus strain ALK061	KC456574.1	100%	99%

strain ID	Sequence length	identification	RDP classifier lca_tax_greengenes lca_tax_rdp lca_tax_slv	BLAST- first described hit description	BLAST- first described hit accession	query coverage	identity
233	999	Lactococcus	Lactococcus99% Lactococcus garvieae Lactococcus Lactococcus	Lactococcus garvieae strain TUB/2013/1-2	KJ909796.1	100%	99%
236	1109	Staphylococcus	Staphylococcus100% Staphylococcus Staphylococcus Staphylococcus	Staphylococcus sp. MOLA 313 partial r gene culture collection MOLA:313	AM945546.1	100%	100%
237	1177	Lactococcus	Lactococcus100% Lactococcus garvieae Lactococcus Lactococcus	Lactococcus garvieae strain TUB/2013/1-2	KJ909796.1	100%	100%
238	1060	Pseudomonas	Pseudomonas100% Pseudomonas Pseudomonas Pseudomonas	Pseudomonas chlororaphis strain HMGU6	HF952533.1	100%	99%
243	1176	Lactococcus	Lactococcus100% Lactococcus garvieae Lactococcus Lactococcus	Lactococcus garvieae strain IMAU60022	FJ215671.1	100%	100%
246	1126	Klebsiella	Enterobacteriaceae100% Klebsiella Enterobacteriaceae Enterobacteriaceae	Klebsiella oxytoca strain ATCC 43863	KC155255.1	99%	99%
248	899	Lactococcus	Lactococcus99% Lactococcus garvieae Lactococcus Lactococcus	Lactococcus garvieae strain TUB/2013/1-2	KJ909796.1	99%	100%
249	1090	Pseudomonas	Pseudomonas100% Pseudomonas Pseudomonas Pseudomonas	Pseudomonas chlororaphis strain BS1393 (rrsA)	FJ652609.1	100%	100%
250	1166	Enterobacter	Enterobacteriaceae100% Enterobacteriaceae Enterobacter Enterobacter	Enterobacter sp. XBGRY7	KJ184972.1	99%	99%
251	1151	Enterobacter	Enterobacteriaceae100% Enterobacteriaceae Enterobacter Enterobacter	Enterobacter sp. XBGRY7	KJ184972.1	100%	99%
252	996	Pseudomonas	Pseudomonas100% Pseudomonadaceae Pseudomonas Pseudomonas	Pseudomonas sp. ESBL391B1_13_4E	KJ831457.1	100%	99%
255	1165	Sphingobacterium	Sphingobacterium100% Sphingobacterium mizutaii Sphingobacterium Sphingobacterium	Sphingobacterium sp. LMG 8346	JF708886.1	100%	99%
256	969	Klebsiella	Enterobacteriaceae100% Klebsiella Enterobacter Enterobacteriaceae	Klebsiella oxytoca strain N8	KM349413.1	100%	99%
258	1086	Comamonadaceae bacterium	Comamonadaceae 100% Comamonadaceae unclassified Comamonadaceae Comamonadaceae uncultured	Comamonas sp. BF1	EU869280.1	100%	99%
259	1093	Comamonadaceae bacterium	comamonadaceae 100% Comamonadaceae Comamonadaceae Comamonadaceae	Comamonas sp. BF1	EU869280.1	99%	98%
260	1117	Alcaligenaceae bacterium	Alcaligenaceae100% Alcaligenaceae Alcaligenaceae Alcaligenaceae	Uncultured Alcaligenaceae bacterium clone F3feb.66	GQ417650.1	100%	99%

strain ID	Sequence length	identification	RDP classifier lca_tax_greengenes lca_tax_rdp lca_tax_slv	BLAST- first described hit description	BLAST- first described hit accession	query coverage	identity
261	1034	Comamonadacea e bacterium	Comamonadaceae100% Comamonadaceae Comamonadaceae Comamonadaceae	Comamonas sp. BF1	EU869280.1	99%	99%
263	899	Enterobacter	Enterobacteriaceae100% Enterobacteriaceae Enterobacter Enterobacter	Enterobacter sp. XBGRY7	KJ184972.1	100%	99%
265	859	Klebsiella	Enterobacter100% Klebsiella oxytoca Enterobacteriaceae Enterobacteriaceae	Klebsiella oxytoca strain NFSt18	GQ496665.1	100%	100%
269	1168	Enterobacter	Enterobacteriaceae100% Enterobacteriaceae Enterobacter Enterobacter	Enterobacter asburiae L1	CP007546.1	100%	99%
273	1160	Pseudomonas	Pseudomonas100% Pseudomonas Pseudomonas Pseudomonas	Pseudomonas sp. XBBRY5	KJ184971.1	100%	99%
276	1110	Ochrobactrum	Ochrobactrum100% Ochrobactrum Ochrobactrum Bacteria	Ochrobactrum pseudogrignon ense strain NG-T7	KF844052.1	100%	100%
277	1133	Comamonadacea e bacterium	Comamonadaceae100% Comamonadaceae Comamonadaceae Comamonadaceae uncultured	Comamonas sp. BF1	EU869280.1	100%	99%

Table S1: List of the sequenced isolates. In the forth column, a summary of the result of identification with two different tools: RDP classifier, and SINA classifier based on three different specialized databases: Greengenes, RDP and Silva.

gel band	Sequence Length	identification	rdpclassifier lca_tax_greengenes lca_tax_rdp lca_tax_slv	BLAST- first described hit	accession	query coverage	identity
F 10	529	Bacillus	Bacilli100% Bacillaceae Bacillaceae1 Bacillus	Bacillus anthracis strain UASWS0900	KF525802.1	100%	98%
F 11	538	Bacillus	Bacilli100% Bacillaceae Bacillaceae2 Bacillus	Bacillus anthracis strain UASWS0900	KF525802.1	100%	98%
F 12	501	Bacillus	Bacilli100% Bacillaceae Bacillus Bacillus	Bacillus cereus strain L-05	KJ534398.1	99%	99%
F 13	517	Bacillus	Bacilli100% Bacillaceae Bacillus Bacillus	Bacillus cereus strain WBD10B	KF550442.1	100%	98%
F 14	491	Bacillus	Bacillus100% Bacillaceae Bacillus Bacillus	Bacillus sp. 7S7	KM374750.1	100%	100%

gel band	Sequence Length	identification	rdpclassifier lca_tax_greengenes lca_tax_rdp lca_tax_slv	BLAST - first described hit	accession	query coverage	identity
f 16	508	Propionibacterium	Propionibacterium100% Propionibacterium acnes Propionibacterium Propionibacterium	Uncultured Propionibacterium sp. clone BDV8-11	KM355729.1	100%	99%
f 20	428	Lactobacillus	Lactobacillales99% Lactobacillales Lactobacillus Lactobacillus	Lactobacillus composti for rRNA strain:NRIC 0690	AB268119.	100%	97%
f 21	536	Leuconostoc	Leuconostoc100% Leuconostoc Leuconostoc Leuconostoc	Leuconostoc mesenteroides strain DISSPA71	KJ187156.1	99%	99%
f 22	529	Enterococcus	Enterococcus100% Enterococcus Enterococcus Enterococcus	Enterococcus sp. 15A	HQ289888.1	99%	100%
f 31	499	Acetobacter	Acetobacter100% Acetobacter Acetobacter Acetobacter	Acetobacter tropicalis strain HT-Z39-B1	KJ526825.1	100%	99%
f 34	532	Enterococcus	Enterococcus100% Enterococcus Enterococcus Enterococcus	Enterococcus gallinarum strain LMG 13129	NR_104559.2	99%	99%
f 3	519	Ralstonia	Ralstonia100% Ralstonia Ralstonia Ralstonia	Uncultured Ralstonia sp. clone BDV1-23	KM355741.1	100%	99%
f 40	434	Enterococcus	Enterococcaceae99% Enterococcus Enterococcus Enterococcus	Uncultured Enterococcus sp. clone Enter-4	JX679639.1	99%	99%
f 8	505	Erythrobacteraceae bacterium	Erythrobacteraceae95% Sphingomonadales Altererythrobacter Erythrobacteraceae	Erythrobacteraceae bacterium K-2-3	JQ963327.1	99%	99%
h 10	466	Klebsiella	Klebsiella97% Klebsiella Klebsiella Enterobacteriaceae	Klebsiella variicola strain A6128	KM275666.1	100%	100%
h 15	519	Enterococcus	Enterococcus100% Enterococcus Enterococcus Enterococcus	Enterococcus sp. ZGZ2	KF747765.1	99%	100%
h 16	524	Enterococcus	Enterococcus100% Enterococcus Enterococcus Enterococcus	Enterococcus casseliflavus strain Z6006	KC212047.1	99%	100%
h 19	517	Enterobacter	Enterobacteriaceae100% Enterobacteriaceae Enterobacteriaceae Enterobacteriaceae	Enterobacter sp. DLB27	KF791515.	99%	100%
h 1	518	Klebsiella	Klebsiella99% Klebsiella Klebsiella Enterobacteriaceae	Endosymbiont of Sphenophorus levis clone Field_clone_C03	FJ626291.1	99%	99%
h 21	514	Propionibacterium	Propionibacterium100% Propionibacteriumsacnes Propionibacterium Propionibacterium	Propionibacterium sp. B4	KJ741207.1	99%	99%
h 22	320	Enterobacter	Enterobacteriaceae100% Enterobacteriaceae Enterobacteriaceae Gammaproteobacteria	Enterobacter sp. C1D	JN936958.1	99%	99%
h 23	522	Enterococcus	Enterococcus100% Enterococcus Enterococcus Enterococcus	Enterococcus sp. ZGZ2	KF747765.1	99%	99%

gel band	Sequence Length	identification	rdpclassifier lca_tax_greengenes lca_tax_rdp lca_tax_slv	BLAST - first described hit	accession	query coverage	identity
h 24	533	Enterococcus	Enterococcus100% Enterococcus Enterococcus Enterococcus	Enterococcus sp. ZGZ2	KF747765.1	99%	99%
h 25	483	Flavobacterium	Flavobacterium100% Flavobacterium Flavobacterium Flavobacterium	Uncultured Flavobacterium sp. clone XJ67	EF648136.1	100%	99%
h 28	519	Staphylococcus	Staphylococcus100% Staphylococcus Staphylococcus Staphylococcus	Staphylococcus succinus strain EGY-SCC2	KJ524461.1	99%	100%
h 2	390	Klebsiella	Enterobacteriaceae100% Klebsiella Klebsiella Bacteria	Klebsiella variicola strain A6128	KM275666.1	100%	99%
h 30	514	Enterococcus	Enterococcus100% Enterococcus Enterococcus Enterococcus	Enterococcus gallinarum strain LMG 13129	NR_104559.2	100%	100%
h 32	519	Klebsiella	Klebsiella100% Klebsiella Klebsiella Klebsiella	Klebsiella variicola strain A6128	KM275666.1	99%	100%
h 33	441	Klebsiella	Enterobacteriaceae100% Klebsiella Klebsiella Klebsiella	Klebsiella variicola strain A6128	KM275666.	100%	100%
h 3	491	Klebsiella	Klebsiella97% Klebsiella Klebsiella Bacteria	Klebsiella sp. 2.1T	AY918477.1	99%	99%
h 6	529	Erythrobacteraceae bacterium	Erythrobacteraceae96% Sphingomonadales Altererythrobacter Erythrobacteraceae	Erythrobacteraceae bacterium K-2-3	JQ963327.1	95%	99%
h 8	433	Klebsiella	Enterobacteriaceae100% Klebsiella Klebsiella Enterobacteriaceae	Uncultured Klebsiella sp. clone GCL11	JX310748.1	100%	100%
h 9	438	Klebsiella	Enterobacteriaceae100% Klebsiella Klebsiella Enterobacteriaceae	Klebsiella variicola strain A6128	KM275666.1	99%	100%
39	522	Enterococcus	Enterococcus100% Enterococcus Enterococcus Enterococcus	Enterococcus casseliflavus strain Z6006	KC212047.1	99%	100%
m 14	491	Erythrobacteraceae bacterium.	Erythrobacteraceae95% Sphingomonadales Altererythrobacter Erythrobacteraceae	Erythrobacteraceae bacterium K-2-3	JQ963327.1	100%	99%
m 15	504	Methylobacterium	Methylobacterium100% Methylobacterium Methylobacterium Methylobacterium	Methylobacterium sp. DDW-1	FJ225120.1	100%	99%
m 17	521	Comamonadaceae bacterium	Comamonadaceae98% Comamonadaceae Comamonadaceae Comamonadaceae	Burkholderiales bacterium TP402	EF636177.1	100%	96%
m 18	531	Acidobacteria Gp6	AcidobacteriaGp6100% Acidobacteria6 AcidobacteriaGp6 AcidobacteriaSubgroup6	Uncultured Acidobacteria bacterium clone AEG_08_210	HQ597176.1	99%	98%
m 19	539	Oxalobacteraceae bacterium.	Oxalobacteraceae93% Oxalobacteraceae Oxalobacteraceae Noviherbaspirillum	Uncultured Oxalobacteraceae bacterium clone PSB011.C21_P19	GU300288.1	99%	94%

gel band	Sequence Length	identification	rdpclassifier lca_tax_greengenes lca_tax_rdp lca_tax_slv	BLAST - first described hit	accession	query coverage	identity
m 22	496	Acetobacter	Acetobacter100% Acetobacter Acetobacter Acetobacter	Acetobacter tropicalis strain HT-Z39-B1	KJ526825.1	99%	100%
m 24	531	Enterobacter	Enterobacteriaceae100% Enterobacteriaceae Enterobacteriaceae Enterobacter	Uncultured Enterobacteriaceae bacterium clone SKF008	JF733252.1	100%	97%
m 26	410	Leuconostoc	Leuconostoc100% Leuconostoc Leuconostoc Leuconostoc	Leuconostoc pseudomesenteroides strain FT268	KM207839.1	100%	99%
m 27	525	Leuconostoc	Leuconostoc100% Leuconostoc Leuconostoc Leuconostoc	Leuconostoc mesenteroides strain DISSPA73	KJ187158.1	99%	100%
m 28	498	Pseudomonas	Pseudomonadaceae100% Pseudomonasstutzeri Pseudomonas Pseudomonas	Uncultured Pseudomonas sp. clone 1 (12)	HQ018608.1	99%	99%
m 2	409	Methylobacterium	Rhizobiales100% Methylobacterium Methylobacterium Methylobacterium	Methylobacterium sp. DDW-1	FJ225120.1	100%	96%
m 30	397	Pseudomonas	Gammaproteobacteria99% Pseudomonas Pseudomonas Pseudomonas	Pseudomonas putida	L37365.1	98%	97%
m 32	370	Ralstonia	Ralstonia100% Ralstonia Ralstonia Ralstonia	Uncultured Ralstonia sp. clone BDV1-23	KM355741.1	99%	100%
m 33	390	Enterococcus	Enterococcus100% Enterococcus Enterococcus Enterococcus	Enterococcus sp. DMB7	KM203631.1	100%	100%
m 34	396	Enterococcus	Enterococcus98% Enterococcus Enterococcus Enterococcus	Enterococcus sp. DMB7	KM203631.1	99%	100%
m 35	492	Gluconobacter	Acetobacteraceae100% Gluconobacter Gluconobacter Gluconobacter	Gluconobacter oxydans strain T0-PCP05	HM562996.1	99%	98%
m 3	485	Erythrobacteraceae bacterium	Sphingomonadales100% Sphingomonadales Altererythrobacter Erythrobacteraceae	Erythrobacteraceae bacterium K-2-3	JQ963327.1	99%	94%
m 40	511	Enterococcus	Lactobacillales98% Enterococcus Enterococcus Lactobacillales	Enterococcus avium for isolate: 3-1-58	AB932529.1	99%	98%
m 5	519	Klebsiella	Klebsiella100% Klebsiella Klebsiella Klebsiella	Endosymbiont of Sphenophorus levis clone Field_clone_C03	FJ626291.1	99%	99%
m 8	493	Erythrobacteraceae bacterium	Erythrobacteraceae98% Sphingomonadales Altererythrobacter Erythrobacteraceae	Erythrobacteraceae bacterium K-2-3	JQ963327.1	99%	99%

Table S2: List of the sequenced DGGE bands. In the forth column, a summary of the result of identification with two different tools: RDP classifier, and SINA classifier based on three different specialized databases: Greengenes, RDP and Silva.

Publications & training

Papers

- Prosdocimi EM, Novati S, Bruno R, Bandi C, Mulatto P, Giannico R, Casiraghi M, Ferri E, Errors in ribosomal sequence datasets generated using PCR-coupled 'panbacterial' pyrosequencing, and the establishment of an improved approach. Mol Cell Probes. 2013 (1):65-7.
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- Montagna M, Chouaia B, Mazza G, Prosdocimi EM, Vacchini V, Crotti E, Merighetti V, Giorgi A, De Biase A, Longo S, Cervo R, Lozzia GC, Alma A, Bandi C, Daffonchio D, Effects of the diet on the microbiota of the red palm weevil. Under revision by PLoS ONE, 2014
- Conference Paper: Chouaia B, Montagna M, Vacchini V, Epis S, Mazza G, Prosdocimi EM, Crotti E, Longo S, Sacchi L, Giorgi A, Cervo R, Daffonchio D, Lozzia G, Bandi C, Microbial symbionts of the red palm weevil, *Rhynchophorus ferrugineus*: Insights from 16S rRNA pyrotag studies. Entomological Society of America Annual Meeting 2013; 11/2013

Posters

- Microbial Community Associated With The Red Palm Weevil, *Rhynchophorus ferrugineus.* Chouaia B, Montagna M, Mazza G, Epis S, Crotti E, Prosdocimi EM, Vacchini V, Daffonchio D, Cervo R, Bandi C. ICE 2012, XXIV International Congress in Entomology. Korea, 19 - 25 August 2012.
- Acetic acid bacteria and the factors driving their roles as insect symbionts. Crotti E, Chouaia B, Vacchini V, Prosdocimi EM, Sansonno L, Daffonchio D. EU US Environmental Biotechnology Workshop. St. Louis, Missouri, USA 5 - 7 November 2012
- Investigation of the microbial symbionts of the red palm weevil, *Rhynchophorus ferrugineus.* Chouaia B, Montagna M, Mazza G, Crotti

E, Epis S, Prosdocimi EM, Vacchini V, Cervo R, Longo S, Bandi C, Daffonchio D. BIODESERT International Conference. Tunis 16 – 19 December 2012.

- Acetic acid bacteria and the factors driving their roles as insect symbionts. Crotti E, Gonella E, Vacchini V, Prosdocimi EM, Mazzetto F, Chouaia B, Mandrioli M, Sansonno L, Daffonchio D, Alma A. BIODESERT International Conference. Tunis - 16 – 19 December 2012
- Acetic acid microbiome associated to the spotted wing fly *Drosophila suzukii.* Crotti E, Gonella E, Vacchini V, Prosdocimi EM, Mazzetto F, Chouaia B, Mandrioli M, Sansonno L, Daffonchio D, Alma A. BIODESERT International Conference. Tunis- 16 – 19 December 2012
- Analysis of bacterial communities involved in the parasite symbiosis between *Apis mellifera* and *Varroa destructor.* Sandionigi A, Prosdocimi EM, Casiraghi M. MD 2013, Turin, Italy, 23 – 25 October 2013
- A hypothesis on the interactions between microbiomes in a parasitic relationship: The case of *Apis mellifera* and its parasite *Varroa destructor.* Sandionigi A, Prosdocimi EM, Casiraghi M. BioSyst.Eu 2013, 18 – 22 February 2013

Meetings attended

- IV Congresso SIBE (Società Italiana di Biologia Evoluzionistica) Milan, 2-4 September 2010. (Organizing staff)
- Next Generation Sequencing applications and future perspectives, F.I.Bio. course, Naples, 27 April 2012.

  Oral presentation: "Pyro-tag for the characterization of bacterial communities"
- "Cortona procarioti 2012" Cortona 3 – 5 May 2012
- VII workshop of the PhD course "Chemistry, Biochemistry and Ecology of pesticides" DeFens, Università degli studi di Milano 21 January 2013
- "Symbiomes: systems metagenomics of host microbe interactions" Edmund Mach Foundation, San Michele all'Adige 11 – 14 March 2013
- VIII workshop of the PhD course "Chemistry, Biochemistry and Ecology of pesticides" DeFens, Università delgi studi di Milano 26-27 January 2014

Partecipation to courses

- FACILIS 2014 Summer School on microbially-driven bioremediation, Milan, 12-25 July 2014. (Organizing staff)

- 5th international course in Microbial Ecology, “Deciphering microbial diversity and function in the environment through Next Generation Sequencing” Venice, 6-10 October 2014
- ”Bioinformatics: life sciences on your computer” John Hopkins University e-learning course, accomplished with distinction, July 2014

International Training

- Molecular cloning techniques and *Drosophila* rearing, prof. Won-Jae Lee laboratory at the Seoul National University (South Korea), May to June 2013

Teaching

- Technical microbiology classes for the Microbial Biotechnology course. Academic years 2012-2013, 2013-2014, 2014-2015
- Technical advising for students doing their master thesis on microbial symbionts of insects.

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Short communication

Errors in ribosomal sequence datasets generated using PCR-coupled 'panbacterial' pyrosequencing, and the establishment of an improved approach

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ABSTRACT

Universal bacterial primers are often used in PCR-coupled sequencing approaches to investigate environmental and host-associated bacterial communities. Some of these primers can also amplify eukaryotic DNA. This is leading to the submission of datasets to public databases which are erroneously annotated as prokaryotic sequences. The present note sends a message about the risk of submitting incorrectly annotated sequence data and suggests a reliable approach for the sequencing of 16S rRNA genes and identification of bacteria within complex communities.

Oligonucleotide primers can be designed to amplify, in a single PCR reaction, 16S rRNA genes from a broad range of bacteria from environmental samples. Using tagged PCR primers, it is possible to analyze PCR products from different sources within single pyrosequencing runs and then trace the origin of the sequences using these tags [1]. It is important that primer pairs are carefully designed or selected, in order to generate sequences of a length that is adequate for the technology employed, and to amplify at least one of the variable regions of 16S rRNA gene. The variable region V3 is regarded as the most appropriate for the analysis of bacterial communities [2]. A pitfall of the panbacterial primers flanking the V3 region (e.g., 341F and 518R; Table 1) is that they also amplify 18S rRNA genes from eukaryotes [3]. This is critical for studies of bacteria associated with eukaryotes, e.g., the microbiota of the gut.

In the present study, two pyrosequencing runs, using Roche 454-FLX titanium, were performed on amplicons produced with primers 341F [4] and 518R [5] (Table 1) in PCR from genomic DNA from human blood and mite (*Varroa destructor*) samples. Ninety blood samples and 67 mites were analyzed simultaneously following chemical multiplexing. Total DNA was extracted using the DNeasy blood and tissue kit (Qiagen), following the pretreatment steps for bacteria, and a pretreatment of the columns to remove contaminating DNA ([6,7]). Primers 341F and 518R were used for the first PCR; products from the first PCR were used as templates for a second one. The first reaction was conducted in 20 μl volumes [8] using the following protocol: 90 s at 94 °C; 29 cycles of 20 s at 94 °C, 30 s at 58 °C, 20 s at 72 °C; 10 min at 72 °C; 5 min at 60 °C. The second PCR was performed using 52 bp primers, comprising pyrosequencing adapters A or B, MID tags (sequences provided by Roche, [9]) and 518R or 341F (Table 2). PCR conditions were as above, but with 40 cycles and primers at 0.5 pmol/μl. For each sample, we used a unique combination of MID tags on the forward and reverse primers. Amplicons were quantified (Bioanalyzer 2100, Agilent) and pooled in order to have in the final mixtures the same quantity of each (blood: 20 ng DNA; mites: 100 ng DNA). The two pools were then run in agarose gel, and the DNA purified from the band.

After pyrosequencing, a total of 64,853 and 70,671 sequences were obtained respectively from the human blood and from the mites. Sequences were assigned to the samples according to the MID-tagging, and assigned to particular operational taxonomic units (OTUs). The majority of the sequences belonged to a few

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Table 1
Universal bacterial primers used in this work.

Name	Orientation	Sequence (5′→3′)	Amplified region/length[a]
341F	Forward	CCTACGGGAGGCAGCAG	V3/194 bp
518R	Reverse	ATTACCGCGGCTGCTGG	V3/194 bp
27F	Forward	AGAGTTTGATCMTGGCTCAG	V1, V2, V3, V4/791 bp
778R	Reverse	AGGGTATCTAATCCTGTTTGC	V1, V2, V3, V4/791 bp
7F	Forward	GAGAGTTTGATCCTGGCTCAG	Almost entire 16S/1508 bp
1513R	Reverse	CTACGGCTACCTTGTTACGA	Almost entire 16S/1508 bp
10F	Forward	AGTTTGATCMTGGCTCAG	V1, V2, V3, V4/796 bp
807R	Reverse	GGACTACHAGGGTATCTAAT	V1, V2, V3, V4/796 bp

[a] Amplification lengths are those in *Escherichia coli*.

OTUs. BLAST-search revealed 100% identity for the majority of the sequences with human or mite 18S rRNA. The RDP classifier tool [10] was unable to reliably identify suspected eukaryotic sequences. Less than 0.2% and 2% of the sequences, respectively for blood and mite samples (corresponding to 2% and 8% of the OTUs), were of bacterial origin. In summary, ribosomal genes were amplified almost exclusively from humans and mites.

Moreover, using the BLAST tool, we discovered that several sequences in the GenBank database are annotated as 16S rRNA, but are actually eukaryotic 18S rRNA gene fragments. Except for the conserved priming sites, the 18S and the 16S rRNA sequences are clearly distinguishable, and can be localized to two separate branches on a neighbor-joining tree (Fig. 1). When the human 18S rRNA sequence, obtained from blood samples, was examined, restricting the search to only bacteria, 289 hits were found. We observed that all of them were annotated as partial 16S rRNA sequences from uncultured bacteria, but were in fact eukaryotic (see also Fig. 1). These 289 misidentified sequences had been deposited in the database from May 2005 to January 2011 and derive from studies of microbial communities in various samples (soil; gut content; oral cavity). Compared with ~3 million 16S rRNA sequences presently in public databases, the proportion of these misannotated entries is very small. Nevertheless, 289 sequences are sufficient to produce a list of incorrect BLAST results when one compares an eukaryotic 18S rRNA sequence (obtained with 341F and 518R or similar bacterial primers) to the GenBank bacterial sequence collection. Most of these erroneous sequences have been published, and have been generated by pyrosequencing. For example, 239 of the sequences published as bacterial by Humblot and Guyot (2009) [11] can be identified as being of plant origin. Also in this study, the amplicons had been produced using primers flanking the V3 region. We emphasize that other primers, in addition to those surrounding V3, likely amplify eukaryotic 18S rRNA genes. Indeed, when we queried the whole human 18S rRNA sequence (GenBank ID: 225637497), using the "discontiguous megablast" tool, restricting the search to the bacterial sequences, we retrieved several hits. Most of them were amplicons similar to the 18S rRNA region 469–631 (i.e. the fragment amplified by 341F-518R), but some sequences annotated as being of bacterial origin had a sequence identity greater than 75% to regions 797–1708 (for example GenBank ID: 160338043), 1187–1692 (for example GenBank ID: 187692214), 1314–1711 (for example GenBank ID: 326648193). It is obvious that the presence of eukaryotic sequences deposited as prokaryotic can led to major misinterpretations of results in studies of bacterial communities.

Therefore, we established a nested PCR method to selectively amplify products from only bacterial 16S rRNA, representing the V3 region, which are suitable for pyrosequencing. The method was tested on templates prepared from human blood and from arthropods (*V. destructor* and *Apis mellifera*). On each of the selected templates (DNAs from 2 blood samples, 2 mites, and 2 honeybees) we first performed three separate PCR reactions, using primer pairs that we assessed *in silico* as being specific for bacteria: 10F/807R, 27F/778R and 7F/1513R (Table 1); conditions were as in the first PCR above, but with the annealing at 55 °C. These three primer pairs amplify a fragment of the 16S rRNA gene comprising the V3 region. The amplification products were then subjected to a second PCR, using MID-tagged primers (Table 2, PCR conditions as described above for tagged primers). The final PCR products (obtained from the combination of two PCR reactions, with primers: 10F-807R followed by MID-tagged primers; 27F-778R followed by MID-tagged primers; 7F-1513R followed by MID-tagged primers) were cloned into the pGEM-T-Easy vector (Promega); the insert was amplified using the M13 forward and reverse primers, and then sequenced. All the sequences were trimmed, BLAST-searched and identified as bacterial by the use of the RDP classifier tool, with a confidence level of 95%.

For the blood project, we sequenced 72 clones. One sequence was unintelligible, while all the others were identified as bacterial: mainly Proteobacteria (90.1%), and a smaller proportion of Bacteroidetes (7%) and Firmicutes (1.4%). From 85 clones successfully sequenced from the arthropods, we obtained 77 intelligible bacterial sequences: Proteobacteria (57.1%) Firmicutes (7.8%), Bacteroidetes (32.5%), Actinobacteria (2.6%). The identifications to the

Table 2
Pyrosequencing primers used in this work.

Name	Orientation	Sequence (5′→3′)[a]
b1f	Forward	CTATGCGCCTTGCCAGCCCGCTCAGacgagtgcgtCCTACGGGAGGCAGCAG
b2f		CTATGCGCCTTGCCAGCCCGCTCAGacgctcgacaCCTACGGGAGGCAGCAG
b4f		CTATGCGCCTTGCCAGCCCGCTCAGagcactgtagCCTACGGGAGGCAGCAG
b8f		CTATGCGCCTTGCCAGCCCGCTCAGctcgcgtgtcCCTACGGGAGGCAGCAG
b9f		CTATGCGCCTTGCCAGCCCGCTCAGtagtatcagcCCTACGGGAGGCAGCAG
b10f		CTATGCGCCTTGCCAGCCCGCTCAGtctctatgcgCCTACGGGAGGCAGCAG
b14f		CTATGCGCCTTGCCAGCCCGCTCAGcgagagataCCCTACGGGAGGCAGCAG
b15f		CTATGCGCCTTGCCAGCCCGCTCAGatacgacgtaCCTACGGGAGGCAGCAG
a1r	Reverse	CGTATCGCCTCCCTCGCGCCATCAGacgagtgcgtATTACCGCGGCTGCTGG
a2r		CGTATCGCCTCCCTCGCGCCATCAGacgctcgacaATTACCGCGGCTGCTGG
a3r		CGTATCGCCTCCCTCGCGCCATCAGagacgcactcATTACCGCGGCTGCTGG
a4r		CGTATCGCCTCCCTCGCGCCATCAGagcactgtagATTACCGCGGCTGCTGG
a5r		CGTATCGCCTCCCTCGCGCCATCAGatcagacacgATTACCGCGGCTGCTGG
a6r		CGTATCGCCTCCCTCGCGCCATCAGatatcgcgagATTACCGCGGCTGCTGG
a7r		CGTATCGCCTCCCTCGCGCCATCAGcgtgtctctaATTACCGCGGCTGCTGG
a8r		CGTATCGCCTCCCTCGCGCCATCAGctcgcgtgtcATTACCGCGGCTGCTGG
a9r		CGTATCGCCTCCCTCGCGCCATCAGtagtatcagcATTACCGCGGCTGCTGG
a10r		CGTATCGCCTCCCTCGCGCCATCAGtctctatgcgATTACCGCGGCTGCTGG
a11r		CGTATCGCCTCCCTCGCGCCATCAGtgatacgtctATTACCGCGGCTGCTGG
a13r		CGTATCGCCTCCCTCGCGCCATCAGcatagtagtgATTACCGCGGCTGCTGG

[a] Sequences in lowercase represent the MID identifiers.

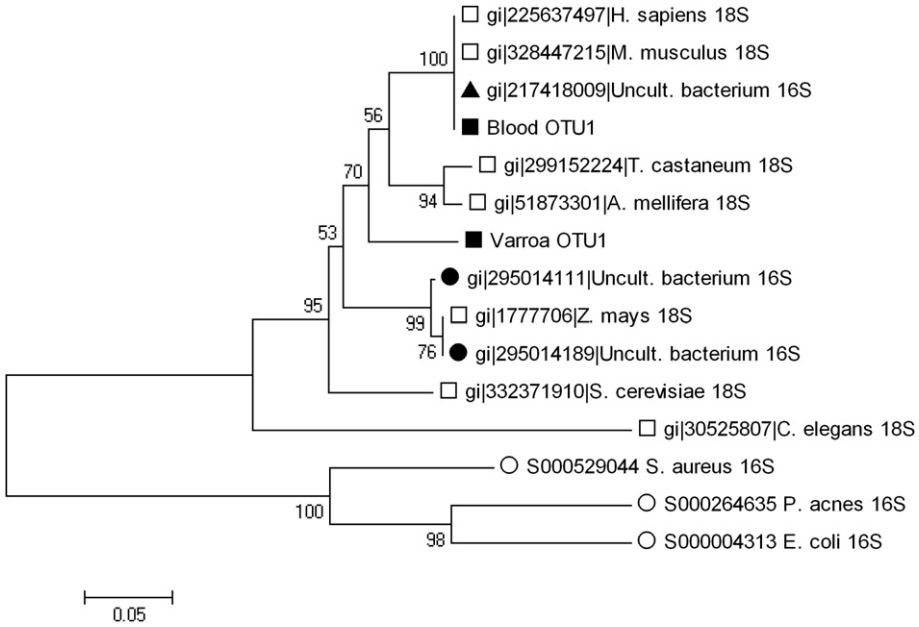

Fig. 1. Neighbor-joining tree showing the clustering with eukaryotes of sequences obtained following PCR using 16S rRNA V3 bacterial primers (341F-518R), from human blood and *Varroa destructor* samples (black squares). In addition to the above sequences, generated in our study, sequences from the databases which have been annotated as bacterial cluster with eukaryotic 18S rRNA (black circles: sequences from [11]; black triangle: unpublished sequence). White squares: reference sequences for eukaryotes (extracted from GenBank). White dots: reference sequences for bacteria (extracted from RDP database). The tree was generated with MEGA4 [12]; model: P-distance, insertion/deletion not taken into account; bootstrap: 500 replications.

phylum and class levels had a confidence greater than 95%, according to the RDP classifier.

In conclusion, the nested PCR approach established allowed us to generate almost exclusively V3 16S rRNA bacterial sequences, starting from animal-associated microbial communities, the same biological samples that led to almost exclusively eukaryotic sequences after the use of standard 'bacterial' V3 primers.

The rapid development of pyrosequencing techniques will probably lead, in the next few years, to an exponential growth of bacterial 16S rRNA sequences in public databases, coming from bacterial community studies of a variety of environmental samples. The generation of large datasets from pyrosequencing often comes with a risk of submitting erroneous sequences to public databases, which do not undergo subsequent curation. The present study sends a message about this risk of submitting incorrectly annotated sequence data, and recommends that reliable methods, such as that established here, should be used for the sequencing of 16S rRNA genes and identification of bacteria within complex communities.

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

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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 large-scale 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

Non-conventional 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 well-studied conventional habitats, such as soil- and water-associated matrices. Nonetheless, they represent a considerable source of compounds and microorganisms with interesting biological and biotechnological potential [1,2]. Growing attention has been recently directed to the study of these niches and, among these various non-conventional habitats, to the animal gut or, in general, body intended as niches in which microorganisms survive and flourish [3].

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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 [3,4]. 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 [see the review 5]. Microorganisms also played a major role in insect adaptation and evolution [6].

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

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productive agriculture and for the maintenance of the non-agricultural 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 [7]. 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 [8]. The dependence of worldwide crops on pollinators is extremely deep and during 2005 the global economic value of insect pollination was estimated to be €153 billion a year, which corresponds to 9.5% of the total economic value of agricultural crops for human consumption [9].

Since few years, concerns are rising over honeybee health and, consequently, over its impact on economy [10]. Large-scale 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 [10]. 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 [8,9,11,12]. 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 [13,14].

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 [15]. 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 [4].

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 [16]. Six phylogenetic groups, i. e. α-, β- and γ-Proteobacteria, 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 [16].

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 [17,18]. Also honeybee microbiota has been evaluated by the use of these techniques [19–23, Table 1]. Interestingly, eight bacterial phylotypes have been retrieved as major constituents of honeybee bacterial community, i. e. Alpha-1, Alpha-2, Beta, Gamma-1, Gamma-2, Firm-4, Firm-5 and Bifido, which correspond to the six phylogenetic groups mentioned above.

The metagenomic survey on honeybees from CCD-affected and not affected hives performed by Cox-Foster *et al.* [19] revealed that in non-affected honeybees Firmicutes and α-Proteobacteria are more abundant than in CCD colonies. Similarly, in the work by Cornman *et al.* [20], deep sequencing on honeybees showed a high proportion of Alpha-1, Alpha-2 and Bifido phylotypes in individuals from not affected hives compared to those from CCD-affected hives. Cloning libraries of 16S rRNA by Martinson *et al.* [21] revealed that the most abundant taxon in *A. mellifera* samples was represented by Firm-5 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, Firm-5 and Gamma-1 phylotypes (BFG phylotypes) as main members of *A. mellifera* microbiota, with a characteristic distribution along the gastrointestinal tract [22]. 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 microbial distribution with Gamma-1 phylotype gathered in a thick mat, between Beta phylotypes and the ileum wall, and with Firm-5 phylotype located in small pockets along the ileum wall. The rectum showed the majority of BFG phylotypes together with the majority of bacterial diversity [22].

A deep sampling of gut microbiota from 40 individuals has been performed by Moran *et al.* [23]. Four phylotypes were present in all samples, even if with different frequencies, i. e. one γ-Proteobacterium, classified as *Gilliamella apicola* [24], one β-Proteobacterium corresponding to *Snodgrassella alvi* [24] and two Firmicutes classified in *Lactobacillus* genus.

Yeasts, wide spread microorganisms in the honeybee environment, such as flowers, fruits and plant leaves [25,26], 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 [20], confirming previous results obtained by cultivation-dependent methods that showed the association of yeasts with honeybee [27].

TABLE 1

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.* [50]. Other cultivation-dependent data are from studies that identified the isolates by partial or complete 16S rRNA gene sequencing

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.* [51]	South Asia	Dissected guts	Sanger	8	n/a	+(3)	+(1)	+(2)	+(2)	–	–	+(1)	+(1)	[b]
Mohr and Tebbe [52]	Germany	Dissected guts	Sanger	13	n/a	–	+(1)	+(1)	+(2)	–	–	–	–	[b]
Babendreier *et al.* [53]	Switzerland	Midgut and hindgut	Sanger	27	n/a	+(3)	+(2)	+(6)	+(8)	+(1)	+(2)	+(4)	–	[b]
Disayathanoowat *et al.* [54]	Thailand	Midgut	Sanger	17	n/a	–	–	+	+(1)	–	–	+(2)	+(1)	[b]
Cox-Foster *et al.* [19]	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.* [21]	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.* [21]	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.* [22]	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.* [50]	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.* [23]	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.* [37]	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 [55]	USA	Individual larvae	Sanger	11	n/a	–	–	–	–	–	–	+(1)	–	[b]
Olofsson and Vasquez [56]	Sweden	Guts	Sanger	17	n/a	–	–	–	+(3)	+(1)	+(1)	+(4)	+(5)	[b]
Vásquez and Olofsson [57]	Arizona	Guts	Sanger	11	n/a	–	–	–	+(1)	–	+(1)	+(2)	+(4)	–
Sabaté *et al.* [58]	Argentina	Pooled intestines	Sanger	1	n/a	–	–	–	–	–	–	–	–	[b]
Loncaric *et al.* [59]	Austria	Honey sac	Sanger	11[e]	n/a	–	–	–	–	–	–	–	–	[b]
Carina Audisio *et al.* [60]	Argentina	Intestines	Sanger	5	n/a	–	–	–	–	–	–	–	–	[b]
Vásquez *et al.* [44]	Sweden and Kenya	Dissected honey crops	Sanger	137[e]	n/a	–	–	–	v	–	+(4)	+(7)	+(29)	[b]

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 number 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.*

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 [14,28].

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 [28]. Honeybees are exposed to neonicotinoids at sublethal doses, and this results in insect behavioral disturbances, orientation difficulties and impairment in social activities [28,29]. Experiments to prove these difficulties have been performed not only in laboratory conditions – by ingestion tests and indirect contact tests [29] – 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 pesticide-contaminated materials stored in the hive or exchanged with the sister bees [28]. 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 [30].

The worldwide-spread, obligate-ectoparasitic mite *Varroa destructor* represents a severe threat for apiculture. It can lead to a colony collapse within a two- to three-year 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 [31]. Moreover, *Varroa* mites act as disseminators of viruses between and within bee colonies [32]. 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 [14]. When DWV dynamics are destabilized, a host immunosuppressive status with the down-regulation of the transcriptional factor NF-kB is recorded. The authors suggest that the DWV-mediated immunosuppressive effect shows a DWV-threshold dependency; below a certain threshold, DWV infection is maintained under control. If a stress factor, like *Varroa*, subtracts the transcriptional factor NF-kB, the concentration of the latter becomes too low to keep under control DWV that can finally outbreak, bringing to the collapse the bee population [14].

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 [33]. 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* [34]) to immunity [5]. 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 food-borne pathogens [35].

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 [36,37].

While nutritional symbioses between insects and bacteria are well documented [5], 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 [38–40].

In their work Ryu *et al.* [38] 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 symbiosis at the base of tsetse–*Wigglesworthia* interactions goes beyond the nutritional role: larvae deprived of *Wigglesworthia* are immunocompromised when they reach the adult stage. Weiss *et al.* [39] 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 individuals 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* [40]. *V. fisheri* is the exclusive partner of the squid light organ and the symbiosis follows a dynamic balance of symbionts expulsion and regrowth. The well-known 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 [41]. 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 [16]. Cox-Foster *et al.* [19] showed that CCD non-affected honeybees are mainly colonized by Firmicutes and α-Proteobacteria, while in CCD-affected bees a high abundance of γ-Proteobacteria 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.

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 [4,42,43]. In *A. pomorum*, the acetic acid produced by the activity of the pyrroloquinoline quinone-dependent alcohol deydrogenase (PQQ-ADH) 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 [42]. By contrast, *L. plantarum* promotes protein assimilation from the diet, regulating diet-derived branched-chain 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 [43]. In fat bodies TOR pathway normally acts stimulating the systemic production of insulin-like 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 [4]. 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 [15], and have been indicated as major modulators of honeybee health [44]. Like LAB, well-known for their ability to produce antimicrobial factors, other symbionts such as sporeforming bacteria are indicated as producers of peptide antibiotics and antibiotic-like compounds, which in some case possess antagonistic activity [45,46]. Finally, acetic acid bacteria (AAB), widespread in nature [47], 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 [48,49].

Perspectives

There is increasing evidence that there is a strict interconnection between the intestinal microbiota balance and the health status of the host [4]. Commensal microbiota drives immune and health

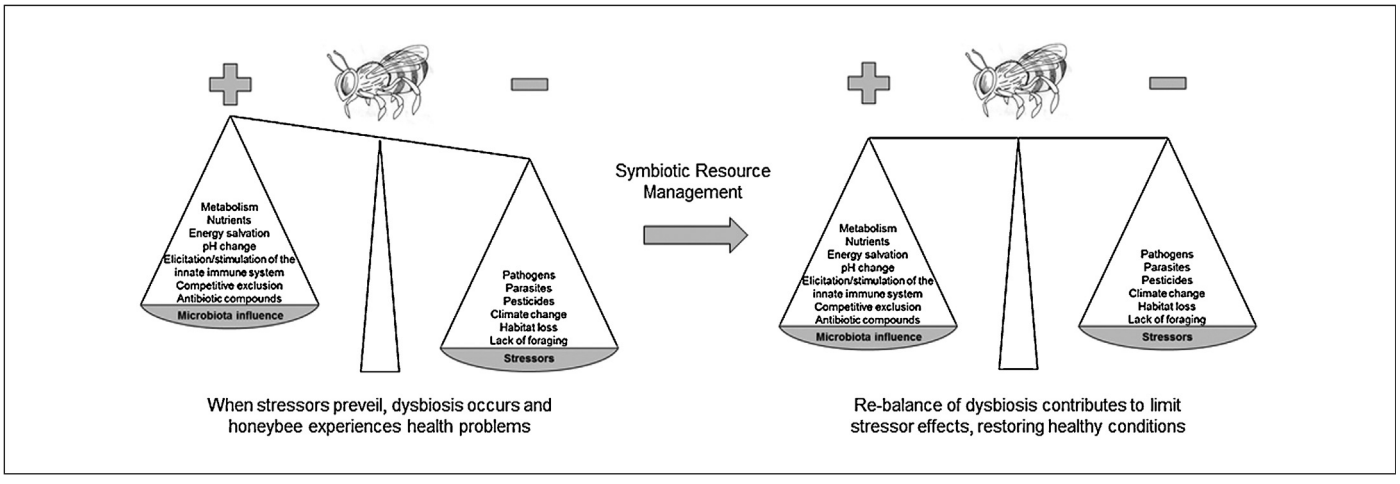

FIGURE 1

A graphical representation of the ecological concept of Symbiont Resource Management (SRM) 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 [4]. 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 [3]. A novel MRM application, the Symbiont Resource Management (SRM), can be defined as the application of microbial symbionts to manage insect-related problems [3; 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.

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Towards a better understanding of *Apis mellifera* and *Varroa destructor* microbiomes: introducing 'PHYLOH' as a novel phylogenetic diversity analysis tool

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Abstract

The study of diversity in biological communities is an intriguing field. Huge amount of data are nowadays available (provided by the innovative DNA sequencing techniques), and management, analysis and display of results are not trivial. Here, we propose for the first time the use of phylogenetic entropy as a measure of bacterial diversity in studies of microbial community structure. We then compared our new method (i.e. the web tool PHYLOH) for partitioning phylogenetic diversity with the traditional approach in diversity analyses of bacteria communities. We tested PHYLOH to characterize microbiome in the honeybee (*Apis mellifera*, Insecta: Hymenoptera) and its parasitic mite varroa (*Varroa destructor*, Arachnida: Parasitiformes). The rationale is that the comparative analysis of honeybee and varroa microbiomes could open new perspectives concerning the role of the parasites on honeybee colonies health. Our results showed a dramatic change of the honeybee microbiome when varroa occurs, suggesting that this parasite is able to influence host microbiome. Among the different approaches used, only the entropy method, in conjunction with phylogenetic constraint as implemented in PHYLOH, was able to discriminate varroa microbiome from that of parasitized honeybees. In conclusion, we foresee that the use of phylogenetic entropy could become a new standard in the analyses of community structure, in particular to prove the contribution of each biological entity to the overall diversity.

Keywords: bioinformatics, high-throughput DNA sequencing, microbial community structure, phylogenetic entropy, symbioses

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Introduction

In ecology, the analysis and interpretation of community diversity is a hot topic. In particular, with the advent of high-throughput DNA sequencing (HTS), the attention of the researchers on this topic dramatically increased. Among communities, symbioses, and particularly host–parasite interactions, are intriguing themes. In a symbiotic relationship, the partners reciprocally influence their physiology and, in general, their evolution. Nowadays, the characterization of the microbiome (intended as the sum of microscopic living beings found in a symbiotic relationship in different host body compounds, ranging from the gut to the skin) is considered pivotal to understand physiological changes occurring in a symbiosis (Mazmanian *et al.* 2005). In recent years, the scientific researchers focused on microbiome composition and variation in different hosts or physiological/environmental conditions using HTS (Sanchez *et al.* 2012; Dimitriu *et al.* 2013; Meriweather *et al.* 2013). The link between microbiome diversity and host health condition was discernible since the first published manuscripts. For instance, there is evidence that humans and mice subjected to different kind of stresses (such as diseases, parasites or ecological factors) show intense modifications in their own microbiomes in terms of initial colonization, final composition and overall stabilization (Candela *et al.* 2012; Lozupone *et al.* 2012).

However, the comprehension of mechanisms and dynamics influencing microbial diversity in hosts and

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symbionts is much more complicated due to (i) the occurrence of several interacting variables (both abiotic and biotic) and (ii) the neglected contribution of the evolutionary history of single biological entities on the overall diversity when conventional analytical methods are applied (Fig. S1, Supporting information). Currently, researchers have just started incorporating historical constraints (represented as phylogenies) into their analyses. This innovation is motivated by the aim of filling the gap between evolutionary and ecological analyses of microbial communities (Lozupone *et al.* 2007, 2011).

Here we tested two classes of approaches for microbial community analysis: distance method approaches (DMAs) and partitioning phylogenetic diversity (PPD). DMAs became a standard in microbiome analyses, whereas PPD, which uses the phylogenetic entropy as a measure of microbiome diversity (Jost 2007; Chao *et al.* 2010), is here applied for the first time. The phylogenetic entropy is a generalization of Shannon entropy based on the fact that different observed categories are not all equally different from each others, having a similar structure that could be modelled using a phylogenetic tree. We implemented PPD in the user-friendly web application, PHYLOH.

We applied DMAs and PPD to characterize microbiomes in the model honeybee (*Apis mellifera*) and its parasitic mite varroa (*Varroa destructor*, Arachnida: Varroidae). The rationale is that the comparative analysis of both honeybee and the parasitic varroa microbiomes could open new perspectives about the role of the parasite on health of honeybee colonies. Indeed, *Varroa destructor* is considered responsible of the increasing incidence of deformed wing virus (Möckel *et al.* 2011), and it was reported as a vector of bacterial pathogens causing for example the European foulbrood (e.g. *Melissococcus plutonius*) (Forsgren 2010; Evans & Schwarz 2011).

Varroa destructor parasites honeybee larvae in their brood cells, where female mites feed on honeybee haemolymph and lay eggs. Mites have a large dispersal capability and, in absence of reiterate chemical and/or antibiotic treatments, infested honeybee colonies typically collapse in few years. For these reasons, the occurrence of varroa has serious consequences on ecological, social and economic contexts (Rinderer *et al.* 2010; Rosenkranz *et al.* 2010; Annoscia *et al.* 2012; Guzman-Novoa *et al.* 2012).

In spite of the interest, the ecological dynamics of the honeybee–varroa parasitic symbiosis are still largely unknown. Studies conducted on adult honeybees showed a characteristic microbiome (Jeyaprakash *et al.* 2003; Dillon & Dillon 2004; Mohr & Tebbe 2006; Martinson *et al.* 2011; Sabree *et al.* 2012). However, the microbiome of the larval stages, as well as that of the parasitic mite, remains largely unexplored, excluding few researches on the transmission of specific pathogens (Mouches *et al.* 1984; Cornman *et al.* 2010; Forsgren 2010; Martinson *et al.* 2012). At the light of present knowledge, alterations of honeybee microbiome due to the symbiosis with varroa are expected, even if several aspects influence the final outcome. The honeybee microbiome undergoes a peculiar dynamic over the life cycle of the insect: the pupa is almost sterile, as a consequence of the physiologic characteristics of the gut tract and the diet of mature larvae during the 6 days before capping (i.e. the closure of the brood cell) (Martinson *et al.* 2012). It is reasonable to assume that the bacterial load within the brood cells partially reflects the total bacterial count of the hives and that microbial communities characterizing the hives are partially present in the cells even after capping (Martinson *et al.* 2012). But, what happens when varroa alters this equilibrium? The perturbation caused by varroa in the developmental phase of honeybee larvae, and the consequent formation of the nutrition hole caused by the parasite, could lead to the intrusion of external bacteria (both from a potential vector or from the environment) into the larva, with a substantial modification of the original microbial community. The hypothesis is that varroa mites play a fundamental role in the alteration of bacterial composition of honeybee larvae, acting not only as a vector, but also as a sort of 'swing door' through which exogenous bacteria can enter into the larva and alter the mechanisms of primary succession of honeybee microbiome.

To validate our hypothesis, we studied varroa and honeybee bacterial communities through DNA-barcoded amplicon pyrosequencing, taking advantage of the HTS methods (Blow 2008; Metzker 2009), which also allow the detection of uncultured bacteria. We compared the results of the phylogenetic entropy-based approach (implemented in PHYLOH) with a classical method based on pairwise distances. We critically evaluated the strength and weakness of both approaches and the importance of phylogenetic constraint. At the same time, we aimed to reach a more complete vision of the relationships between honeybee and varroa in the microbiome interchange.

Materials and methods

A schematic overview of the experimental pipeline is shown in Fig. 1. Our work is divided into three sections as follows: (i) laboratory procedures including sampling; (ii) DNA extraction, amplification and pyrosequencing; (iii) sequence analysis and microbial community differential analysis.

Laboratory procedures

Sampling—Honeybee larvae and varroa mites were sampled directly from capped brood cells in eight

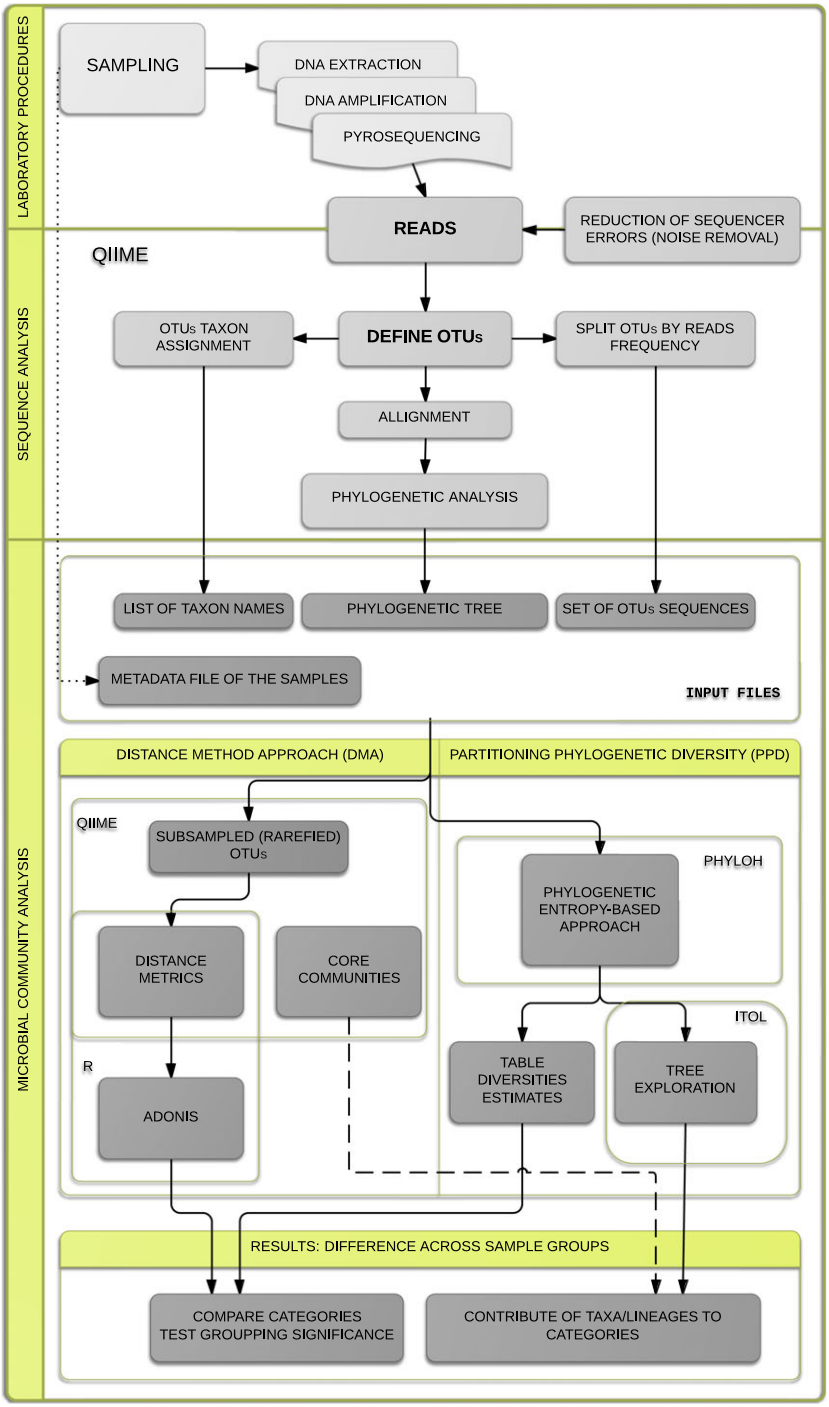

Fig. 1 Workflow of the procedures used in our project. Our work is divided into three sections: (i) laboratory procedures; (ii) sequence analysis; and (iii) microbial community analysis. In particular, the microbial community analysis was conducted following two approaches: microbial diversity analyses and partitioning phylogenetic distances.

apiaries in Northern Italy. We performed our analyses on a total of 21 individuals of honeybee larvae from seven different apiaries, and 21 varroa mites found in the same brood cells. As a negative control, a pool of five healthy honeybee larvae from a noninfected site was analysed.

Opercula of cells were opened with sterile instruments. Honeybee larvae and varroa were immediately removed and put in 2-mL tubes filled with absolute ethanol. The samples were stored at −20 °C until DNA extraction. In the study area, *V. destructor* is abundant and widespread, and consequently, we found only one noninfested apiary. This apiary was determined to be healthy after a careful inspection of all the hives by expert bee-keepers. The same experts determined that the presence of varroa in the other seven apiaries was

high. In absence of preventive acaricide treatments, these honeybee colonies would have certainly collapsed before winter.

DNA extraction—All the extraction steps were performed in a sterile laminar flow cabinet. After the removal of the head, only the first segments of *A. mellifera* specimens (after cuticular removal) were used for the DNA extraction, while for *V. destructor*, DNA was extracted from the whole organism. The dissections were made in sterile conditions with a scalpel in a Petri dish. Each sample was then rehydrated for 4 h in sterile water at room temperature, and mechanically grinded with the scalpel. Total DNA was then extracted using a commercial kit (DNeasy Blood and Tissue Kit; Qiagen, Milan, Italy) and eluted in 50 μL sterile water. A pretreatment of Qiagen columns was performed to wash away any trace of contaminating bacterial DNA (Evans *et al.* 2003; Mohammadi *et al.* 2005). DNA extracts of the five larvae from the noninfested apiary were pooled.

16S rRNA amplification and pyrosequencing—The 16S rDNA gene fragment corresponding to the V3 hypervariable region was PCR-amplified with Roche 454 FLX (Titanium reagents) using the primer pair 341F (5′-CC TACGGGAGGCAGCAG-3′) and 518R (5′-ATTACCGC GGCTGCTGG-3′) (Watanabe *et al.* 2001). The reaction was performed in a 20 μL volume with the following reagents: 1X Taq-buffer with $MgCl_2$ 1,5 mM, dNTPs 2 mM, forward and reverse primers 1 μM each, Taq polimerase 0.5 U, DNA 50 ng, milliQ H_2O to the volume. The thermal cycle was: 94 °C for 90 s, 29 cycles at 94 °C for 20 s, 58 °C for 30 s, 72 °C for 20 s followed by a final extension at 72 °C for 10 min and 60 °C for 5 min.

A subsequent nested PCR step using the products of the first one as template was performed with standard 52 bp primers, comprising pyrosequencing primers A or B, multiplex identifiers (MID) and 518R or 341F primers. PCR mix and reaction volumes were the same as described above, except for the primers (10 μM). The thermal cycle was 94 °C for 90 s, 40 cycles at 94 °C for 20 s, 58 °C for 30 s, 72 °C for 20 s and a final extension at 72 °C for 10 min and 60 °C for 5 min.

For each sample, we used a unique combination of MIDs on the forward and reverse primers. PCR products were quantified using Bioanalyzer 2100 (Agilent) and normalized for quantity. Pyrosequencing was then performed on Roche 454 GS-FLX titanium by BMR Genomics Service at the Interdepartmental Biotechnology Centre of the University of Padua (CRIBI).

Sequences analysis—Sequence analysis was performed according to the following steps: (i) reads denoising; (ii) operational taxonomic units (OTUs) definition; (iii) OTUs taxon assignment; (iv) phylogenetic analysis; (v) OTUs splitting based on reads frequencies.

(i)All reads were trimmed, filtered and assigned to the corresponding sample according to their tag. Sequences shorter than 100 bp with quality average <30 or containing unresolved nucleotides were removed from the data set. ACACIA software version 1.52 (Bragg *et al.* 2012) was used for pyrosequencing noise removal considering Balzer error model and a maximum k-mer distance between reads of 13 (default parameter for error correction). The detection of chimera reads was performed using a pipeline based on USEARCH (Edgar 2010) and UCHIME (Edgar *et al.* 2011) included in Quantitative Insights Into Microbial Ecology (QIIME) software suite (version 1.7.0) (Caporaso *et al.* 2010).

(ii)UCLUST wrapper was used to cluster sequences into OTUs, based on 97% sequence similarity. For each OTU, a cluster centroid (i.e. a representative sequence) was chosen. To estimate diversity and reduce noise in patterns of beta diversity, singleton OTUs (i.e. OTUs represented by a single sequence) were removed before community analysis (Zhou *et al.* 2011).

(iii)Using a PYTHON2.7 script, we merged the Greengenes 16S rRNA database prefiltered at 97% identity (McDonald *et al.* 2011) with a bacterial OTUs data set constituted by symbionts previously described in studies conducted on *Apis mellifera* (Mohr & Tebbe 2006; Martinson *et al.* 2011, 2012; Mattila *et al.* 2012; Moran *et al.* 2012; Sabree *et al.* 2012). To create a reference database for taxonomic assignment, bacterial 16S rRNA sequences were retrieved from GenBank and clustered using UCLUST at 97% sequence similarity. The taxonomic attribution of cluster centroid sequences was carried out using RDP Bayesian classifier (Wang *et al.* 2007) with the new merged data set obtained using a 0.8 confidence level. OTUs were assigned by the RDP classifier, considering the fifth and sixth taxonomic levels wherever possible, which, in most cases, corresponded to family and genus ranks. If RDP assignment was uncertain (probability between 0.8 and 0.9), the QIIME-selected representative sequence was used to query with *blastn* algorithm on NCBI nucleotide database. Only in case of perfect match (i.e. max identity 100%, query coverage 100%), the NCBI taxonomy was used (results are shown in Table S1 in Supporting information and/or DRYAD entry doi:10.5061/dryad.j4d15).

(iv)A maximum-likelihood (ML) phylogenetic tree was built according to default parameters using FASTTREE software (Price *et al.* 2010) integrated in QIIME.

(v)The community abundance profile, produced by UCLUST and labelled by RDP Bayesian classifier, was split into two groups, as their global frequency was lower or higher than 1%. This threshold is the advised value in QIIME manual; it is often used in the literature and

matches a gap in the frequency distribution of this data set (see Supporting information and/or DRYAD entry doi:10.5061/dryad.j4d15).

After this step, three data sets were produced: 'All Frequency Cluster' (i.e. AFC, including all OTUs), 'Low Frequency Cluster' (i.e. LFC, including OTUs with frequency lower than 1%) and 'High Frequency Clusters' (i.e. HFC, including OTUs with frequency higher than 1%). This partitioning allowed us to explore the effect of dominant and rare taxa among the microbiomes of honeybee and varroa.

Microbial community analyses

Microbial communities were examined using two approaches: the conventional distance matrices analysis (DMA) and the partitioning phylogenetic diversity (PPD). The last method was here applied for the very first time to the analysis of microbiomes.

Distance matrices analysis, coupled with ANOVA family statistics, is a well-known statistical framework in microbial community analysis that allows comparing the effect of different explanatory variables. This method is sensitive to unbalanced sampling, and it requires data rarefaction. In a HTS framework, the term 'unbalanced sampling' refers to the amount of sequences generated for each biological sample (e.g. if you get 100 000 sequences from the organism 1 and 50 000 sequences from the organism 2, the randomization leads to a loss of information from the larger sample). The principal pitfall of this approach is that it does not take into account the influence of rare OTUs on the global diversity of a sample (see also Fig. S1, Supporting information). To overcome this limit, we here propose the use of a PPD approach (Chao *et al.* 2010). PPD is being framed within information theory and can deal directly with discrete values, without producing distance matrices. It can also incorporate information deriving from unbalanced sampling, therefore avoiding a preliminary step of data rarefaction. Finally, being based on the phylogenetic structure of the data, PPD takes into account the influence of rare lineages on the microbial composition. In fact, similar sequences with low counts can build up consistent contribution to beta diversity on the branch leading to their most recent common ancestor.

The distribution of variability among bacterial communities was described for three environmental variables: (i) 'Cells' to show differences between single honeybee and the corresponding parasite found in the same brood cell, highlighting possible relationships between host and parasite microbiomes; (ii) 'Localities' to draw attention to differences existing between the microbiome of the seven apiaries; (iii) 'Status' to show microbiome differences among the pools of healthy honeybees, parasitized honeybees and mites.

Distance method approach

All the analyses were performed on the rarefied OTU tables to permit comparisons of diversity patterns within and between communities. The number of OTUs (based on the 97% sequences similarity) was determined for each sample. As depicted in Fig. 1, community analyses were performed with qualitative *(jaccard* and *unweighted UniFrac* (Lozupone *et al.* 2011) and quantitative distance metrics [*squared chord* (Cavalli Sforza & Edwards 1967; Orloci 1967) and *weighted UniFrac* (Lozupone *et al.* 2011)] using QIIME and R for statistical computing (R Development Core Team 2012).

Jaccard and *squared chord* were chosen as complementary metrics to *unweighted* and *weighted UniFrac* to test how the community pattern changes with or without phylogenetic information. We chose the *squared-chord* distance because it was identified in previous works as a metric fitting well at an exploratory analysis of communities where sampling was conducted blindly [see for example (Legendre & Gallagher 2001)].

To interpret the distance matrix, we used UPGMA hierarchical clustering method and we tested the robustness of results with jackknife analysis (1000 permutations). Further, to determine whether the grouping of samples by a given category was statistically significant, we used *adonis* (Oksanen *et al.* 2007) a permutational MANOVA allowing the use of distance matrix as dependent variable. This procedure is included within the QIIME suite and was implemented in *vegan* R package. The model used in *adonis* was the following:

$$\mathrm{Dist}\,(i, j) \sim Status + Localities + Cells$$

where i and j are all possible pairs of samples without redundancy and identity.

In this model, distances were considered as the response variable, whereas each of the environmental variables was considered as predictor.

We identified the microbial core community in honeybee and in the mite using *compute_core_microbiome.py* script from QIIME. OTUs were grouped according to their occurrence in a specific percentage of the total samples. The grouping steps were defined as elevens threshold between 0.5 and 1, corresponding to the 50% and 100% of the samples, respectively. This allowed defining the core community of each host species and more specifically to recognize the OTUs present in the majority of the samples of a given host.

Figures S2 and S3 (Supporting information and/or DRYAD entry doi:10.5061/dryad.j4d15), showing the

taxonomic assignment and the abundance distribution, were generated with *phyloseq* (McMurdie & Holmes 2013) and *ggplot2* (Wickham 2009) R packages.

Partitioning phylogenetic diversity

Following the framework proposed by Jost (2007), it is possible to parse the total phylogenetic entropy of a data set (γ component), in intragroups entropy (α component, and intergroups entropy (β component). Jost (2007) distinguishes between entropy measures, having bit has unit (or nats, or bans, depending on the logarithm base), and diversity measure having as unit the number of equally abundant categories that would produce the same amount of entropy.

Partitioning operations are performed using entropy components, while the final result is transformed into diversity by elevating to the base of the used logarithm. Assuming that cluster label of observation is collected in vector $\boldsymbol{X}$ and that group label is collected in vector $\boldsymbol{Y}$, this framework allows to define H_γ as entropy of $\boldsymbol{X}$, H_α as entropy of $\boldsymbol{X}$ conditional to $\boldsymbol{Y}$, and H_β entropy as $H_\gamma - H_\alpha$, defined also as the mutual information between $\boldsymbol{X}$ and $\boldsymbol{Y}$ (MacKay 2003).

It is important to notice that *beta* diversity (D_β), the exponential of H_β, has as unit the number of equally abundant and different samples (the categories of $\boldsymbol{Y}$), while D_γ and D_α are measured in number of equally abundant clusters (the categories of $\boldsymbol{X}$). Within microbial community analysis, the interest lays generally in estimating D_β. To assess whether this measure was significantly different from 1 (i.e. the diversity value under the hypothesis of no difference among groups), we compared the realized statistics with a null distribution obtained by a permutation of $\boldsymbol{X}$ values onto $\boldsymbol{Y}$ ones. This procedure keeps a constant number of observations per group, allowing accounting for different sampling efforts. As described here, this procedure does not consider the phylogenetic structure that links the categories of the vector $\boldsymbol{X}$. This limitation is critical for biological data. Indeed, radically different findings from a biological perspective would produce the same Shannon-based beta diversity (see Fig. S1, Supporting information). This becomes possible using the phylogenetic entropy. The phylogenetic entropy is a generalization of Shannon entropy where the different observed categories are not all equally different from each other, but have a similarity structure that could be modelled using a phylogenetic tree. Following Chao *et al.* (2010), we assume that variable $\boldsymbol{X}$ is the abundance distribution of the clusters defined by UCLUST, and its similarity structure can be modelled with a phylogenetic tree t. The phylogenetic entropy measure could be defined as follows:

$$H_p(\boldsymbol{X}) = -\sum_{i \in B_t} \frac{L_i}{T} p_i \log p_i$$

where L_i is the value of the branch length for the ith branch while T is the average distance from tip observation to root in the tree, as defined by the formula

$$T = \sum L_i p_i$$

where $i \in B_t$ is the set of branches of the tree t, and p_i is the frequency of the descendant of branch i. Once this point is set, to generalize the partitioning of diversity to include phylogenetic information, it is sufficient to apply the previous definition of γ, α, β diversity using the phylogenetic entropy instead of the Shannon entropy. Phylogenetic entropy γ (H_γ) is equal to H_p ($\boldsymbol{X}$), while phylogenetic entropy α is equal to the weighted mean of the phylogenetic entropies per group, where weights are proportional to the number observation carried out in each group. More formally, this can be written as follows:

$$H_{p\alpha} = \sum_{y \in \boldsymbol{Y}} p_y \sum_{x \in \boldsymbol{X}} H_p(\boldsymbol{X} \mid \boldsymbol{Y} = y) \quad (1)$$

where p_y is the relative frequency of observation in each group while

$$\sum_{x \in \boldsymbol{X}} H_p(\boldsymbol{X} \mid \boldsymbol{Y} = y) \quad (2)$$

is the phylogenetic entropy measured in the different group y defined in vector $\boldsymbol{Y}$. Phylogenetic entropy beta ($H_{p\beta}$) remains defined as the $H_{p\gamma} - H_{p\alpha}$. This phylogenetic entropy beta, or phylogenetic mutual information, behaves in analogous way as Shannon-based mutual information given the Kullback–Leiber divergence. This matches the different way to estimate the classical mutual information (Marcon *et al.* 2012).

Given that the $H_{p\beta}$ is a difference of two summations in which each term is relative to a branch in the tree, it is possible to reorder the terms and obtain the contribution of each branch in the final $H_{p\beta}$.

The mutual information $H_{p\beta}$, or its exponential D_β, cannot be directly compared across different grouping variable $\boldsymbol{Y}$, given that the cardinality of each variable $\boldsymbol{Y}$ (the number of possible states) defines a different upper boundary to the value of each β diversity. To normalize diversity measures across different partitioning variables, it is necessary to transform diversity in overlap or effective average proportion of shared lineages in an

individual environment. The value ranges from 1 (all lineages are shared) to 0 according to the following formula:

$$\text{Overlap} = \frac{(H_\beta - \max H_\beta)}{\max H_\beta}$$

where the maximum value of H_β is the logarithm of the number of groups or the entropy of $\boldsymbol{Y}$ depending on whether differential sampling needs to be included in the measure or not.

In this work, this approach was applied using the tree obtained from FASTTREE (Price *et al.* 2010) and the tree with the same topology, but internal branches with length 0 and terminal branches with length 1. The latter modified tree is used to perform the Shannon entropy analysis without taking into account the phylogenetic information, using the same software implementation. In fact, setting all internal branch lengths to zero leaves in the summation only the terms present in Shannon entropy formula. These two alternative settings allow to better evaluating the importance of phylogeny when interpreting the results.

Note that fractions of overlap are always higher in the phylogenetic entropy case, given that there are some phylogenetic similarities between different OTUs, while the classic Shannon-based approach assumes that each OTU is totally different from the others.

The mutual information (i.e. the beta entropy) deals with the covariation between two variables, so we explored the relationship between sequences and a single explanatory variable at the time. Within the framework of information theory, it could be possible to take into account the network of multiple interactions, but its application to phylogenetic entropy requires further investigations.

Partitioning phylogenetic diversity approach was implemented as a stand-alone Python script (PHYLOH available at https://github.com/svicario/phyloH) and includes a visualization routine based on ITOL (Letunic & Bork 2007) that allows to visualize the distribution of diversity and the contribution of the different OTUs to the partition as an html file. Input format follows PHYLOCOM input standard (Webb *et al.* 2008). To facilitate the use for the microbiologist community, we wrapped in a Web Service (https://www.biodiversitycatalogue.org/rest_methods/143) the script coupling it within a workflow (http://www.myexperiment.org/workflows/3570.html) with some parsing script enabling the use of output files from QIIME suite as input. The workflow could be run locally using a TAVERNA desktop engine (Wolstencroft *et al.* 2013) or as web application in the BioVeL portal. Access to the portal could be obtained from the BioVel website (www.biovel.eu) or contacting directly the authors.

Results

Results are organized according to the pipeline showed in Fig. 1.

Sequence analysis

Define OTUs—After sorting sequence reads for quality scores, sequencing errors and chimeras, we obtained 34 816 sequences. UCLUST returned 295 OTUs (data set AFC).

Taxonomic assignment—The complete list of OTUs, with corresponding taxa names and acronyms, is provided as Supporting information and/or DRYAD entry doi:10.5061/dryad.j4d15 (Table S1, Supporting information). Taxon assignment at family level of the three types of organisms involved (healthy honeybee, parasitized honeybee and varroa) is shown in Fig. 2.

Split OTUs by sequence frequency—Of 295 OTUs, 21 exceeded the threshold of 1% of minimum total observations (data set 'HFC', total sequences: 24 005), and 274 were defined as a rare OTUs not reaching the 1% threshold (data set 'LFC', tot sequences: 10 811).

Phylogenetic analysis—The maximum-likelihood tree generated by FASTTREE is shown as internal tree in PHYLOH output (see Supporting information and/or DRYAD entry doi:10.5061/dryad.j4d15).

Microbial community analysis

Distance method approach—The UPGMA analysis, considering *jaccard* distances for all the three sets of observations (AFC, HFC, LFC), shows a single cluster including all samples belonging to parasitized honeybees and mites and a separate cluster including the pool of healthy honeybees. On the contrary, with *squared-chord* metrics, which consider abundances information of OTUs in HFC and AFC, the analysis shows two different groups between parasitized honeybees and mites. These results are partially replicated with *UniFrac* (both *weighted* and *unweighted*), although separation is less sharp (see Fig. S4, Supporting information).

The *adonis* test was performed separately for each variable (*Cells*, *Localities* and *Status*) and using all variables together without interactions. Results were highly similar; for this reason only the coefficients of the model with all predictors combined are reported (Supporting

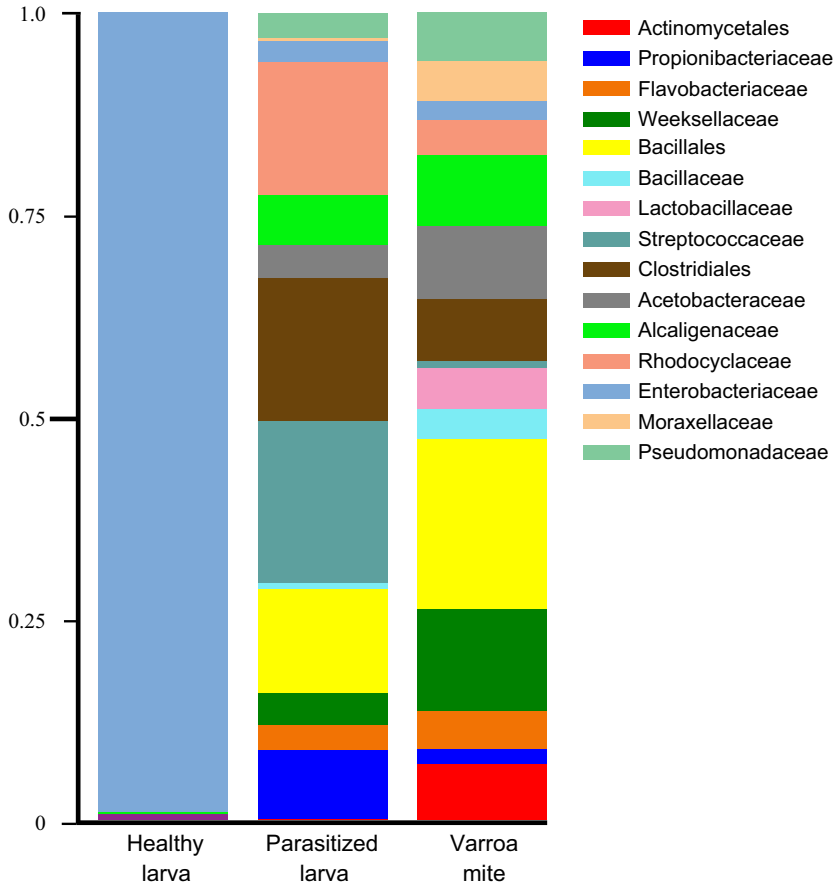

Fig. 2 Structure of microbiomes from healthy and parasitized honeybees and varroas. The histogram shows the 15 families detected and relative abundances. In two cases (Bacillales and Clostridiales), the family rank was not assigned, and consequently, the order rank is shown.

information Table S2 and/or DRYAD entry doi:10.5061/dryad.j4d15).

The *adonis* test grouping the samples for brood cells (*Cells*) has limited significance (*P*-values between 0.05 and 0.01) and small effect compared to degrees of freedom used up (34–35% explained variance, 1.6% the mean value for single cell). In addition, *adonis* test is significant only using the *jaccard* distance and *unweighted UniFrac* in the LFC data set. We found the same variance in the AFC data set, but only using the *unweighted UniFrac* (35% variance explained and *P*-value of 0.04).

A slightly stronger signal is detected in *Localities*, where *jaccard*, *unweighted UniFrac* and *squared chord* show a significant grouping in the AFC data set (*P*-value <0.01 and effect 17–20% with per-*Localities* mean variance explained between 3% and 2%). Similar signal is also visible with HFC and LFC, but only *jaccard* is significant (Supporting information Table S2 and/or DRYAD entry doi:10.5061/dryad.j4d15).

The *Status* grouping shows a very different pattern. The mean variance explained per state of *Status* builds up to 4–5% in the AFC data set and 9% for the *weighted UniFrac* in HFC. Only the LFC subset is not significant for the majority of distances used, and in any case the mean variance explained is quite low (1%).

Microbial core communities. In healthy honeybees, we found only three OTUs, one of them (Proteo-7, a member of the genus *Serratia*) accounting for the 99% of sequences. The remaining 1% is shared between two OTUs: Proteo-2 and Firmi-7, respectively, identified as *Achromobacter* sp. and *Lactobacillus* sp.

On the curves returned by QIIME script, we defined the host and parasite core microbiomes using a 0.8 threshold (Fig. S5, Supporting information). The threshold was chosen according to the steepest point in the curve, namely the point in which the least addition of OTU in the core ensures the largest fraction of samples to be compliant with the core representation. This means that a single OTU has to be present in the 80% of samples of a certain species to be considered 'core' for that species.

The OTUs considered as microbial core for honeybee and varroa are shown in Table 1.

Partitioning phylogenetic diversity—We used PHYLOH to perform partitioning tests on three environmental variables (*Cells*, *Localities* and *Status*) for the three frequency data sets (AFC or LFC or HFC) considering the

Table 1 OTUs associated with healthy and parasitized honeybees and varroa by *compute_core_microbiome.py* script

Parasitized honeybee	Varroa mite	Shared
Propionibacterium sp. (Actino-1)	*Chryseobacterium* sp. (Bacte-1)	*Chryseobacterium* sp. (Bacte-1)
Chryseobacterium sp. (Bacte-1)	Flavobacteriaceae (Bacte-2)	*Streptococcus* sp. (Firmi-1)
Streptococcus sp. (Firmi-1)	*Streptococcus* sp. (Firmi-1)	*Lactobacillus* sp. (Firmi-7)
Clostridiales (Firmi-3)	*Bacillus* sp. (Firmi-2)	*Hydrogenophilus* sp. (Proteo-1)
Lactobacillus sp. (Firmi-7)	*Geobacillus* (Firmi-5)	*Achromobacter* sp. (Proteo-2)
Hydrogenophilus sp. (Proteo-1)	*Anoxybacillus* (Firmi-6)	
Achromobacter sp. (Proteo-2)	*Lactobacillus* sp. (Firmi-7)	
Escherichia sp. (Proteo-6)	*Hydrogenophilus* sp. (Proteo-1)	
	Achromobacter sp. (Proteo-2)	
	Pseudomonas sp. (Proteo-4)	
	Escherichia sp. (Proteo-6)	

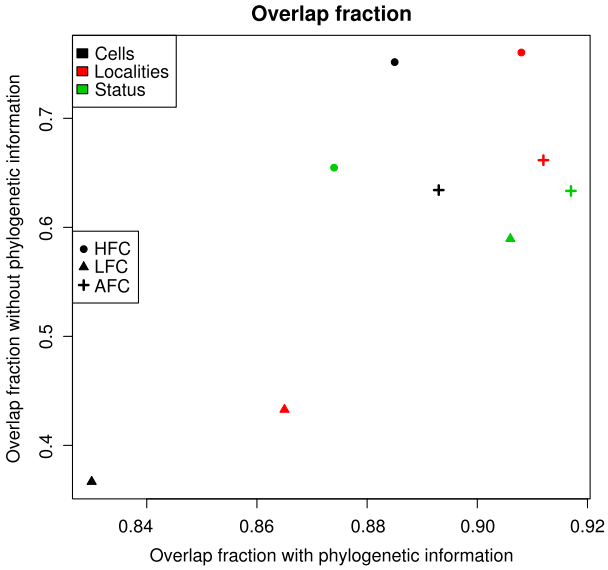

Fig. 3 Comparison of the percentage of overlap of the six data sets with or without taking into account the phylogenetic information. The percentage of overlap was calculated using estimate of beta diversity and phylogenetic beta diversity for AFC, HFC, LFC cluster frequencies and *Cells*, *Localities*, *Status* environmental variables.

phylogenetic information available or not. We can observe several qualitative differences among the 18 analyses transforming the mutual information into percentage of overlapping to allow a comparison among variables. As shown in Fig. 3, *Localities* and *Cells* variables are not heavily influenced by phylogenetic information. The relative position of LFC, HFC and AFC, measured in fraction of overlap counted in OTUs or lineages, is similar. In fact, LFC has, in both cases, low overlap, whereas AFC and HFC data sets show the maximum overlapping.

The variable *Status* describes a very different pattern from the previous two. Taking into account phylogeny, HFC differentiates more among groups (overlap 0.87), with the other two data sets showing about 0.91 overlapping. On the contrary, if phylogenetic information is not considered, the pattern is similar to the other two variables. The biggest difference is observable in LFC, while the smallest difference is observable in HFC.

The contribution of the branches to the mutual information between sequences and the Status *variable*. The experimental design output and the numerical partitioning of phylogenetic diversity for *Status* variable considering all OTUs found (AFC) are shown in Table 2. A general overview of the branches contribution to beta diversity across groups is shown in Fig. 4. The 295 AFC OTUs are well distributed on the phylogenetic tree, and consequently, their impact on the phylogenetic index is higher. It is observable that Bacte-1, -2, -3 are more typical of the varroa and are all grouped in the same lineage (L208). On the contrary, in Proteobacteria and Firmicutes, it is possible to find OTUs preferentially found in honeybee or varroa. Proteo-12, Proteo-14, Proteo-46, Proteo-24 and Proteo-17 OTUs belong to lineage L268 and are preferentially present in honeybee. There are three isolated OTUs preferentially found in varroa (Proteo-3, Proteo-4 and Proteo-5). Firmi-1, Firmi-9 and Firmi-4, typical of honeybee, are mixed with varroa's OTUs Firmi-6, Firmi-14, Firmi-2, Firmi-8 (both descending from lineage L387).

This lack of strong phylogenetic signal could be caused by recent specialization or random community assembly, given that Firmi-6 has a lower frequency sister taxa Firmi-14 also present mainly in varroa we prefer the first hypothesis.

Discussion

The analysis of complex communities (such as microbiomes) is today standard in different fields of biology and medicine. The number of published works is increasing daily, but there are some concerns on the real quality of the results showed. We are here comparing a 'traditional' approach to the community analysis [i.e. distance method approach (DMA)], vs. the innovative partitioning phylogenetic diversity (PPD, here implemented in

Table 2 Variability in reads count for each sample. Variability in reads count and sample number in groups. Overall gamma in each group. Overall alpha and contribution to alpha in each group. Beta across samples within groups and between groups. E is always within S, given that each sample belongs to only one environment type or sample group

Experimental design diversity: entropy and diversity of observation in the different groups		
H_Environment		
H(E)	MaxDiversity	Diversity
0.691	2	2

Experimental design diversity: entropy and diversity of observation in the sample within the groups		
H_Sample		
H(E)	MaxDiversity	Diversity
3.68	42	39.5

Gamma diversity: diversity using all data and in each group					
H_gamma		H_gamma_parasitized_honeybee		H_gamma_mite	
H(T)	alpha_Diversity	H(T | E = host_p)	gamma_Diversity	H(T | E = pars)	gamma_Diversity
1.76	5.83	1.75	5.73	1.69	5.42

Alpha diversity: mean within group diversity			
H_alphaByEnvironment		H_alphaBySamples	
H(T | E)	alpha_Diversity	H(T | S)	alpha_Diversity
1.72	5.56	1.35	3.85

Beta diversity or mutual information between the phylogeny and a given grouping: diversity across group and across sample within same group							
MI_treeAndEnvironment				MI_treeAndSampleGivenEnvironment			
I(T,E)	beta_Diversity	Percentage_Overlap	*P* value	I(T,S | E)	beta_Diversity	Percentage_Overlap	*P* value
0.0465	1.05	0.933	0	0.369	1.45	0.466	0

Difference of each group from total: phylogenetic Kullback–Leiber divergence between each group and the overall sample	
KL_of_host_p Observed	KL_of_pars Observed
0.0162	0.0303

H_gamma = H_alphabyEnvironment + H_beta = H(T) = H(T | E) + I(T,E) while taking into account sample info: H_gamma = H_alphaBySample + H_betabySamplegivenEnvironment+H_beta = H(T) = H(T | S) + I(T,S | E) + I(T,E).

our newly proposed software PHYLOH). The final aim is to propose our approach as a new standard in community diversity analysis. We tested the performance and utility of DMAs and PPD in the case of honeybee and varroa microbiomes, a biological scenario intriguing and complex at the same time. Our results showed that healthy honeybees have a simplified microbiome, constituted of few bacterial OTUs, while varroa is characterized by a more complex microbiome, qualitatively not different from that of parasitized honeybee. A simple conclusion could be that varroa microbes infected honeybees. However, the most abundant OTUs of parasitized honeybee do not derive directly from the mite, but are generalist or environmental bacteria. This is undoubtedly a peculiar result suggesting that these microbes could play a role of pioneer species, with a potential pathogenic activity (i.e. Firmi-1, -9, L387, *Streptococcus*; Firmi-4, -14, L387, Clostridiales) (Lozupone *et al*. 2012).

Our results show that both DMAs and PPD approach give comparable results, but only PPD shows explicit support and allows, within the same statistical framework, to observe our data both from the general pattern to the contribution of single or group of OTUs.

We partitioned data according to three environmental variables, which represent three different forces shaping microbiome diversity: *Localities*, *Cells* and *Status*. When *Localities* variable is considered, in DMAs approach, the UPGMA method and the *adonis* test analyses show a weak, but significant signal for most of the distances and data set used. When all OTUs (AFC) combined with *unweighted UniFrac* are considered, the signal is stronger.

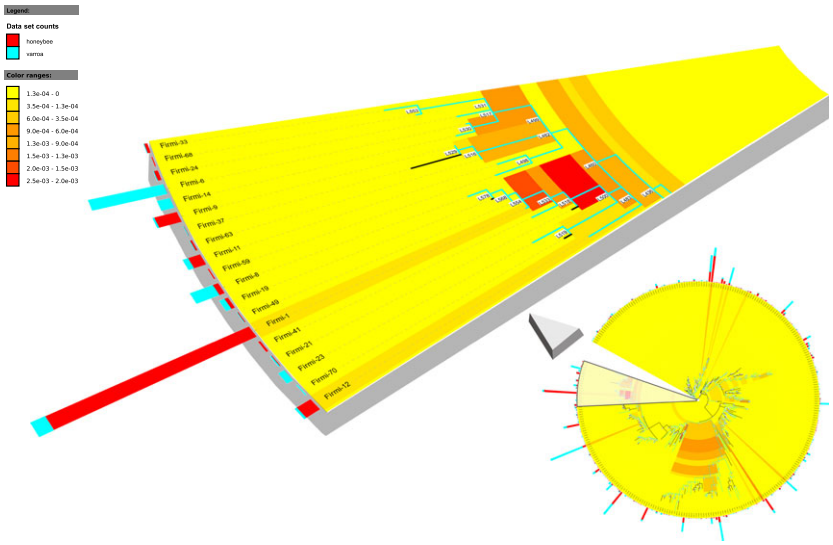

Fig. 4 In the figure are shown the main tips and relative branches of one of the most discriminating lineages across *Status* groups [L387, see tree.html (Appendix S1) in Supporting information and/or DRYAD entry doi:10.5061/dryad.j4d15 for further details] as example of the 'hairy pacman graphical output' derived from a PHYLOH analysis. Three types of data are shown on the tree: (i) the cyan colour of the branches indicates a significant contribution to I(T | E) (beta entropy or mutual information); (ii) the background of each branch is a gradient (colour range) from yellow to red for increased contribution to I(T | E); (iii) bar plot on each tip indicates the number of reads count in each group. For more details, see README file (Appendix S1) in Supporting information and/or DRYAD entry doi:10.5061/dryad.j4d15.

The results are similar using PPD, but with a stronger support. In fact, the variable *Localities* produces a significant effect, although this is better appreciable for rare OTUs (LFC) rather than considering the most abundant OTUs (HFC). In other words, low-frequency OTUs (better defined using PPD) represent the fraction of bacteria characterizing the single apiary. This finding is in agreement with previous results, showing that geographical distance is not crucial in shaping the core microbiome of beehives (Sabree *et al.* 2012).

In our hypothesis, the bacteria are transmitted from varroas to the honeybee larvae. This is corroborated by the analysis of the variable *Cells*, where differences between each larva and the corresponding mite were estimated. This grouping has limited effects in terms of mean explained variance when the DMA approach is used, but it is still significant for some distances in AFC and LFC data sets. In PPD, the overlap across categories is obtained by *Cells* with the LFC data set, showing a strong effect. This situation is difficult to explain: only the more frequent bacteria are shared among cells, whereas rare bacteria are not. However, the rare bacteria are more interesting because they act as a clear signature to discriminate different apiaries.

Considering the variable *Status*, both DMAs and PPD approaches can discriminate the three different categories: healthy honeybees, parasitized honeybees and varroas. Healthy honeybees have a simply and distinctive community with only one dominant OTU and two other low-frequency OTUs. The most representative phylotype (Proteo-7) belongs to the genus *Serratia* that was isolated from the intestinal contents of healthy foraging worker honeybees (Jeyaprakash *et al.* 2003), a well-known symbiont (generally harmless) in many insect taxa (Dillon & Dillon 2004).

The scarcity of bacteria in the healthy larvae could be attributed to their particular gut morphology, physiology and nutrition (Martinson *et al.* 2012). Indeed, the larva retains its faeces from the early days of development, due to the temporary absence of a connection between the large mid-gut and the hindgut. The mature larva defaecates just before spinning a cocoon, when the capping has already happened. As the cocooned pupa does not eat, we can assume that there is no further colonization by bacteria present in the brood cell. Through these mechanisms, the early microbiome characterizing honeybee larvae is maintained constant in composition and ubiquitous in space (Jeyaprakash *et al.* 2003; Mohr & Tebbe 2006).

The higher level of bacterial communities diversity showed in varroa and parasitized larvae suggests a transmission from parasite to the host. These two microbiomes are identical from a qualitative point of view (taking into account the HFC), but the relative abundance of the different OTUs clearly differentiates the two bacterial communities. When phylogenetic information is included, the level of discrimination between parasitized larvae and varroa is even higher. In fact, only using

PPD and *weighted UniFrac* in DMA (i.e. the approaches that take into account the phylogenetic signal), the two clusters are highly discriminated. However, only in PPD, it is possible to show the contribution of the single OTU and groups of OTUs to the differences observed among samples.

The enhanced performance of PPD is exemplified in PHYLOH output tree [Supporting information tree.html (Appendix S1) and/or DRYAD entry doi:10.5061/dryad.j4d15 and as an example Fig. 4] where the lineages L208, L268 and L387 are those discriminating the microbiome of varroa and infected honeybee. In particular, L208 lineage encompasses OTUs present in varroa, while L268 and L387 contain OTUs present in both varroa and honeybees. The most discriminating OTUs of lineage L208 belong to the genus *Chryseobacterium* (Bacte-1, -2, -3). In contrast to the majority of bacteria belonging to Flavobacteria, typically found in soil and water environments, two (Bacte-1, -2) of these three OTUs were found as pathogens of soft ticks (Burešová *et al.* 2006). Given the phylogenetic closeness between mites and ticks, it would be interesting to investigate the role of this genus in mites. The OTUs belonging to the lineage L268 were assigned to genus *Haemophilus*, in which bacteria recognized as pathogenic to bees but not associated with the presence of varroa were found. Indeed, there are numerous studies related to the antimicrobial properties of honey with references to *Haemophilus* (i.e. Jeffrey & Echazarreta 1996; Antúnez *et al.* 2008; Al-Waili *et al.* 2011).

Proteo-4 (genus *Pseudomonas*) is strongly present in varroa, probably because it is common on the mite cuticle (Tang *et al.* 2012), which had not been removed in our study.

Lineage L387 (Bacillaceae: Firmi-6, -8) includes OTUs associated with honeybee (Mohr & Tebbe 2006; Evans & Schwarz 2011; Moran *et al.* 2012), but here we found associated mainly with varroa. It is possible that these bacteria may be generalist present in the hive.

In conclusion, we would like to underline that the partitioning of phylogenetic diversity is a powerful method to analyse community diversity. Using PPD, it is possible to evaluate the different analyses using the percentage of overlap across groups as a comparable statistic. The use of relative read frequencies as distance among samples, instead of the summary statistics typical of DMAs, allows to identify which lineages, or groups of lineages, generate the significant differences. Furthermore, the permutation procedure on all sequences prevents any subsampling procedure (i.e. rarefaction), a practice that has been recently criticized because it reduces the resolution power hiding the signal coming from rare OTUs (McMurdie & Holmes 2014).

Recently, Chiu *et al.* (2014) proposed a different formulation for alpha diversity than the one proposed in Jost (2006, 2007). Here, the alpha entropy becomes the joint entropy of observation and environment minus the logarithm of the number of environments. The new formulation allows beta diversity to reach its theoretical maximum (number of environments) whatever unbalanced design is used, but it causes the lower bound of the beta diversity to be higher than 1 depending on sampling design. Furthermore, the alpha diversity does not match anymore the concept of mean diversity within each environment. Consequently, we are not following this new formulation because it is distant from the canonical information theory and further evaluations are needed.

A brief example could illustrate the reason of our preference. Let us assume two communities with the same six equal abundant species, but one locality has 100 observations and the other 1000. According to our definitions, alpha and beta diversities values are, respectively, 6 and 1, while according to Chiu *et al.* (2014) definition corresponding values are 4.07 and 1.475. We think much more logic, and close to the original information theory realm, the values of 6 and 1 that reflect the fact that in each environment we expect to find six species and that the two samples behave as 1. Chiu *et al.* (2014) could oppose that using a very similar example, but with the six species being different in the two environments, the value of beta diversity would be 1.35 for the formulation of this article and 2.00 for theirs. This apparent mismatch could be corrected by realizing that the real maximum beta diversity is given by the exponential of the entropy of the sampling vector that is exactly 1.35.

Partitioning phylogenetic diversity method was here implemented in our newly proposed tool PHYLOH. We tested our analytic software using large trees (i.e. constituted by several thousand tips) with no significant delay. The only problem we observed is related to the number of permutation that is proportional to the number of observations following the rule of $N\log(N)$ permutation for N observations. There are no possibilities to avoid this, and several thousands of observations require hours of computations. However, the service, given by the National Institute for Nuclear Physics (INFN), allows 24 h as maximal running time, sufficient to cope with medium–large data sets. Larger data sets could be handled downloading the software and using it in local computation facilities.

The software is not parallelizable, and replicates cannot be distributed on several CPUs. We do not think that parallelization is needed, but we will work on distributing permutations on several CPUs. A complete tutorial of PHYLOH is available on Biovel web page. Input data used in the present article are available on DRYAD entry doi:10.5061/dryad.j4d15.

On the whole, we think that PHYLOH will be a relevant innovation to study community diversity, a field where data analysis is complex, but representation of results is even more difficult. Indeed, PHYLOH produces clear vectorial graphical outputs, in html format, that are easy to explore using functions such as text finder and zoom without reducing image quality. PHYLOH shows its performances when large amounts of interlinked data are available. This is the case of the researches involving high-throughput DNA sequencing, such as our pilot study on microbiomes. Furthermore, it can be used whenever a depth analysis of diversity distribution, from microorganisms to macroorganisms, is required.

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A.S., S.V. and M.C. wrote the manuscript. E.M.P., A.G., E.F., A.B. and B.B. implemented the manuscript. E.M.P., E.F. and M.C. designed the project. S.V. designed and implemented the software PHYLOH. A.S., S.V. and B.B. evaluated the codes. A.S., A.G., E.F., A.B., V.M. and M.C. provided biological background.

Data Accessibility

DNA sequences: NCBI SRA: SRP046312.

Source code of PHYLOH, user manual and example data set are available on github: https://github.com/svicario/phyloH.

Access to the web page of PHYLOH: https://www.biodiversitycatalogue.org/rest_methods/143.

Input and output data of PHYLOH, Supporting information Figures and Tables: DRYAD entry doi:10.5061/dryad.j4d15.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 The figure depicts how Shannon entropy is totally blind to some meaningful biological pattern.

Fig. S2 Distribution of OTUs of High Frequency Cluster (HFC) between parasitised honey bee and varroa considering relative abundance.

Fig. S3 Distribution of OTUs of High Frequency Cluster (HFC) between healthy and parasitised honey bee and varroa.

Fig. S4 UPMGA trees showing the different relationships among samples for all the distance metrics considered in DMA (*jaccard*, *squared-chord*, *unweighted UniFrac* and *weighted UniFrac*), considering Localities and Status variables.

Fig. S5 Output of the script *compute_core_microbiome.py*.

Table S1 Summary of all OTUs taxon assignment.

Table S2 Summary of results of adonis statistic.

Appendix S1 PHYLOH output in html format for 'Status' variable.

Appendix S2 Phylogenetic tree used in PHYLOH analysis in Newick tree format.