Comparison of the gut microbiome of a generalist insect, *Spodoptera littoralis* and a specialist, leaf and root feeder one, *Melolontha hippocastani*

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1. General Introduction

1.1 Insect-bacteria associations

Insects account for the most diverse group of animals inhabiting the planet. Almost 1 000 000 of all described species are insects [1]. Their presence has been noticed in almost all environments. Moreover, insects correlate and establish relationships with a very ample number of other organisms with them associated [2]. Among them, it is possible to mention fungi, viruses, protozoa and the most common ones, prokaryotes. In a broad extend, the association between unicellular and multicellular organisms has strongly contributed to the evolution of life on our planet [3]. Moreover, the rather unlimited metabolic capabilities of prokaryotes have provided insects with strategies to access and conquer diverse ecological niches [4]. In that sense, the ancient 'domestication' of bacteria harbored in the insect body (i.e. bacteryocites or digestive tract) have benefited insects principally through nutritional enrichment by supplying them with limiting nutrients as nitrogen, vitamins and essential nutrients [5].

Using the symbiosis definition given by Zook [6] as a base; we can state a symbiotic relationship of microbes with its insect host as the acquisition and maintenance of the microorganism that results in novel structures or have metabolic capabilities for the insect. Leaving parasitism aside, most of all documented insect-bacteria symbiosis relationships, either obligate or facultative, improve the fitness of the host [7]. Endosymbionts exploitation is among the most widely known symbiosis existing. Beside this, lesser attention has been given to the study of the insect gut-bacteria associations. This likely relates to the complexity while analyzing the effect of the whole bacterial community in the insect physiology [8]. To disentangle the contribution that each of the gut community members represents for the development and fitness of the insect as well as the internal interactions within the bacterial community is really challenging. In this dissertation, bacterial communities associated with

the gut of insects having different ecological niches, a generalist vs. a specialist, are compared.

1.1.1 Intracellular endosymbiotic associations

Intracellular symbiosis where bacteria are accommodated in insect cells are widely spread [9]. Approximately 15% of all insects harbor such kind of endosymbionts [7].

Most of the known beneficial obligate symbioses (P-symbiosis) involve nutritional enrichment of the host [10]. This is particularly important for insects thriving on deficient substrates as sap of plants or blood. They depend on P-symbionts in order to supplement their diets with essential nutrients. This type of symbiosis is broadly distributed among sap-sucking insects. Within this group the most studied example is that between aphids and their mutualistic symbiont- *Buchnera aphidicola*. The bacterium is providing the insect with the essential amino acids that are not available from its plant host [11]. This is an obligate association. Aphids die or do not reproduce when treated with antibiotics, and the bacterium is unable of growing outside the living host [12].

Another type of symbiont-nutritional upgrading, is the contribution to the nitrogen economy of the insects [13]. The latter is common for cockroaches, where nitrogen is recycled from urea and ammonia by its endosymbiont, *Blattabacterium* sp. [14]. Another example is the supplement of Vitamin B that *Wigglesworthia glossinidia*, the P-symbiont of the tsetse, provides [13,15]. Finally, sterols are supplied to certain insects by yeasts or fungi. Examples are the anobiid beetles, *Lasioderma serricorne* and *Stegobium paniceum* which utilize intermediate sterol metabolites from their yeast-like-symbionts (YLS) to further synthesis their own [16].

As a common denominator with few exceptions, all mentioned associations result in mutualist obligate symbiosis [17]. In that sense, host and P-symbiont are commonly unable to survive without each other, or at least the elimination of the bacterium has deleterious

effects on the host [4]. Typically, the bacteria cells are restricted to the cytoplasm of an insect cell type of characteristic morphology and location called bacteriocyte [7,13]. Its position in the insect body varies between insects. They might be associated to the insect gut, lie in the haemocoel or are in the fat body [13]. Most identified insect symbionts belong to the γ-3 subdivision of the class Proteobacteria and are close relatives of the Enterobacteriaceae [4]. Additionally, typical features of the P-symbionts are their maternal inheritance (typically via egg) and the co-speciation commonly existing between both organisms (bacterium and insect) due to the obligatorily of the relationship [17].

In addition to P-symbionts, there are facultative symbionts (S-symbionts) also inhabiting their host intracellularly. They are not always restricted to specialized organs and not strictly necessary for host survival. Similar to pathogens, they are able to engineer their own invasion of host cells and tissues. Additionally, they display a combination of vertical and occasional horizontal transmission across host populations or species [18,19]. Moreover they can be harbored in the same host bearing P-symbionts (in tissues beside each other but also in the same ones) and can be common across diverse arthropod lineages (review by Oliver et al. [10]). Generally, this kind of endosymbionts are mainly contributing to the host by playing important roles on insect defense (i.e. resistance to natural enemies), enhancement of host specialization and reproduction, resistance to abiotic stress (i.e. heat) and detoxification of toxins. Examples of all mentioned benefits for the insect after association with S-symbionts are typically found among aphids (review by Oliver et al. [10]).

Both P- and S- symbionts belong principally to the Flavobacteria and Proteobacteria clades and are closely related to intracellular pathogenic species. When compared to their close free living relatives, they possess a distinctive set of genomic traits, that include reduced genome size, biased nucleotide base composition and fast polypeptide evolution. Most of them, have lost certain functional categories of genes necessary for their independent life, retaining at times only 10-20% of their original genomes [18,20].

1.1.2 Exoskeleton-ectosymbiotic associations

Oppose to the intracellular localization of the P-symbionts in the host cells, some symbionts locate outside of the insect body or on the surface of insect tissues (gut association will be discussed in next sections in detail). The benefits these symbionts provide to the insect are of diverse nature. One of them is insect defense. A famous case involving a tripartite symbiosis is that of attine leaf-cutting ants which harbor antibiotic-producing bacteria (*Pseudonocardia* sp.; Actinomycetales) in cuticular crypts on their exoskeleton to help defend their fungus garden from their garden parasite, *Escovopsis* sp. [21]. Similarly, *Streptomyces* spp. (Actinomycetales) has been found associated with the European beewolf [22]. In this association the bacteria are cultivated by the insect females on antennae glands. Once the brood cell is ready, the insects apply the bacteria on its surface prior to oviposition. In that fashion, the structure is then protected by an antibiotic cocktail mixture (nine different antibiotics) against infection from ubiquitous entomopathogens [23].

1.1.3 Gut lining ectosymbiotic symbiosis

Gut digestive system: morphology, function and special features

The general appearance of the insect gut is depicted in Figure 1 [24]. The main regions of the gut are: the foregut, or stomodeum, of ectodermal origin; the midgut, or mesenteron which is endodermal and the hindgut; or proctodeum, which again is ectodermal. Those sections of ectodermal origin are thus lined with a fine cuticle layer, intima, which continues outside the body and is mostly unsclerotized and flexible. In some areas, sclerotized spines or teeth project from its surface. In many insects, the midgut rather than having a cover of cuticle, it contains a fine envelope named peritrophic membrane, surrounding the food [25]. This separates the luminal contents into two compartments, the endo- and ecto-peritrophic space [24].

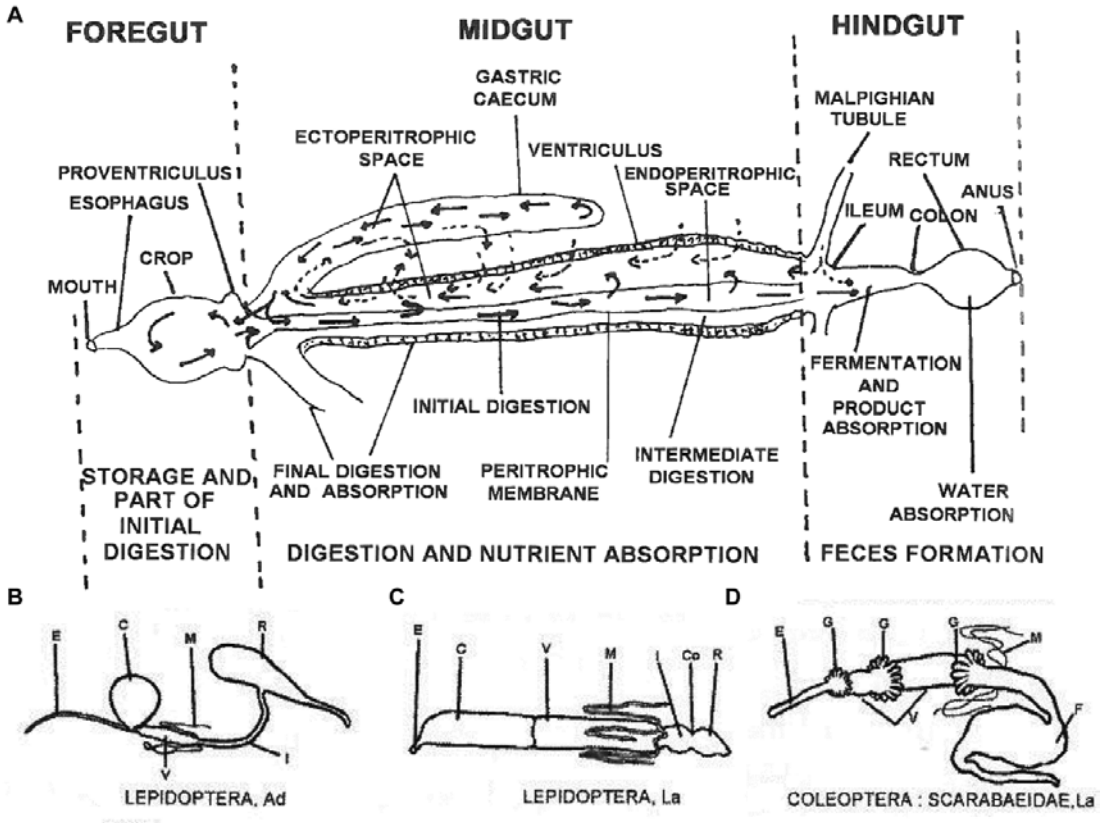

Figure 1. Insect gut morphology. (A) General insect gut feature. (B) Gut of an adult and (C) of a larvae of lepidopteran insects. (D) Gut of a scarabeidae larva. Ad adult, La larva, E esophagus, C crop, V ventriculus, M midgut, I ileum, R rectum, Co colon, G midgut (gastric) ceca, F filter chamber.

As presented in Figure 1A, the crop is any foregut structure involved in food storage. Its main function is concerned with ingesting the food and passing it forward to the midgut. The major digestive region is the midgut, which produces and secretes the digestive enzymes into the gut lumen but also absorbs part of the nutrients [24]. The pH of the midgut is usually in the range of 6-7.5. However, certain groups namely, Lepidoptera [25], scarab beetles [26] and higher termites [27] possess very high alkalinity in the midgut contents or in other sections of the gut. In that sense whilst the tubular midgut of *S. littoralis* have a pH ranging from 8-10, the gut of *M. melolontha* is characterized for having moderate alkalinity (pH of 7.9-8.6) in both the midgut and the hindgut [28]. This feature, seems to correlate with the dietary preferences of the respective taxa, and may contribute to the digestion in various manners. Soil-termites and humivorous scarabs seem to depend on this to sequester

organic matter from the inorganic soil matrix [27]. For Lepidoptera as well as other insects, this is probably necessary to extract hemicelluloses from the ingested plant cell walls [29]. On the other hand, that would also prevent the development of microbial pathogens [25]. The final section of the gut is the hindgut. This includes the ileum, colon and rectum, terminates with the anus and the malpigian tubules arising from it. The main functions of the hindgut are the absorption of water and salts from the urine and feces [24].

As a result of the sources of food on which the insect develops, adaptations on the morphology of the intestinal tract arose in different insect orders and groups. The main groups compared in this dissertation, Lepidoptera and Scarabeidae, are shown in Figure 1B-D [30]. The gut of Lepidopteran larvae (Fig. 1C) consists basically of a long tube composed of a big crop or foregut, a tubular long midgut and a reduced hindgut. When turning molting to an adult, the gut of Lepidoptera looks as in Fig. 1B. This simple structure is likely developed to enhance a rapid passage while processing solid plant foods in big amounts [31]. The gut food passage takes less than two hours for *S. littoralis*. On the other hand, the gut of scarabs' larvae, i.e*. M. hippocastani*, (Fig.1D) posses a very short crop, a long tubular midgut, containing numerous hydrolytic enzymes [32] surrounded by three rows of cecae and finally, an enlarged hindgut, the fermentation chamber with a dense bacterial community associated. In *M. melolontha*, food remains 8-16 h in the midgut and requires a total of 96 h to be out of the fermentation chamber [32]. After metamorphosis the insect gut modifies to a long thing organ composed of a very long midgut which winds up in the insect body and a reduced almost rudimentary hindgut as compared to that of the larvae.

Governing the fermentative processes taking place in the hindgut of scarabs, a steep gradient of O_2 over the gut wall that penetrates less than 100 µm in the lumen is present. This suggests largely anoxic conditions in the midgut and the hindgut. Along with the O_2 radial arrangement, a slight accumulation of H_2 restricted to the central part of the midgut prevails [28]. Similar oxygen regimes as mentioned for scarabs, are characteristic of lepidopteran guts since anterior and posterior midgut are nearly anoxic [33].

Gut symbiosis general features

Gut microbes of insects are composed of an ample variety of species that includes bacteria, archaea and fungi among others [34]. In most of the insects, which posses a pronounced gut microbiome, the microbes are harbored in the hindgut. This is particularly true for termites and scarabs, which hindguts are specifically enlarged and adapted in one or more segments in order to accommodate gut symbionts. As for vertebrates, gut microbes of most insects are extracellular, either lying free in the gut lumen or adhering to the gut wall. In the gut of insects it is possible to find many bacterial cells belonging to different species that could only be transient. Thus, if among all these species, it is possible to distinguish a core group that is typically found in the next generations, harbored in particular organs or sections of the gut (where the bacterial cells are intimately associated with the gut epithelium), it would be proper to speak of indigenous or autochthonous microbiota associated.

The origin of those bacterial species is likely to be the insect surrounding environment as well as the food on which it is feeding. Despite this, in scarabs, i.e. *Costelytra zealandica,* the existing transference routes for the autochthonous harbored bacteria seems to be independent of the diet [28,35].

As for vertebrates, the gut microbiota of the insect may contribute to food digestion and better use of available resources, production of essential vitamins for the host (as mentioned for P-symbionts), keep out potential pathogens, as well as detoxification of allelochemicals [8].

An example of insect nutrition upgrading is the supplement of vitamins. This is observed in the better fitness and reproductive rate observed in *Erwinia*-associated thrips fed under suboptimal diets as compared to their aposymbiont homologous [36]. That the bacteria associated with the insect gut protects it from invasion of pathogens and therefore

hampers the development of diseases or onset of negative effects, appears as one of the main functions of the gut microbiome. The former is attained via diverse mechanisms. One of them is the mere displacement of pathogens by competence since the indigenous microbiome is adapted to the harsh conditions of pH, enzymes and oxygen regimens present in the gut. Moreover, the present bacterial species might be involved in the generation or secretion of antimicrobial products as antifungal phenols [37] or generating antibiotic bio-molecules [38]. Additionally, it is possible that the present bacterial species help detoxifying secondary metabolites, i.e. glucosides [39]. Finally, one of the most important roles of bacteria present in the gut is through food digestion. Indeed, gut bacteria enhance the accessibility to and better use of certain polysaccharides. Examples of this among insects with relatively simple gut are house crickets [40] and omnivorous beetles [41]. In both cases, antibiotic treated insects decreased the amount of food consumed as a consequence of the impossibility for digesting it. Furthermore, hindgut bacteria present in the gut of the crickets, supply most of the degradation of heterogenous class of soluble plant storage/structural polysaccharides. In this regard, the most widely studied case is the one established between termites and its gut bacteria in order to digest lignocellulose materials. This event occurs principally in the hindgut of these insects [42] where bacteria release cellulolytic enzymes supplementing those from the insect or performing the total digestion of the eaten food on behalf of the insect [43,44]. This organ constitutes a fermentation chamber of anaerobic characteristics. Here the gut biota begins a depolymerization of cellulose and hemicelluloses to further ferment the resulting carbohydrates into easily absorbable nutrients that would supply the required energy demands of the insects. These are later absorbed and oxidized by the host. In *M. melolontha*, both midgut and hindgut compartments contain many different types of these products, but mainly: acetate, lactate, and succinate in decreasing order of abundance and in higher concentrations in the midgut. This event suggests that, despite the presence of great number of bacterial species with fermentative metabolism in the hindgut, most of the generated fatty-acids are reabsorbed through the epithelium [28]. Additionally, a great number of termites' hindgut bacterial

species is not involved in fiber digestion. Moreover, they participate in other functions as methanogenesis (performed by Archaea), homoacetogenesis, sulfate reduction and recycling and fixation of nitrogen [45]. Many of the main bacterial species present in termites are not associated with other insects and have apparently established a very specific symbiosis with their host. Despite this, similar roles as those they serve in termites are displayed by homologous species found in other insects. This is particularly true for insects that posses similar anatomical adaptations in their guts in order to harbor bacteria, i.e. scarabs. A schematic description of this process in humivorous beetles is presented in Figure 2. After mastication, the food is moved forward to the midgut, in which digestive enzymes are secreted. If the products are solubilized i.e. monomers are directly absorbed. At this point the same products could be generated by the fermentative effect of microbes also, as indicated by the accumulation of fermentation products in the midgut [26]. If this is the case and the products are not immediately consumed by the insect they could be subjected to further microbial degradation in the hindgut [46]. Indeed, several bacterial species, i.e. *Promicromonospora pachnodae* isolated from the gut of *P. marginata* [47] and *Serratia* sp., *Citrobacter* sp. and *Pseudonomonas* spp. isolated from the gut of *M. hippocastani* [48], displayed cellulolytic and xylanolytic properties respectively. Finally, it seems to be that sulfate-reducing bacteria, present in gut of termites are also commonly found in abundant amounts in the gut of scarab larvae, i.e. *P. marginata* [49] and in the gut of *M. melolontha* [28], where they account up to 10-15% of the total of cells.

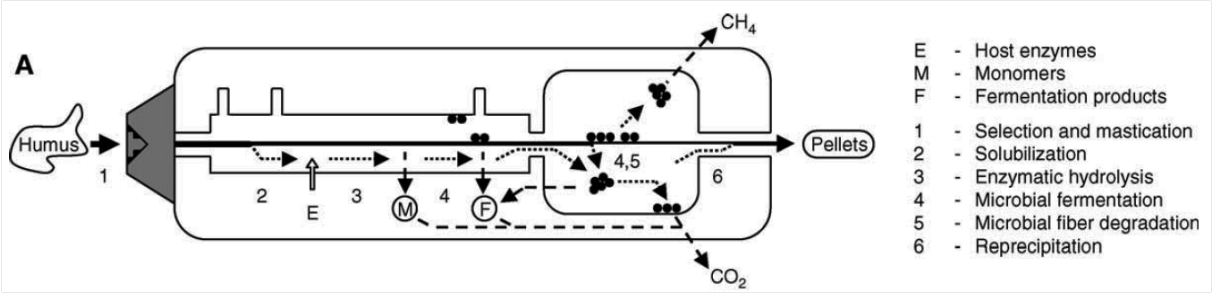

Figure 2. Synoptic scheme of digestion process in a humivorous scarabeidae larva. Solid and dotted lines represent the path of insoluble and soluble material, respectively. Dashed lines symbolize assimilation of

nutrients and their mineralization. The exact location of the gut microbiota (Filled circles) and the individual processes within the midgut and the hindgut are not known [46]

Bacterial communities in scarabs, the specialists that thrive below and above ground

Despite the strong association of the gut of scarabs with microbes and the particular microenvironment that prevails in the intestine of these insects, this is a poorly explored field. Following, the main findings of the few existing studies characterizing the gut biota present in the root/soil feeding stage (larvae) of some cockchafers are summarized. Consensually, Egert et al. [28], Egert et al. [50] and Huang et al. [51], defined the bacterial community associated with the guts of larvae of *M. melolontha*, *P. ephippiata* and *C. zealandica* as highly diverse. Furthermore, they agree that major differences in the species inhabiting midgut and hindgut exist, and that this last part contains the most structured and diverse associated bacterial community. Additionally, it is clear, that the hindgut harbor autochthonous or true insect symbionts since its composition remains constant despite diet changes and different insect origins. The differences in community similarity are not only valid for midgut and hindgut, but also within hindgut lumen and wall as in *M. melolontha*. This is related to the differences observed in the physio-chemical conditions that dictate diverse niches within this organ and consequently a radial arrangement of the bacterial species [28]. Indeed, it has been proposed that some aerobic or facultative aerobic bacterial species as *Serratia* sp., but also sulfate-reducing-bacteria species as i.e. *Desulfovibrio* spp. grow at the very edge of the hindgut epithelium removing the penetrating oxygen, maintaining an anoxic milieu [52].

Regarding to the composition of the bacterial community in the humivorous larvae of *P. marginata,* the midgut is principally inhabited by Actinobacteria, Bacillales, Lactobacillales Clostridiales and few amounts of α-, β-, γ- and δ-proteobacteria. On the other hand, the hindgut is greatly colonized by Lactobacillales, Clostridiales and representatives of the CFB

phylum. These last three groups together with a big proportion of δ-proteobacteria were also the main ones observed in the hindgut of the larvae *C. zealandica* [35].

Bacterial communities in generalist insects, lepidopteran larvae

Contrasting the high species richness and diversity of the bacterial communities associated with the gut of larvae of scarabs, lepidopterans associated gut's bacterial community is simple. This has been confirmed for larvae belonging to different species and genera, since in most of the cases not more of 15 different phylotypes of bacteria have been found [53-56]. Along with this, only a small number of them seems to be metabolically active inside the insect gut [54]. The reason for this limited diversity is likely bound to the fast gut passage, the high alkalinity, and multiple and quick molting cycles between larval stages. In some lepidopteran species, the bacterial community might be strongly reduced till the point of no detection as reported for *Heliothis virescens* [57]. Again, studies focusing on the bacterial community associated with these insects as for scarabs have focused only on the characterization of the most voracious stage that feeds exclusively on leaves, the caterpillars and lack of any reports in adults.

After surveying the bacterial community associated with the guts of several lepidopteran species, it is clear that in most cases the same phylum and subphylum are present. The main groups reported in different larvae species involve γ-, α- and β-proteobacteria, Bacterioidetes, Actinobacteria and Firmicutes [53,55,58]. Out of this group, it seems that γ-proteobacteria species are often found as the most abundantly associated with the insect gut as reported in gypsy moth, cabbage white butterfly, and Pacific Coast Wireworm [56]. From the mentioned phyla and sub-phyla the following genera and other groups emerge as the most frequently found among them: *Enterobacter*, *Pantoea*, *Bacillus*, *Acinetobacter*, *Lactobacillus*, *Pseudomonas*, *Serratia*, *Enterococcus* and *Staphylococcus* [53,55,58]. Groups not so frequently found are: Flavobacteriales, Brucella, *Stenotrophomonas*, Bacterioides, *Cytophaga* and *Bordetella* among others [58].

An important feature of the bacterial communities associated with the gut of lepidopterans is that it alters upon changes of the insect's diet. Its species richness is greater in insects directly collected from field than those reared in the laboratory [58]. Major changes occur at the bacterial species or genera level, but conserving the membership at the phylum level [53]. This suggests a continuous substitution of functions by different bacterial species from the same taxonomical genus in order to fulfill the insect host requirements. Furthermore, it exits a very intimate microbe-microbe structure among the bacterial species composing the gut community, which dictates the response and resistance to invasion when the community is challenged, i.e. by mean of antibiotics. In the cabbage white butterfly, it was demonstrated that an input of antibiotics leads to the risk of invasion of other bacterial species normally not abundant members of the gut bacterial community [53].

Finally, little is known about the function that gut biota associated with lepidopteran is serving. Albeit, it seems that in many cases the gut bacterial community allows the insect to cope up and successfully thrive on diverse plant materials despite the potential toxins present. One example is the success of the white cabbage butterfly on efficiently reproducing and growing in food containing sinigrin, toxin abundantly found in Brussels sprouts [53]. Another example is the increase on mortality of the larvae of *Pectinophora gossypiella* when exposed to *Bacillus thuringensis* treatment in absence of its gut biota [57].

1.2 Description of the insect species

1.2.1 Biology of *Spodoptera littoralis*

Spodoptera littoralis (Lepidoptera: Noctuidae) commonly known as Cotton leafworm, Egyptian cottonworm or Mediterranean brocade moth among others, is an insect of great economic importance since it is polyphagous (Figure 3). The species origins in Africa, but distributed to Asia, Africa and Europe. The host range covers over 40 families of plants, containing at least 87 species of economic importance (i.e. cotton, egg plant, potato, red pepper and tomato among others) [59].

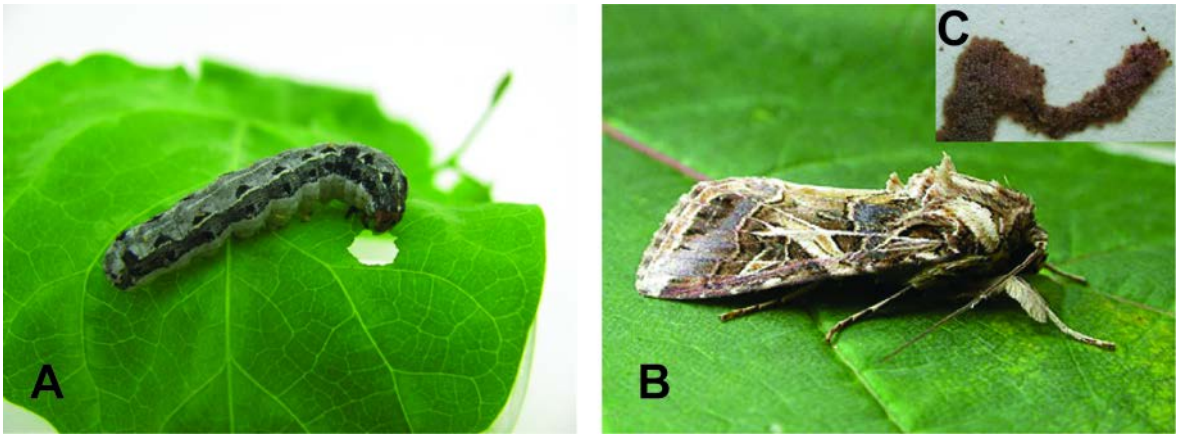

Figure 3. *Spodoptera littoralis* insect instars. (A) L4 larva instar feeding in a bean leaf. (B) Adult moth instar. (C) Egg mass. Moth picture source: http://ukmoths.org.uk/images/.

After 2-5 days after emergence, female moths lay most of their eggs (1000-2000) in egg masses of 100-300 on the adaxial-side of the host leaves. The egg masses contain from 25 to several hundreds of eggs (25-1000). The masses are covered by hair-like scales from the end of the insect's abdomen [60]. Fecundity is adversely affected by high temperature and low humidity. Larvae are quite sensitive to climatic conditions, especially to combinations of high temperature and low humidity; temperatures above 40°C or below 13°C may increase mortality [61]. The eggs hatch in about 4 days under warm conditions or up to 11-12 in winter. The larvae pass through six instars in 15-23 days at 25-26°C. During 3-6 days after hatching the larvae remains to eat on a leaf, but after a week they will spread all over the plant [60]. Observations in cotton have determined that the 1^{st}-3^{rd} larval instars feed mainly on the lower surface of the leaves. The following 4^{th}-6^{th} instars feed on both sides of the leaf. Feeding is taking place before the warmer day hours, earlier than 10 o'clock and after sunset [62]. The pupal period is spent in earthen cells in the soil and lasts about 11-13 days at 25°C. Longevity of the adults is about 4-10 days, being reduced by high temperature and low humidity. Females are living longer than the males [63]. The adults have a flight range of 1.5 km during a period of 4 h overnight [61] In summary, the whole life cycle can be completed in five weeks. In countries like Japan a total of four

generations develop between May and October, and in the humid tropics there may be eight annual generations [60].

1.2.2 Biology of *Melolontha hippocastani*, the forest cockchafer

Melolontha hippocastani (Coleoptera: Scarabeidae), belongs to the subfamily Melolonthinae, whose beetles are known as chafers, maybugs or may beetles, in German language Maikäfer. In general this is a scarabeidae member commonly known as the European Forest Cockchafer (Figure 4).

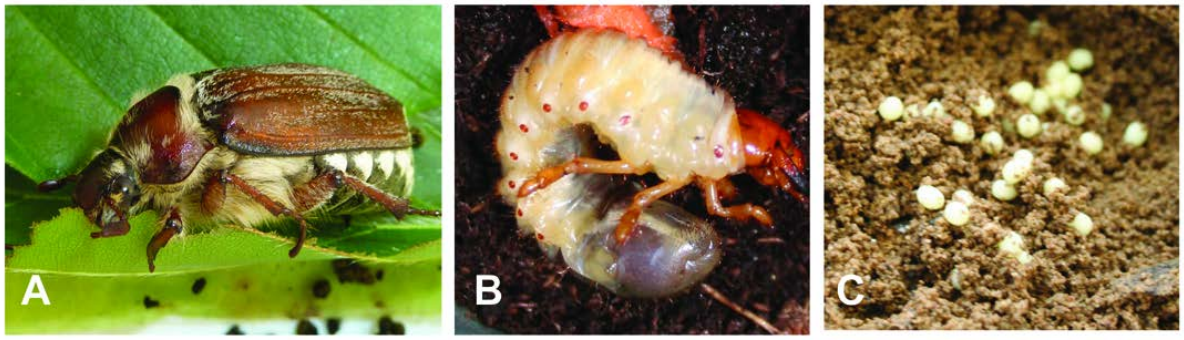

Figure 4. *Melolontha hippocastani* life cycle. (A) Beetle feeding on a birch leaf. (B) L3 instar larva in soil, feeding on carrot roots. (C) Eggs laid in soil.

Together with its close relative *M. melolontha*, the field chafer, they are species of great economic importance due to the damage to field crops and forest stands they infringe. Main hosts of *M. hippocastani* in order of preference are: British oak (*Quercus robur* L.), Mapple (*Acer* spp.), Hornbeam (*Carpinus betulus* L.), Beech (*Fagus sylvatica* L.), Chestnut (*Castanea vulgaris* LAM), Conker (*Aesculus hippocastanum* L.) [64].

M. hippocastani and *M. melolontha* are Euro-Asian endemic species. Among the main countries where significant populations can be found are Switzerland, Hungary, Austria and Germany [65].

Life cycle, reviewed by Reinecke [64]

Depending on the geographical distribution as well as the changes in climatic conditions, the whole life cycle of the insect could last 3, 4 or 5 years (South, North and East Europe respectively). The development of the larvae as well as the metamorphosis into beetles takes place in the soil. After maturation, the beetles emerge by mid of April to beginning of May. The beetles feed on the leaves of the same trees where the larvae developed and fed on. After 2-3 weeks of a so-called 'eating-maturation period', the females start to lay eggs, digging themselves for 3-4 days into a depth of 10-40 cm. Clutches of big eggs (2-3 mm diameter) are laid next to tree roots from which the larvae will feed, up to 2 times in the whole 4-6 weeks that the female lives. After 6-10 weeks new L1 larvae begin to hatch and further developing through two larvae stages, L2 and L3. During winter the insects go to a winter-rest phase, digging themselves deeper in the soil in order to withstand the cold winter temperatures. At the end of the last year of the insect's development during summer, the L3 larva digs deeper into the soil (up to 100 cm) to form a prepupae stage. After that the insect enters into the pupae stage which elapses for up to 2 months. Approximately at the end of August, the beetle is already formed but remains protected and hidden in the pupae case in a diapauses stage until begin of the new flight season in May. During this time the insect maturates its gonads and the sex is determined.

1.3 Goals of this study

In the present thesis the association between prokaryotes and eukaryotes under study involves only insects, a fraction of all the multiple associations that can be observed in nature and that could involve vertebrates or plants. During evolution, due to the incapability of multicellular organisms for acquiring external genes that would enable them to perform multiple biochemical and biosynthetic processes, they opted to establish symbiotic associations with bacteria and other microbes. These associations have been the engine driving a rapid adaptive extension on their phenotypic capabilities [66]. Considering these

facts, it is indisputable the enormous importance of studying bacteria-insect symbiotic associations and interactions.

On the last pages ample evidence of the common symbiotic associations that are established between insects and bacteria excluding parasitism is presented. The level of intimacy of these relationships ranges from very close and intimae as for P-symbionts, located intracellularly to those extracellular like the gut biota. The intracellular symbionts have been estimated to be present in close to 15% of all insect species. Regarding gut bacterial associations, although a lack of extensive surveys across insect taxa impede to demonstrate its importance, the growing number of cases of insects harboring gut associated bacteria suggest that this association prevails across the whole Insecta order. Even when it has been claimed that certain lepidopteran species (i.e. *Heliothis virescens*) [57] harbor no bacteria associated with their guts, this may be caused by limitations on the application and use of molecular techniques employed for the detection. Indeed, depending on the residues of plant-food as well the presence of very acidic or basic juices from the insect gut, PCR reactions (the base for sequencing techniques) might result impaired.

The association between bacteria and the gut of insects confer benefits to the latter ones in order to access and conquer new niches. Considering this, the overarching aim of the present thesis was to analyze and compare the differences of the bacterial communities associated with the insect gut of species thriving on different niches. For that comparison, the gut bacterial communities associated with the gut of a generalist leaf feeder insect, *Spodoptera littoralis*; and a specialist herbivorous insect living above and below ground, *Melolontha hippocastani* were studied. Furthermore across the main research line, the thesis had the following specific aims:

1- To describe the taxonomic composition of the bacterial community associated with the gut of a generalist leaf feeding insect, *S. littoralis* (larvae). More in detail, to describe the

dynamics of gut microbiota in the course of larvae development and the impact of the diet on its composition.

2- To describe and compare the composition of the bacterial community associated with the gut of a specialist insect (*M. hippocastani*) feeding on two different planes of its host, roots and leaves. Additionally, to further unravel the function that this community may be serving the insect.

3- To compare and define the main differences existing between the bacterial communities associated with the mentioned specialist and generalist insects and correlate them to the differences in habitat and ecological niches which they inhabit.

4- To set up and standardize a methodological approach enabling the characterization of metabolically active bacteria in the gut of a generalist insect *Spodoptera littoralis* as model organism to be further applied to other insect species.

2. List of articles with author's contribution

Article I

Complexity and Variability of Gut Commensal Microbiota in Polyphagous Lepidopteran Larvae

Xiao Shu Tang, Dalial Freitak, Heiko Vogel, Liyan Ping, Shao, Yongqi, Erika Arias-Cordero, Gary Andersen, Martin Westermann, David Heckel

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In this article, a survey of the gut bacterial community associated with the gut of the two lepidopteran pest *Spodoptera littoralis* was performed. Applying cultivation-independent techniques based on 16S rRNA gene sequencing and microarray the composition of the community was revealed. Further experiments based Fluorescence in situ Hybridization (FISH) were performed. A core gut bacterial community, consisting of Enterococci, Clostridia, and Enterobacteriaceae was evident. The core community was identified after processing samples from different larvae developmental stages and upon changes in the insect diet. Conclusively, changes in food and host physiology modulate the bacterial community in the gut.

Conceived and designed the experiments: LP HV WB DH. Performed the experiments: XT DF GA MW. Analyzed the data: XT DF HV YS LP EC. Wrote the paper: HV LP WB.

Article II

Identification of the metabolically active bacteria in the gut of the generalist *Spodoptera littoralis* via DNA stable isotope probing using ^{13}C-glucose

Yongqi, Shao, Erika M. Arias-Cordero, Wilhelm, Boland

Journal of Visualized Experiments (*in press*)

In this paper, the metabolically active bacterial community associated with the gut of the generalist insect (our model system) *Spodoptera littoralis* (Lepidoptera, Noctuidae), was determined by stable-isotope-probing (SIP). The technique was applied *in vivo* using ^{13}C-labeled glucose as substrate and coupled to pyro-sequencing. Due to the novelty of the technique a detailed description of the methodology by using a video explanatory resource aims to make it reproducible for others. After successful application of the methodology to our model system, the pyrosequencing profile showed an increased abundance of certain species including *Enterococcus and Pantoea* in the labeled sample (^{13}C Labeled DNA) compared with that in the control group (^{12}C Control DNA). As metabolically active players of the *S. littoralis* gut bacterial community, these bacterial species will be in future further studied.

Conceived and designed the experiments: EA, YS and WB. Performed the experiments: EA, YS. Analyzed the data: EA, YS. Wrote the paper: EA, YS and WB.

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Article III

Comparative Evaluation of the Gut Microbiota Associated with the Below- and Above-Ground Life Stages (Larvae and Beetles) of the Forest Cockchafer, *Melolontha hippocastani*

Erika Arias-Cordero, Liyan Ping, Kathrin Reichwald, Horst Delb , Mattias Platzer, Wilhelm Boland

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In the present study a comparison of the diversity of the bacterial communities associated with the larval midgut and unfed diapausing beetle gut of the European forest cockchafer (*Melolontha hippocastani*) is presented. The former was achieved by; cultivation depended and independent approaches. Furthermore, using Fluorescent *in situ* hybridization, spatial localization of selected bacterial species in gut tissues was possible. Further functional characterization of some isolates is also described. The results suggest a complex rich and diverse bacterial community in the cockchafer larval midgut and the beetle gut. Notorious differences among both bacterial communities are evident. However, a core group of bacterial phylotypes seems to be common to them despite metamorphosis and diapauses.

Conceived and designed the experiments: LP WB. Performed the experiments: EA. Analyzed the data: EA LP. Contributed reagents/materials/analysis tools: KR MP HD WB. Wrote the paper: EA LP WB.

Unpublished results

Bacterial community and novel structures associated with the hindgut of the forest cockchafer (*Melolontha hippocastani*),

Erika Arias Cordero, Liyan Ping, Eiko Wagenhoff, Jürgen Rybak, Martin Kaltenpoth, Martin Westermann, Wilhelm Boland

In preparation

In the unpublished results part, a summary of the experimental outcome after an extensive anato-microscopical examination of the hindgut tissue of larvae and beetles of *Melolontha hippocastani* to find new bacterial niches (beyond the lumen and surface of the hindgut wall) is presented. The exploration of the hindgut lobes, the hindgut epithelium and a pair of novel structures (larvae hindgut pockets), found bacterial cells in all these places. Further phylogenetic identification of the bacteria cells in the hindgut pockets, defined few genera belonging to α-, β- γ-proteobacteria (mostly *Pseudomonas* spp.). The species appears to have low abundance in hindgut of larvae and beetles but is higher in the egg. This leads to the hypothesis that they might represent key functions in the insect physiology and that are likely vertically transferred along the life cycle. Further experiments are being designed to test this hypothesis.

Conceived and designed the experiments: EA LP WB. Performed the experiments: EA MW. Analyzed the data: EA MK Contributed reagents/materials/analysis tools: EW MK MW JR. Wrote the paper: EA WB.

3. Articles

3.1 Article I

Complexity and Variability of Gut Commensal Microbiota in Polyphagous Lepidopteran Larvae

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Complexity and Variability of Gut Commensal Microbiota in Polyphagous Lepidopteran Larvae

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Abstract

Background: The gut of most insects harbours nonpathogenic microorganisms. Recent work suggests that gut microbiota not only provide nutrients, but also involve in the development and maintenance of the host immune system. However, the complexity, dynamics and types of interactions between the insect hosts and their gut microbiota are far from being well understood.

Methods/Principal Findings: To determine the composition of the gut microbiota of two lepidopteran pests, *Spodoptera littoralis* and *Helicoverpa armigera*, we applied cultivation-independent techniques based on 16S rRNA gene sequencing and microarray. The two insect species were very similar regarding high abundant bacterial families. Different bacteria colonize different niches within the gut. A core community, consisting of Enterococci, Lactobacilli, Clostridia, *etc*. was revealed in the insect larvae. These bacteria are constantly present in the digestion tract at relatively high frequency despite that developmental stage and diet had a great impact on shaping the bacterial communities. Some low-abundant species might become dominant upon loading external disturbances; the core community, however, did not change significantly. Clearly the insect gut selects for particular bacterial phylotypes.

Conclusions: Because of their importance as agricultural pests, phytophagous Lepidopterans are widely used as experimental models in ecological and physiological studies. Our results demonstrated that a core microbial community exists in the insect gut, which may contribute to the host physiology. Host physiology and food, nevertheless, significantly influence some fringe bacterial species in the gut. The gut microbiota might also serve as a reservoir of microorganisms for ever-changing environments. Understanding these interactions might pave the way for developing novel pest control strategies.

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Introduction

Microorganisms play important and often essential roles in the growth and development of insect species. Many insects that harbour endosymbionts depend on them for reproduction, digestion, supply of essential nutrients and pheromone production, etc. [1,2]. The bacteria in the gut of some specialized niche feeders, such as termites and aphids, have attracted wide attention because of the microbial enzymes achieving particular biochemical transformations [3,4,5]. However, relatively little is known about insects feeding on foliage, where no strict symbiotic interaction has been proposed so far. In fact, most lepidopteran larvae are herbivores [6,7] and their gut content (food bolus) is not sterile [8]. Indigenous gut bacteria of lepidopteran and other insects have been found to detoxify harmful secondary metabolites [9] and to protect the host against the colonization of pathogens [8]. They are also involved in formation of the aggregation pheromones of locusts [10], maintenance of the host fitness [11,12] and the homeostasis of plant defense elicitors in certain lepidopteran larvae [13,14,15].

For a long time, studying insect gut microbiota was mainly performed by cultivation and isolation. These studies formed the basis of our current understanding but often led to a biased description [8]. Less than half of the bacterial phylotypes identified with terminal-restriction fragment-length polymorphism of 16S rRNA genes from gypsy moth (*Lymantria dispar*) were viable on Petri dishes [16]. None of the bacteria isolated from the laboratory-bred tobacco hornworm (*Manduca sexta*) [17] belong to the abundant phylotypes revealed by PCR-single-strand conformation polymorphism of the 16S rRNA genes [18]. A

denaturing gradient gel electrophoresis coupled with 16S rRNA gene sequencing has revealed that 72% midgut bacteria of the "old world" cotton bollworm (*Helicoverpa armigera*) shared less than 98% sequence identities to known species [19].

The larvae of African cotton leafworm (*Spodoptera littoralis*) and the cotton bollworm (Lepidoptera; Noctuidae) are generalist herbivores and devastating agricultural pests, feeding on more than a hundred plant species [6]. The uptake food passes through the larval gut quickly, normally within a few hours. Whether autochthonous bacterial strains exist in these insect guts is largely unknown [8]. Here we ask the following questions: i) the taxonomic composition of bacteria living in lepidopteran larval gut; ii) the dynamics of gut microbiota in the course of larval development; iii) the influence of diet on gut microbiota.

Results

Bacteria Enumeration

Both *S. littoralis* and *H. armigera* were maintained in the laboratory on heat- and UV-sterilized artificial diet [15]. To rule out the possibility that laboratory conditions have long-term effects on the midgut bacterial community, we compared the *H. armigera* strain TWB that was collected in 2004 in Australia with the strain HELIVI that has been maintained under artificial condition for many years. However, no significant difference between the two *H. armigera* strains was observed.

By cloning and sequencing PCR products, we obtained 1473 high-quality bacterial 16S rRNA gene sequences from the *S. littoralis* gut (Figure 1) and 1245 from the *H. armigera* gut. Most of the 18 operational taxonomic units (OTUs) in *S. littoralis* larvae can be classified to known genus based on 99.5% similarity threshold (Table S1). If the sequence is highly similar to one known species, it was named after that species; if the sequence shares equal similarity to two or more species belonging to the same genus, it was regarded as an unknown species of the genus. In addition, sequence heterogeneity exists in several species, which might be attributed to strains or ecotypes. *Clostridium* and *Enterococcus* constitute 42.2% and 42.3% of the final dataset, respectively (Figure 1). Enterobacteriaceae represent the remaining 14.6%. Most of the dominant species in *H. armigera* larvae were identical to those found in *S. littoralis* (Table S2). Furthermore, we could not detect any Archaea in the insect samples.

Spatial Distribution

In Lepidoptera, the larval alimentary canal is composed of three morphologically distinguishable segments [7]: the foregut and the hindgut derived from ectodermal ingrowth and the midgut from the endoderm (Figure 2A). For microbiota analysis, the gut of 5th-instar *S. littoralis* larvae feeding on artificial diet was cut into three segments at the two visible constricting sites on the midgut. In section I, *E. mundtii* is the most dominant species, whereas in section III, *E. casseliflavus* is more dominant. *P. acnes* was only found in section I, and *E. termitis* was only identified in section III. Only one species, namely *Clostridium* sp. SL01 was detected in section II. Rarefaction analyses confirmed that the sequencing is deep enough to reveal high abundance species in section I and III (Figure 2C). Fluorescent in situ hybridization (FISH) using probes designed from the cloned 16S rRNA gene sequences (Table S3) revealed that *Clostridium* sp. SL01 form large aggregates in the deep anoxic area of the food bolus, and small satellite aggregates already exist at 50 µm away from the gut wall. Other species attached to the gut peritrophic membrane (Figure 3).

Temporal Variation

In the course of larval development, the body length of *S. littoralis* larvae increases from 1.5 mm to ca. 40 mm, and the diameter of its gut increases from 0.5 mm to ca. 7 mm. We monitored the change of dominant species at different instars feeding on artificial diet. The microbiota of the freshly emerged larvae mainly comprised *E. faecalis* and *E. casseliflavus* (Figure 4A). *E. casseliflavus* was also detected on the eggs (data not shown). In older larvae, bacterial diversity increased and *E. mundtii* became very abundant. *E. casseliflavus* was no longer detectable by sequencing but was found with the more sensitive PhyloChip (see discussion below). The *Clostridium* sp. began to appear in 6-day-old larvae. On the larval cuticle, 75% bacterial species were *Pseudomonas*, and *E. casseliflavus* was the only gut inhabitant detected. Statistical analysis with two richness indices Chao1 and ACE (abundance-based coverage estimator) and the α-diversity indices Shannon and Simpson supports the conclusion that the composition of the dominant bacteria in *S. littoralis* larval gut is not complex (Figure 4B).

The Impact of Food

The influence of food plant on the gut microbiota was also investigated by feeding *S. littoralis* with either Lima bean or barley, and feeding *H. armigera* with cabbage, cotton and tomato. In addition, *E. coli* were doped to the artificial diet of *H. armigera* larvae to mimic food born non-pathogenic bacteria. When the young *S. littoralis* larvae were supplied with the toxic Lima bean containing cyanogenic glycosides [20], a high mortality and a transient growth retardation was observed (Figure 5A). The same phenomenon was observed when *H. armigera* larvae fed on the toxic tomato which contain other alkaloids [21].

The bacterial composition in these plant-feeding insects was dramatically different from artificial diet-feeding insects (compare Figure 4A and Figure 5B). When the larvae suffered from intoxication, their gut microbiota was composed of 25% *E. mundtii* and 50% of *P. agglomerans* (Figure 5B). When the larvae recovered after four days, *Clostridia* and *E. casseliflavus* became dominant. In the Barley feeding insects, Clostrida and *K. pneumonia* were most abundant. Even with the slightly more complex microbiota, our sequencing approach is deep enough to cover the dominant species (Figure 5C). A similar pattern was observed when the frass and gut of *H. armigera* larvae were analyzed. Furthermore, in the frass of *H. armigera*, the plant-derived *Burkholderiaceae* sp. was identified in high abundance (Table S2).

Microarray Analysis

Direct cloning is particularly useful to uncover new and dominant bacterial species, while microarray-based PhyloChip can identify thousands of OTUs simultaneously [22]. The 10-day-old *S. littoralis* larvae that fed on artificial diet, Lima bean, and barley, as well as the *H. armigera* larvae that fed on artificial diet, tomato, and cabbage and the food plants were also subjected to analysis with Affymetrix PhyloChip arrays. 55 OTUs were obtained from *H. armigera* larvae and 46 OTUs from *S. littoralis* larvae. Among them, 39 OTUs belonging to 22 families were common (Table 1). It is worth noting that the microarray OTUs were different from those of the sequencing, because it is based on hierarchical clustering of the fluorescence signals generated with group-specific probes. However, most of the ubiquitous bacterial families were detectable in all larvae and independent of diet. In general, microarray confirmed the results of cloning and sequencing, and some low abundant species were only detected by microarray.

Figure 1. Phylogenetic tree of bacterial divisions retrieved from *S. littoralis* larval gut based on sequence similarity. The 16S rRNA gene sequence of the cyanobacterium *Synechococcus elongatus* PCC 6301 (NC_006576.1) and the Armatimonadetes *Chthonomonas calidirosea* T49 (AM749780.1) were used as the out groups. A detailed description of the phylotypes and accession numbers of the most closely related reference sequences can be found in Table S1. The accession number of the other reference sequences are: *Enterococcus durans* Ed-02 (HM130537.1), *Lactobacillus brevis* T9 (JQ301799.1), *Staphylococcus aureus* subsp. aureus JH1(CP000736.1), *Micrococcus luteus* NCTC2665 (CP001628.1), *Corynebacterium diphtheriae* 31A (CP003206.1), *Burkholderia pseudomallei* K96243 (NC_006350.1), *Rhizobium etli* CFN 42 (CP000133.1), *Desulfobacterium autotrophicum* HRM2 (CP001087.1). The two digit bootstrap number and the three decimal posterior probabilities are shown on major nodes. The bottom bar represents substitution rate per site.
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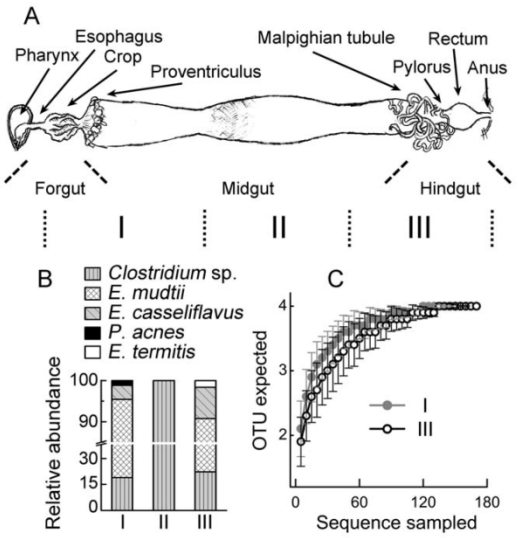

Figure 2. Change of bacterial composition along the digestive tract of 5th-instar larvae of artificial food-feeding *S. littoralis*. (a), The structure of the alimentary canal. The digestive tract was cut into three segments (I, II, and III) for sampling as indicated by the dotted lines. (b), Relative abundance of bacteria in the three segments revealed by cloning and sequencing. (c), Rarefaction curves of the bacterial diversity in gut section I and section III.
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Discussion

The gut microbiota of lepidopteran insects was studied with two complementary and cultivation independent approaches: direct cloning and sequencing that uncovers unknown and dominant bacterial species [23] and a microarray-based approach that monitors low abundant species [22]. Our results clearly showed some dominant bacterial species are shared by two lepidopteran insects. Bacterial species constantly present in the gut are considered as members of the "core set of bacterial community."

Core Community

The composition of dominant species of insect gut microbiota can be very simple. A recent survey using 454 sequencing revealed 5dominant OTUs in the gut of the fruit fly (*Drosophila melanogaster*) [24]. In the gut of the gypsy moth and cabbage white butterfly (*Pieris rapae*) were found 23 and 15 OTUs, respectively [16,25]. We detected 36 dominant OTUs in *S. littoralis* larvae and a similar composition in *H. armigera* larvae. It has been shown that the gut microbiota of laboratory-reared insects is much simpler than those of the insects collected from the field [19,26].

The fact that insects maintain a stable gut microbiota suggests potential benefits. An *Enterococcus* sp. had been detected in gypsy moth larvae independent of the plant diet [16]. It was the major and the only metabolically active bacterium in the gut and eggs of *Manduca sexta* [18]. *Enterococci* are also prominent in the gut of insects such as *Drosophila*, ground beetle, and desert locust [26,27,28]. We detected several *Enterococcus* species in the two lepidopteran larvae, with *E. casseliflavus* being the most widely distributed. The most abundant sequence type in the two lepidopteran larvae belongs to an unknown *Clostridium* species. Clostridia are the dominant bacteria in the guts of termites [5]. We did not detect any Archaea in the lepidopteran insects, in good agreement with the observation on another lepidoteran species *Calyptra thalictri* [29]. Lactobacilli have been detected in the gut of both lepidopteran insects. They were also present in the guts of the fruit fly and the ground beetle [26,27,30]. It has been shown that bacteria isolated from other Lepidoptera performed various hydrolytic activity under aerobic conditions [31]. We believe that the core set microbiota would play important roles in host physiology other than digestion.

Spatial and Temporal Distribution

The tubular lepidopteran midgut is structurally simple, and with a pH gradient from the highly alkaline (ca. 10) anterior end to the nearly neutral posterior ends [14]. The spatial distribution of some bacterial species might reflect their pH tolerance (Figure 2). A strain showing high sequence similarity to *E. termitis* isolated from termite gut was found specifically in the hindgut [32]. *Clostridium* sp. was the most dominant species in the midgut of 6-day-old larva (Figure 4). They were also the most dominant linage in the gut of the European cockchafer, where 100 μm away from the gut all becomes completely anoxic [33]. In the lepidopteran larval gut, *Clostridium* sp. was only detectable about 50 μm inside the gut wall (Figure 3), in accordance with its anaerobic nature. As the insects grew bigger, the ratio of gut volume to the gut surface increased with a factor of D/4 (here D is the diameter of the gut). As a consequence, anaerobic species like *Clostridia* became more dominant. Besides the change of the *Clostridium* sp., the overall composition of the gut microbiota change significantly as the insect ages (Figure 6), suggesting the involvement of other host-derived factor(s) shaping the gut community.

Impact of Food

Most lepidopteran herbivores are highly polyphagous and naturally exposed to bacteria via food consumption. However, the bacteria on the food plant were very different from those in the guts (Table 1), which are again different from those in frass (Table S2). The alkaline pH, digestion enzymes, reactive oxygen species produced by cells of the gut membrane [34] along with the ionic strength in insect gut generally kill the ingested bacteria [35]. Persisting bacteria might become gut colonizers, or remain as transient passengers [18]. We found examples of all, e.g. *X. campestris* from the artificial diet of *S. littoralis* were not detectable in the insect guts. A bacterium belonging to *Anammoxales* was detected in both plant and insects, while *C. maltaromaticum* was abundant in *H. armigera* frass (Table S2).

The gut bacterial communities in insects feeding on different diet are dramatically deferent (Figure 6). It has been shown that the gut microbial composition was different between crickets feeding on protein-rich diet and those feeding on fiber-rich diet [36]. *P. agglomerans* that was also found in gypsy moth larvae [16] and in locust hindguts [28] was also detectable in our plant-fed larvae (Figure 5). In the *S. littoralis* larvae that ingested Lima bean, many low-abundant species began to bloom. The dominance of some species such as *Enterococci* and *Lactobacilli* can be explained by their cyanide resistance [37]. When a large amount of *E. coli were* ingested, the gut microbiota of *H. armigera* became more complex. Whether this is due to a probiotic effect or dysbacteriosis needs further investigation.

Conclusions

The comprehensiveness of the current study on microbiota of lepidopteran gut is only comparable by few studies performed on termites [38], and fruit flies [24,39]. Demonstrating the existence

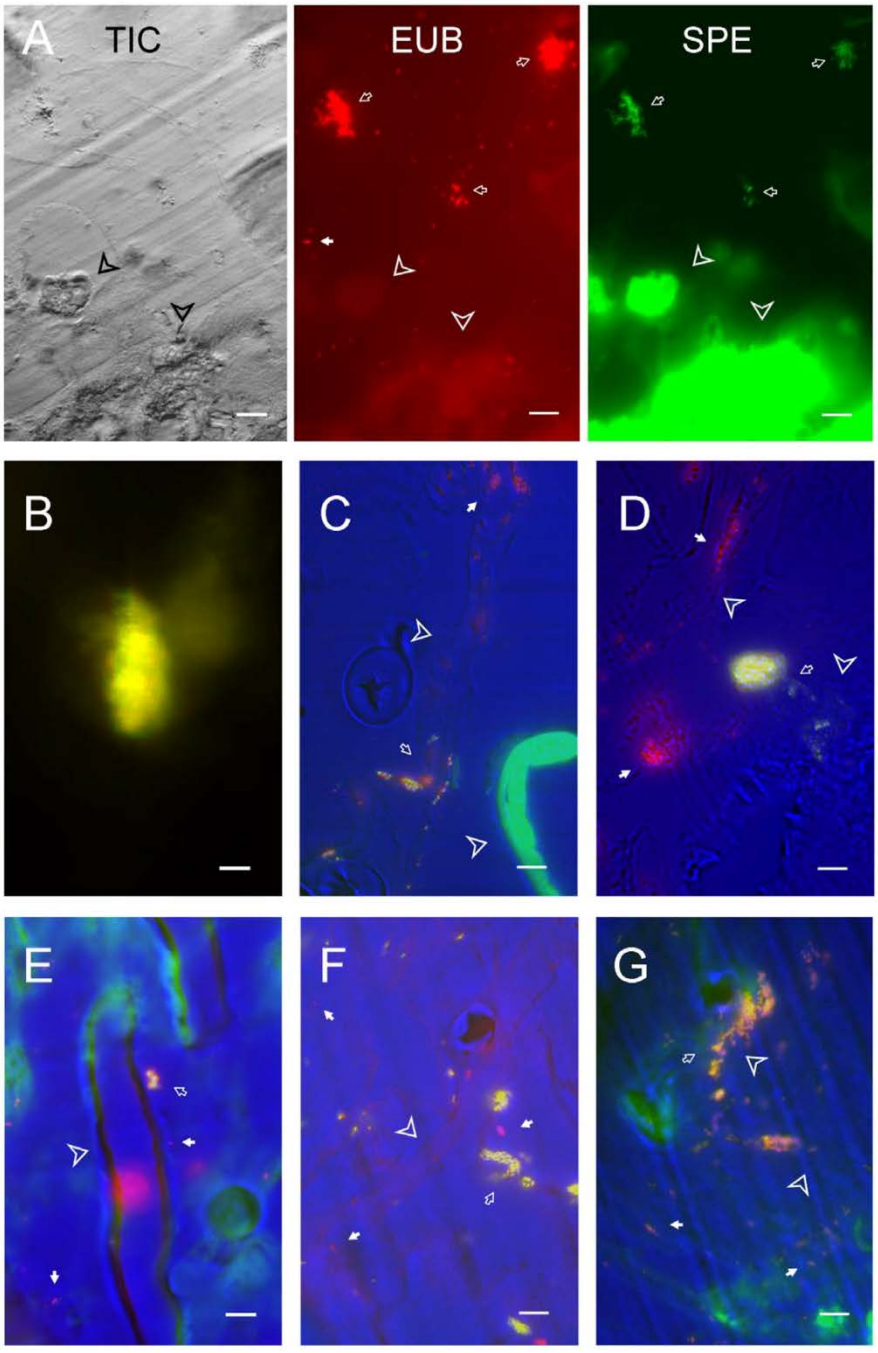

Figure 3. Bacterial localization in the gut of S. littoralis larvae with Fluorescent In Situ Hybridization. Scale bar equals 10 µm. A, Detection of *Clostridium* sp. In the midgut. The three images shown are TIC image, fluorescent image of universal probe (EUB, red) and of specific probe (SPE, green). B to G are merged images of TIC, EUB and SPE. The bacteria detected only with universal probe are red, and the bacterial with both probes are green. B, a large aggregate of *Clostridium* sp. deep in the gut lumen. C, Detection of *E. mundtii*. D, Detection of *E. casseliflavus*. E,

P. acnes in the midgut. F, *E. coli* detected in the midgut; G, *K. pneumonia* detected in the midgut. Bacteria detected only by universal probe are highlighted with white arrows; Bacteria stained by sequence-specific probes are pointed by open arrows. Insect tissue is indicated by arrow heads.
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of the core bacterial community established a platform for further evaluation of the tritrophic bacteria-insect-plant interaction. Further research on each individual species as well as genetic and chemical manipulating the insect and bacteria partners will advance our knowledge on the role of lepidopteran gut microbiota far beyond the old assumption as neutral commensals. As microbiota contribute substantially to insect nutritional ecology and other processes, understanding the physiological role of gut microbiota could potentially pave the way for novel pest control strategies.

Materials and Methods

Insects and Plants

S. littoralis eggs were purchased from Syngenta Crop Protection Münchwilen AG (Münchwilen, Switzerland). The artificial food made of white bean and some essential nutrients was prepared according to [15]. Eggs were hatched at 14°C. Larvae were transferred to room temperature (24°C). Neonatal larvae (400), 2-day-old (400) and 6-day-old (50) larvae were used to prepare the DNA template, while the 10-day-old (20) and 14-day old (7) larvae were dissected, the whole gut was used for DNA preparation. The cuticle of 10-day-old larvae was collected as control. After starvation for 4 hours, larvae were rinsed 3 times alternatively with water and 70% ethanol before dissection. Samples were stored at −20°C before DNA extraction.

H. armigera strain TWB (from laboratory stock) and strain HELIAR (Bayer CropScience, Monheim, Germany) were grown on artificial diet or on plants until the beginning of the final instar as described previously [40]. Artificial diet doped with *E. coli* was performed as described before [12]. Midguts (3×5 larvae per diet) were dissected from freeze-killed larvae in ice-cold phosphate-

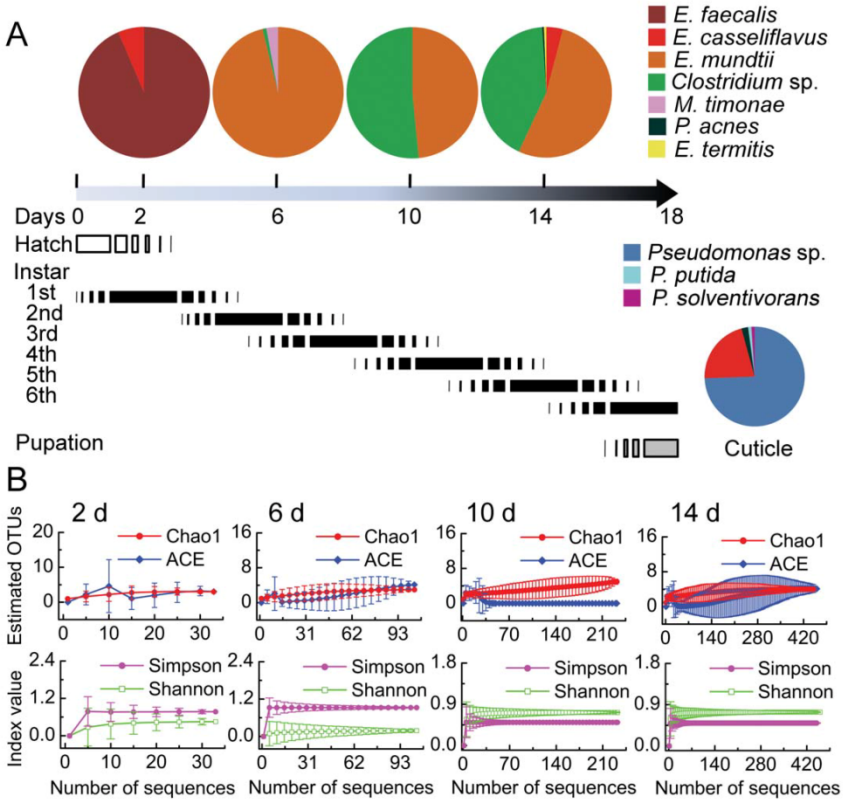

Figure 4. Different gut bacterial community structures in *S. littoralis* larvae of different instars feeding on artificial diet. A, The bacterial community compositions detected by cloning and sequencing from insects that are 2-days (n = 33), 6-days (n = 104), 10-days (n = 232), and 14-days (n = 490). The arrow represents the life span of an *S. littoralis* larva. The developmental stages, hatch, pupation, and larval instars are represented by bars. The inset shows the relative abundance of bacteria detected on the epithelium of 10-day old larvae (n = 94). B, The rarefaction curves of the richness indices Chao1 and ACE, and the diversity indices Shannnon and Simpson based on sequences retrieved from larvae. Indices were calculated using 95% confidence level and 0.03 distance cutoff for OUT clustering.
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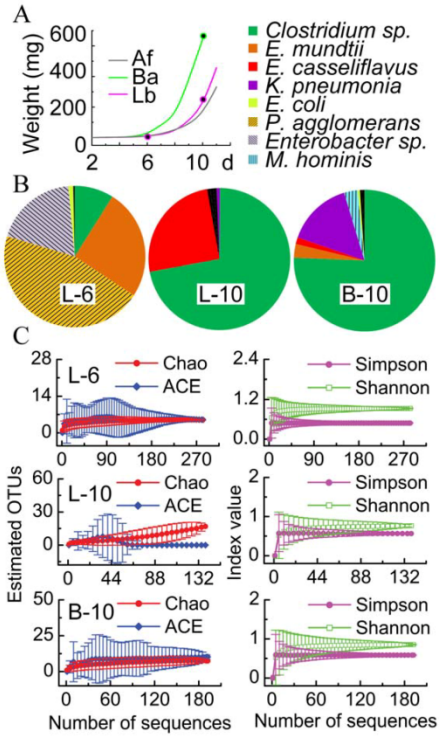

Figure 5. The impact of diet on the gut community in *S. littoralis* larvae revealed by cloning and sequencing. A, Growth curve of the insects. Black dots indicate where the insect gut was sampled. Af, artificial food; Ba, barley; Lb, Lima bean. B, Gut bacterial composition of 6-day-old larvae feeding on Lima bean for 4 days (n = 283, L-6), in 10-day-old larvae feeding on Lima bean (n = 139, L-10), and in the gut of 10-day-old larvae feeding on barley (n = 192, B-10). Case-specific species are shadowed. Singletons are black. C, The rarefaction curves of the richness indices Chao1 and ACE, and the diversity indices Shannnon and Simpson. Indices were calculated using 95% confidence level and 0.03 distance cutoff for OUT clustering.
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buffered saline solution (PBS), immersed in ice-cold balanced salt solution (BSS) and kept at −20°C.

Tomato (*Solanum lycopersicum*), cabbage (*Brassicae oleraceae*), cotton (*Gossypium hirsutum*), barley (*Hordeum vulgare* subsp. *vulgare* Cultivar: Barke) and lima bean (*Phaseolus lunatus* strain CV_JWBJ A) were cultivated in the greenhouse [20,37]. Small larvae were reared in a box and supplied with fresh cuttings of plant shoots on a daily basis.

16S rRNA Gene Library and Sequencing

Frozen samples were thawed on ice and dried at 45°C in a speedvac (Concentrator 5301, Eppendorf). The dried samples were crushed in a 1.5 ml tube with a plastic pestle. Plant material was ground in liquid nitrogen. DNA was extracted with the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to protocol provided by the manufacturer. 240 ng of purified DNA was used as template for a temperature gradient PCR. The primer pairs used to amplify the eubacterial 16S rRNA gene genes were 27f (5′-AGAGTTT-

Table 1. Bacterial families and genus detected with phylochip in the larvae of H. armiggera (HA) and S. littoralis (SL) and plant.

Phylum/Class	Family/Genus	HA	SL	Plant
Bacteroiddetes	Sphingobacteriaceae	+++	ND	ND
	Flexibacteraceae	+++	+++	ND
	Flavobacteriaceae	ND	ND	ND
	KSA1	+++[1]	+++[1]	+
Acidobacteria	Acidobacteriaceae	ND	+	+
Actinobacteria	Corynebacteriaceae	+	+	ND
	Micrococcaceae	+	+	ND
	Propionibacteriaceae	+	+	ND
	Unclassified	+	+	+
Chloroflexi	Anaerolineae	+++	+++	+
	Thermomicrobia	+	ND	ND
Cyanobacteria	Chloroplasts	+	ND	+++
Deinococcus	Unclassified sf1	+	ND	ND
Firmicutes/Bacilli	Enterococcaceae	+++	+++	ND[2]
	Bacillaceae	+++	+++	ND
	Halobacillaceae	+[3]	+[3]	ND
	Aerococcaceae	+++	ND	ND
	Lactobacillaceae	+++	+++	ND
	Streptococcaceae	+++	+++	ND
Molicutes	Erysipelotrichaceae	+++	+++	ND
Clostridiales	Clostridiaceae	+++	+++	ND
	Lachnospiraceae	+	+	ND
	Catabacter	+++	+++	ND
	Symbiobacteria	ND	ND	+
Planctomycetes	Planctomycetaceae	+[4]	+[4]	ND
	Annamoxales	+++[5]	ND	+++
α-proteobacteria	Caulobacteraceae	+[6]	+[7]	ND
Rhodobacterales	Rhodobacteraceae	+	+	ND
γ-Proteobacteria	Enterobacteriaceae	+[8]	+	ND
	Alteromonadaceae	+[9]	+[9]	ND
δ-Proteobacteria	Desulfovibrionaceae	ND[10]	+	+++
ε-Proteobacteria	Campylobacteraceae	+	ND	ND
Verrucomicrobia	Xiphinematobacteraceae	ND[11]	ND[11]	+
Thermodesulfobacteria	Thermodesulfobacteriaceae	+	ND	ND
OP9/JS1	Unclassified	+++	ND	ND
Unclassified	sf160	+	+	+
	sf156	ND	+	ND
	sf95	ND	+	ND

"+", low abundance (Z score < 2); "+++", high abundance (Z score > 2); "ND", not detected.
[1]not found in all insect samples;
[2]low abundance only in tomato plant;
[3]*S. littoralis* and *H. armigera* possibly contain different species;
[4]Found in all plant materials and insects except those feeding on arificial diet;
[5]Only detected in plant-feeding *H. armigera*;
[6]high abundance in plant feeding larvae and low abundance in artificial diet feeding larvae;
[7]only found in one *S. littoralis* sample;
[8]not detected in *H. armigera* feeding on cabbage;
[9]not in *S. littoralis* eeding on aritficial diet and only in *H. armigera* feeding on artificial diet;
[10]high abundance in tomato-feeding *H. armigera*;
[11]detected in artificial diet-feeding *S. littoralis* and tomato-feeding *H. armigera*.
doi:10.1371/journal.pone.0036978.t001

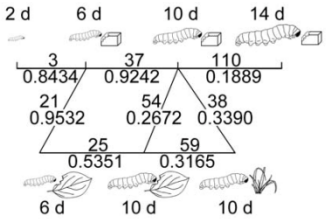

Figure 6. The phylogeny-based β-diversity values between bacterial communities detected in the gut of *S. littoralis* larvae at different instars and after feeding on different diets by cloning and sequencing. The upper values are the parsimony scores and the lower values are the weighted UniFrac scores. Higher score indicates that the two samples are more different on bacterial composition. All significance are lower than 0.001. Artificial food was depicted as cubes; Lima bean as a single leaf; barley as a whole plant.
doi:10.1371/journal.pone.0036978.g006

GATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′). The primer pairs used to amplify archaeal sequences were either 4fa (5′-TCCGGTTGATCCTGCCRG-3′) and 1492r or Ar109f (5′-ACKGCTCAGTAACACGT -3′) and Ar912r (5′-CTCCCCCGCCAATTCCTTTA -3′).

The PCR of each sample was performed with 8 tubes. Every tube contained 0.4 mM of each primer, 30 ng template, 300 mM dNTP, 2.5 units Taq polymerase (Invitrogen), and the buffer from the manufacturer. The annealing temperatures on each tube were 47.5°C, 49.0°C, 50.5°C, 52.0°C, 53.5°C, 55.0°C, 56.5°C, and 58.0°C, respectively, to ensure equally efficient amplification of templates with different GC content. Denaturation was achieved by heating at 94°C for 3 min, and followed by 25 cycles: 94°C for 45s, annealing for 30s, and 72°C for 1.5 min. The final elongation was at 72°C for 10 min. Pooled PCR products were concentrated using the QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany), and further cleaned by running 0.8% agarose gels and cutting out bands of the correct size. Gel slices were purified using the QIAquick Gel Extraction Kit (QIAGEN).

The purified PCR product was cloned with pCR2.1 TOPO TA Cloning Kit (Invitrogen). Colonies were picked and sequenced as described before [41]. DNA sequences were cleaned and assembled with DNASTAR Lasergene software package (DNASTAR, Inc., Madison, WI, USA). Chimeric sequences were discarded. Consensus sequences were used for blast search in databases at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and Greengenes (http://greengenes.lbl.gov). Phylogenetic analyses were first performed with ARB 5.3 software package [42]. The obtained tree was compared with the tree generated with the maximum-likelihood algorithm using Phylip3.67 (http://evolution.genetics.washington.edu/phylip.html) and with Bayesian Inference using the software package BEAST v1.6.2 [43]. Rarefaction, the richness indices (abundance-based coverage estimator (ACE), bias-corrected Chao1), the two α-diversity indices (Shannon and Simpson), and the two β-diversity indices (Parsimony and UniFrac) were calculated using the software mothur [44]. The bacterial partial 16S rRNA gene sequences have been deposited at the National Center for Biotechnology Information with accession numbers HQ264061 to HQ264097.

PhyloChip Analysis

Purified PCR products of 500 ng from each set of pooled samples were used for phylogenetic microarray analysis. Fragmentation and terminal labeling were performed according to the Affymetrix protocol as described in [22]. DNA fragmentation, hybridization and data analysis were performed as previously reported [45]. An OTU was considered to be present in the sample when the positive fraction was larger than 0.90. For each sample, all operational taxonomic units (OTUs) intensity measurements were normalized by a scaling factor such that the overall chip intensity was equal. Raw data output files were analyzed using the Graphical User Interface (LimmaGUI) version of the software Limma and Phylotrac. Each taxon detected was described by a single species.

Fluorescence in situ Hybridization

5th-instar *S. littoralis* larvae were washed 3 times with 70% ethanol and water. The anesthetized insects were briefly frozen at −20°C and were dissected under microscope. Gut was cut into three pieces (Figure 2A). Different parts of gut were fixed with 4% formaldehyde overnight. After washing 3 times with 1× phosphate buffered saline (PBS), the samples were embedded with Technovit 8100 according to the protocol provided by manufacturer (Heraeus Kulzer GmbH, Wehrheim, Germany). Embedded samples were cut into 5 µm thin sections. The thin sections were mounted on SuperFrost Ultra Plus glass slide (Thermo Scientific) and treated with 5 mg/ml lysozyme for 15 min at 37°C. After washing away the lysozyme, the slide was dried by blowing with air. The side was hybridized with 1.5 µM of each probe (Table S3) in hybridization buffer containing 900 mM NaCl, 0.02 M Tris-HCl (pH8.0), 20% formamide, 1% SDS. Hybridization was performed at 46°C for 4 hours on the Advalytix slide booster (Beckman Coulter Biomedical GmbH, Munich, Germany). Afterward, the slide was washed in 50 ml washing buffer containing 0.02 M Tris-HCl (pH 8.0), 0.2 M NaCl, 0.05 M EDTA, 1% SDS at 48°C for 20 min. Slide was then washed with running water for 30 sec and dried with blowing air. Images were taken with an Axio Imager Z1 microscope (Carl Zeiss) equipped with an AxioCam MRM camera.

Supporting Information

Table S1 Bacterial partial 16S rRNA gene sequences cloned from *S. littoralis* larvae and the BLAST results.
(DOC)

Table S2 Bacteria detected in *H. armigera* larval gut and frass based on cloning and sequencing.
(DOC)

Table S3 FISH probes used to detect bacteria in *S. littoralis* gut.
(DOC)

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Author Contributions

Conceived and designed the experiments: LP HV WB DH. Performed the experiments: XT DF GA MW. Analyzed the data: XT DF HV YS LP EC. Wrote the paper: HV LP WB.

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Table S1. Bacterial partial 16S rDNA gene sequences cloned from *S. littoralis* larvae and the BLAST results.

Isolation No.	known species			Most similar sequences[b]		
	Organism	Accession No.	% identity	Organism	Accession No.	% identity
SL01	*Clostridium ramosum*	M23731.1	92.30	Soil clone from tallgrass prarie FFCH17703	EU134663.1	99.11
SL02 - SL06[a]	*Enterococcus mundtii* str. ATCC43186	AF061013.1	99.93			
SL07	*Enterococcus casseliflavus* str. F32	AF039903.1	99.87			
SL08 - SL17[a]	*Klebsiella pneumonia* str. 342	CP000964.1	99.93			
SL18	*Escherichia coli* O157:H7 str. EC4115	NC_011353.1	99.87			
SL19 - SL21[a]	*Pantoea agglomerans* str. ChDC YP1	AY691543.1	99.73			
SL22	*Enterobacter asburiae* str. J2S4	EU221358.1	99.60	*Enterobacter* sp. str. J11	EU099377.1	99.80
	Pantoea agglomerans str. ChDC YP1	AY691543.1	99.60	microbiota antlions	DQ068844.1	99.73
SL23	*Enterococcus faecalis* str. EC-12	AB154827.1	100			
SL24	*Microbacterium hominis* str. DSM 12509	AM181504.1	99.86	Contaminated soil bacterium rJ6	AB021324.1	100
SL25	*Massilia timonae* OS-71	AM237371.1	98.78	Indoor dust clone BF0002D02	AM697512.1	99.12
				Cave-wall biofilm clone LKC_Acid_11	EU038009.1	99.13
SL26	*Enterococcus termitis* str. LMG 8895	AM039968.1	99.20			
SL27	*Propionibacterium acnes* #4584	AB042287.1	99.66	soil microbial clone 227	DQ158099.1	99.66
SL28	*Clostridium ramosum*	M23731.1	90.22	Soil clone from tallgrass prarie FFCH17703	EU134663.1	97.07
SL29	*Clostridium piliforme* str. RJ	D14638.1	88.43	Solid waste clone G35_D8_H_B_C11	EF559167.1	89.12
SL30	*Escherichia coli* K12 str. K-12	NC_000913.2	98.74			
SL31	*Clostridium cocleatum* CM972	AF028350.1	89.67	Soil clone from tallgrass prarie FFCH17703	EU134663.1	96.20
SL32	*Dehalococcoides* sp. str. CBDB1	AF230641.1	78.07	Contaminated soil bacterium TF7	DQ248299.1	98.61
SL33	*Clostridium ramosum*	M23731.1	87.29	Soil clone from tallgrass prarie FFCH17703	EU134663.1	93.75
SL34	*Pseudomonas oryzihabitans* str. IAM 1568	AM262973.1	99.86	*Pseudomonas* sp. CYN01B	AB175661.1	99.93
	Pseudomonas psychrotolerans str. C36	AJ575816.1	99.86			
SL35	*Pseudomonas putida* str. 32zhy	AM411059.1	99.93			
SL36	*Paracoccus solventivorans*	Y07705.1	100			
SL37	*Xanthomonas campestris* pv. *vesicatoria*	AM039952.1	100			

. **Table S2**. Bacteria detected in *H. armigera* larval gut and frass based on cloning and sequencing.

Phylum/Class	Family/Genus	Species (best hit)	Larvae	Frass
Firmicutes/Bacilli	Enterococcaceae	*E. mundtii*	Y/H	Y/H
Firmicutes/Bacilli	Enterococcaceae	*E. casseliflavus*	Y/H	Y/H
Firmicutes/Bacilli	Enterococcaceae	*E. faecium*	Y/L	Y/H
Firmicutes/Bacilli	Streptococcaceae	*Lactococcus* sp.	Y/H	ND
Firmicutes/Bacilli	Staphylococcaceae	*Staphylococcus* sp. HT10 (HM446261)	Y/L[1]	Y/L[1]
Firmicutes/Bacilli	Clostridiaceae	*Clostridium* sp.	Y/H	Y/H
Planctomycetes	Carnobacteriaceae	*Carnobacterium maltaromaticum*	Y/H	Y/H
Gammaproteobacteria	Enterobacteriaceae	*Enterobacter* sp.	Y/L	ND
Gammaproteobacteria	Rhodobacteraceae	*E. coli*	ND	Y/H
Gammaproteobacteria	Enterobacteriaceae	n.a.	Y/L[2]	Y/L[2]
Cyanobacteria	Chloroplasts	*Burkholderia* sp.	Y/L	Y/H
Betaproteobacteria	Burkholderiaceae	Uncultured Sphingobacteriaceae clone EW2-005 (FJ764597.1)	ND	Y/H
Bacteroidetes	Sphingobacteriaceae	Uncultured *Propionibacterium* sp.	Y/L	Y/L
Actinomycetales	Propionibacterineae	*Micrococcus* sp.	Y/L	Y/L
Actinomycetales	Micrococcaceae	*Rhodococcus baikonurensis*	Y/L	Y/L
Actinomycetales	Rhodococcaceae	*Paracoccus carotinifaciens*	Y/L	Y/L
Alphaproteobacteria	Rhodobacteraceae	*Brevundimonas diminuta* (HM446258)	ND	Y/L
Alphaproteobacteria	Caulobacteraceae	Uncultured bacterium clone nbt120c04 (EU540585)	Y/L[1]	ND
Unclassified Bacteria	n.a.		Y/L	ND

1 = identical to NCBI Genbank entries: Madhusudan,S., *et al*. "Gut bacterial flora of insecticidal resistance larvae of ***Helicoverpa armigera*** collected from tomato ecosystem". Unpublished.

2 = can only be detected in ***Helicoverpa armigera*** fed on artificial diet spiked with *E. coli*.

Table S3. FISH probes used to detect bacteria in *S. littoralis* gut.

Probe	Target	Sequence	Labeling
EUB338	all	5’-GCTGCCTCCCGTAGGAGT-3’	Cy3
sl001	*Clostridium sp.*	5'-CACTGATATACCATTTCCTGC-3'	Fluorescein
sl002	*Enterococcus mundtii*	5'-AGGGGTGAACAGTTACTCTC-3'	Fluorescein
sl003	*Enterococcus casseliflavus*	5'-GGGATGAACATTTTACTCTCA-3'	Fluorescein
sl004	*Escherichia coli*	5'-CAATGAGCAAAGGTATTAACTT-3'	Fluorescein
sl005	*Klebsiella pneumonia*	5'-CAATCGGTGAGGTTATTAAC-3'	Fluorescein
sl006	*Propionibacterium acnes*	5'-ACTCACGCTTCGTCACAG-3'	Fluorescein
Sl007	*Pantoea agglomerans*	5'-TGCTGCGGTTATTAACCG-3'	Fluorescein

3.2 Article II

Identification of the metabolically active bacteria in the gut of the generalist *Spodoptera littoralis* via DNA stable isotope probing using ^{13}C-

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Keywords:

Lepidoptera, *Spodoptera littoralis,* stable-isotope-probing (SIP), pyro-sequencing, ^{13}C-glucose, gut, microbiota, bacteria

Short Abstract:

The active bacterial community associated with the gut of *Spodoptera littoralis*, was determined by stable-isotope-probing (SIP) coupled to pyro-sequencing. Using this methodology, identification of the metabolically active bacteria species within the community was done with high resolution and precision.

Abstract:

Guts of most insects are inhabited by complex communities of symbiotic non-pathogenic bacteria. Within such microbial communities it is possible to identify, commensal or mutualistic bacteria species. The latter ones, have been observed to serve multiple functions to the insect, i.e. helping in insect reproduction[1], boosting the immune response[2], pheromone production[3], as well as nutrition, including the synthesis of essential amino acids[4] among others.

Due to the importance of these associations, many efforts have been made to characterize the communities down to the individual members. However, most of these efforts were either based on cultivation methods or relied on the generation of 16S rRNA gene fragments which were sequenced for final identification. Unfortunately, these approaches only identified the bacterial species present in the gut, and provided no information on the metabolic activity of the microorganisms.

To characterize the metabolically active bacterial species in the gut of an insect, we used stable-isotope probing (SIP) *in vivo* employing ^{13}C-glucose as a universal substrate. This is a promising culture-free technique that allows the linkage of microbial phylogenies to their particular metabolic activity. This is possible by tracking stable-isotope-labeled atoms from

substrates into microbial biomarkers, such as DNA and RNA[5]. The incorporation of ^{13}C isotopes into DNA increases the density of the labeled DNA compared to the unlabelled (^{12}C) one. In the end, the ^{13}C-labeled DNA or RNA is separated by density-gradient ultracentrifugation from the ^{12}C-unlabeled similar one[6]. Subsequent molecular analysis of the separated nucleic acid isotopomers provides the connection between metabolic activity and identity of the species.

Here, we present the protocol used to characterize the metabolically active bacteria in the gut of a generalist insect (our model system), *Spodoptera littoralis* (Lepidoptera, Noctuidae). The phylogenetic analysis of the DNA was done using Pyro-sequencing, which allowed high resolution and precision in the identification of insect gut bacterial community. As main substrate, ^{13}C-labeled glucose was used in the experiments. The substrate was fed to the insects using an artificial diet.

Introduction:

Insect-bacterial symbiotic associations are known for a great number of insect species[7]. In these symbiotic associations, microorganisms play important roles in the growth and development of insects. Microbes have been shown to contribute to insect reproduction[1], pheromone biosynthesis[3], nutrition, including the synthesis of essential amino acids[4] and digestion of inaccessible food to the host. Despite the vast variety of gut- bacterial associations, much less is known about the functional role they play in favor of the insect. Only in case of the termites, the symbiotic digestion of lignocellulose carried out by prokaryotes, protozoa and fungi, has been widely studied[8,9]. In contrast to this, little is known about the symbiotic association present in the gut of generalist insects i.e. the cotton leafworm, *Spodoptera littoralis.* Moreover, due to their frequent shift of plant hosts, generalist insects and their gut associated bacterial communities are permanently exposed to new challenges linked to their feeding habits consuming plants with a plethora of phytochemicals. Beside this, the gut environment in lepidopterans represents *per se* a harsh environment for the growth of bacteria because of the high gut pH[10]. Particularly in the case of *S. littoralis*, it

ranges from 10.5 in the foregut, 9 in the midgut until 7 in the hindgut [11]. On the other hand, the bacterial community associated with the gut of *S. littoralis* is simple. Tang, Freitak, et al. [12] reported a maximum of 36 phylotypes, belonging to a total of 7 different bacterial species as the only members of the bacterial community associated with this insect. Besides this, no complicated rearing procedure is required for the insect growth in the laboratory. Furthermore, this and the short life cycle of the insect facilitate multi-generational studies, turning this species into an ideal model system for studying gut-microbe interactions.

With the advent of PCR-based sequencing technologies, the number of studies dealing with gut biota of several organisms (i.e. humans, insects or marine organisms) has increased. Moreover, the results are independent from isolation and cultivation of the gut harbored bacteria as in the past. Almost 99% of bacteria are not cultivable and the simulation of the environmental conditions prevailing in the gut is difficult[12]. By using PCR, 16S rRNA gene fragments (a widely used phylogenetic gene marker among bacteria) could be selectively amplified from a mixed DNA template of gut bacterial communities, sequenced and cloned. With this information, the user is able to identify the bacterial species after retrieving the sequence information from public databases[13,14]. Nevertheless, the sequencing approaches to describe bacterial communities remain insufficient due to lack of information on the intrinsic metabolic contribution of the individual species within the community.

Stable-isotope probing (SIP) is a promising culture-free technique. It is often used in environmental microbiology to analyze microbial phylogenies linked to particular metabolic activities. This is achieved by tracking stable-isotope-labeled atoms from substrates into microbial biomarkers, such as phospholipid-derived fatty acids, DNA and RNA[5]. When considering nucleic acids, the methodology is based upon the separation of ^{13}C-labeled DNA or RNA from the unlabeled DNA by density-gradient ultracentrifugation[6]. Due to this direct connection between the DNA label and metabolic activity, a downstream molecular analysis of the nucleic acids identifies the species and provides information on metabolic activities. Moreover, combination of DNA-SIP and pyrosequencing as applied by Pilloni, von Netzer, et al. [15], permits a particular simple and sensitive identification of the bacterial species present

in the heavy ^{13}C-labeled DNA fraction. Up to now, this technique has been applied to describe the bacterial communities involved in biogeochemical processes in the soil under aerobic[16,17] and anaerobic conditions[18,19]. Besides of the use in environmental science, the technique has been applied in medical sciences as reported by Reichardt, et al. [5], who described the metabolic activities of different phylogenetic groups of the human intestinal microbiota in response to a non-digestible carbohydrate.

Here we use 13C-glucose to 'label' the DNA of the metabolically active bacterial species in the gut. Glucose is a sugar utilized by most bacterial species along the widespread Entner-Doudoroff (ED) pathway, although exceptions are known[20]. This justifies the use of ^{13}C glucose as a reliable metabolic probe. Depending on the scientific question, other substrates, i.e. ^{13}C-methane, $^{13}CO_2$, etc., addressing the particular metabolic activity of the microorganisms can be used.

At this point, we present the protocol applied in the metabolic characterization of the gut bacterial community of a generalist insect, namely S*. littoralis* (Lepidoptera, Noctuidae). Moreover, the technique was coupled to Pyro-sequencing, which in turn allows the identification of insect gut bacterial community with high resolution and precision. As the main substrate, ^{13}C-labeled glucose was utilized during the experiments.

Protocol Text:

1.) Insect rearing

1.1) Purchase or take eggs clutches of *Spodoptera littoralis* from your own rearing. Maintain them in sterile Petri dishes at room temperature (RT) until hatching.

1.2) Prepare the artificial diet for the insects rearing as follows:

1.2.1) Soak 500 g ground white beans overnight in 100 mL of water.

1.2.2) Add 9.0 g ascorbic acid and 75 g agar to 1000 mL distilled H_2O and afterwards boil it.

1.2.3) Let the mixture to cool down and when it solidifies (to a white waxy solid mixture) store it at 4 °C until use.

1.3) Transfer the newly hatched larvae to a clean plastic box and rear them on the artificial diet at 23 - 25 °C under a long-day regime with 16 h of illumination and 8 h of darkness. Change the food every day until the larvae go through all six larval instars.

2.) ^{13}C-labeling of DNA in metabolically active gut bacteria

2.1) Dissolve 186.1 mg of fully ^{13}C-labeled glucose with distilled and deionized water (ddH_2O) and mix it well with 100 g of artificial diet for the SIP experiment. Ensure a final ^{13}C-glucose concentration of 10 mM in the artificial diet. This mimics the *in situ* glucose concentration in the gut of *S. littoralis* larvae feeding on cotton plants according to Shao *et al* (submitted).

2.2) Transfer 9-15 healthy second instar larvae from the rearing box to sterile plastic Petri dishes (one larva per Petri dish) containing fresh ^{13}C-glucose spiked artificial diet. Use artificial diet containing the same amount of native glucose (^{12}C-glucose) as described, for feeding the control group.

2.3 Renew the artificial diet frequently during the incubation period (1 day). Use rearing conditions as in point 1.3.

2.4) Collect nine larvae from each treatment for later processing (DNA extraction). Each replicate is constituted by three individuals; make at least three replicates per treatment.

3.) Insect dissection.

3.1) Clean and disinfect the dissecting tools at the beginning of the work with ethanol 70% (Tools= vannas scissors, forceps and scalpel with fine disposable blades).

3.2) Take the insects with a soft forceps and wash their skin superficially with distilled water. Place them for at least 30 min on ice so that they get anesthetized.

3.3) Disinfect the insects superficially by immersing them in ethanol (70%) for a couple of minutes. While still anesthetized place them at 0°C for one hour to kill them.

3.4) Perform the insect dissection into a volume of approximately 15 ml of 1x PBS deposited on a sterile Petri dish (1XPBS= 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, adjust the pH to 7.4 , autoclave). Be sure of having the whole insect body fully immersed in the solution during the whole dissecting period.

3.5) Begin the dissection by making an incision in the skin along the right and left sides of the insect, commence on the head and finish on the anus. Remove the whole skin, malpighian tubules, and fat body. Change to fresh buffer before cutting open the gut. Section the gut in fore-, mid- and hindgut when required. Store the tissue in the freezer (-80 to -20°C) until DNA extraction.

4.) DNA extraction and amplification

4.1) Place the insect tissue in a 1.5 ml sterile centrifuge tube and dry all samples at 45°C in a vacuum concentrator device. Crush dried tissue with a sterile plastic pestle.

4.2) Extract the genomic DNA using a suitable kit for soil DNA extraction. Transfer the dry tissue to the reaction tube of the kit and follow the instructions as indicated by the producer.

4.3) Determine the concentration of the final eluted DNA using a spectrophotometer.

4.4) Verify a successful extraction of the microbial metagenomic DNA from the insect gut by preparing a diagnostic PCR assay using Eubacterial general primers 27f (5- AGAGTTTGATCCTGGCTCAG -3) and 1492r (5- GGTTACCTTGTTACGACTT -3). Set the PCR reaction as follows:

4.4.1) Set a 50-µl reaction mixture containing 1X Buffer, 1.5 mM $MgCl_2$, 10 mM four deoxynucleoside triphosphates (dNTPs), 2.5 U of Taq DNA Polymerase, 0.5 mM of each primer and 60 ng of the extracted DNA as template.

4.4.2) Run the PCR reactions using a thermocycler as follows: initial denaturation at 94°C for 3 min; followed by 35 cycles: 94°C for 45s, annealing at 55°C for 30s, and extension at 72°C for 1 min. Use a final extension step at 72°C for 10 min.

4.4.3) Verify the successful PCR amplification in a 1% agarose electrophoresis ethidium bromide (EB) stained gel (dissolve 1 g of agarose in 100 ml of 1XTAE buffer and stain with 1µl EB). Deposit 5 µl of the PCR product + 1µl of 6X loading dye into the gel pockets. (1X TAE= 40 mM Tris-Base, 20 mM glacial acetic acid, 1 mM EDTA, adjust to pH 8)

4.4.4) Run the gel using 1xTAE buffer at 300 V, 115 mA for ca. 20 min in an electrophoresis chamber. By using a gel documentation chamber (UV transilluminator connected to a digital camera and computer) observe the gel and compare the size of your final product with the also loaded gene marker; the correct size of the amplicons must be of approximately 1.5 kb.

4.5 Store the PCR products under deep freezing conditions, preferentially -80°C, until use.

5.) DNA separation-CsCl gradient ultracentrifugation

5.1) Prepare the reagents for the gradients for ultracentrifugation as follows:

5.1.1) Prepare a 7.163 M cesium chloride (CsCl) solution by gradually dissolving 603.0 g of CsCl in ddH_2O and fill up to a final volume of 500 ml.

5.1.2) Accurately determine the density of the prepared CsCl solution by weighing a 1.0 ml aliquot on a three digit balance by triplicate. This usually ranges from 1.88 and 1.89 g ml^{-1} at RT.

5.1.3) Prepare the gradient buffer (100 mM Tris, 100 mM KCl and 1 mM EDTA) by combining 50 ml of 1M Tris–HCl (pH 8.0), 1 ml of 0.5 M EDTA (pH 8) and 3.75 g of KCl in a final volume of 500 ml using ddH_2O. Filter (0.22 µm) sterilize and autoclave this solution.

5.2) DNA Separation

5.2.1) Once knowing the individual DNA concentration of the treated insects, measured as in point 4.3, pool those corresponding to a replicate together and normalize their DNA concentration to ensure that each one, equally contributes to the pooled sample. A final concentration of 500 – 5000 ng of DNA (measure again the final concentration) is suitable for the ultracentrifugation process[21].

5.2.2) For the gradient medium setting up, combine the quantified DNA, gradient buffer and 4.8 ml of 7.163 M CsCl solution together to a mixed volume of 6.0 ml in a sterile 15-ml screw-cap tube (Generation of a gradient medium-DNA mixture).

5.2.3) Place carefully the prepared gradient medium-DNA mixture into a 5.1 ml ultracentrifuge tube (fill in until the base of the tube neck) using a syringe and needle. Avoid pumping or forming any air bubbles. Include at least one blank gradient (using ddH_2O instead of DNA) in each centrifuge run as a reference gradient.

5.2.4) Balance tube pairs to within 10 mg of weight after each required gradient medium is filled into the respective ultracentrifuge tube. Seal the tube with a "Tube Topper" and ensure that the seal is leak-free by inverting the tube and applying moderate pressure by hand.

5.2.5) Use a near vertical rotor with eight wells for holding 5.1 ml ultracentrifugation tubes for the ultracentrifugation. Carefully seal the rotor wells and load the rotor into the ultracentrifuge according to the manufacturer's instructions. Set the ultracentrifugation conditions: spin at 50,000 r.p.m. (around 177,000 gav), temperature at 20°C, and ultracentrifugation running time for 40 h with vacuum. Select maximum acceleration and the deceleration without brake.

5.2.6) Once finished, remove carefully the tubes from the centrifuge rotor using forceps. Place them in a rack without disturbing the formed gradients within the tubes. Process the gradient fractionation samples as soon as possible to minimize any diffusion.

5.3) Retrieval of DNA from isopycnic separated gradients by fractionation

5.3.1) Use an accurate HPLC pump system to carry out the gradient fractionation, which equally separates the formed gradients from the ultracentrifuge tube top displacement method. Connect the HPLC pump (100% flow gradient) to the bottle filled with 1 L displacement ddH_2O and tightly attach to the open pump tubing with a 23-gauge 1'' syringe needle. Add sufficient bromophenol blue dye to the ddH_2O to give it a dark blue color. This facilitates the visualization of the interface between the gradient medium and the displacement of ddH_2O.

5.3.2) Set the pump to a flow rate of 850 µl min^{-1} and equilibrate the system until a single drop of the blue-colored ddH_2O emerges out of the syringe needle.

5.3.3) Carefully fix the ultracentrifuge tube to a clamp stand and pierce the bottom of the tube with a syringe 23 gauge 1'' long needle. Connect the pump to the ultracentrifuge tube by inserting the needle attached to the pump tubing into the top of the tube, and collect drops from the bottom directly into sterile 1.5 ml microfuge tubes. Collect a fraction every 30 s, amounting to approximately 425 µl per fraction and a total of 12 fractions per gradient. Note that the last fraction (fraction 12) usually contains the displacement ddH_2O and should be discarded.

5.3.4) After fractionation, measure the density of all separated fractions using an analytical balance as mentioned in 5.1.2.

5.3.5) Precipitate the DNA from each fraction (around 425 µl) by first adding 1 µl glycogen (20 µg $µl^{-1}$ in ddH_2O, autoclaved) as a carrier for the precipitation of low amount of DNA. Mix it well by inversion.

5.3.6) Add two volumes (850 µl) of polyethylene glycol (PEG) solution (dissolve 150 g of polyethylene glycol 6000 and 46.8 g of NaCl in ddH_2O to a total volume of 500 ml. Filter, sterilize and autoclave. The PEG solution is 30% PEG 6000 and 1.6 M NaCl.), and mix again by inverting ten times.

5.3.7) Leave the tubes at RT for at least two hours (if necessary, overnight) to precipitate the DNA.

5.3.8) Centrifuge the precipitates at 13,000 g for 30 minutes at RT. A visible pellet should form.

5.3.9) Remove carefully the supernatant with a pipette and wash the pellet with 500 µl of 70% ethanol. Centrifuge under the same conditions for 10 minutes again.

5.3.10) Discard carefully the supernatant and air-dry the pellet at RT for 15 minutes.

5.3.11) Re-dissolve the dried DNA pellet in 30 µl of ddH_2O and mix well by gently tapping the tube. Quantify the DNA in each gradient fraction as in point 4.3 Store the samples under -20 or -80°C if long term storage is required.

6.) DNA characterization by pyrosequencing

6.1) Ensure that the unlabeled native (^{12}C) DNA occupy the density range from 1.705 g ml^{-1} to 1.720 g ml^{-1}, whereas ^{13}C-labeled DNA has a density of around 1.720-1.735 g ml^{-1}. Note that both the native and ^{13}C-labeled DNA extracted from environmental samples can span several gradient fractions. Expect the light DNA to be associated with fractions 9-11 and the heavy DNA to be associated with fractions 4-6.

6.2) Combine key fractions to create a single “compiled” representative fraction according to the specific peak allocation of the density-resolved DNA. Alternatively, other means to examine the separated fractions by using the isotopic ratio mass spectrometry (IRMS)[22] or a fingerprinting method, such as denaturing gradient gel electrophoresis (DGGE)[23], can help to choose appropriate fractions before the sequencing.

6.3) Perform pyrosequencing of the PCR-amplified bacterial 16S rRNA genes from the compiled heavy, middle and light fractions of each individual gradient..Using this method, identification of the lineage and relative abundance of metabolically active bacteria species in the SIP incubated samples is possible. Perform pyrosequencing as follows:

6.3.1) Perform Amplicon pyrosequencing using a Roche 454 FLX instrument with Titanium reagents (454 Life Sciences, Roche Applied Science, USA) and applying particular procedures developed by the laboratory or company you are submitting your samples (i.e. Research and Testing Laboratory (RTL) (Lubbock, TX, USA) as in our case).

6.3.2) Prepare Barcoded amplicons for multiplexing using the fusion primer set Gray28F (5-GAGTTTGATCNTGGCTCAG -3) and Gray519r (5- GTNTTACNGCGGCKGCTG -3)[24,25] extended with the respective primer Adaptor A/B and sample-specific multiplex identifiers (MID).

6.3.3) Apply a one-step PCR with 30 cycles, using a Hot Start Taq polymerase, to generate the sequencing library.

6.3.4) Sequence the amplicons based upon the supplier protocol, and extend the reads from the forward direction (Gray28F) as described in http://www.researchandtesting.com/

7.) Pyrosequencing data analysis

7.1) Analyze the raw sequence reads generated by the 454 pyrosequencing with the "quantitative insights into microbial ecology", QIIME software pipeline[26]. Perform the processing as follows:

7.1.1) Initially, convert the 454-machine-generated sff file to FASTA, QUAL and Flowgram files with the **process_sff.py** script.

7.1.2) Validate the mapping file (based on **check_id_map.py**) and assign multiplexed reads to biological samples with the **split_libraries.py** script. Trimm the low-quality ends with a sliding window size of 50 and an average quality cut-off parameter of 25, eliminate also short ambiguous sequences (the length < 200 bp) from the dataset.

7.1.3) Denoise of the 454 data using the **denoise_wrapper.py**.

7.1.4) Next, cluster the high-quality reads into operational taxonomic units (OTUs) using the multiple OTU picking method (**pick_otus.py** with 97% sequence similarity cut-offs).

7.1.5) Pick a representative sequence from each OTU using **pick_rep_set.py**.

7.1.6) Assign taxonomy to the representative sequence set based on **assign_taxonomy.py.** Use the Ribosomal Database Project (RDP) classifier with a minimum confidence to record assignment set to 0.80.

7.1.7) Align the representative sequence set against the pre-aligned Greengenes core 16S sequences using the algorithm PyNast (**align_seqs.py** with the minimum sequence identity percent set to 75).

7.1.8) Additionally, deplete of chimeras from further analysis by running **identify_chimeric_seqs.py**.

7.1.9) Remove all gap positions (not useful for phylogenetic inference) with the lanemask template (**filter_alignment.py**) prior to build a phylogenetic tree (**make_phylogeny.py**).

7.1.10) Finally, generate OTU tables with **make_otu_table.py**, describing the occurrence of bacterial phylotypes within each sample. The OTU table is then used to construct the heatmap for comparing the bacterial abundance and activity.

7.1.11) If required, computer the alpha and beta diversity within and between samples respectively. In the same manner, rarefaction curves (graphs of diversity versus sequencing depth) and Principal Coordinates Analysis (PCoA) plots representing the relationships among microbial communities can be also prepared.

Representative Results:

To achieve sufficient labeling of the metabolically active bacteria present in the insect gut, the insect must be exposed to the ^{13}C-rich substrate for a previously optimized period sufficient to allow the separation of the labeled heavier fraction easily from the unlabeled lighter one. In our case, ^{13}C-glucose was supplemented in the artificial diet at a final concentration of 10 mM for 1 day (Figure 1A). The same amount of normal glucose (Figure 1B) was supplied in the artificial diet of the control insects. As mentioned before, separation of the DNA fractions (heavier from lighter) is the basis of the whole process. Therefore, the first step to be performed is the extraction of DNA from the tissue of interest; in this case, the bacterial community associated with the insect gut. To confirm its quality it is important to run

an electrophoresis gel to determine if any DNA at all was obtained from the analyzed tissue. In Figure 1C, it is observable a strong band of genomic DNA at the top of the image confirming the positive DNA extraction. In addition, it is necessary to perform a diagnostic PCR, using bacterial universal primers, to determine the presence of bacterial DNA (Figure 1D). This positive result is important to decide if further continue with the separation of the extracted metagenomic DNA.

After ultracentrifugation, once the quantity of the DNA in each separated fraction is confirmed, and the measurement of the density is done, plot the average fraction density in g ml^{-1} over fraction number to confirm the proper gradient formation in tubes, which normally covers a density range from 1.690 g ml^{-1} (for fraction 12 or 11) to 1.760 g ml^{-1} (for fraction 1). Quantitative pyrosequencing is performed directly on the representative SIP gradients to reveal species lineage and relative abundance (Figure 2). Here we sequenced the heavy fractions from both the ^{13}C-glucose amended sample and the unlabeled control, which served for profiling active populations in the community. After sequencing, a total of 120,045 high quality reads with an average length of 404 nucleotides were generated. The pyrosequencing profile showed an increased abundance of certain species including *Enterococcus* and *Pantoea* in the labeled sample (^{13}C Labeled DNA, Figure 3) compared with that in the control group (^{12}C Control DNA, Figure 3). Therefore those bacteria are considered to be metabolically active, and merit further study.

Tables and Figures:

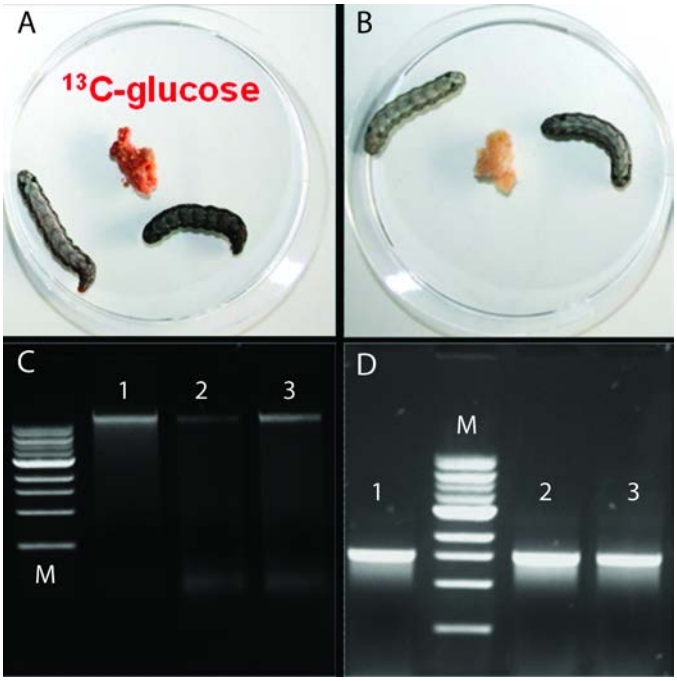

Figure 1: ^{13}C-glucose feeding experiment, DNA extraction and PCR amplification of the bacterial 16S rRNA gene. (A) ^{13}C-glucose amended artificial diet consumed by *Spodoptera littoralis* larvae. (B) Native glucose (^{12}C-glucose) amended artificial diet consumed by larvae from the same batch of insects used in (A), which serves as the control. (C) Typical metagenomic DNA extract from the gut tissue of larvae in the ^{13}C-glucose treatment group and in the ^{12}C-glucose control group. Three biological replicates (lane 1, 2 and 3) are included in each group. M, stands for 1 kb DNA marker. (D) PCR amplification with a universal bacterial primer set generates the correct 1.5 kb 16S rRNA gene products by using the extracted metagenomic DNA as the template. Lanes 1 is the PCR positive control. Lane 2 and 3 represent the ^{13}C-glucose treated sample and the ^{12}C-glucose control respectively.

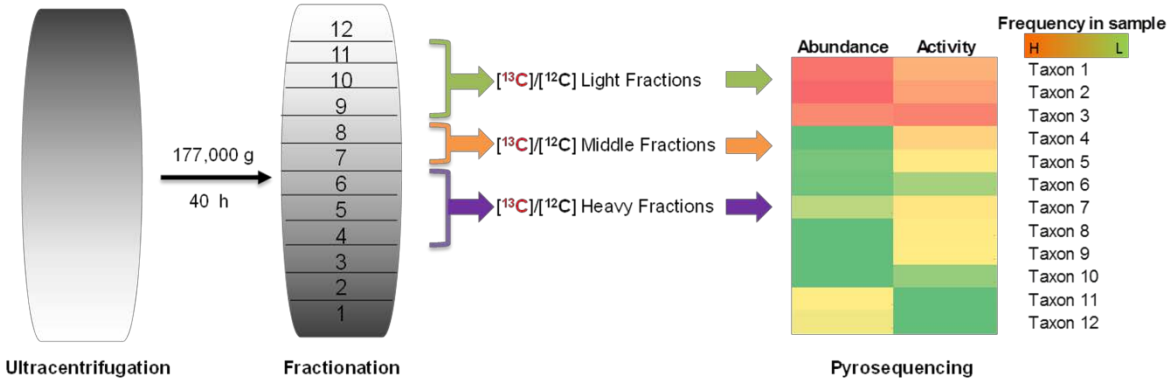

Figure 2: Outline of a Pyro-SIP experiment involving CsCl density gradient ultracentrifugation and fraction characterization with pyrosequencing of the separated DNA. H= higher abundance, L= lower abundance.

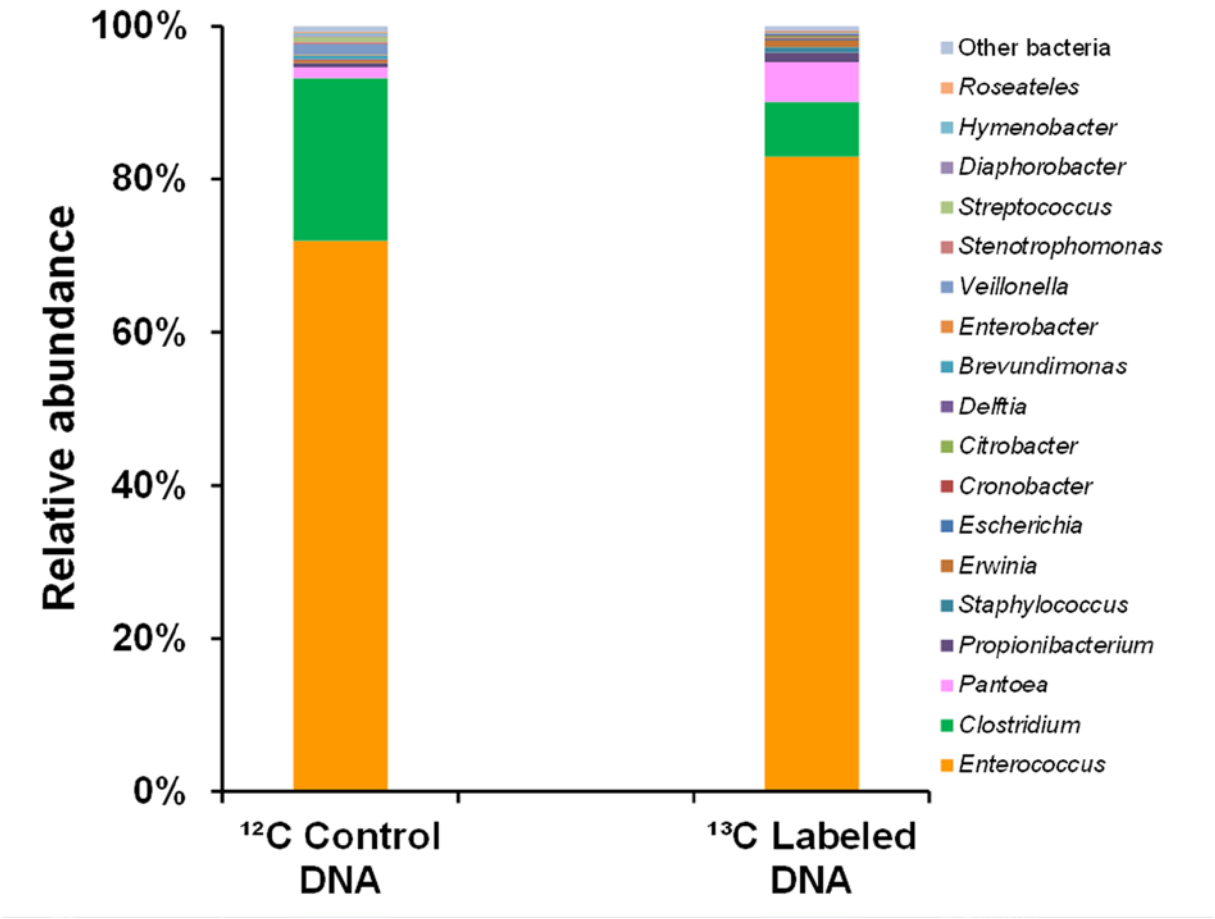

Figure 3: Bacterial diversity and relative abundance in the heavy fractions of the native glucose fed larvae (^{12}C Control DNA) and ^{13}C-glucose fed larvae (^{13}C Labeled DNA).

Discussion:

The gut of most insects harbors a rich and complex microbial community, typically 10^7 – 10^9 prokaryotic cells lodge there, outnumbering the host's own cells in most cases. Thus, the insect gut is a "hot spot" for diverse microbial activities, representing multiple aspects of microbial relationships, from pathogenesis to obligate mutualism[27]. Although many studies have described an amazing variety of insect gut microbial communities, characterization of the metabolic activity of gut microbiota is rare. Stable isotope probing approaches offer now a possibility for distinguishing the active members from a complex microbiota background independent of cultivation and even *in vivo*. We applied this valuable tool to study the gut microbiota in an insect model system, cotton leaf worm (*Spodoptera littoralis*). Since glucose is the dominant sugar in the gut of cotton leafworm, in this demonstration we fed the larvae

with an artificial diet spiked with an extra but physiological dose of ^{13}C-glucose to track the metabolically active bacteria in the gut flora. An identical incubation established with native glucose provided a critical control for subsequent comparison to ensure that any apparent labeling of nucleic acid was not an artifact of the method itself, such as high G+C content in DNA contributing to separation. The near *in situ* concentration of glucose additionally reduced experimental bias. Other considerations related to a rigorous experimental setup of the DNA-SIP study has been discussed elsewhere[28,29]. The gut bacteria normally are metabolically highly active and have short generation time compared to the microorganisms from most terrestrial and aquatic environments. Thus a 24 hours continuously feeding caused already a significant labeling. Note that a large amount of host genomic DNA was also co-extracted from the gut tissues, which should be taking into consideration when discussing the results.

The typical gradient fraction characterization relies on the low-resolution fingerprinting methods, such as terminal restriction fragment length polymorphism (T-RFLP), and time consuming clone library construction to link the data. It is only recently that the advent of high-throughput second generation sequencing technique allows for rapid, cost-effective and detailed analysis of complex microbial communities. Here we applied this new technology to directly survey the genetic composition of density gradient fractions and identify metabolically active bacteria, which gave enhanced sensitivity compared with the gel electrophoresis-based methods, and facilitated subsequent phylogenic classification. By comparing equivalent heavy fractions from both the ^{13}C-labeled and ^{12}C-control samples, we find that *Pantoea* and *Enterococcus* became labeled upon isotope probing. Once the metabolically active bacteria have been identified, other analysis such as the fluorescence *in situ* hybridization (FISH) using specific probes and metagenomic analysis of the labeled DNA recovered from active community members can be conducted to provide a comprehensive insight into the true symbionts associated with the host. Based on the established system here, other ^{13}C-labeled carbon sources could also be fed into the host to dissect the bacteria involved in the targeted gut metabolic pathway, for instance, labeling the

cellulose to identify active bacteria involved in the host digestion process. With the development of such new approaches, the role of insect gut microbiota will become more apparent than currently.

Collectively, the protocol described here represents a straightforward, rapid and effective method for determining metabolic activities of the gut microbiota, which further could be applied to assess the specific role of bacterial symbionts in the host nutrition, detoxification, and defense.

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Disclosures: We have nothing to disclose.

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3.3 Article III

Comparative evaluation of the gut microbiota associated with the below- and above-ground life stages (larvae and beetles) of the forest cockchafer, *Melolontha hippocastani*

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Comparative Evaluation of the Gut Microbiota Associated with the Below- and Above-Ground Life Stages (Larvae and Beetles) of the Forest Cockchafer, *Melolontha hippocastani*

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Abstract

A comparison of the diversity of bacterial communities in the larval midgut and adult gut of the European forest cockchafer (*Melolontha hippocastani*) was carried out using approaches that were both dependent on and independent of cultivation. Clone libraries of the 16S rRNA gene revealed 150 operational taxonomic units (OTUs) that belong to 11 taxonomical classes and two other groups that could be classified only to the phylum level. The most abundant classes were β, δ and γ-proteobacteria, Clostridia, Bacilli, Erysipelotrichi and Sphingobacteria. Although the insect's gut is emptied in the prepupal stage and the beetle undergoes a long diapause period, a subset of eight taxonomic classes from the aforementioned eleven were found to be common in the guts of diapausing adults and the larval midguts (L2, L3). Moreover, several bacterial phylotypes belonging to these common bacterial classes were found to be shared by the larval midgut and the adult gut. Despite this, the adult gut bacterial community represented a subset of that found in the larvae midgut. Consequently, the midgut of the larval instars contains a more diverse bacterial community compared to the adult gut. On the other hand, after the bacteria present in the larvae were cultivated, eight bacterial species were isolated. Moreover, we found evidence of the active role of some of the bacterial species isolated in food digestion, namely, the presence of amylase and xylanolytic properties. Finally, fluorescence *in situ* hybridization allowed us to confirm the presence of selected species in the insect gut and through this, their ecological niche as well as the metagenomic results. The results presented here elucidated the heterogeneity of aerobic and facultative bacteria in the gut of a holometabolous insect species having two different feeding habits.

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Introduction

In nature one of the largest classes of living organisms is Insecta. Its diversity is made particularly obvious by the enormous and various microbial communities found in the guts of insects [1]. The bacteria-insect interaction encompasses not only nutrition but also behavior. This is exemplified by the fact that the relationship between gut microbiota and host depends on the niche that the insect host occupies [2]. One of the widely studied cases involves termites: the bacteria and protozoa present in termites' hindgut paunches allows them to degrade recalcitrant polymers, such as cellulose and hemicellulose, into soluble compounds easily absorbed by their intestinal epithelia [3,4]. These studies have also demonstrated the existence of microbial lineages that apparently showed co-evolution with their termite hosts [5].

Insects belonging to the Scarabeidae (Coleoptera) family have habits similar to termites. Examples of these habits include saprophagous beetles that thrive on carrion, dung, humus or decaying matter, as well as phytophagous beetles that feed on the seeds, roots and foliage of plants [6,7]. The forest cockchafer, *Melolontha hippocastani*, is a member of this family and is a pest of European forests. More than three-quarters of its life cycle is spent in the soil where it feeds on roots. Adults of *M. hippocastani*, on the other hand, feed on tree leaves [8]. After pupation, the insect changes its feeding habits from rhizophagous to grazing. Considering this, the whole system represents a fascinating research field for the study of bacterial populations associated with the guts of the insects inhabiting both environments. Despite this opportunity, the field remains an almost neglected research area about which little is known.

The guts of *M. hippocastani* larvae consist of two large compartments: a tubular midgut and an enlarged hindgut [9] (cf. Figs. 1A and 2A). The midgut releases a large amount of hydrolytic enzymes [10] into an alkaline and oxidative environment, the characteristics of which threaten the development of bacterial species [11]. The second section is an expanded organ specialized for anaerobic fermentation [11,12], which resembles in functionality the paunch of termites. Both regions are associated with diverse bacterial communities. Studies focused on the bacterial community associated with the midguts of insects

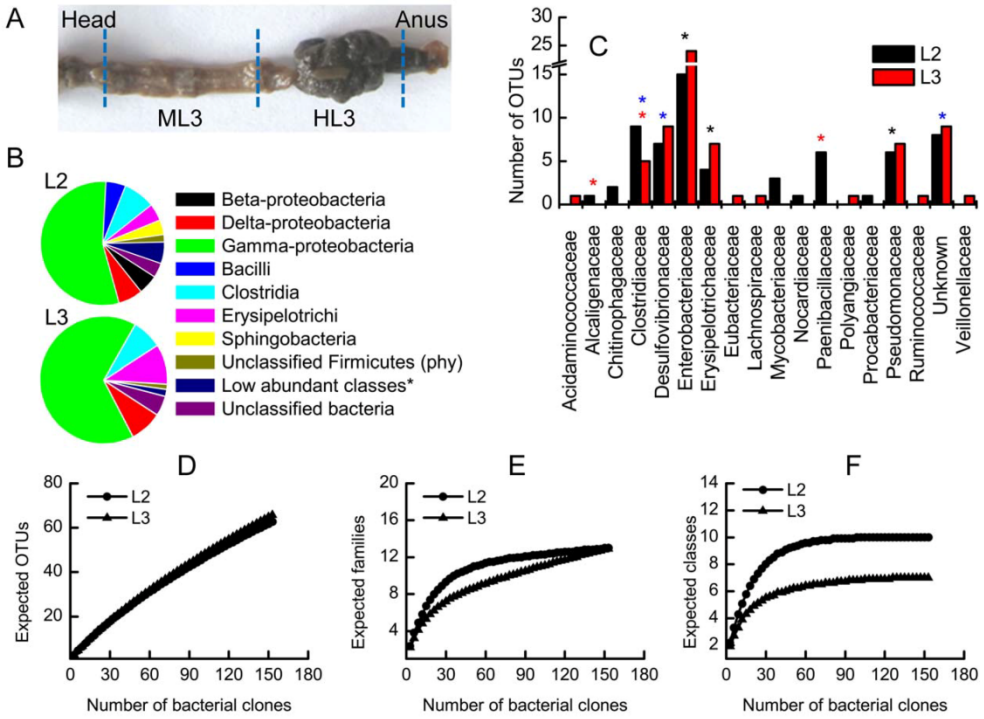

Figure 1. Composition of the bacterial community present in the larval midgut of *M. hippocastani* revealed by cloning and sequencing. (A) Image of the digestion tract of the L3 larvae. Sections used for fluorescence *in situ* hybridization (FISH) analysis, labeled as ML3 and HL3 are shown. (B) Relative abundance of bacterial classes found in the L2 and L3 larvae. *Low abundant classes: Actinobacteria, Negativicutes and unclassified Bacterioidetes. (C) Total number of operational taxonomic units (OTUs) belonging to each taxonomical family. Asterisks represent groups (phylotypes) shared among samples: black, common to all; red, shared by L2 larvae and adult; blue, shared by L2 and L3 larvae. (D) Rarefaction curve of the total number of OTUs identified in the midgut of the larvae. (E) Rarefaction analysis of the number of bacterial families identified in the midguts of the larvae. (F) Rarefaction curve of the total number of bacterial classes found in the data set against the total number of clones sampled.
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involved mostly Lepidopteran species. The latter systems are characterized for having simple bacterial assemblages [13,14]. Despite this, the bacterial community seems to be responsible for gut pH modification, the detoxification of plant allelochemicals and the maintenance of the microbial community structure [13].

Although in scarab beetles, the role of bacteria associated with the midgut is poorly understood, gut microbiota research in scarabs have mainly focused on bacteria that are harbored in the hindgut chamber [11,15,16]. Furthermore, to our knowledge, all the studies used larvae exclusively [16–19]. In this contribution, the bacterial communities associated with the midguts of larvae of *M. hippocastani* and the adult guts are systematically investigated and compared. The potential contribution of larval midgut and hindgut microbiota to the food digestion process is demonstrated in some isolated bacterial species.

Results

Larval Midgut Microbiota: Culture-independent Approach

A total of 309 high-quality sequences were retrieved from second- (L2) and third-instar (L3) larvae midguts. The 16S rRNA gene libraries revealed the presence of 9 bacterial classes (β-proteobacteria, δ-proteobacteria, γ-proteobacteria, Actinobacteria, Bacilli, Clostridia, Erysipelotrichi, Negativicutes, and Sphingobacteria) and 2 other groups that can be classified only to the phylum level (Firmicutes and an unknown phylum). These groups can be further classified to 18 families (Fig. 1C). The most abundant classes, including β-proteobacteria, δ-proteobacteria, γ-proteobacteria, Bacilli, Clostridia, Erysipelotrichi and Sphingobacteria, represented approximately 90% of the total sequences found in the two larval midgut samples (Fig. 1B).

The midguts of L3 larvae carry the highest amount of operational taxonomic units (OTUs; Table 1), many of which were also found in the midguts of the L2 larvae. Despite the high amount of OTUs observed, the size of our libraries was not sufficient to unravel the richness of the samples suggested by the rarefaction curve (Fig. 1D, no saturation reached) and species richness index, Chao1. Nothwithstanding the in-saturation of the rarefaction curve at the OTUs level, when applying the analysis at the family (Fig. 1F) and class levels (Fig. 1E) saturation was reached. γ- proteobacteria were the most abundant class of bacteria detected in larvae. In both samples, this class comprised only three families. The most abundant of these was Enterobacteriaceae, composed basically of a consortium of *Serratia* species (Table S1). Overall, the midgut microbiota of both instars of larvae

are almost identical. Slight differences could be seen in the presence of few OTUs detected only in the L2 midgut. The similarity in the diversity of both samples was further confirmed by the Chao1 estimator, since almost identical values were reported for the two instars. On the other hand, the highest ACE estimator value was obtained in the L3 midgut, because the relative abundance of many OTUs was higher than in the L2 larva.

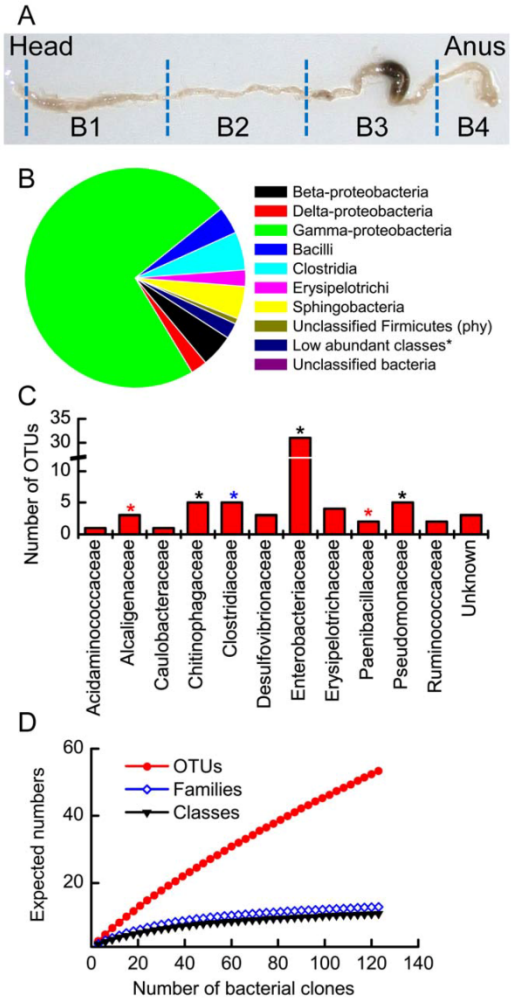

Figure 2. Composition of the bacterial community present in the guts of adult insects. (A) Image of the digestion tract of the adult gut. Sections used for fluorescence *in situ* hybridization (FISH) analysis were labeled B1 to B4. (B) Relative abundance of bacterial classes. *Low abundant classes: Acidobacteria, Negativicutes and Alpha proteobacteria. (C) Total of operational taxonomic units (OTUs) belonging to each taxonomical family. Asterisks represent groups (phylotypes) shared among samples: black, common to all; red, shared by L2 larvae and adult; blue, shared by L2 and L3 larvae. (D), (E), and (F) Rarefaction curves of the total number of OTUs, bacterial families and bacterial classes identified in the guts of adult insects.
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Table 1. Richness and diversity indices calculated with the 16S rRNA gene libraries.

Sample	Sequences sampled	Number of OTUs	Richness indices		Diversity indices	
			Chao1	ACE	Shannon	Simpson
L2	154	63	134.00	259	2.72	0.194
L3	155	67	134.25	431	2.68	0.205
Adult	125	54	80.85	88	2.37	0.262
Soil	85	61	n.d.	n.d.	n.d.	n.d.
Root	60	46	n.d.	n.d.	n.d.	n.d.

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Root and soil metagenomic libraries of the insect's host plant were generated to determine the extent to which the bacterial species of the insect gut could reflect the environmental and food bacterial communities. The size of the sequencing sample and the number of OTUs found are shown in Table 1. The greatest proportion of the species found, particularly in roots, corresponded to unknown bacteria. Additionally, many bacterial classes not described for the gut community or observed in low proportion, such as i.e., Planctomycetia, TM7 phylum, Actinobacteria and α-proteobacteria, were present in the soil and in the roots (Fig. S2A). Few of the bacterial OTUs described in the guts of larvae and adults were found either in the soil or in the roots, i.e., *Serratia* spp., *Cohnella* spp and an Acidobacteria class clone. The sequence similarity of clones belonging to those genera found in the gut (i.e. MH-05, MH-154 and MH-220 OTU) and in the soil or in the roots (S-46, S-16 and R-04) was greater than 97%. Nonetheless, the abundance of these bacteria in the soil was very low. For instance, γ-proteobacteria accounted for less than 3% of sequences. This opposes the trend observed in the gut, where this was the most abundant bacterial class. In both cases, the main bacterial species was represented by *Serratia* spp. On the other hand, all the OUTs belonging to this class found in the roots, were absent from gut. Overall, the overlap among the bacterial species found in guts and those detected in the soil and in the roots is minimal.

Gut Microbiota of Adult Insects: Culture-independent Approach

To avoid the complication of encountering transient environmental bacteria from food residues, only the guts of unfed beetles were analyzed. These adults had emerged recently from the pupae but remained in the soil in their diapause period. A total of 125 high-quality sequences were recovered. The adult gut bacteria were very similar to the samples of larval midgut on the class level. The most abundant class, γ-proteobacteria, was composed mainly of *Serratia* sp. and *Pseudomonas* sp. (Fig. 2B). Only Sphingobacteria and γ-proteobacteria showed greater abundance in adult guts than in larval midguts. The number of families (Fig. 2C) was similar to the number observed in the larval midgut. Only the number of OTUs differed, in adult, it was the lowest of all samples (Table 1). The rarefaction analysis suggests that the sample size was large enough to fully describe the number of classes and families (Fig. 2D, 2F) present in the sample but not the OTUs (Fig. 1E). Finally, all estimated indices confirmed a low richness and diversity of bacterial species present in the adult gut (Table 1) as compared to the richness and diversity of the midgut of larval samples.

Comparison of the Gut Microbiota from Roots and Leaf Feeders

When the compositions of the larval midgut microbiota and the adult gut microbiota were compared, an astounding similarity was observed (asterisks in Figs. 1C and 2C). Particularly for the larval instars, OTU-based pairwise comparisons using the weighted UniFrac test; found no statistical significant difference among samples. The opposite was observed when the gut microbita of the larval instar was compared to the corresponding of one of the adult, particularly for the L2-adult comparison ($p<0.05$) and, to a lesser extent, for the L3-adult analogy ($p = 0.07$). These results suggest differences in the microbial communities associated with the insect gut directly proportional to the insect maturity. Thus, the bacterial community of younger larvae instars will differ greatly from that of the adult stages. Despite these differences, a core group of bacterial species seems to be common to larvae and adult instars (Figure S1). This group of species was detected in the insect gut of the adults despite pupation, molting and metamorphosis. In that sense, the phylotypes, *Cohnella* sp., *Achromobacter* sp., and Clostridiales clone MH-141, were present in the L2 midgut and the adult gut. The latter two groups were more abundant in the L2 midgut than in the adult gut. In the same fashion, the unknown Chitinophagaceae species were present only in the adult gut the L2 midgut. In contrast, *Pseudomonas* spp. were detected in all samples. Another group abundant in the L3 midgut but less abundant in the other two samples was *Turicibacter* sp. Finally, the phylotypes Clostridiales clone MH-146 and the Delta proteobacteria were observed only in the larvae.

Larval Gut Microbiota: Culture-dependent Approach

The bacteria present in the gut of L3 larvae that were cultivable on brain heart infusion (BHI) media are shown in Table S1. From the midguts, only 5 species were obtained: *Serratia* spp., *Acinetobacter* spp., *Ralstonia* sp., *Citrobacter* spp. and *Stenotrophomonas maltophilia*. They were all γ-proteobacteria representatives, except for *Ralstonia* sp. In the hindgut homogenates, Enterobacteriaceae species including *Serratia* spp. and *Citrobacter* sp., represented 99.5% of all colonies; the remaining 0.5% consisted of a Bacillus species, *Viridibacillus arenosi*.

Various studies have reported that similar bacterial species, such as those identified in the current work (Table S1), degrade recalcitrant materials, i.e. polysaccharides such as cellulose, xylan, pectin and starch [20,21]. Some bacterial species isolated from the larval gut showed xylanolytic activity. Those included *Serratia* sp., *Pseudomonas* and *Citrobacter* sp. (Table S2). Moreover, when incubating the culture supernatant on media containing xylan, a degradation halo surrounding the deposit was observed. This demonstrates that the secretion of extracellular xylanolytic enzymes in the media since degradation occurred despite the absence of bacterial cells. Some isolates, including *Serratia* sp., *Citrobacter* sp. and *Viridibacillus arenosi*, were also able to degrade starch. Unlike in the xylanolytic isolates, no degradation of the substrate was observed when the culture supernatant of the isolates degrading starch was incubated on the media. This suggests that the enzymes responsible for that remain intracellular.

Bacterial Localization with Fluorescent Probes

To further evaluate the most abundant phylotypes in all gut sections of L3 larvae (Fig. 1A) and inactive (diapausing) and active (flying) adults (Fig. 2A), fluorescence *in situ* hybridization (FISH) was conducted using the probes described in Table S3. When FISH was performed on the guts of diapausing insects, almost no fluorescence signal was detected. The low hybridization signal intensity may be due to the small amount of ribosomes in the bacterial cells (which could be dormant) or to the shading of the insect tissue in which bacterial cells were embedded. The abundance of bacteria did not differ between males and females. All probes (Fig. 3A) showed positive foci in the midguts of the adults (sections B1 & B2) and the guts of the larvae (midgut, ML3, and hindgut, HL3). In section B3, the larval hindgut paunch vestige, and in B4, the anal conduit, few loci of hybridizing probes were observed. The reason for that might be the microenvironmental conditions prevailing at the interior of these organs. Probe p01, for detecting Chitinophagaceae species, revealed a large population of bacteria in the midguts of larvae (ML3). In all sections in which this probe's signal was observed, bacterial cells were distributed evenly on the gut epithelium, lumen and food bolus. *Achromobacter* spp., detected by probe 03, was very abundant in the adult gut sections B1 and B2 but not in the midguts of larvae. This species was mostly attached to the peritrophic membrane or to the gut epithelium (Table S5).

The bacteria detected by probes p08 and p09 targeting different groups of Clostridiales were observed in nearly all tissue sections in both adults and larvae, except in the B3 section of the adult gut (larval fermentation chamber vestige). In this section, only probe p09 detected a few cells. There, the bacteria cells were associated mainly with the chitinous protusions of the lobe (gut epithelium) but were also found in the gut lumen and on the food bolus of the adult hindgut. In the larvae midgut, bacterial cells were most abundantly associated with the peritrophic membrane and occasionally attached to the epithelium. Probe 06, targeting δ-proteobacteria, specifically *Desulfovibrio* spp., detected cells in the adult midgut (sections B1 and B2). They were located mostly in the gut lumen and in the peritrophic membrane but, occasionally, also attached to the gut epithelium. When hybridized to the larval midgut section, ML3, many foci (Fig. 3A) were observed as big aggregates on the food particles and on the epithelium. In the hindgut section, many cells were observed attached to the food bolus (Table S5).

Discussion

Insects' abilities to conquer diverse niches and therefore have particular life habits are closely linked to their interactions with microbes [2]. *M. hippocastani* is permanently exposed to the rhizosphere environment as eggs, larvae, pupae and diapausing adults. The larvae feed on tree roots that may consist of up to 50% of cellulose and a large amount of lignocellulose and lignin as well as associated humic materials. On the other hand, adults feed on tree foliage. Despite the obvious contribution of midgut microbes to fermentation processes in the gut [16,22], studies dealing with scarabs' microbiota focused mainly on hindgut-associated bacteria. In this study, we detected a complex bacterial community associated with the rhizophagous (larvae) and grazing (adults) life stages of *M. hippocastani*. Despite the different feeding habits of both stages, they seem to have a particular bacterial community in common. Moreover, part of this bacterial community prevails in the gut of the adult instar after metamorphism.

The Composition, Richness and Comparison of Bacterial Communities: a Culture-Independent Approach

About 90% of the species found in larvae midguts and adult guts were β-, δ-, and γ-proteobacteria, Bacilli, Clostridia, Erysipelotrichi and Sphingobacteria (Figs. 1B, 2B). These data coincide with some of the results of previous gut studies of other scarab insects, i.e. *Pachnoda ephippiata*. The midgut clones of this insect are affiliated mostly with Actinobacteria, followed by Clostridiales,

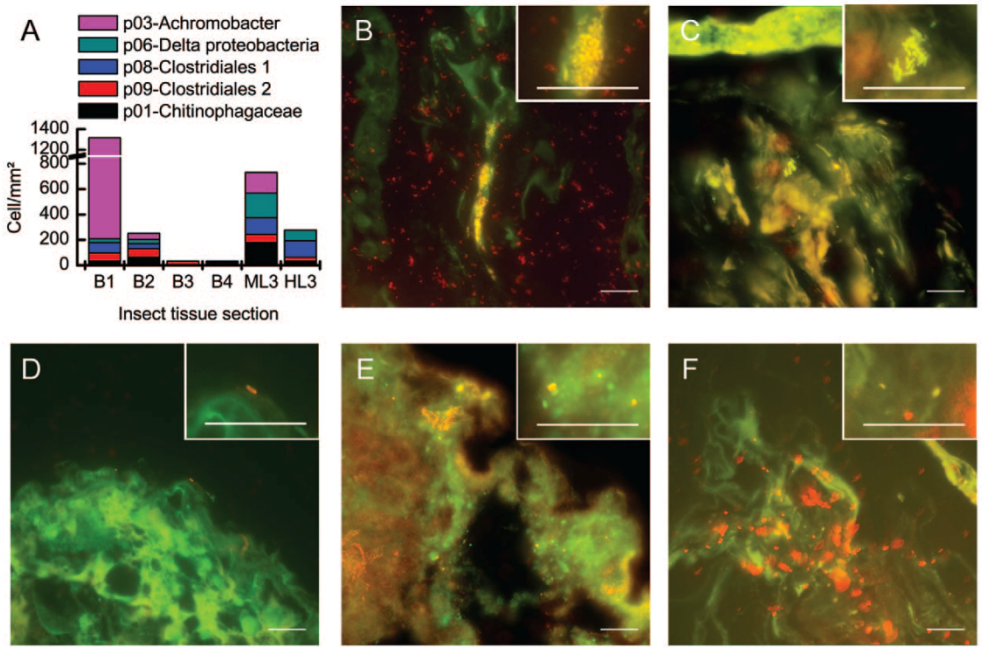

Figure 3. Bacteria in the gut of *M. hippocastani*, L3 larvae and adult insects. (A) Number of bacterial foci detected (cell/mm^2); probes are listed in Table S3. (B) An image showing *Sediminibacterium* sp. in the female beetle gut section B1. (C) Image of *Achromobacter* sp. in female beetle gut section B1. (D) Image of *Desulfovibrio* sp. in the midguts of L3 larvae. (E) Image of Clostridiales-related clone (p-08) in the hindguts of L3 larvae. (F) Image of another Clostridiales species (p-09) in female beetle gut section B2. Scale bars 20 µm.
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Lactobacillales, Bacillales, and *Turicibacter sanguinis* (Eysiopelotrichi). Additionally, in the hindgut, the majority of species were Lactobacillales and Clostridiales [22]. In the hindgut of different scarab species, such as *M. melolontha* [16] and *Dermolepida albohirtum* [23], the taxa commonly found were Clostridiales, *Turicibacter sanguinis* (Eysiopelotrichi), β- and δ- protebacteria (the most abundant). Overall, the most abundant group found in the midgut of *M. hippocastani* was γ-proteobacteria; this group consisted of two large families, Entobacteriaceae and Pseudomonaceae (Figs. 1C and 2C), coincide with observations in other beetles [24–26].

The richness and diversity analysis applied to our data (Table 1) suggests no difference between larvae samples but greater diversity and bacterial species richness of the larvae midgut bacterial community than of the adult gut bacterial community. Moreover, after comparing the evaluated larval instars with the adults using the Unifrac test, greater difference among the bacterial communities is observed when differences in the maturity of the insects are greater. The bacterial communities of immature insects (L2) appear to differ more compared to those of adults than when compared to those of the next larvae instar (L3). Different relations seem to be established between bacterial communities and the guts of different insect instar stages. That is the case for the subcortical beetle *Agrilus planipennis* [25] in which the microbial population in larvae was a subset of the adult and the pre-pupae. Like *M. hippocastani*, this insect feeds on different parts of the plants as immature and adults. The larvae bore in the cambium and phloem of the trees, whereas the adults thrive on the leaves.

The adult samples used for the metagenomic libraries were collected during January when the insect does not feed because it is diapausing. Our results detected a bacterial community present in the diapausing adults, although they had gone through molting and metamorphosis. The bacterial community in these insects was detected only after sequencing, not during FISH hybridization when the signal obtained was dim. This might indicate that the bacterial population in the gut of the diapausing insects was dormant or that their growth had slowed in response to the unfavorable environmental conditions, which occurs in other ecosystems [27]. This assumption was supported by the fact that the FISH hybridization signal obtained on actively feeding beetles was restored and, moreover, very strong.

This study is among the few comparing bacterial communities of the guts of different insect stages with different feeding habits. That a core group of species common was observed in the bacterial community of both insect stages (larvae and adults) confirms findings in the study involving the emerald ash borer, where 22 OTUs were shared by the gut samples of larvae, prepupae and adults [25]. On the domestic fly, a similar phenomenon was observed. The four most abundant bacterial species in the larval guts were the only inhabitants of the newly emerged adults [28]. A total of nine different bacterial phylotypes (Table S4) belonged to a core of bacterial species common to all three samples analyzed (Fig. S1). The most abundant phylotypes in after both the approaches followed in our study was *Serratia* sp. Among other functions, *S. marcescens* is suggested to serve as an oxygen scavenger, creating anerobic conditions for other bacterial species, i.e. those responsible for digesting cellulose in the gut of the termite *Coptotermes formosanus*, [29].

Another group abundantly detected was *Turicibacter* sp., a genus often found in the gut of other scarabs (see Table 2) as *P. ephippiata* [22] and *M. melolontha*. This species produces lactate, a fermentation product present in the midgut of *M. melolontha* [16]. Beyond this, nothing further is known about the role this species plays in the gut of insects. Interestingly, a greater abundance of *Turibacter* sp. was observed among the larvae than in the adults. The association of this species with *M. hippocastani* grubs suggests that it is playing important roles in the physiology of the rhizophagous instars. However, further research is required to fully elucidate what is the main contribution of Turicibacter to the insect digestion or physiology in general. Another group common to all gut samples is *Cohnella* spp. The genus possesses facultative anaerobic metabolism, and is often found in the soil, in the phyllosphere or in the rhizosphere. Species closely related to the clones found in this study, such as *C. thailandensis* [30] and *C. panacarvi* [31], display xylanolytic activity that may be involved in the hydrolysis of hemicellulose. Some other common phylotypes shared by gut samples were Enterobacteriaceae clone MH-043, Clostridiales clone MH-146 and the Clostridiales clone MH-141. However, their relative abundance may vary. For example, the amount of Clostridiaceae and Desulfovibrionaceae decreased in the adults compared to in the larvae. The decrease in the abundance of Desulfovibrionaceae in the adult gut might be directly linked to the changes in feeding habits that occur in adulthood. Actually, the digestion recalcitrance of the insect feeding materials decreases, and moreover, their nutritional richness increases. In oak roots, an amount of up to 128 mg g^{-1} of dry weight (DM) was found [32].In contrast, the amount of lignin in leaves is only 32 mg g^{-1} DM [33]. Furthermore, the content of protein and nitrogen in the roots of woody plants such as oak, tends to be lower than that in leaves. Bacterial species such as *Desulfovibrio* spp. as well as *Citrobacte*r spp. and *Enterobacter* spp. (not only detected in our metagenomic libraries but also isolated from the larval gut) may be actively contributing to the supplement of nitrogen in larvae as occurs in termites [34,35]. Furthermore, Clostridiales related species as well as many other having celullolitic and/or hemicellulolytic properties, may more abundantly present in the larvae than in adults since the food contents greater contration of those components.

As pointed out in Table 2, many of the main phylotypes and bacterial species detected in this study have close phylogenetic relationships or were previously detected associated with the guts of other insects, principally scarab larvae and termites. The frequent association of these bacterial species with such insect species denotes a clear symbiotic relationship and may indicate that co-evolution is taking place. Nevertheless, further research is required to confirm this.

While feeding on roots, the larvae introduce a significant amount of soil and humic materials into the gut. In order to determine the extent to which the gut bacterial community resembles the environment and food on which the insect thrives, metagenomic bacterial characterization of roots and soil was done. Few bacterial OTUs present in the gut of the insect were phylogenetically closely related to those detected in the roots and in the soil. That is true for *Serratia* spp., *Cohnella* spp. and an OUT belonging to the Acidobacteria class (Fig. S2). Despite this close phylogenetic relationship between OTUs, the relative abundance of those bacterial species was quite different in the soil and in the roots than in the gut. For example, *Serratia* spp., which was the most abundant species in the gut, was present in less of 3% of the sequences retrieved from soil. Our results suggest that although some bacterial species originating in food and soil were observed in the gut of the insect, no large overlap among the bacterial species abundantly found in the gut and the environment and food seems to occur.

Larval Gut Microbiota: Culture-dependent Approach

Beyond describing the bacterial species associated with the gut of the larvae and adults of *M. hippocastani*, our objective was to unravel the function that the bacterial community serves in the insect gut. Therefore, a group of bacteria was isolated from L3 larvae gut homogenates (Table S1). Many of the identified γ-proteobacteria, i.e. *S. liquefaciens*, *P. fluorescens* and *C. freundii* have proven to degrade xylan, cellulose, pectin and starch [21,26,36]. Since there are very few examples of insects possessing hydrolytic enzymes such as cellulases or xylanases, most of the degradation of such polymers during insect digestion relies on microbes. Xylanolytic isolates were identified among the *Serratia* sp., *Pseudomonas* sp. and *Citrobacter* sp. cultures (Table S2) obtained. These species were among the most abundant ones in the culture-dependent and -independent surveys performed. This abundance suggests an active role of these bacteria in the degradation of the xylan content of the insect food. In nature, xylan is one of the main components of the hemicellulose fraction of wood. For instance, sugar hydrolyzates from the bark of oak can make up to 20% of xylan [37]. Furthermore, the assumption that the xylanolytic bacterial species could be actively serving this function in the gut of the insect is supported by their ability to secrete extracellular xylanases in the media (Table S2). The latter was obvious after a degradation halo formed around the supernatant of the isolates when incubated in media containing xylan. Indeed, our results are supported by the findings of Emami et al. [38]. They demonstrated that ca. 65% of the xylanolytic activity of *P. cellulosa* was present in the culture supernatant and that the remainder was associated with the bacterial cell. Additionally, two of the xylanolytic isolates, one from *Serratia* spp. and the other from *Citrobacter* spp., were also able to degrade starch. Nonetheless, the degradation of the substrate by the isolates supernatant was not observed. This suggests production of extracellular amylases was negligible. Thus far, amylases of insect origin have not been found in the gut of *Melolontha* spp. [39].

Bacterial Localization in the Gut

To confirm our metagenomic results and to localize the bacterial species in the gut and thus to identify their niches, we conducted FISH with specifically designed probes (Table S3). The probe p-01 targeting Chitinophagaceae species revealed bacteria attached to the gut epithelium in the adult midgut, whereas in the L3 larvae midguts and hindguts, the bacteria were associated with the food bolus (Table S5 and Figure 3). In the larval hindgut, only a few foci were observed. Their location correlated with the oxygen regimes existing in each organ. A radial gradient of oxygen has been reported in the guts of termites [40] and *M. melolontha* [16]. Some areas are microoxic or completely anoxic. The arrangement of the microorganisms in the gut presumably obeys this oxygen gradient, depending on the ability of the microorganisms to respire oxygen [41]. No information about the physiological function of the Chitinophagaceae clone identified in our samples (SM44, Table 2) is yet available. The only knowledge of this clone that we have is restricted to its origin and phylogeny; it was found inhabiting the gut of the yellow catfish and it is known to be a close relative of *Sediminibacterium* sp. The Chitinophagaceae family belongs to the Sphingobacteria class. This class has frequently been found in insect species feeding on wood [24]. *Sphingobacterium* sp. TN19 was isolated from the gut of a member of the cerambycidae, which possesses xylanases directly involved in hemicellulose digestion [42]. On the other hand, the *Achromobacter*

Table 2. Comparison of the bacteria found in *Melolontha hippocastani* in this study and those described previously in other organisms.

Phylotype	Closest link BLAST	Accession number	Report	Reported species host	Eating habits
Achromobacter clones	*Achromobacter* sp.	FJ828885.2	[55]	Human	omnivorous
Alcaligenaceae	Clone PeHg37	FJ374254.1	[18]	*Pachnoda* spp.	humus-feeding
Chitinophagaceae	Clone SM44	GU293236.1	n.p.	*Pylodictis olivaris*	humus-feeding
Clostridiales	Clone PCH-24	EF608542.1	[56]	*Poecilus chalcites*	omnivorous
	Clone RS-E61	AB0808987.2	[57]	*Reticulitermes speratus*	fungus-growing
	CloneMGMjD-018	AB234447.1	[58]	*Macrotermes gilvus*	humus-feeding
	Clone PeH56	AJ576369.1	[22]	*P. ephippiata*	humus-feeding
	Clone PeHg78	FJ374197.1	[22]	*P. ephippiata*	humus-feeding
	Clone PeHg58	FJ374218.1	[22]	*P. ephippiata*	humus-feeding
δ-proteobacteria clones	Clone Cf6-11	GQ502596.1	n.p	Formosan termite	n.d
	Clone PeHg02	FJ374259.1	[22]	*Pachnoda* spp.	humus-feeding
	Clone PeHg87	FJ354258.1	[22]	*Pachnoda* spp.	humus-feeding
	Clone MG MjD-065	AB234531.1	[58]	*Macrotermes gilvus*	humus-feeding
Enterobacteriaceae	Clone Hg14	EF675596.1	[59]	*Hepialus gonggaensis*	roots of weeds
Turicibacter	*T. sanguinis*	HQ428099.1	[22,60]	Human and *P. ephippiata*	Omnivorous/humus-feeding
	Clone PeM71	AJ576424.1	[22]	Human and *P. ephippiata*	Omnivorous/humus-feeding
Veillonellaceae clone	*Sporomusa sphaeroides*	NR025417.1	[61]	n.d.	n.d.

n.p., not published; n.d., not described.
doi:10.1371/journal.pone.0051557.t002

sp. and the Chitinophagaceae species shared a similar distribution. *Achromobacter* sp. are obligate aerobes [43] and common inhabitants of insect [44,45] and human guts. Our data confirmed that they are unable to survive in the anoxic regions of the gut. The cells localized only to certain regions of the midgut and were absent from the hindguts of larvae and adults.

A large amount of *Desulfovibrio* sp. and related species existed in the guts of larvae and adults. They were more often observed in the larvae, the bacterial cells located on the epithelium in the midgut and on the food bolus in the hindgut. This bacterial species is likely located at the oxygen interface (lowest concentration of oxygen), since it is regarded as strictly anaerobe but also oxygen tolerant [41]. Moreover, at that position it is likely to be involved in removing oxygen from the environment. This bacterium represents 10–15% of the total bacterial count in the hindgut of M. *melolontha* [16]. As a sulfate-reducing bacterial species, it seems to participate actively in decreasing the concentration of sulfates between midgut and hindgut. Moreover, *Desulfovibrio* sp. and related species could be actively participating in the generation of acetate (as for xylophagous termites [34]), the main fermentation product observed in the gut of *M. melolontha*.

Finally, the clones of Clostridiales (Table S5) were mainly found in the midguts of both adults and larvae and also in the hindguts of larvae. In the hindguts they are mainly found on the epithelium. It is generally believed that Clostridiales are obligated anaerobes, but certain species, i.e. *Clostridium novyi*, are known to tolerate an atmosphere of up to 3% oxygen [46]. This wide range of tolerance may explain their distribution in the guts of the insects we observed.

Conclusions

Our data revealed the presence of a complex bacterial community in the cockchafer larval midgut and the adult gut. Moreover, despite the harsh environment that the midgut represents, the richness and diversity of the bacterial community in the midguts of larvae was superior to the community present in the guts of the diapausing adult insects. In addition, a core group of bacterial phylotypes seems to be shared among the samples, despite the different feeding habits of larvae and adults. Moreover, it is evident that no great alteration of the bacterial diversity in the insect occurs as a result of the input of bacteria that entering the intestine via food or soil contamination, since little overlap of the bacterial species present in all the environments was observed. In general, the gut bacterial community of the adult stage seems to be a subset of the larval midgut. This community prevails into adulthood, despite the molting and metamorphosis of the larvae. Interestingly, many of the bacterial species detected were reported to be associated with the guts of other insects, particularly scarabs and termites. This fact suggests their active participation in roles which ultimately benefit insect physiology, i.e. digestion, nitrogen supply and hormone production among others. Moreover, when the functionality of some of the abundant gut bacterial species present in the L3 larvae was characterized, it became clear that some of the isolates displayed amylase and xylanolytic properties. Finally, the application of FISH permitted us to locate selected bacterial species in the gut, confirming the results of the 16S rRNA gene libraries and, moreover, defining the niche that each species occupies.

Materials and Methods

Sample Collection and DNA Extraction

Second- and third-instar larvae (L2 and L3) of *M. hippocastani* and newly emerged, unfed (diapausing) adult insects were collected in forests of red oak in Mannheim (49°29′20″N 8°28′9″E), and Iffezheim (48° 48' 00" N 8° 08' 00" E), Germany, in December 2009 and January 2010. Actively feeding (flying) adults were obtained at Iffezheim, Germany, in April and May 2010. The insects were transported live in boxes with soil or tree leaves. Soil

and root samples were collected in the Iffezheim forest in April 2012. Once in the laboratory, before dissection, the insects were kept at 0°C for an hour to kill them and then rinsed alternately with water and 70% ethanol, 3 times. Dissection was performed in a phosphate-buffered saline (PBS) solution. The guts from the larvae were sectioned in two parts as shown in Figure 1A. The midgut section was defined as the region between the first caecae after the head, excluding therefore the foregut, and the second region shortly before the pyloric sphincter. The midguts from four larval specimens from the same instar were pooled together. The digestion tracts of adult insects were obtained in the same way. Samples were stored at −20°C before DNA extraction. Frozen samples were thawed on ice and dried at 45°C for 90 min in a Speedvac (Concentrator 5301, Eppendorf). Dried samples were crushed in a 1.5 ml tube with a sterile plastic pestle. The DNA extraction of the tissue, as well as the soil and root samples, was carried out using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the protocol provided by the manufacturer.

One gram soil samples stored at 4°C were washed 3× with 1 ml distilled water and dried for approximately 18 hours at 80°C. Four hundred mg were used to extract DNA. A hundred mg fresh roots were cut in very small pieces after the surface soil was brushed off. The root slices were mixed with AP1 lysis solution from the DNAeasy Plant Mini kit from Qiagen and ground in a Precelly Homogeniser (91-PCS24, PeqLab). The supernatant was incubated for 10 minutes at 65°C and transferred to a bead tube from the PowerSoil™ kit for the DNA extraction. DNA solutions of several replicates were pooled and purified 2 times using an Invisorb Fragment CleanUp kit (STRATEC Molecular GmbH, Berlin, Germany) before being used as templates for the PCR reaction.

Cultivation of Gut Bacteria and Substrate Screening

For cultivating the gut bacteria, the insects were paralyzed at 4°C and washed alternately with water and ethanol before dissection. The midgut and hindgut sections from three larvae (L3) were collected and submerged in 15% glycerol. After being vortexed at maximum speed for 2 minutes, the samples were stored at −80°C. The glycerol suspension was diluted 5 times with 0.9% NaCl solution and spread on brain heart infusion (BHI) media plates. DNA purified from pure bacterial cultures was used as a template for PCR identification. Colony-forming units of each isolate were estimated by serial dilutions of the glycerol stock. The result was presented as a percentage of relative abundance.

For testing the capability of degrading different polysaccharides, the isolates were grown in minimal media supplemented with four different materials; xylan, cellulose, potato starch and pectin as described in Anand et al. [21]. Rapidly, the media was supplemented with the polysaccharides as carbon source, and an aliquot of 8 µl of LB-culture of each isolate was deposited on top of a sterilized circle of filter paper placed on a petri dish plate. The plates were incubated for 24 h under 37°C. Cultures showing degradation capacity were revealed using the Congo red overlay method (cellulose, xylan and pectin) and the iodine method (starch). To identify the presence of excreted sugar hydrolases in the media, the supernatant of the liquid culture of the isolates degrading each polysaccharide was used. For that, the supernatant of the culture was 10 times concentrated with a column VIVASPIN 6 (Sartorius AG, Goettingen, Germany). The test was performed using the methodology described for the liquid culture screening.

16S rRNA Gene Amplification

16S rRNA genes were amplified directly from gut DNA preparations using the universal primers 27f (5_- AGAGTTTGATCCTGGCTCAG -3_) and 1492r (5_- GGTTACCTTGTTACGACTT -3_) with temperature gradient PCR (SI M+M) on a GeneAmp 9700 Thermocycler (Applied Biosystems). The 50-µl reaction mixture contained 1× Buffer, 1.5 mM $MgCl_2$, 10 mM four deoxynucleoside triphosphates (dNTPs), 2.5 U of Taq DNA Polymerase (Invitrogen), 0.5 mM of each primer and 60 ng of DNA as a template. To further clean up the PCR product, a nested PCR was performed using the primers Bac357f (5_ -CTCCTACGGGAGGCAGCAG-3 _) and the Bac 1392r (5_-ACGGGCGGTGTGTRC-3_) with 5 µl of combined temperature-gradient-PCR products used as a template. The PCR reactions were performed as follows: initial denaturation at 94°C for 3 min; 35 cycles of denaturation, 94°C for 45s), annealing, 45°C-55°C for 30s, and extension at 72°C for 1 min. The final extension step was at 72°C for 10 min. were amplified The 16S rRNA genes amplification from the soil sample were performed with a two-step PCR using nested primers, as for the insect gut. For the oak root sample, the primers 799f (5_ -AACAGGATTAGATACCCTG-3 _) and the 1492r (5_- GGTTACCTTGTTACGACTT -3_) were used.

The PCR products were separated on a 1.5% agarose gel, and correct bands excised and purified using an Invisorb Fragment CleanUp kit (STRATEC Molecular GmbH, Berlin, Germany). The purified DNA was cloned with a pCR2.1 TOPO TA Cloning Kit (Invitrogen) with TOP 10 *E. coli* competent cells. Colonies were chosen randomly and then sequenced. Sequencing was carried out at the Leibniz Institute for Age Research, Fritz Lipmann Institute –FLI (Jena, Germany) following the procedure described by Ping et al. [47].

Phylogenetic Analyses and Calculating Indices of Diversity

DNA sequences were cleaned and assembled using the DNASTAR Lasergene software package (DNASTAR, Inc. Madison, WI, USA). The initial assembling of the sequences was performed with a 99% threshold. Consensus sequences were used for BLAST search at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) and Greengenes (http://greengenes.lbl.gov). Chimeric sequences were identified using bellerophon [48] and further confirmed by comparison to the BLAST results. Phylogenetic analyses of the OTUs observed in gut, soil and roots were performed using Bayesian inference with the software BEAST 4.1 [49]. For convenience, the sequence classification threshold was arbitrarily assigned as 100–97% identity, species; 96–95%, genus; 94–90%, family; and below 89 to 80%, class. Rarefaction analyses were performed with Analytic Rarefaction 1.3 (http://www.uga.edu/strata/software/index.html).

Species richness was defined as the number of OTUs present in each sample. The Chao estimator, the abundance-based coverage estimator (ACE) [50] and the α-diversity estimators, the Shannon and Simpson indices, were calculated using Mothur [51]. Finally, community similarity or β-diversity was estimated using the UniFrac-weighted significance test [52] from the online open source UniFrac [53]. Partial bacterial 16S rRNA gene sequences have been deposited at the National Center for Biotechnology Information with accession numbers JQ683506-JQ683656 for gut OTUs and JX427407-JX427503 for soil and root OTUs.

Fluorescence *in situ* Hybridization

L3 larvae as well as male and female adults (either active or diapausing) were dissected as mentioned. The gut tissues were fixed in 4% of paraformaldehyde in phosphate-buffered saline (PBS) overnight. After being rinsed 3 times with PBS and dehydrated in acetone, the samples were embedded with Technovit 8100 (Heraeus Kulzer GmbH, Wehrheim, Germany). Sections of 5 µm in thickness were mounted on SuperFrost Ultra Plus glass slides (Thermo Scientific) and treated with 5 mg/ml lysozyme for 15 min at 37°C. After the lysosyme was washed away with distilled running water for 30 seconds, the slide was dried by blowing it with compressed air. The sections were double-hybridized with 1.5 µM of each specific probe (Table S3) and an Eubacterial probe EUB 338 [54]. The hybridization buffer contained 900 mM NaCl, 0.02M Tris-HCl (pH8.0), 20% Formamide and 1% SDS. Hybridization was performed at 46°C for 4 hours on an Advalytix slide booster (Beckman Coulter Biomedical GmbH, Munich, Germany). Subsequently, the slide was washed in 50 ml washing buffer containing 0.02 M Tris-HCl (pH8.0), 0.2 M NaCl, 0.05 M EDTA, 1% SDS at 48°C for 20 min. Finally, after being rinsed with water and 70% ethanol for 30 sec each, the sections were mounted with Citifluor (London,UK). Images were taken with an Axio Imager Z1 microscope (Carl Zeiss) equipped with an AxioCam MRM camera.

Supporting Information

Figure S1 Relative abundance of the bacterial phylotypes shared by midguts of L2 and L3 larvae and the whole adult gut.
(TIF)

Figure S2 Composition of the bacterial community present in the guts of *M. hippocastani* (larvae and adult pooled), soil and roots revealed by cloning and sequencing. A. Relative abundance (by percentage) of bacterial classes found in the insect guts (sequences from larvae and adult pooled), roots and soil. Names displaying a star on the right side correspond to classification at the phylum level, since the bacterial class classification is not available. **B.** Phylogenetic tree of bacterial divisions retrieved from *Melolontha hippocastani* gut, soil and roots based upon sequence similarity. Code color for designation of the OTUs of different samples: black, gut; red, soil; and green, root. A list of the OTUs' clone names, the accession names and the closest related BLAST reference sequences can be found in Table S6. Numbers in front of groups indicate the number of OTUs grouped. The numbers displayed next to the branches indicate the two decimal posterior probabilities. The bottom bar represents the substitution rate per site.
(TIF)

Table S1 Abundance of bacteria isolated from the gut of L3 larvae.
(DOCX)

Table S2 Degradation of xylan and starch in minimal media by the bacterial isolates obtained from the L3 larvae homogenates.
(DOCX)

Table S3 FISH probes designed.
(DOCX)

Table S4 Most abundant phylotypes found in the 16S rRNA gene libraries of *Melolontha hippocastani*.
(DOCX)

Table S5 Localization of the bacteria in different insect sections with FISH.
(DOCX)

Table S6 List of bacteria used for phylogenetical tree; clone name, accession number and closest related sequence deposited in GenBank.
(DOCX)

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Author Contributions

Conceived and designed the experiments: LP WB. Performed the experiments: EA. Analyzed the data: EA LP. Contributed reagents/materials/analysis tools: KR MP HD WB. Wrote the paper: EA LP WB.

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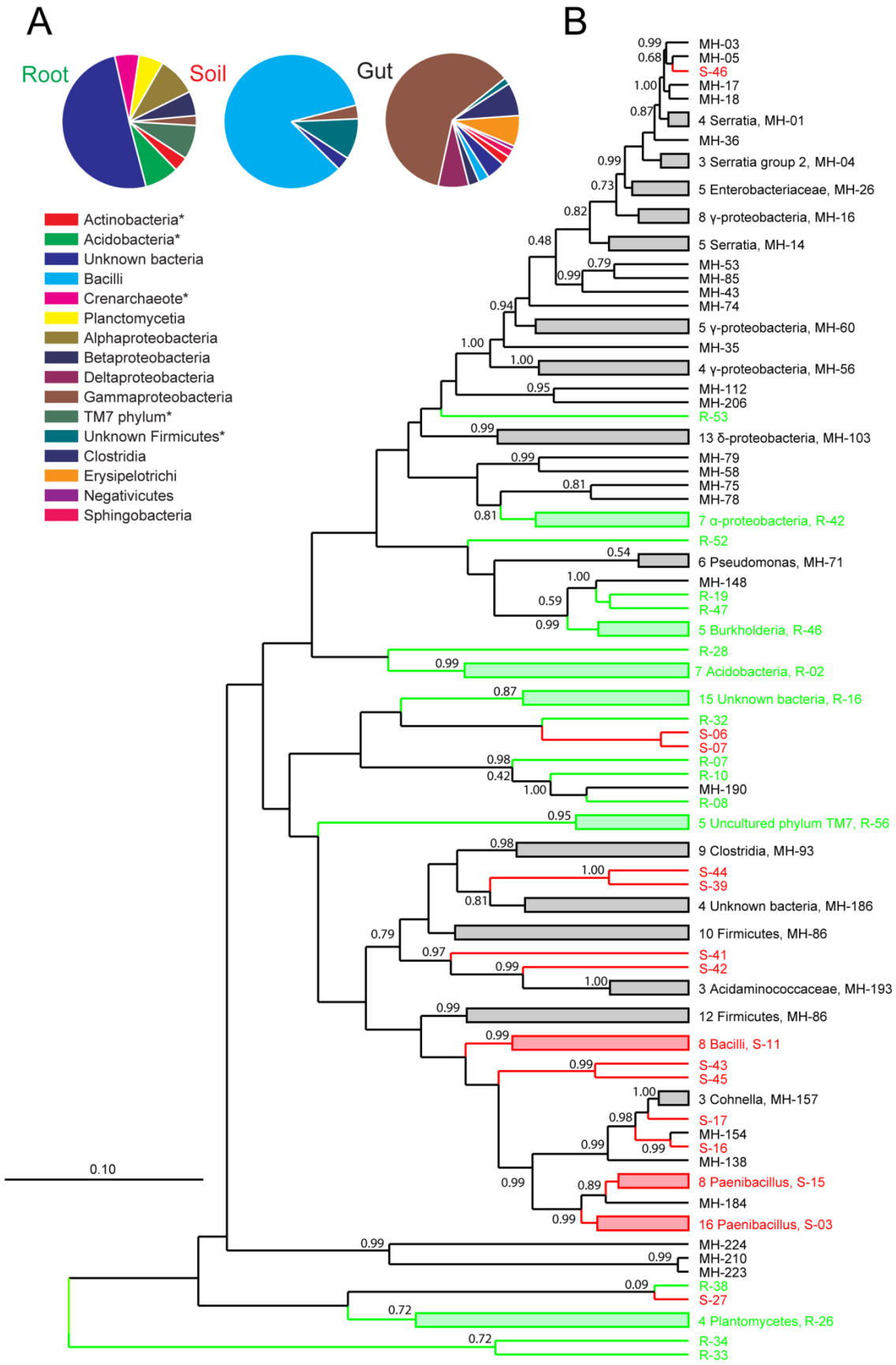

Figure S2. Composition of the bacterial community present in the guts of M. hippocastani (larvae and adult pooled), soil and roots revealed by cloning and sequencing.

A. Relative abundance (by percentage) of bacterial classes found in the insect guts (sequences from larvae and adult pooled), roots and soil. Names displaying a star on the right side correspond to classification at the phylum level, since the bacterial class classification is not available.

B. Phylogenetic tree of bacterial divisions retrieved from Melolontha hippocastani gut, soil and roots based upon sequence similarity. Code color for designation of the OTUs of different samples: black, gut; red, soil; and green, root. A list of the OTUs' clone names, the accession names and the closest related BLAST reference sequences can be found in Table S6. Numbers in front of groups indicate the number of OTUs grouped. The numbers displayed next to the branches indicate the two decimal posterior probabilities. The bottom bar represents the substitution rate per site.

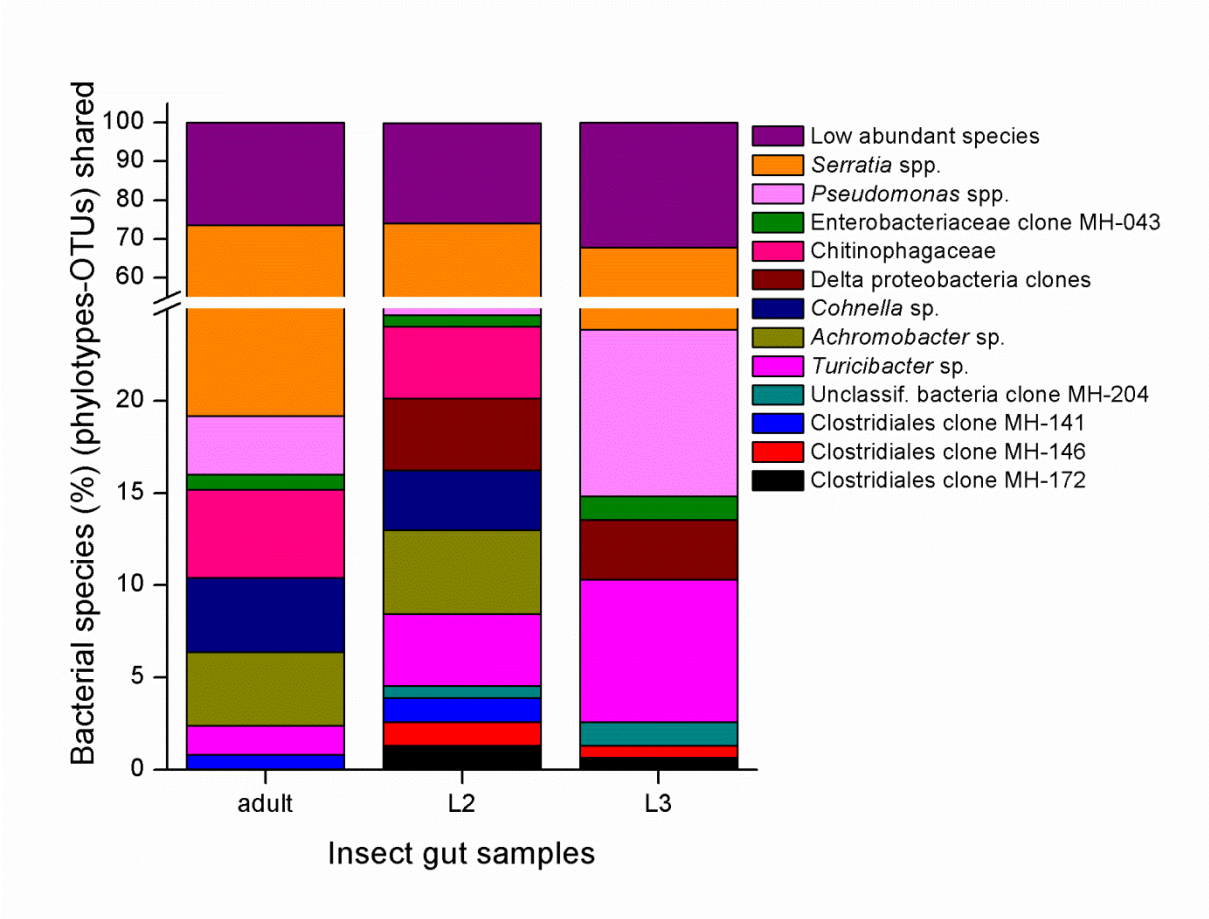

Figure S1. Relative abundance of the bacterial phylotypes shared by midguts of L2 and L3 larvae and the whole adult gut.

Table S1. Abundance of bacteria isolated from the gut of L3 larvae.

Tissue	Bacteria name	Similar sequence found by BLAST	Identity (%)	Accession number	Relative abundance (%)
Midgut	*Serratia* spp.	*S. liquefaciens*	99	DQ123840.1	34.6
		S. proteamaculans	99	CP000826.1	
		S. grimesii	99	HQ 42737.1	
	Acinetobacter spp.	*A. rhizosphaerae*	99	DQ536511.1	27.2
		A. calcoaceticus	99	AM157426.1	
	Ralstonia sp.	*Ralstonia* sp.	98	FJ193255.1	27.2
	Citrobacter spp.	*C. freundii*	98	AF025365.1	8.6
		C. murliniae	98	DQ068811.1	
		C. koseri	98	CP000822.1	
	Stenotrophomonas maltophilia	*Stenotrophomonas maltophilia*	99	GU385870.1	1.2
	Pseudomononas sp.	*Pseudomononas* sp.	98	GQ478265.1	1.2
Hindgut	*Serratia* spp.	*S. proteamaculans*	99	CP000826.1	73.2
	Citrobacter sp.	*Citrobacter* sp.	98	HQ399664.1	26.3
	Viridibacillus arenosi	*Viridibacillus arenosi*	99	EU741070.1	0.5

Table S2. Degradation of xylan and starch in minimal media by the bacterial isolates obtained from the L3 larvae homogenates.

Isolate	Origin	Polysaccharide source			
		Xylan		Starch	
		LC[a]	Supernatant[b]	LC[a]	Supernatant[b]
Serratia sp. 1	midgut	**+**	**+**	-	-
Serratia sp. 2	hindgut	**+**	**+**	-	-
Serratia sp. 3	midgut	**+**	**+**	**+**	-
Serratia sp. 4	midgut	**+**	**+**	-	-
Serratia sp. 5	midgut	**+**	**+**	-	-
Serratia sp. 6	hindgut	**+**	**+**	-	-
Citrobacter sp. 1	midgut	**+**	**+**	-	-
Citrobacter sp. 2	midgut/hindgut	**+**	**+**	-	-
Citrobacter sp. 3	hindgut	**+**	**+**	**+**	-
Pseudomonas	midgut	**+**	**+**	-	-
Viridibacillus	Midgut	-	-	**+**	-

[a] LC, stands for the liquid culture of the isolate pure

[b] Supernatant refers to a concentrated solution (10x) of the isolate liquid culture supernatant to evaluate the presence extracellular enzymes

Table S3. FISH probes designed.

Probe	Target	Sequence (5'-…-3')	Label	Corresponding OTUs
EUB-338	all eubacteria	GCTGCCTCCCGTAGGAGT	Cy3[a]	General probe
p-01	Chitinophagaceae-*Sediminibacterium* sp.	TGGTACCGTCAAGTGGGA	FITC[b]	MH-210, MH-223, MH-234, MH-174
p-03	*Achromobacter* sp.	TCAGTTTCACGGGGTATTAG	FITC	MH-148, MH-194
p-06	Deltaproteobacteria-*Desulfovibrio* sp.	CAAGTAAAGGCTGATTAGCAC	FITC	MH-142, MH-137, MH-144
p-08	Clostridiales 1	GTCACTTTATTCTTCCTTGAGG	FITC	MH-172
p-09	Clostridiales 2	CATTATCGTCCCCCACC	FITC	MH-141

[a] Fluorescent cyanine Cy3

[b] Fluorescein isothiocyanate, FITC

Table S4.1. Most abundant phylotypes found in the 16S rRNA gene libraries of *Melolontha hippocastani*.

Denomination	Assigned name	Closest BLAST Identified record	Accession	Identity	Closest BLAST unidentified record	Accession	Identity
Phylotype	*Achromobacter* sp. clones group 1	*Achromobacter* sp.	GU254016.1 FJ828885.2 DQ414679.1	95-100			
Clone	Alcaligenaceae clone MH-180c231				Clone PeHG37	FJ374254.1	95
Phylotype	*Citrobacter* sp.	*Citrobacter* sp. *C. freudii*	GQ416174.1 HQ324431.1	99 98			
Phylotype	Clostridiales clones	*Ruminococcus gauverauii*	EF5296201	96	 Clone PCH-24 Clone 3T-1 Clone PeH56 Clone FF_h08 Clone RS-E61 CloneMGMjD-018 Clone ZSB-C8	 EF608542.1 EF404556.1 AJ576369.1 EU469620.1 AB0808987.2 AB234447.1 GU205581.1	 96 96 95 97 96 96 98
Phylotype	*Cohnella* sp. clones	*C. soli* *Cohnella* sp.13-25 *Cohnella sp.* M36	EF368009.1 EU912527.1 HM624040.1	97 98 96			
Phylotype	Delta proteobacteria clones group 1				Clone Cf6-11 Clone PeHg87	GQ502596.1 FJ374258.1	95 95
Phylotype	Desulfovibrionaceae clone	*Desulfovibrio* sp. Z1RB	AY532164.1	96	 Clone MG MjD-065 Clone MgMjD073	 A234531.1 AB234528.1	 96 95
Phylotype	*Mycobacterium* sp. clones group 1	*Mycobacterium* sp. NMR17-6 *M. peregrinum*	AB286061.1 AM884591.1	100 97			

Table S4.2. Most abundant phylotypes found in the 16S rRNA gene libraries of *Melolontha hippocastani*.

Denomination	Assigned name	Closest BLAST Identified record	Accession	Identity	Closest BLAST unidentified record	Accession	Identity
Phylotype	*Paenibacillus* sp.	*Paenibacillus* sp.	HM162341.1	97	Unc.*Paenibacillus* sp. Clone S9ABac	EU669180.1	98
Phylotype	*Pseudomonas* sp. clone group 1	*Pseudomonas* sp. ML1-2010	FN825677.1	98-96	Clone PBXB4	GU569130.1	98
		Pseudomonas sp.	EU438852.1	97			
		Pseudomonas sp.	AF351240.1	96-95			
		Pseudomonas sp. strain LS197	FJ937924.1	98-96			
		P. putida strain PC36	DQ178233.1	100			

Table S4.3. Most abundant phylotypes found in the 16S rRNA gene libraries of *Melolontha hippocastani*.

Denomination	Assigned name	Closest BLAST Identified record	Accession	Identity	Closest BLAST unidentified record	Accession	Identity
Phylotype	*Serratia* sp. clones group 1	*S. proteamaculans* strain wg-2	EU627690.1	99-97			
		S. grimesii str. ZFX-1	AY789460.1	99			
		S. proteamaculans strain BXCC-35	JF431270.1	99			
		Serratia sp. SES-01	EU414474.1	99			
		S. grimessii	HM217122.1	99-96			
		Serratia sp. B-136-2	EU557341.1	99			
		S. liquefasciens strain 19-CDF	FJ811866.1	98-95			
		Serratia sp. B-1123	DQ347536.1	96			
		S. proteamaculans subsp. quinovora LMG 7887	AF286867.1	95			
		S. grimesii	DQ991163.1	96			
		Serratia sp. BZ65	HQ588852.1	97-95			
Phylotype	Chitinophagaceae clones group 1				Clone SM44	GU 293236.1	99-97
Phylotype	*Turicibacter* sp. clones group 1	*T. sanguinis* strain MOL361 *T. sanguinis strain PC909*	NR028816.1 HQ428099.1	97-95 96-95			

Table S5. Localization of bacteria in different insect gut sections with FISH.

Organ section	Probes														
	p01-Chitinophagaceae			p03-Achromobacter			p06- Deltaproteobacteria (*Desulfovibrio* sp.)			p08-Clostridiales 1			p09-Clostridiales 2		
	L	E	FB	L	E	FB	L	E	FB	L	E	FB	L	E	FB
MiL3	+	+	+	+	+	+	+	+	+	-	+	-	-	+	+
HiL3	-	-	+	-	-	-	-	-	+	+	-	-	-	-	+
B1	-	+	-	+	-	-	-	+	-	+	+	-	+	+	-
B2	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-
B3	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-
B4	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-

L=gut lumen, E= gut epithelium and FB= food bolus or residues of food.

3.4 Unpublished results

Bacterial community and novel structures associated with the hindgut of the forest cockchafer (*Melolontha hippocastani*)

Erika Arias Cordero, Liyan Ping, Eiko Wagenhoff, Jürgen Rybak, Martin Kaltenpoth, Martin Westermann, Wilhelm Boland

In preparation

Variation in the food and habitat of insects generates great diversity in their gut morphology. Generally, the gut of insects consists of three sections: foregut, midgut and hindgut. Most of the anatomical diversity is centered in the hindgut. The principal function of this gut section is the absorption of water and salts from the urine and feces. Despite this, in specialists' insects, especially in beetles of the scarabeidae family the same site is devoted to help digestion. Their hindgut is composed of an enlarged organ called fermentation chamber that is lined with bacteria. In that chamber, the recalcitrant fraction of the insect food is processed by the microorganisms. That is the case in *Melolontha hippocastani*, the forest cockchafer, a member of the scarabeidae family. It feeds on a poor and recalcitrant diet, namely the roots of forest species as oak and birch. In order to succeed while thriving in such food, it has established symbiotic relations with bacteria harbored in its gut. The diversity of the bacterial community associated with the insect gut has been suggested to be as high as for termites. Previous gut bacterial studies in other scarabeidae beetles suggest the presence of bacteria mostly as free cells on its surface and lumen. Since a very intimate and close relationship between the insect and its microbiome is established, presence of bacteria beyond the surface must exists. With help of microscopic techniques i.e. light, epifluorescence and electron; fluorescent in situ hybridization-FISH, and 16SrRNA 454-pyrosequencing; the hindgut of below and above ground stages of the forest cockchafer, *Melolontha hippocastani* were studied. The main objective was to describe the bacterial arrangement in hindgut.

Results

Surface arrangement of the bacterial community associated with the hindgut of the forest cockchafer. Assessment of the bacterial community on the surface of the lobe structures (Fig. 1C) lining the hindgut (Fig. 1B) of a L3 larva of *M. hippocastani* (Fig. 1A) was performed by SEM. Field collected L2 larvae were used for the experiment. After dissection, in the prepared gut of the larva is possible to identify the hindgut fermentation chamber as the tissue between slashed lines in Fig. 1D top image. For comparative purposes, the morphology of the beetle gut is presented in the bottom image of Fig.1D (between dashed

lines the hindgut fermentation chamber). After dissection, to remove the food and fecal contaminants , the tissue was washed several times with a Phosphate Buffer Saline Solution (PBS). Despite this, it was impossible to get completely rid of the contaminants, and a layer of matrix of bacteria and vegetative material remained. During tissue dissection and macroscopical examination of hindgut, a couple of novel structures, named from here on as "pockets" were spotted (Fig. 1C,1E, 1G for *M. hippocastani* and 1F for *M. melolontha*). In literature no direct report of this is known. Further details are presented in the next section.

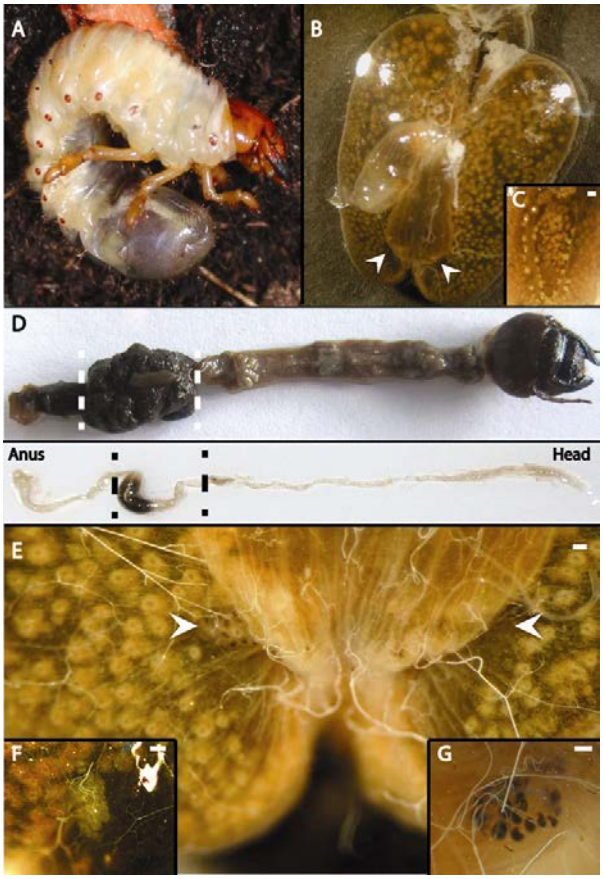

Figure 1. Gut anatomy of larvae and beetle of *Melolontha hippocastani*. (A) L3 larva instar living in soil. (B) Hindgut -fermentation chamber. (C) Close up of the hindgut lobe lining the organ. (D) Whole gut preparation of a L3 larva instar (top image) and a female adult beetle (bottom image). Between dashed lines is the hindgut section used for microscopy and pyrosequencing. (E) The fermentation chamber and the pocket position (pointed with arrows). (F) Close- up of the *Melolontha melolonthas*' pocket and (G) close- up of the *M. hippocastanis'* pocket. White arrows at the hindgut pockets. Scale bar, 100 µm.

In Fig. 2A the bacterial arrangement on top of the hindgut lobes (Fig. 1C) is shown. Over these structures, the bacterial cells pack densely, forming biofilm layers that covers the

complete lobe surface. After treatment with PBS, some naked lobes were observed (Fig. 2B). The lobes increase the surface area of hindgut, accommodating higher amount of bacteria cells as compared to a flat surface. They are not only covered by one layer of bacteria cells, but a strata of different bacterial types that deposits one over the other (Fig. 2C). Finally, when the lobe is completely covered the bacterial biofilm continues forming tridimensional arrangements as displayed in Fig. 2D.

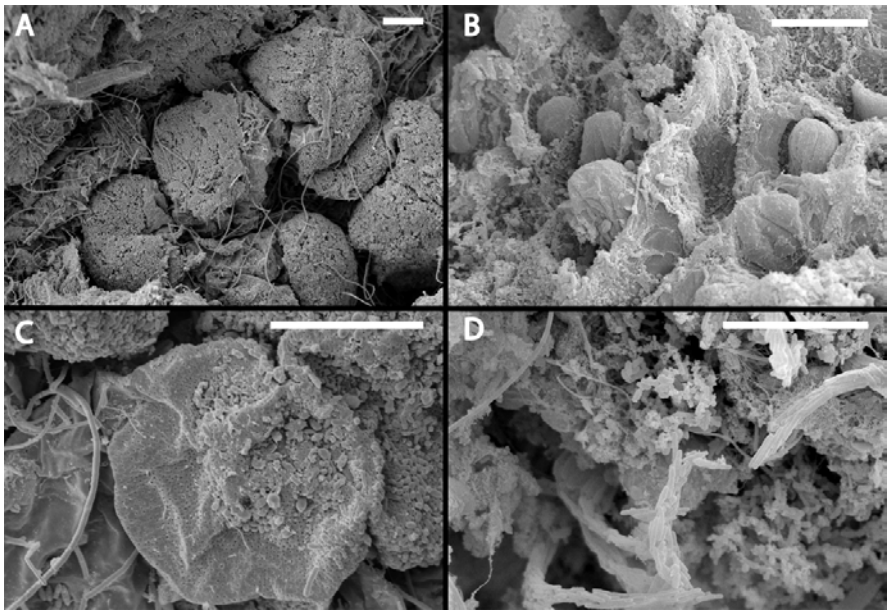

Figure 2. Bacterial surface arrangement of a L2 larva hindgut-lobe, visualized by scanning electron microscopy-SEM. (A) Hindgut lobe surface densely packed with bacteria and food residues, (B) Naked lobes, (C) Close up of the bacterial cell strata on a hindgut lobe. (D) Close up of the matrix lining the hindgut of a L2 larva instar. Scale bar 100µm.

Novel spatial niches of bacteria harbored in the hindgut of the forest cockchafer. As pointed in previous section, a couple of small dark structures (white for *M. melolontha*, Fig. 1F) attached to the hindgut of only larvae instars were spotted. They were situated outside this gut section, specifically, at its terminal point (Fig.1E). Since only one report of this tissue is documented in literature [67], a deeper characterization was carried out. It is around 500 µm long. For the first exploration of the tissue, scanning using laser confocal microscope with a spectral laser line of 488 nm was done. Due to the high self-fluorescence of the tissue, no further staining was required to capture its complete architecture (Fig.3 B).To

further characterize the cells constituting the tissue, laser confocal imaging using various fluorescent stains was performed. Using SYTOX Orange nucleic acid stain (Invitrogen) it was possible to determine that the hindgut pockets are superficially covered with tracheoles all over (see stained nuclei of the tracheole cells, Fig.3 C). Furthermore, after labeling the tissue with Alexa Fluor 488 nm phalloidin (Phallotoxin, Invitrogen) the presence of a fine layer of muscle tissue covering it was evident (Fig. S1). Finally, a plasma membrane stain (CellMask 554nm) was also applied to the tissue (data not shown). Unfortunately, as in case of the other applied stains, the labeling was restricted to the surface only. Hence, in order to deeply explore the tissue, light microscopy was performed (Fig. 3C). A cross semi-thin section allowed the observation of each of the poles constituting the hindgut pocket anatomy. Moreover, the connection of each of them to the hindgut lumen was visualized. Additionally, it was observed that each pole is lined with high amount of bacteria cells (Fig.3D). Further anatomical description after transmission electron microscopy was also performed. As shown in Fig. S2, each pole is surrounded by a thick a-cellular tissue layer (likely mucous-like), likely responsible for the stain penetration blockage. Close by the a-cellular layer, three layers of gut typical tissue, characterized for a very dense amount of mitochondria and tracheole connections are identified. Apparently, a higher density of the cells concentrates at the distal section of each pole (Fig. 3D). A gradual decrease on the density of cells is achieved the closer the hindgut wall. When the bacterial cells are much more densely packed in pole, they are embedded together in a sort of membrane enveloping many cells at once. Finally, some of the bacteria cells display cytoplasmic inclusions. Positive Nile blue staining [68] of the inclusions permitted their identification as poly-β-hydroxybutyrate granules (PHB) (Fig. S3). This is a kind of polyhydroxyalkanoates (PHAs), a polymer commonly stored by eubacteria and Archaea. They serve as reserve materials of the cells, which are stored in water insoluble inclusions in their cytoplasm [69].

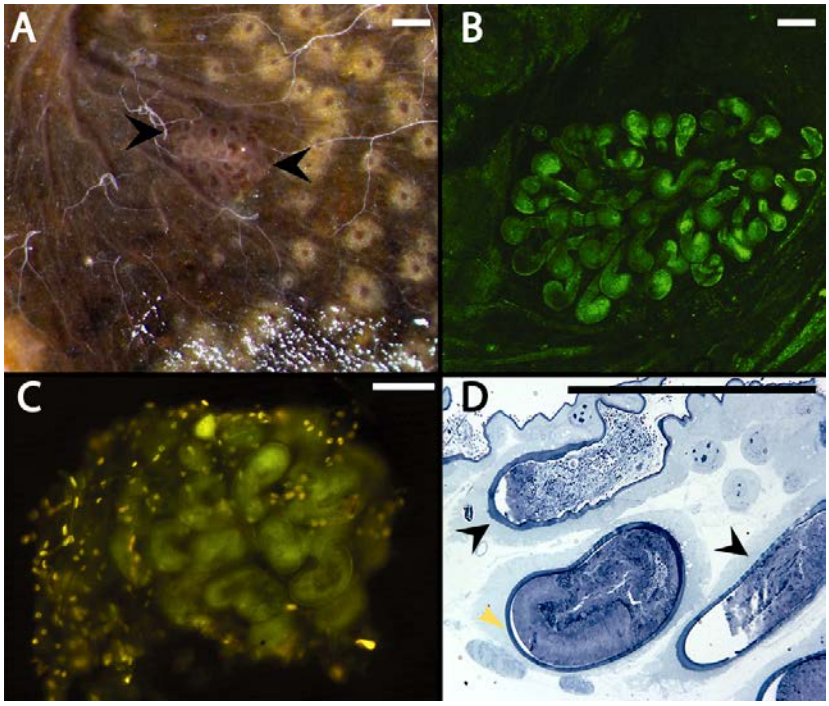

Figure 3. Appearance of the hindgut pocket. (A) Attached pockets on the hindgut external surface-pointed with black arrow heads. (B) Auto fluorescence picture of the pocket tissue using a 488 nm laser in a confocal microscope. (C) Autofluorescence-Sytox DNA stained image of the pocket tissue using a 488/543 nm laser in a confocal microscope. (D) Cross section of a larva hindgut pocket stained with Richards's solution. Black arrow heads pointing the single poles of the pocket, yellow arrow head points the a-cellular layer enveloping every pole. Scale bar 100µm.

In addition, after light and TE microscopy examination, morphologically similar bacterial cells were found in the pockets and hindgut lobes (Fig. 4A and B). Moreover, it seems that these bacterial cells move between among the tissues as they are also present in the intercellular space where the muscle cells of the hindgut wall converge (Fig. 4C and D). The cross section of the pockets shows a great amount of bacterial cells with the same morphology along the poles (Fig. 4E). Furthermore, its central part is densely lined with at least two anatomically different types of gram negative bacterial cells (Fig.4F). To further identity the bacterial species in pockets and lobes, a hierarchical FISH approach was carried out. With probes targeting the β-, and γ-subclasses of Proteobacteria it was possible to identify the bacterial cells as members of both groups. Hybridization of hindgut pocket section found some γ-proteobacteria (Fig. 5A) and larger numbers of β –proteobacteria (Fig. 5B) Contrary to this, most of the observed bacteria either in hindgut lobe of beetles and larvae or the

intercellular space in the tissue are γ-proteobacteria (Fig.5C, E and F). Only a small number of cells was identified as β -proteobacteria (Fig. 5D). Since it is difficult to distinguish the bacterial cells when densely packed next to each other, a proper method allowing higher resolution is required. To solve the problem, gold-in situ hybridization visualized with TEM is planned.

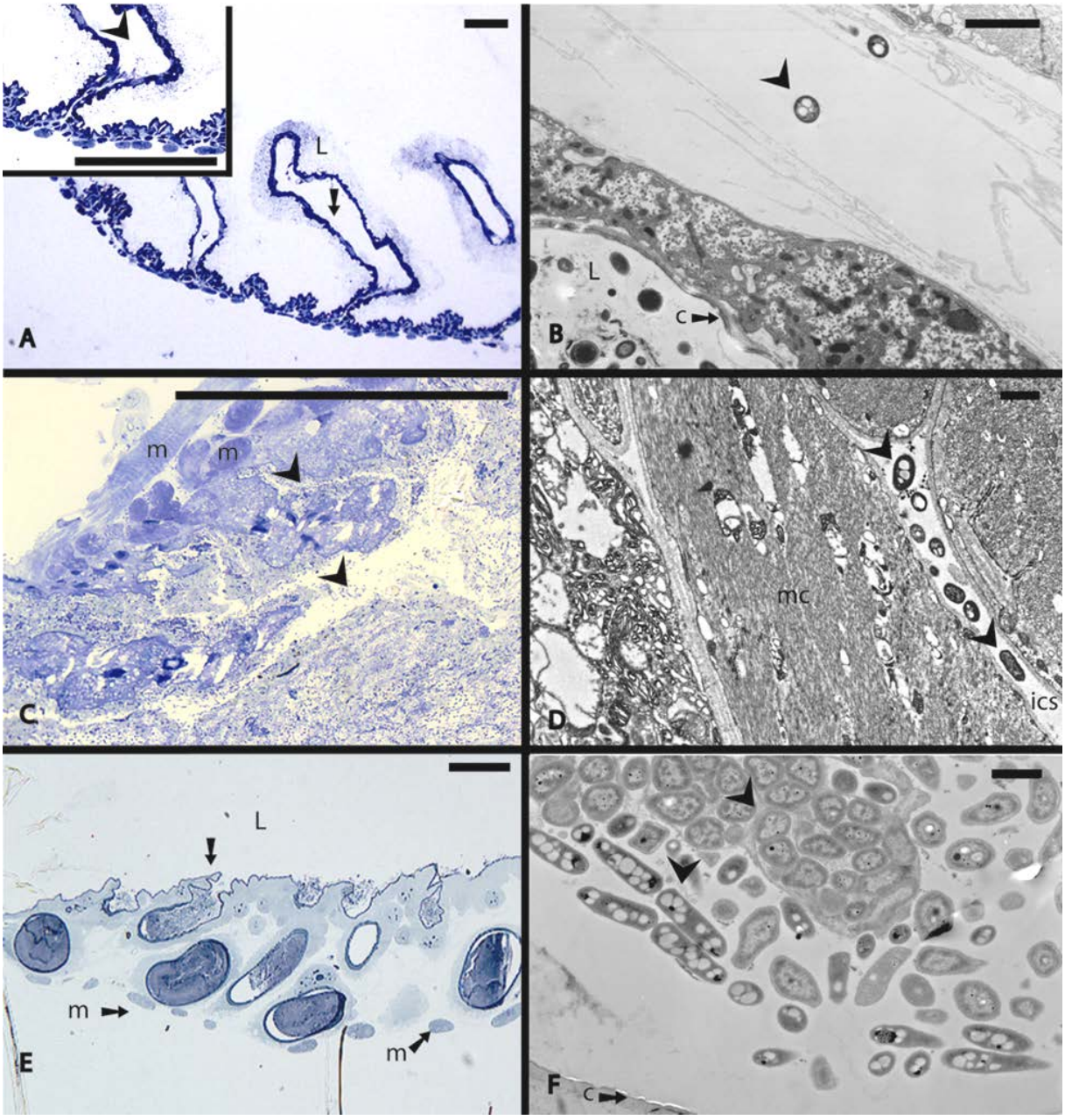

Figure 4. Light and transmission electron microscopy (TEM) of hindgut: lobe, wall and pockets of larvae and beetles. (A) Overview of the lobe of a female hindgut. Insert: close up pointing the bacteria localization by black arrows. (B) TEM image of the bacteria localization inside the hindgut lobe of a female. Note the polyhydroxybutyrate granules (PHB) inclusions in cell, pointed with black arrows head. Lumen (L) and hindgut

cuticle (c). (C) Cross section of the hindgut wall of a L3 instar larva, not the bacteria localization. m= muscle fibbers (D) Distribution of the bacterial cells in the intercellular space (ics) between the muscle cells (mc). (E) Overview of the hindgut pocket of a L2 instar larva. Black arrows point the muscle fibbers (m) and the linkage region to the hindgut lumen (L). (F) Cross section of one of the poles composing the hindgut pocket. c= cuticle surrounding the structure lined with bacterial cells either single or embedded in a membrane. Arrow heads points bacterial cells. Scale bar, left panel 100 µm, right panel 1µm.

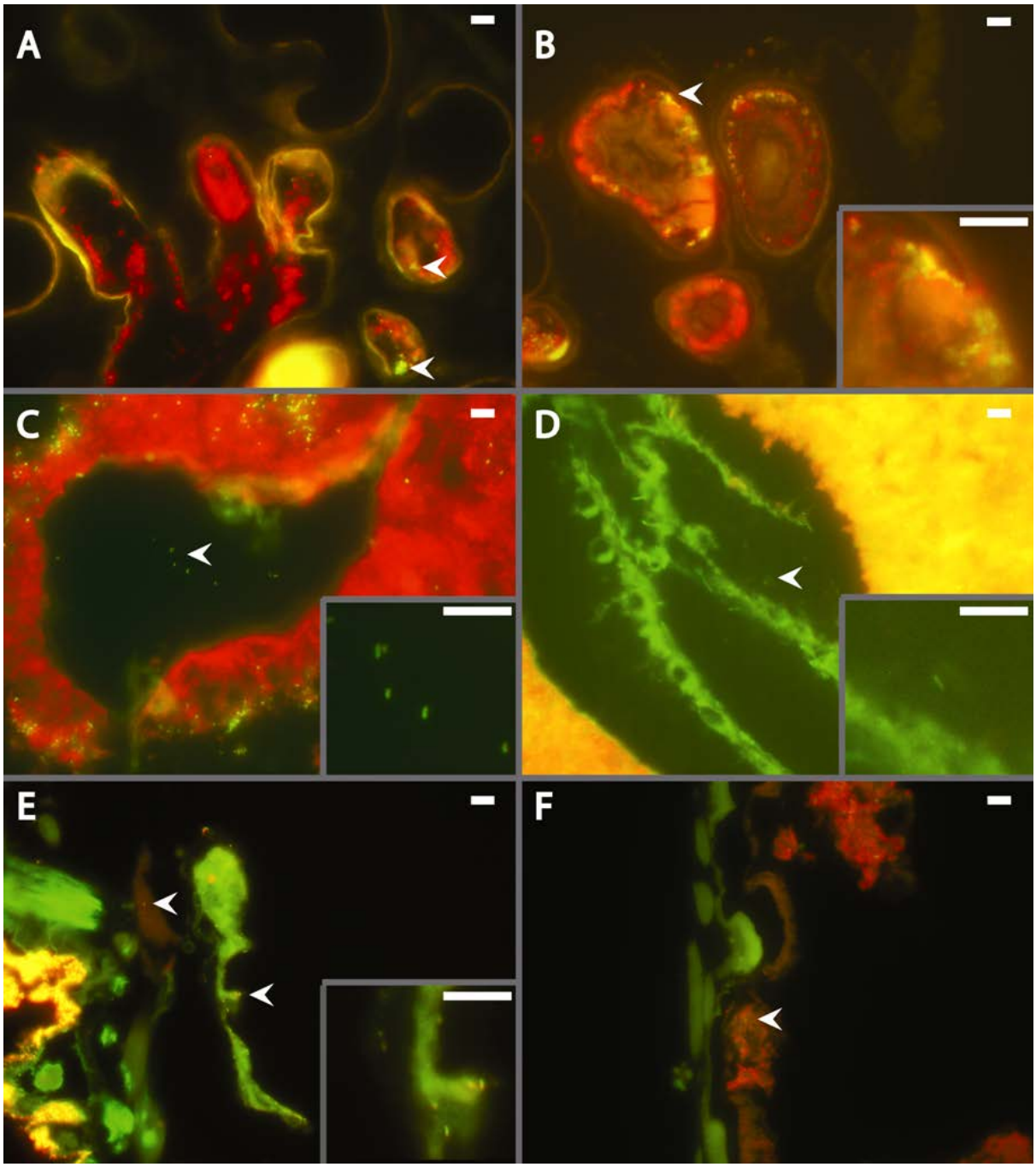

Figure 5. Fluorescent *in situ* hybridization of bacterial cells present in hindgut pockets and wall of a L3 larva instar, and hindgut lobe and wall-epithelium of a female. (A) and (B) Cross section of the hindgut pocket of a L3

instar larva displaying the foci of cells hybridized to GAM42a (identifies γ-proteobacteria) and BET42a (identifies β-proteobacteria), respectively. (C) and (D) Foci of bacteria cells hybridized with probes as in A and B at the inner of the hindgut lobe of a female. (E) and (F) Foci of bacteria cells hybridized as in A with GAM42a to hindgut wall of a female and L3 instar larva respectively. White arrow heads point the bacteria cells. Scale bar 10 µm.

Bacterial taxonomic survey of whole hindgut, hindgut pockets and eggs pyrosequencing. To further identify the bacterial species of the hindgut pockets and the lobe epithelium, pyrosequencing of the pockets was performed. Furthermore, to establish if some of the bacterial community members are vertically transmitted from the mother to the eggs, hindgut of larvae and beetles as well as eggs were processed (Fig. 6 and 7A). The samples included were: hindgut of L2 larvae, female, soil laid eggs and the hindgut pockets. In the final output, a total of 85,233 high quality reads (hqr) were obtained. Individual sampled tissue number of sequences is shown in Table 1.

The results suggested the following bacterial classes as the larger components of the bacterial community in the hindgut of the larvae: Bacteroidia (37%), Clostridia (31%), and a large amount (24%) of unknown bacteria (Fig. 6). For beetles and eggs, same bacteria taxonomical classes with different abundance were obtained. In the beetles, the largest represented class was Bacteriodia (65%), followed by Clostridia (9.7%) (Fig. 6). In most of the cases, the classification remained at the family level only. Estimation of alpha-diversity in these samples was done using rarefaction methods (Fig. S4). Additionally, richness and diversity indexes were also prepared (Table 1). After the rarefaction analysis, saturation was reached for the hindgut of larvae and beetle as well for the pockets samples only. An insufficient amount of reads did not allow reaching saturation for the egg sample (Fig. S5). Despite the large number of Operational Taxonomical Units (OTUs), many still remain to be described (Table 1). The large amount of unidentified OTUs as predicted by the Chao1 richness estimator may be generated for identification of many bacterial species in contaminants carried along with the egg since it is laid in soil. For hindgut of beetle and larvae, the number of OTUs observed was very close to the estimation. This must be is interpreted as saturation too. On the other hand, diversity values as estimated with Shannon

and Simpson indexes pointed the following decreasing diversity order in the samples: hindgut of beetle<egg<and <hindgut of larvae.

By means of pyrosequencing of the bacterial community present in the hindgut pockets the identity of the bacteria present was confirmed. As observed in Figure 6B, the main taxonomical bacterial classes were Actinobacteria and α-, β-, and γ-proteobacteria. This coincides with our FISH results. Moreover, *Pseudomonas* spp. is the species with the highest frequency (>65% of total of sequences). When comparing the abundance of this species in the whole hindgut of larvae and beetles, the group was less frequent (Fig.7A). Surprisingly, this is not the case for the egg sample, where the same genera as found in the egg (i.e. *Achromobacter* sp. -Alcaligenaceae, and *Pseudomonas* spp.-Pseudomonaceae) are abundantly present. This may suggest that key physiological species playing important functions in the insect physiology are vertically inherited. Indeed, when hybridizing recently laid eggs with the EUB-338 general probe, a fine layer of bacterial cells on its surface is observed (Fig. S5). As previously mentioned the anatomical appearance of the bacterial cells in the pockets and the other novel niches are high. In order to confirm that the bacterial cells in all mentioned spaces are the same, further confirmation is required. In order to achieve this, Gold-FISH employing specifically designed probes out of the pyrosequencing results is planned. In order to get a better resolution, visualization of the hybridized cells is planned using TEM.

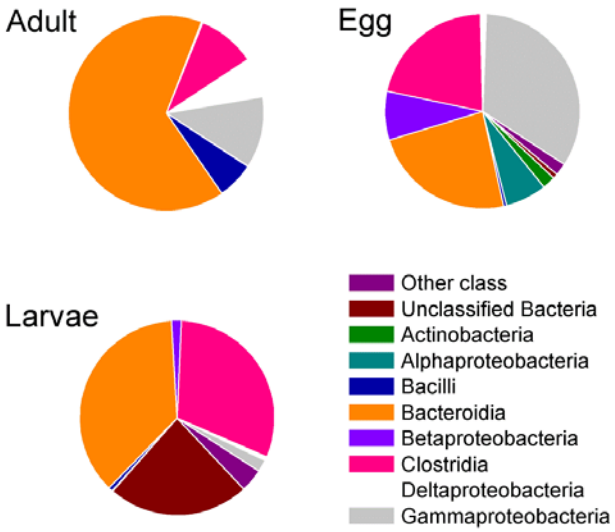

Figure 6. Relative abundance of the main bacterial taxonomical classes associated with hindgut of L2 larvae and female and egg of *Melolontha hippocastani*, determined by 454-pyrosequencing. Other class: Erysipelotrichi, Flavobacteria, Opitutae and Sphingobacteria.

Table 1 Richness and diversity indices calculated at the OTUs level from the pyrosequencing data of samples of hindgut and eggs of *Melolontha hippocastani*.

Sample	**Total of high quality reads**	**OTUs**	**Richness index± S.D.**	**Diversity indexes ± S.D.**	
			Chao1	**Shannon**	**Simpson**
Egg	8 494	287±0.00	609.57±0.00	4.15±0.00	0.88±0.00
Beetle-hindgut	16 016	74±2.65	105.67±22.90	3.06±0.02	0.71±0.00
L2-hindgut	85 233	572±9.43	705.91±28.57	6.52±0.03	0.96±0.00

S.D. standard deviation. Simpson expressed as 1-D, the bigger the number the greater diversity.

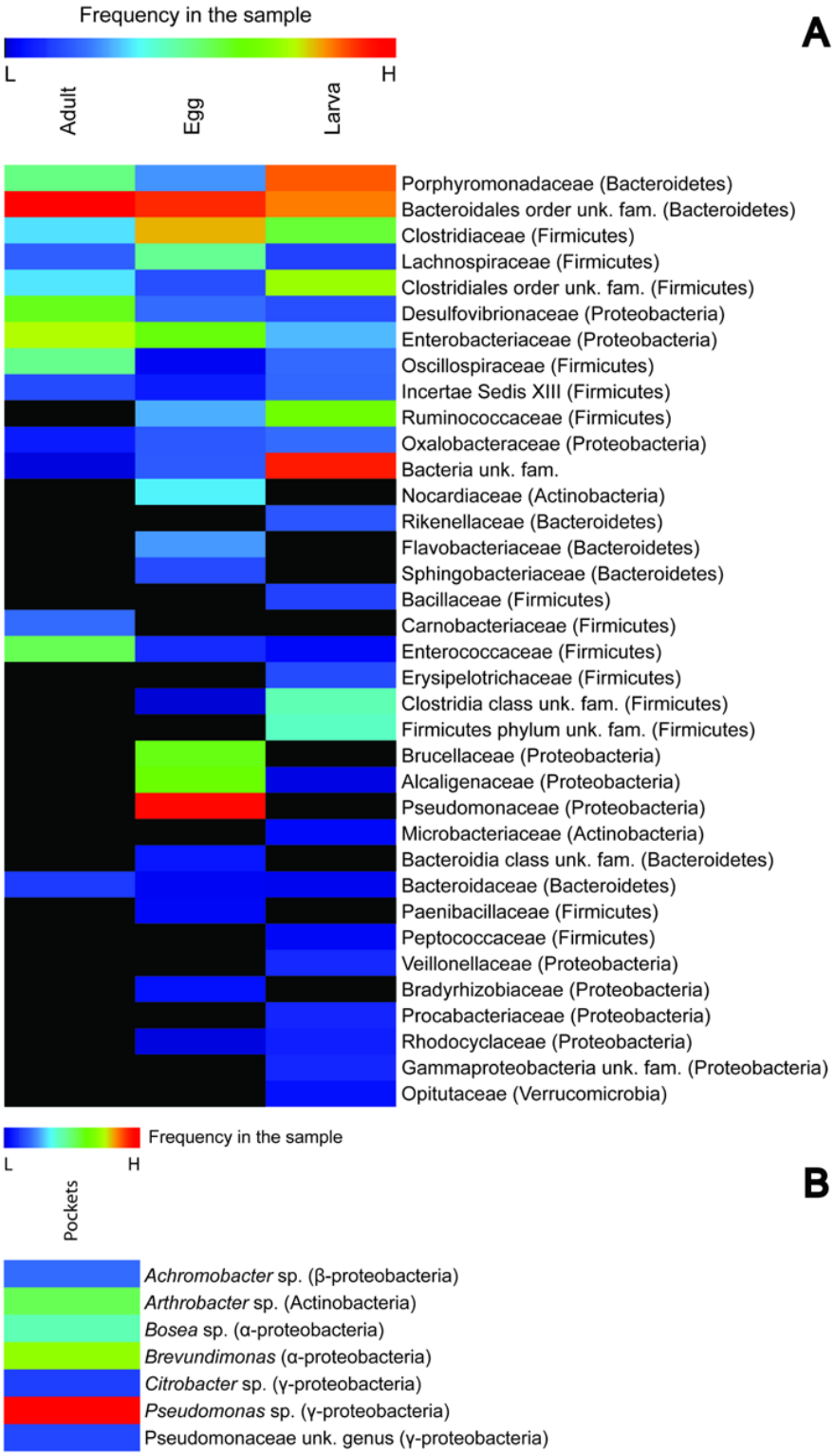

Figure 7. Gut bacterial community composition of gut sections of *Melolontha hippocastani.* (A) Frequency of bacterial taxa from 454 pyrosequencing data (109, 743 sequences in total) displayed as map based on the log-transformed values indicating low (L) and high (H) abundance values with cold and warm colours. Processed samples: egg, hindgut of L3 larvae and female. (B) Same variable as in (A) but displaying the bacterial community of the hindgut pocket of a L3 instar larva (3, 723 sequences).

Summary and preliminary conclusions

Scanning Electron Microscopy (SEM) permitted the visualization of bacterial distribution on top of the gut tree-like-structures (lobes) lining the hindgut. Transmission electron microscopy (TEM) also revealed the novel niches for bacterial accumulation in hindgut. They exist novel places in the hindgut where bacterial cells were identified for the first time: tree-like-structures or lobes, the intercellular spaces of the gut wall epithelium and structures in the fermentation chamber of hindgut - called pockets which are present only in the larvae. The bacterial morphological appearance suggested the presence of 1-2 different cell types, all gram negative in these specialized niches.

Using Fluorescence in situ hybridization (FISH) with taxonomical hierarchical probes, the bacterial cells located in novel tissue spaces were defined as β- and γ- proteobacteria. To narrow down the bacterial identification to the genus or species level, 454-pyrosequencing was employed. The samples used for the purpose were the hindgut of L3 larvae (85,233 high quality reads-hqr), hindgut of beetle female (16,016 hqr), soil laid eggs (8,494 hqr) and the L3 larvae hindgut pockets (3,723 hqr). The bacterial community of the larval hindgut was mainly composed of Bacteroidia (37%), Clostridia (31%), and a large amount (24%) of unknown bacteria. For beetles and eggs the same bacteria taxonomical classes were present. In the beetles, the largest represented group was Bacteriodia (65%), followed by Clostridia (9.7%). In the hindgut pockets, only few groups of bacteria were indentified. A 65% of the sequences corresponded to OTUs identified as *Pseudomonas* spp., and a γ-proteobacteria belonging genus. This is in line with the results previously obtained with FISH. As reported for many other insects, α- and β- proteobacteria have been identified in many other insects species as endosymbionts or specific symbionts. Considering the presence of the here identified species in novel niches of the hindgut, suggest them as more than simple members of the gut surface community or pathogens. To finally elucidate the function they serve to the insect physiology further research must be conducted.

Material and methods

Sample collection and DNA extraction. Second- and third-instar larvae (L2 and L3) of *M. hippocastani* and actively flying adults were collected in forests of red oak in Mannheim (49°29′20″N 8°28′9″E), and Graben-Neudorf (49° 9' 55" N 8° 29' 21" E) respectively, Germany, in December 2010 and May 2012. Eggs recently laid where collected at the same site as the beetles. The insects were transported alive in boxes with soil or tree leaves. Once in the laboratory, before dissection, the insects were kept at 0°C for an hour to kill them and then rinsed alternately with water and 70% ethanol, 3 times. Dissection was performed in a phosphate-buffered saline (PBS) solution. The guts from the larvae and adults were sectioned and used as required. If processing the hindgut of larva or adult, the enlargement as shown between dotted lines in Fig. 1D, top for larva and bottom for beetle, was processed. The pockets were also excised from the hindgut enlargement and the surrounding epithelium was removed as much as possible. Samples were stored at -20°C before DNA extraction. Frozen samples were thawed on ice and dried at 45°C for 90 min in a Speedvac (Concentrator 5301, Eppendorf). Dried samples were crushed in a 1.5 ml tube with a sterile plastic pestle. The DNA extraction of the tissue was carried out using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the protocol provided by the manufacturer. Single insect gut DNA was extracted. A sample for sequencing was composed by the pooled DNA of a total of six insects. In the case of eggs, a total of five composed a sample. Final DNA concentration was determined using a Nanovue device (GE Healthcare, UK).

In order to test for the quality of the extracted DNA and confirm the presence of DNA from bacteria, a diagnostic PCR reaction as described in Arias-Cordero et al. [48] was carried out.

Bacterial tag-encoded FLX amplicon pyroseqencing (bTEFAP) and data analysis

DNA was sent to an external service provider (Research & Testing Laboratories, Lubbock, USA) for bTEFAP with 16S rRNA primers Gray28F (5'-GAGTTTGATCNTGGCTCA-3') and Gray519R (5'-GTNTTACNGCGGCKGCTG -3') [70,71]. A sequencing library was generated

through one-step PCR with 30 cycles, using a mixture of HotStar and HotStar HiFidelity *Taq* polymerases (Qiagen). Sequencing extended from Gray28F, using a Roche 454 FLX instrument with Titanium reagents and procedures at Research and Testing Laboratory (RTL, Lubbock, TX, USA, http://www.medicalbiofilm.org/). Quality control and analysis of 454 reads, including calculation of rarefaction curves and community richness and diversity indexes was done in QIIME [72]. Low-quality ends of the sequences were trimmed with a sliding window size of 50 and an average quality cut-off of 25. Subsequently, all low quality reads (quality cut-off = 25) and sequences <200 bp were removed, and the remaining reads were denoised using the “denoiser” algorithm as implemented in QIIME [73]. Denoised high-quality reads were clustered into operational taxonomic units (OTUs) using a multiple OTU picking strategy with cdhit [74] and uclust [75], with 97% similarity cut-offs, respectively. For each OTU, the most abundant sequence was chosen as representative sequence and aligned to the Greengenes core set (available from http://greengenes.lbl.gov/) using PyNast [72]. RDP classifier was used for taxonomy assignment [76]. An OTU table was generated describing the occurrence of bacterial phylotypes within the samples.

Fluorescence in situ hybridization (FISH). L3 larvae as well as female beetles were dissected as mentioned. The gut tissues were fixed in 4% of paraformaldehyde in phosphate-buffered saline (PBS) overnight. Afterwards, the tissue was 3 times rinsed with PBS and finally dehydrated for one hour in acetone. In the case of eggs, they were superficially washed in PBS tree times. To quench the strong auto fluorescence of the tissue, the eggs were left overnight in a solution 3-6% H_2O_2 in water. Following, fixation was done in Carnoy's solution for two days. Afterwards the eggs were washed in 80% ethanol two times. At this point further dehydration using 80, 90 and 96% ethanol and acetone solutions for 1 hour each were applied. Next, all samples (egg and gut) were embedded with Technovit 8100 (Heraeus Kulzer GmbH, Wehrheim, Germany) as indicated by the manufacter. Note: in case of eggs the embedding step was prolonged for four days. Sections of 5 µm in thickness were mounted on SuperFrost Ultra Plus glass slides (Thermo Scientific) and treated with 5 mg/ml lysozyme for 15 min at 37°C. After the lysosyme was

washed away with distilled running water for 30 seconds, the slide was dried by blowing it with compressed air. The sections were double-hybridized with 1.5 µM of each specific probe (targeting proteobacteria classes see Table S1) and the control Eubacterial probe EUB 338 [77]. The hybridization was performed as described by Manz et al. [78]. Counterstaining with DAPI at a concentration of 200 nM in PBS was performed for 10 min in dark conditions. Finally this was washed out for 10 min with PBS and in the end the slide was mounted with Citifluor (London,UK). Images were taken with an Axio Imager Z1 microscope (Carl Zeiss) equipped with an AxioCam MRM camera.

Scanning electron microscopy (SEM). Hindguts of larvae were dissected in a chilled solution of 2.5% glutaraldehyde-2% paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.2. Immediately the tissue was transferred to the same solution for overnight fixation. Next day, the fixative was removed and the tissue was further washed three times in sodium cacodylate buffer and dehydrated in rising ethanol concentrations followed by critical point drying using a BAL-TEC CPD 030 Critical Point Dryer (BAL-TEC, Liechtenstein). The dried samples were mounted conductive on aluminum sample holders and gold sputter coated (layer thickness 20 nm) in a BAL-TEC SCD 005 Sputter Coater (BAL-TEC, Liechtenstein). The samples were examined in a Zeiss (LEO) 1450VP scanning electron microscope (Carl Zeiss AG, Oberkochen, Germany) at 10 kV acceleration voltage and a working distance of 12 mm using an Everhard-Thornley secondary electron detector.

Transmission electron microscopy (TEM). Hindguts of larvae and adults as well as pockets of larvae were dissected and fixed as for SEM. Sample embedding and processing was conducted as described by Marquardt et al. [79].

Light microscopy, phalloidin and SYTOX and Nile Blue staining. In all cases the tissue was fixed as for SEM. Light microscopy stained with Richardson's blue solution was prepared prior TEM in order to localize the right position for the examination. The tissues employed were larvae and female hindguts and larvae hindgut pockets. Semi-thin sections of 0.3-0.6 µm (embedded as for TEM) were immersed in a 60°C Richardson staining

solution for 3-5 min. Afterwards the tissue was washed with sterile water two times. Finally, the sections were placed on a glass slide, dried and mounted for microscopic observation. Alexa Fluor 488 nm phalloidin (Phallotoxin, Invitrogen) double stained with SYTOX Orange nucleic acid stain (Invitrogen) was performed in excised complete hindgut pockets that were fixed overnight. For the phalloidin staining the tissue was prepared as described in Rössler et al. [80]. Afterwards a droplet of a 0.1mM of SYTOX in PBS was added to the tissue. After a 10 min incubation time, the sample was placed in a glass slide, covered with PBS and visualized using a LeicaTCS-SP2 confocal microscope using a 10× dry or 40×oil Leica objective (HC PL APO 10×/0.4, Leica, Bensheim, Germany). Laser lines employed were 488 nm and 543 nm. For the Nile Blue staining, same tissue sections as prepared for Fluorescent in situ Hybridization from hindgut pockets were used. For the staining, the protocol as described by Ostle and Holt [68] was applied. Finally, after a sterile water wash, the tissue was blotted and mounted using Citifluor (London,UK). Visualization was done using the same microscope as for FISH.

Supplementary materials

Table S1. FISH probes used

Probe	**Target**	**Sequence (5'-3')**	**Label**	**Reference**
EUB-338	Eubacteria	GCTGCCTCCCGTAGGAGT	Cy3	[77]
BET42a	β-proteobacteria	GCCTTCCCACTTCGTTT	Cy5	[78]
GAM42a	γ-proteobacteria	GCCTTCCCACATCGTTT	Cy5	idem

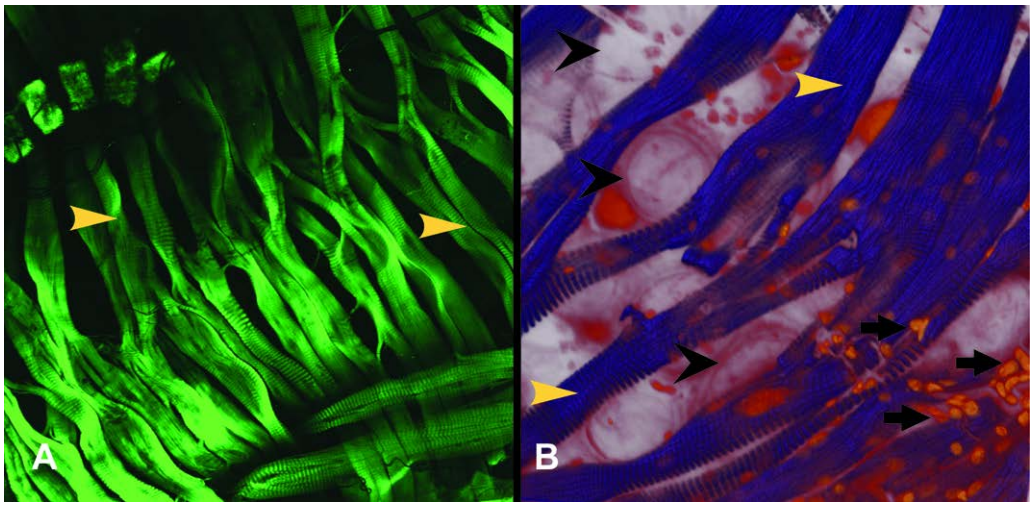

Figure S1. Confocal images of the hindgut pocket tissue of a *Melolontha hippocastani* L2 larva. (A) Staining of the pocket tissue with Alexa Fluor 488 nm phalloidin stain (Phallotoxin, Invitrogen). (B) Double staining with Alexa Fluor 488 nm phalloidin stain and SYTOX Orange nucleic acid stain (Invitrogen), overlaid image. Yellow arrow heads point the muscle fibers that cover the pocket poles; black arrow heads indicate the position of the spheres at the distal point of the poles composing the pocket; black arrows, point the tracheoles that cover the pocket tissue, note the nuclei of these cells.

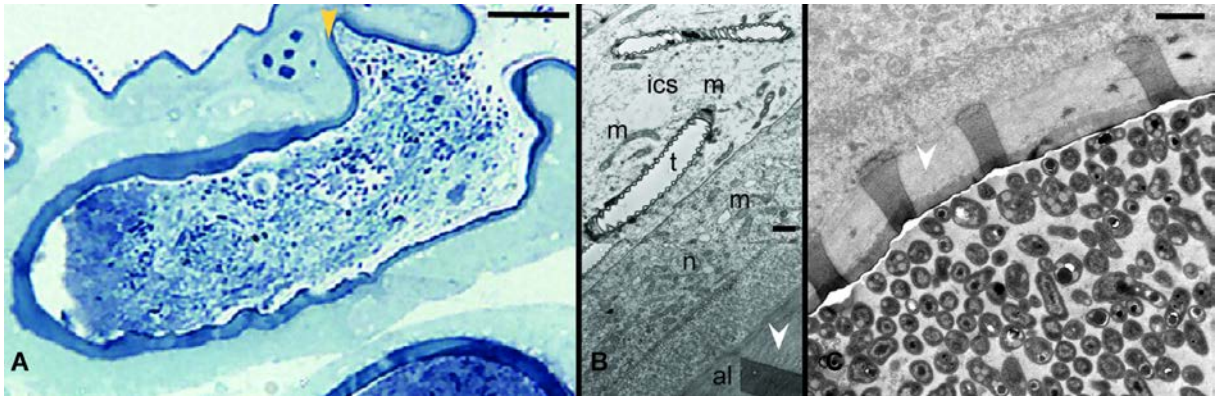

Figure S2. *Melolontha hippocastani* L2 larva hindgut pocket microscopical detail. (A) Cross section of a L2 larva hindgut pocket stained with Richards's solution. (B) Transmission Electron Microscopic (TEM) image of the layers of tissue surrounding the poles of the pocket. (C) TEM image of the layers of tissue surrounding the pole of the pocket and its content, bacterial cells lining the centre of the pocket pole. Yellow arrow head displays the point where TEM images were done; white arrow heads pointing the a-cellular, mucous like layer enveloping the poles and blocking the staining. m mitochondria, t tracheole, n nucleous, ics intercellular space, al a-cellular layer. Scales bar A, 100 µm; B, 0.5 µm; C, 1µm.

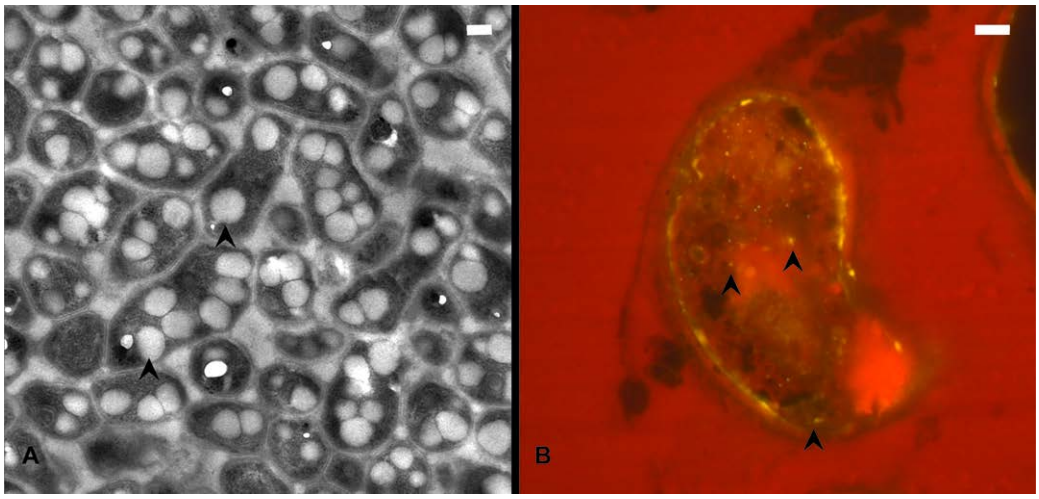

Figure S3. *Melolontha hippocastani* L2 larva hindgut pocket displaying the content bacterial cells and particular features. (A) Transmission Electron microscopy (TEM) image, of the dense bacterial population in the center of the pocket tissue, black arrow heads point to the inclusion granules observed in the cell cytoplasm. (B) Epifluorescent image with a 543 nm microscope filter of a pole of a hindgut pocket, after stained with Nile Blue dye, binding to poly-ß-hydroxybutyrate (PHB), the yellow fluorescent points examples of positive staining, shown with black arrow heads. Scale bar A, 0.2 µm and B, 10µm.

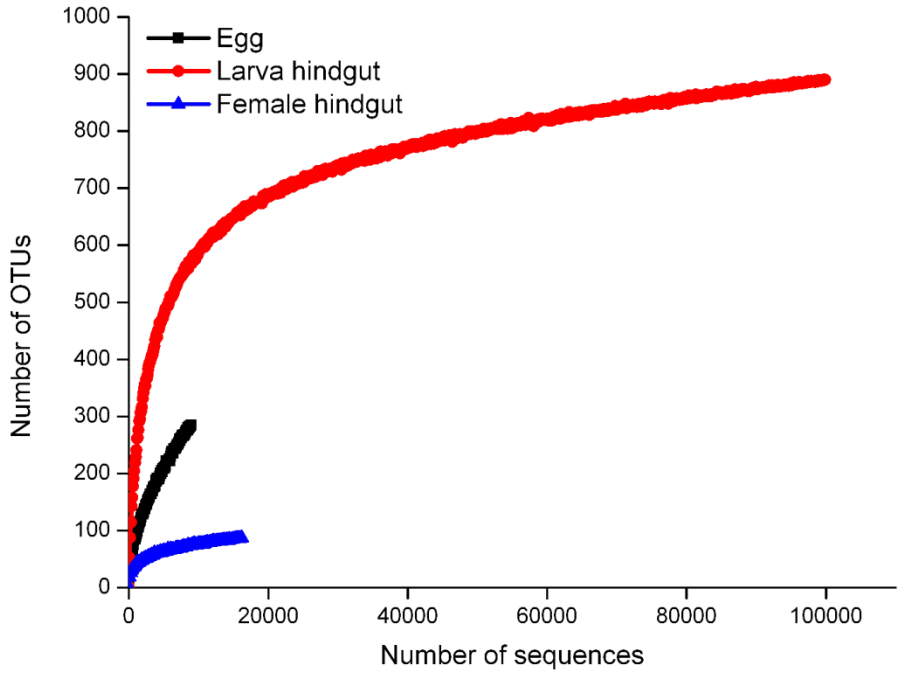

Figure S4. Rarefaction curve of the 454-pyro sequencing performed in the egg, hindgut of larva and hindgut of female beetles of *Melolontha hippocastani.*

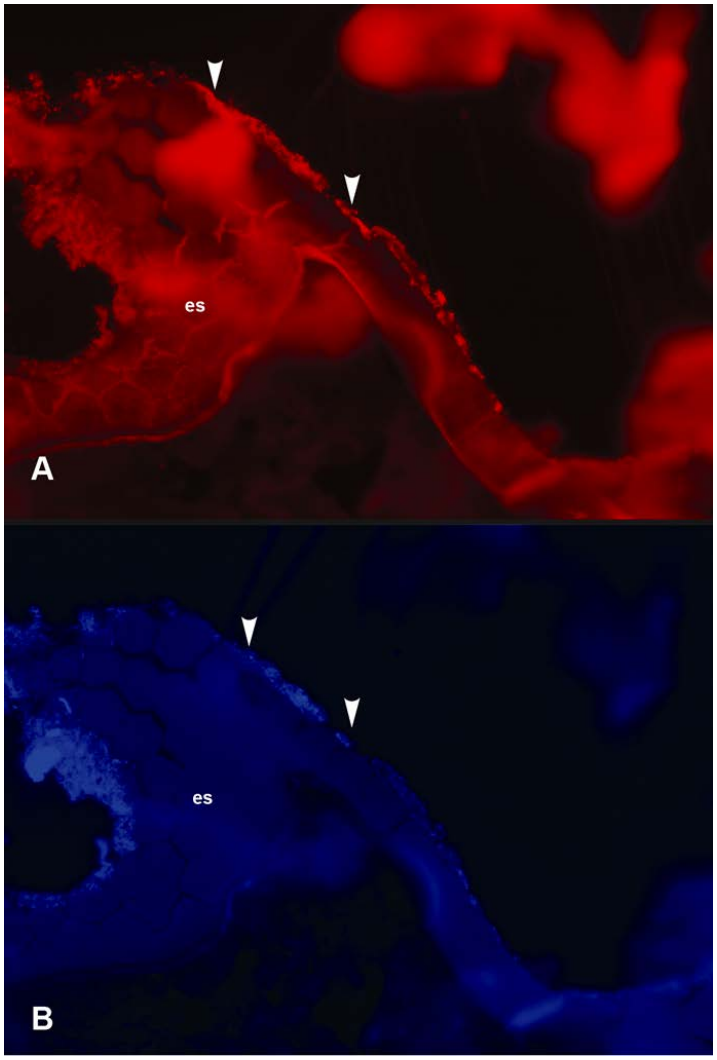

Figure S5. Fluorescent *in situ* hybridization of bacterial cells deposited on the shell of an egg laid in soil. (A) Cross section of an egg laid in soil, bacteria loci hybridized using a EUB-338 universal probe (B) Same as (A) but stained with the general DNA staining, DAPI as control. White arrow heads pointing the some of the bacterial cells on the egg shell surface. eg egg shell. Scale bar 10 µm.

4. General discussion

The thesis deals with identification and characterization of the symbiotic relationships established between insects and their gut microorganisms in nature and their role in plant-insect interactions. After analyzing the composition of the bacterial community associated with the insect guts of two species, namely a leaf feeder generalist insect, *Spodoptera littoralis*, and a specialist, leaf and root feeder, *Melolontha hippocastani*, thriving in different diets and environments the following findings could be summarized. Firstly, from the survey of the gut bacterial community of *S. littoralis*, a simple composition in terms of species richness and diversity became obvious (Article I and II). Secondly, the bacterial community composition varies with food; i.e. different plant species (Article I). Finally, despite of the changes generated with the food, a core group of bacterial species prevails. Additionally, we were able to determine that certain bacterial species belonging to the core group are metabolically active (Article II). Contrary to this, different results were obtained when looking at the gut bacterial community of the specialist species *M. hippocastani* as compared to the generalist, *S. littoralis*. Firstly, the bacterial community is highly complex and different. Secondly, the presence of certain bacterial species with particular characteristics; i.e. xylanase or celullase producers, suggest them as indispensible for insect food digestion. Two clear bacterial communities were defined in the two major sections of the gut - midgut and hindgut (Article III and Unpublished results). Furthermore, after surveying the bacterial community associated with the diapausing, unfed, recently molted beetle, a core community was identified (Article III). Finally, upon a detailed microscopic analysis of diverse tissues of the *M. hippocastani* hindgut, bacterial-insect symbionts localized to certain specialized novel organs was discovered. Moreover, the identity of the members of this community was established (Unpublished results). The results are discussed in detail below.

4.1 Gut bacterial community of a generalist insect, *Spodoptera littoralis*: composition and metabolically active players

As mentioned in the introduction, the bacterial community associated with the anatomically primitive gut of lepidopteran insects is generally simple [53,55]. In the present research using a leaf feeder, *Spodoptera littoralis*, this was confirmed (Article I). In terms of Operational Taxonomic Units (OTUs), the gut bacterial community was composed of only 18 representatives. They were located mainly at the gut epithelium or associated with the food in the gut lumen. Furthermore, only a limited number of bacterial species were observed. Additionally, not all 18 reported OTUs were simultaneously present in all larval instars. Different combinations of them generated particularly different bacterial assemblages across the six caterpillar instars studied. All of them were fed and reared under the same artificial diet and environmental conditions. Considering this, the different gut bacterial assemblages reflect in part the effect of a set of insect physiological related factors mediating the process. Furthermore, microbe-microbe interactions and in addition resource competition are important determinants of intestinal niches too. An example of this is the interspecies cooperation that occurs during the hydrolysis of complex carbohydrates and sequential fermentation of the resulting sugars, i.e. Bifidobacteria a prominent mucin degrader in the human colon, is able to obtain this substrate only after Bacteroides have released it from complex food materials [81].

Lepidoptera larvae eat huge amounts of plant tissue in order to compensate for their low protein content in food and fulfill its nutritional requirements [82]. During *S. littoralis* digestion, only little gut assimilation of the complex polysaccharides (celluloses and hemicelluloses) present in plant cell walls might occur, as the gut food passage is a very fast process. In order to avoid shedding out with the feces during the rapid food throughput, gut bacterial associated species must have short generation times. Under optimal growth conditions, *Enterococcus faecalis*, one of the *S. littoralis* gut core community members has a doubling time of 48 min [83]. This is an ideal case for gut establishment, since at least 4 generations would be generated before the gut food passage is completed. Despite this, it is important to consider the actual generation time of *Enterococcus* spp. under the gut physiochemical conditions. In case that the doubling time would be longer, a greater

probability for the cells to easily detach is possible. Another way for the bacteria to establish in the gut might be via bacteria biofilm formation. As observed by Shao (personal communication), thick biofilms of *Enteroccocus* spp. are recognized on the gut epithelium. Their presence may contribute to a better bacterial anchorage on the gut surface that prevents drastic drops of the number of the cells.

As a consequence of the *S. littoralis* gut physiochemical characteristics, and its feeding habits, a very particular bacterial community associates with its gut. As mentioned in the introduction, the high pH of the Lepidopteran gut, might be a strategy to enhance the solubilization of dietary proteins in the digestive tract [84]. In view of the vast amount of secondary metabolites in the diverse variety of plants consumed by these generalist insects, only those bacterial species able to deal with them, will persist in the insect gut. This is in line with our data (Article I), where dramatic changes in bacterial composition occurred as consequence of the change on the plant species changes on which the insect fed. An already known example is *H. armigera,* where changes in the fed plant species i.e. chickpea, cotton, sunflower and tomato among others [85], generated variation in the gut bacterial community associated. Furthermore, the bacterial community represented a subset of that found on the leaf surface (phyloplane). Despite this, our data points to a core bacterial community that prevails in the gut (*Enterococcus, Clostridium* and *Pantoeae*) and is independent of the food source (Article I and II). It has been reported that variation in the ingested food also changes proteinase expression in order to compensate for variable protein quality and/or quantity in host plants [86,87]. These events may also regulate and modulate the associated bacterial species. Changes in the profile of the digestive enzymes is also a mechanism used by the insects to cope up with the effects of insecticidal proteins of plant origin [82]. Furthermore the differential gut enzymatic expression patterns along the life cycle of insect, and the continuous regeneration of midgut cells during the insect development, may also explain some of the changes in the composition of the gut biota (Article I). Finally, an important factor involved in the change of the bacterial composition from the younger to the older larvae instars, is related to the increase in the size of the

midgut. As the insect grows, the surface area of their epithelia increases steadily [88] and this increases the space for bacterial cells. On the other hand, the oxygen regime changes, and is principally reduced due to the greater volume and position in the gut tube. From foregut to midgut there is a change of 99 to 0 mm Hg of O_2 by the time the food has progressed 1-2 mm beyond the mandible in the gut of *Helicoverpa zea* [33]. Thereby significant differences in the metabolism of the bacterial species colonizing this space are observed. Selectively more anaerobic species are favored and thus established. An example is the abundance of species as the alkaline tolerant *Clostridium* sp. which is dominantly observed in the midgut and whose abundance increases from the 4th larval instar onwards.

Although further investigation is required, one could speculate that some of the metabolically active bacteria of the core community of *S. littoralis* (i.e. *E. mundtii* and *casseliflavus*) (Article I) might be involved in detoxification of compounds consumed with the food. Besides, they might also participate in the production of anti-microbial peptides and other defensives (*P. agglomerans*), and to a small extent in the degradation of food recalcitrant components (*Clostridium* sp.). An example of food detoxification is given by Vanhaecke et al. [89]. They found that *Enterococcus* related species participate in the detoxification of a heterocyclic aromatic amine, PhIP a potent carcinogenic in humans. The bacteria are able of converting the PhIP-M1 derived compound ingested with cooked meat, into its intermediate microbial metabolite, having lesser mutagenic and carcinogenic characteristics than its precursor. In addition, Shao et al. [90] demonstrated the presence of large amounts of *Enterococcus* bacteria associated with ß-carotene crystals formed in the foregut of *S. littoralis* fed with Lima bean, a cyanogenic plant. This permits by an unknown mechanism the insect survival on this toxic diet. On the other hand, it seems that *Enterococcus* is also involved in host protection as it suppresses the growth of pathogens ingested with food in *Galleria mellonella* [91]. Same function serves *Pantoea agglomerans* for its host, *Schitocerca gregaria*. In this case, *P. agglomerans* released phenolic compounds into the insect gut fluid that prevented the growth of the entomopathogen *Metharrizium anisopliae* [37]. As already mentioned, in the case of *S. littoralis*; *Enterococcus*

spp. seems to form biofilms in the insect gut, preventing niche colonization by other species (Shao, personal communication). In *Manduca sexta, Enterococcus* spp. has been found to be metabolically active in eggs and larvae, likely protecting both stages from opportunistic bacteria and fungi. In line with these results, we also found *Enterococcus* spp. as members of the metabolically active core community associated with the gut of *S. littoralis* (Article II). However, none of the mentioned putative roles of the bacterial species composing the gut core bacteria community of *S. littoralis* are confirmed. However, considering the increase in abundance of *Enterococcus* spp. and *Pantoea* spp. (Fig. 2 of Article II) in the ^{13}C-enriched DNA as compared to the control, they appear as very active players in the gut bacterial community. On the other hand, our speculation of little participation of *Clostridium* spp. in the digestion of plant cell-wall components requires further confirmation. Interestingly, representatives of the same genus seem to be the dominant taxa present in the very alkaline P1 compartment of the gut of higher termites [92], likely contributing to the initial food processing. In order to investigate the participation of *Clostridium* sp. in food processing, feeding of the insects with ^{13}C-celullose or any other plant cell wall component assimilated by the bacteria is required. Despite this, looking at the decrease in the abundance of this bacterial species in the ^{13}C-enriched DNA as compared to the unlabeled DNA after the ^{13}C-glucose treatment, and to its reduced abundance in relation to the other metabolically active species, *Clostridium* spp. seems to be less active. This may suggests a more transient habit, that could relate to its niche in the gut, the aggregates of food in lumen (Article I), which are frequently voided with the feces.

In the past, the only possibility to elucidate bacterial metabolism has been through their cultivation. Since less than 1% of microbes are cultivable, molecular technologies have been used in order to study bacterial communities. The main method used is the phylogenetical classification based on the 16S rRNA gene [93]. Although the application of phylogenetic studies permits description of the relationships among microorganisms, it provides little information about the microbe function in the environment. In our case, a solution to this problem is the use of the Stable Isotope Probing (SIP) methodology described in Article II.

By enriching the DNA of the bacterial species able to metabolize the ^{13}C-enriched substrates, its separation and subsequent identification is simple. In our case, as already mentioned, its implementation permitted the identification of the metabolically active bacteria in the gut of *S. littoralis* successfully. The methodology has been widely applied to study environmental processes [94], but its use *in vivo* particularly in arthropods, has been limited to incubation of dissected guts of earth worms only [95]. The application of this method *in vivo* as in Article II, has began our way to fully understand the role that the members of the *S. littoralis* gut bacterial community are playing. Up to now the methodology was applied using ^{13}C-glucose a 'universal' substrate only. However its application can be extended to the use of other labeled compounds in order to identify the specific organism involved [96].

4.2 Gut bacterial community composition of a specialist insect, i.e. *Melolontha hippocastani*

Melolontha hippocastani is a specialist insect that feeds on both leaves and roots of woody trees. The larvae feed on root tissues and the beetles on leaves. As mentioned in the introduction, the bacterial community present in the gut of scarabeidae larvae, in this case *M. hippocastani*, is highly diverse and our data confirmed this. Many OTUs were described for the unstructured bacterial community of the midgut (Article III), but far more for the hindgut. Indeed, the hindgut section displays a much better structured rich and diverse bacterial community as compared to the midgut (Article III and Unpublished results, Table 1). However, the comparison of both datasets must be viewed with caution, since they were generated by different sequencing methodologies having different resolution capabilities (Article III: Sanger, Unpublished results: 454-pyrosequencing). Furthermore, it is important to also be aware of the limitation of the comparison established between taxonomical groups in a sample and between samples, when using 454-pysosequencing abundance results [97]. Yet, the known bias generated while employing a PCR based method [98], added to that introduced while 454-sequencing [99] make the comparison improper. The only possible use of abundance in order to compare samples, is when comparing the same taxa i.e. OTU

across samples [99]. This underlines the importance of data corroboration applying other techniques as FISH (Article I, III and Unpublished results). Returning to our analysis, the gut bacterial community of the beetle remains simpler than in the larva, independently of the sequencing methodology and if processing the whole gut or only the hindgut. The change in complexity in the gut community of the beetle as compared to the larva, is a clear consequence of the change in the insect feeding habits. Plant cell walls consist of cellulose fibrils embedded in a matrix of hemicelluloses (xylan, mannan, xyloglucan and β-glucan) and pectin, with lignin also present in secondary walls [100]. In order to digest all these components, the insect must possess specific enzymes. Particularly cellulases and hemicellulases are not endogenously produced in the gut of *M. hippocastani* (see discussion of Article III). Thus, the insect relies on the set of enzymes that its gut symbionts could supply. As mentioned in the discussion of Article III, the roots on which the larvae feed may consist of up to 50% of cellulose and a large amount of lignocellulose as well as associated humic materials. Less is the content of those materials in tree foliage on which beetles feed. The beetles food (leaf) which is nutritionally richer than that of the larvae (root), requires less participation of microorganisms in order to be digested [42]. This is evident in the reduction of complexity of the gut microbial community associated with the beetle already directly after metamorphosis (Article III). During metamorphosis, a strong selection of the bacterial community carried from the larva is applied. The effect of reconstruction and anatomical transformation of the insect gut during molting and subsequently metamorphosis, affects severely the identity of the final bacterial community able to settle down in this environment [24]. These fascinating events suggest the evolution of insect strategies favoring transmission of selected beneficial/symbiotic gut bacteria, important for the larvae physiology, to successive generations. A process that requires to be unraveled. Along with all these gut anatomical and morphological changes after metamorphosis, modification of physiochemical characteristics innate to the larval gut might occur. One of these modifications may involve that certain gut micro-environments are abolished. The reduced

abundance of certain bacterial species in some beetle gut sections as compared to larvae while performing FISH confirms this (Article III).

Along with the effect of the change of food, the gut bacterial community of the beetle may be reduced as a consequence of the continuous exposure to phenolic compounds i.e. host leaves tannins (from i.e. oak and beech). Generally, phenolics are reported to severely damage insect gut cells [101] and also display strong antimicrobial properties [37,102,103]. Among them, tannins are the most abundant secondary metabolites present in plants and are found in particularly high concentrations in woody species [101]. In case of various *Quercus* species (preferred host of *M. hippocastani*), tannins reach values of up to 55 mg g^{-1} of dry weight (DW) in older leaves. Despite an ubiquitous distribution of phenolics in plant leaves [104], their concentration in roots is lower [105]. This permits less larvae fitness problems when compared to adult beetles. In the beetles, it seems that selection of bacterial species able to tolerate the high amount of phenolics have occurred in order to permit the insect to cope with their negative effects. The gut bacterial communities represent a source of novel genes that permit the insect to overcome the rigors of the environment and persist. An example in snails, is the variety of microbial genes that could assist the host in processes as protection against oxidative and osmotic stress or heat shock among others [106].

There are clear differences in the composition of bacterial communities associated with the beetle and larval gut of *M. hippocastani*. Many of the taxa identified in the larval gut, are not present in the beetle gut. The amount of OTUs present in certain families i.e. Erysipelotrichiaceae, Clostridiaceae and Desulfovibrionaceae are significantly reduced in the beetle gut when compared to the larval midgut. In these groups not only number of OTUs, but also abundance was reduced. Same trend was also observed for some of the taxa found in the hindgut. Contrary to this, the frequency of γ-proteobacteria was greater in adult beetles than in larvae. Furthermore, this bacterial class is very much prominent in the hindgut of beetles (Unpublished results). The beetle gut is thin and lengthy (it reaches 2 times the insect body size) [107] and thus results in greater availability of oxygen favoring

the colonization of aerobes or facultative aerobes i.e. Enterobacteriaceae and Pseudomononaceae.

Beyond the mentioned differences between the midgut bacterial communities of the insect larval and adult stages (Article III), greater differences in their hindgut communities were observed (Unpublished results). The predominant taxonomical classes in the larval hindgut were Bacteroidia, Clostridia, and unclassified bacteria. For the adult beetle, Bacteroidia, γ-proteobacteria, Clostridia and Actinobacteria were predominant. All of them are typical intestine associated bacteria, found either in vertebrates i.e. humans [108] or invertebrates i.e. termites [109] and are common members of fermentative environments [15]. In such typical intestine fermentative environments, i.e. in termites, the task of the gut microbiota is to depolymerize complex food materials like cellulose and hemicelluloses into monomers. Furthermore, the bacteria or gut protist (if present), ferment the monomeric carbohydrates anaerobically, producing acetate (and other short chain fatty acids), H_2, and CO_2. Finally, the acetate is absorbed by the termite host as the main energy and carbon source [110].

As observed for the midgut community (Article III), many taxa present in the larval hindgut, were not present in adult beetles. These dynamics may reflect changes between the distinct community members. These changes can be inferred when looking at the variable composition in short-fatty acids and other fermentation products of the hindgut fluid. [111]. One of the reasons for the changes in the composition of the fermentation products, seems to be the different availability of certain materials, i.e. complex polysaccharides content in the food. Unfortunately, due to the complexity of interactions within the bacterial community is difficult to unravel the action of each of its members [112]. The absence of groups like Actinobacteria and β-proteobacteria in the leaf feeding beetle gut, may be due the changes in the insect habitat. Both bacterial taxa are natural soil inhabitants. Moreover, their presence might be influenced by the reduction in leaf hemicellulosic content (particularly xylan) as compared to roots [113]. The high number of Clostridiaceae and

Ruminococacceae OTUs in the root feeding larvae, hints at their role in the initial breaking down of cellulose. Both bacterial families, represent anaerobes that utilize fermentable carbohydrates to grow [114]. *Ruminococcus flavefaciens*, is among the main cellulolytic rumen active species able to solubilize significant amount of plant cell wall components in pure culture [100]. In the human intestine, the Ruminococcaceae family is responsible for key metabolic conversions within the community. They include for example the major butyrate-producing species, as well as species that convert lactate to butyrate or propionate and species that perform reductive acetogenesis [100]. The reductive acetogenesis or homoacetogenesis represents the process by which bacteria can autotrophically synthesize acetate from H_2 and CO_2 [110]. In *M. melolontha* one of the main fermentation abundant products is acetate [28]. This suggests the possibility that many of the bacterial community members of *M. hippocastani* close relative of *M. melolontha* are homoacetogens. Hence, other members could use their final products, i.e. lactate produced by Clostriaceae, for secondary fermentations [112]. This is documented in termites, where *Bacteroides* spp. grows and produces fermentation products based on the lactate produced by *Streptococcus* spp. [115]. If such primary fermenters and their products are no longer available, changes in the frequency of the secondary fermenters are expected. Among such secondary fermenters are many other members of the Bacteroidia class. Thus, this is a likely reason for the high abundance of Bacteroidia in the gut of the beetles. Bacteroides are one of the most abundant bacteria in the gastrointestinal tract of humans [108]. In this vertebrate environment, the bacterial group is able to degrade diverse plant polysaccharides including pectin, galactomannan, arabionogalactan, alginate, laminarin, xylans, xyloglucan, rhamnogalacturonans I and II, β-glucans and glucomannan [100]. Furthermore, some of its members degrade also proteins displaying proteolytic properties [116]. As leaves are richer in amino acid content than roots, this could be another reason for the higher frequency of Bacteroidia in leaf feeding beetles too. Furthermore, the urolytic activity displayed by some of these bacteria, suggests a potential role in nitrogen cycling [42].

Upon surveying egg bacterial community, we found that many of the bacterial groups already present found in the beetle and larval guts were also in the egg. Particularly, those species abundantly found in the hindgut pockets, i.e. OTUs belonging to Pseudomonaceae, Alcaligenaceae and Enterobacteriaceae families. Interestingly, one of the very abundant groups observed in beetle, Bacteroidetes, was abundantly found in the egg too. This suggests a likely vertical transmission of the bacterial symbionts to the successive insect generations, as previously suggested [117].

Finally, new niches for localization of bacterial symbionts in the hindgut were observed. This is novel for scarabs, where bacteria are identified only on the hindgut epithelium and in the lumen [35,118]. The current work, identifies new niches for bacteria in the hindgut: the intercellular space of the hindgut epithelium, the inner surface of the hindgut lobe of both instars and the pockets of the larval hindgut. The pockets are a dense bacteria lined tissue present only in the larvae and whose arrangement and content, resembles midgut crypts in stinky bugs [119]. Due to the enormous amount of bacterial cells contained in the pockets, the structures appear devoted to the bacterial rearing and maintenance. The main bacterial species in the pockets are γ-proteobacteria (main species; *Pseudomonas* spp.) and β-proteobacteria (*Achromobacter* sp.) and they could be important insect symbionts. An interesting feature of both species is their putative role in xylanolytic and celullolytic processes [120,121]. Albeit the relevance of this feature, the confirmation of their participation in such events requires further research.

Looking back at the discussed results it is evident that there is a compelling need for determining the metabolically active bacteria in the *M. hippocastani* gut. Responding to the urgent need of describing the gut microbiome associated with this ecologically successful insect is that the present work was thought. To begin understanding the active gut biota, the methodology described in Article II is quite promising. Once gut metabolically active bacteria is identified, it will be possible to define the main players in the community. Furthermore, by using different isotopically labeled substrates, i.e. cellulose or secondary fermentation

products as lactate, it will be possible to assign the specific metabolism that each of the members of the community possess.

4.3 Comparison of the gut bacterial community of *Spodoptera littoralis* and *Melolontha hippocastani* and general factors affecting them

As already discussed in sections 4.1. and 4.2, the main differences observed in the bacterial communities of *S. littoralis* and *M. hippocastani* are a result of different feeding habits. Actually the anatomy of insect guts have evolved and adapted in order to process their specific food. At least this is a very striking feature in coleopteran thriving on various food sources [122] and in the case of the insects under study it seems to be a determining factor. The proctodeal dilatation of the gut of *M. hippocastani* slows down the food transit and increases the residence time of the digesta [111]. Contrary to this, the long straight simple shaped gut of *S. littoralis* facilitates the rapid passage of huge amounts of food. Consequently, different bacterial communities are established in the insect gut. As mentioned, *S. littoralis* maintains a very high gut pH [123] to get efficient nutrient assimilation from the plant tissue [124]. Same is the case of soil feeding termites which have a strong alkaline compartment (pH 12) in their gut which results in better assimilation of organic matter fraction from soil [125]. Under such conditions only sparse number of bacterial cells are present [110] as for *S. littoralis* (Article I and II). Additionally, when comparing the *S. littoralis* core bacterial species with literature reports of other lepidopteran species, it is clear that such taxa have established multiple symbiotic associations across the order. Contrary to the digestion process in *S. littoralis*, *M. hippocastani* relies on its bacterial symbionts in order to process their food. The enlargement and modification of the hindgut, which present specialized structures to harbor bacteria confirms this. For instance, the intima, the fine cuticle layer covering the hindgut of larvae and beetles display protrusions-lobes (Unpublished results) which give refuge to greater amount of bacteria cells. On top of this structures, bacteria deposit and form multilayered arrangements. Further, the food passage in *M. hippocastani* gut is a slow and lengthy process. This reflects the long life cycle of the

insect and is a common feature among insects relying on microorganisms to help processing their food, eg. termites and cerambycidae beetles. As opposed to this is the length of the life cycle of *S. littoralis*, which reflects its fast food passage through gut. Another important feature that distinguishes the gut of *M. hippocastani* from *S. littoralis* is the presence of bacterial cells in specialized structures (Unpublished results). Their mere presence, may suggest further nutritional complement and assistance for insects by the bacterial symbionts as done by endosymbionts. If that would be the case, it could represent one of the first cases of such kind of symbiosis in the scarabeidae family of Coleoptera [13]. On the contrary, such specialized gut structures and bacterial associations are rarely found in the lepidopteran order [25].

Many factors affect the bacterial communities associated with the gut of insects if they are generalist or specialists. Among them the physiochemical conditions prevailing in the gut are very important and pH is one such factor. In general, bacteria adapt better to more acid pH than to alkaline ones. This is reflected in the different gut bacterial community composition observed for each of the insect species studied. Furher, the differential pH also selects for differential expression and predominance of certain enzymes. An example is the greater abundance of trypsin and chymotrypsin as main serine proteases in insects with alkaline midgut pH [126]. On the contrary, selection of cysteine or aspartic proteases is common in acid guts [127].

Additional forces determining the differential establishment of bacterial populations generally in insects are: the ionic strength of the midgut, the redox potential in the gut environment, the oxic regime (discussed in 3.1 and 3.2) and the release of reactive oxygen species (ROS) in the insect gut [2]. Furthermore, all these factors seems to be also affected by the food source. The midgut of *S. littoralis* and *M. hippocastani* maintain more oxidative redox status which changes in the hindgut [26,124]. This could significantly influence the activity of plant allelochemicals by altering its protonation and redox status [128]. But the redox adjustment is a process controlled not only by the insects, but also by the bacterial

community associated as reported for termites and scarabs [129]. Besides this, gut oxic regimes determine the bacterial arrangement. Many bacterial species establish their niches as oxygen scavengers, thereby creating anaerobic conditions for others. This is the case in both insects under study as the presence of strict anaerobes was mostly observed in the lumen (Article I and III). Along with this, the levels of ROS produced during cell metabolism and oxidative stress generated while feeding on phenolic rich diets impact the gut bacterial community. ROS generation is a common immunity mechanism displayed by insects in order to control gut invasive microbes. Increased mortality due to high level of opportunistic and pathogenic bacteria was observed in mosquitoes and fruit flies when ROS generation was suppressed [130]. At the same time that such humoral defense immunity mechanisms are triggered, the insect can also control gut microbiota genetically. An example is the cross talk between bacteria and the cells of the gut epithelium reported in *Drosophila melanogaster*. The simple presence of a persistence gene, as the *evf* (*Erwinia* virulence factor), allows *Erwinia* and other gram negative species carried with the food to persist in the host gut [131].

Finally, the greater the number of traits distinguishing the gut bacterial communities of the compared insects, some similarities are also shared. Despite the effect of food on the gut bacterial community composition, in both species, a core community prevails. This indigenous bacteria is not directly observed in the food (pointed in Article I and III) but modulated when changing the main feeding host (for *S. littoralis*). Though the core bacterial community is not carried along with the food, vertical transference to successive generations is suggested to occur with the egg. To finally confirm this, further experiments are required. The main hypothesis is that the gut bacterial community inoculum is carried with the eggs to successive generations in both *M. hippocastani* (Unpublished results) and in *S. littoralis* (Shao, personal communication) when the mother deposits her feces on them, as reported with other insects like *M. melolontha* and *M. manduca* [54,117].

5. Future perspectives

The gut bacterial community associated with the larva of the devastating generalist insect, *S. littoralis* was unraveled in my study. Also identification of the metabolically active and inactive bacteria was performed by Pyro-SIP. Following a logical experimental framework, both studies helped the identification of the insect core gut bacterial community. At this point, it is still unknown if there is a direct interaction between the bacterial species composing the community and the host. A large participation of the gut community in *S. littoralis* digestion is unlikely, and the real benefits provided to the insect have to be examined. As presented in the results, changes in the food crop of insects generates significant changes in the gut bacterial community. Unraveling the role of the gut bacterial community in supporting host plant shifts of the insect is an area of considerable promise. Understanding this could permit the development of tools to control important crop pests. This could be studied through the generation of a gut bacterial community metagenomics. A summary of the set of genes differently expressed among the bacteria community members while changing the food will shed some light in the main functions that the gut microbiome serve the insect. Furthermore, linking the identified genes from the metagenomic approach to the phylogenetical identity of the bacteria species expressing them, would generate a list of candidate bacterial species involved in each of those processes. The gut bacterial community of *S. littoralis* characterized in the study included only unmatured stages like larvae. In future, it is of utmost interest to unravel the gut bacterial community associated with the adult gut. A complete gut microbiome profile covering all life stages of *S. littoralis* life cycle is of utmost importance to identify the functional role of bacteria in the tri-trophic interaction.

In the case of the leaf and root feeder, it is still an open question, how *M. hippocastani* indigenous gut bacterial community overcomes insect metamorphosis and fasting, and is transferred to successive generations. Due to the long life cycle of *M. hippocastani,* the mechanism must be elucidated in a model insect. I currently use *S.*

littoralis as the model system. The strategy involves the generation of GFP-labeled gut indigenous bacteria that could be tracked along the whole insect life cycle and this could throw some light on the mechanism. As in the case of *S. littoralis*, the determination of the metabolically active gut bacteria in *M. hippocastani* is also very important. Pyro-SIP technique as presented in Article II could serve this purpose. Contrary to the model organism *S. littoralis*, *M. hippocastani* cannot be reared in the laboratory nor feed with artificial food. Thus, the substrates for Pyro-SIP technique must be delivered using the natural food, leaves and plant roots.

Finally, the assignment of the species name of the bacterial cells observed with TEM in the novel hindgut niches has to be finished. For this purpose, Gold-FISH detected with TEM is planned. This will be achieved employing specific FISH probes designed out of the 454-pyrosequencing data. Additionally, the study of the function that the bacterial species discovered in the hindgut pockets serve to the insect physiology, will be attempted using cultivation.

6. Summary

6.1 English

Insects are the most diverse group of animals on earth and they interact with large number of other organisms in nature. An example of such associations is the intimate relationship established with bacteria residing in the insects' guts. Through this symbiotic association, insects exploit the unlimited metabolic capabilities of prokaryotes in order to access and conquer diverse ecological niches. As a generalist herbivore, *Spodoptera littoralis* (Lepidoptera: Noctuidae) is a voracious polyphagous insect feeding on leaves of plants. On the other hand, *Melolontha hippocastani* (Coleoptera: Scarabeidae) is a specialist insect feeding principally on woody trees. *M. hippocastani* larvae feed on the roots of the host, while the adults feed on the leaves. Due to their diverse ecological niches, the bacterial communities associated with both mentioned insects are expected to differ significantly. Considering this, the overall goal of the present work was the identification of the main differences existing between the gut bacterial communities associated with each insect.

Gut bacterial community of a generalist insect, *Spodoptera littoralis*, a model organism for the development and application of a novel methodology (SIP-pyrosequencing)

Previous studies characterized gut bacterial communities associated with lepidopteran larvae as simple. The low richness and diversity of the bacterial community associated with the gut of *S. littoralis* larvae, obtained in the current extensive survey, confirm this (Article I). A very low number of Operational Taxonomical Units (OTUs) and furthermore fewer number of bacterial species were identified. However, significant variation in the composition of such communities across different developmental larval stages was noticed. Moreover, *S. littoralis* has rather harsh physiochemical gut conditions and its feeding habit involves a rapid gut passage. Despite all this, a core group of bacterial species prevailed in all cases. Altogether, this constitutes strong selection pressure upon bacterial

gut colonizers. This explains the simple *S. littoralis* gut bacterial community composition. Since the results of Article I described all bacterial species present in the insect gut, a methodology suitable for the identification of the metabolically active species was essential. A novel protocol described in Article II known as stable-isotope probing (SIP) *in vivo*, was established. In this method ^{13}C-glucose was used as universal substrate, linking the microbial phylogenies to their particular metabolic activity, in this case the assimilation of glucose. Identity of the species involved was revealed after analyzing the enriched ^{13}C–DNA of the bacterial species by 454-pyrosequencing (Article II). Metabolically active species include *Enterococcus*, *Clostridium* and *Pantoeae.* That many of the gut community members identified in the general survey (Article I) were found as metabolically active in the gut of *S. littoralis*, suggest them as important bacterial symbionts of insects.

Gut bacterial community composition of a specialist insect, *Melolontha hippocastani*

A common feature of the scarabeidae beetles family is their modified gut anatomy in order to accommodate bacterial symbionts. In such symbiotic associations, the insect, i.e. M. hippocastani, relies on the digestion that such bacterial symbionts perform, in order to process the recalcitrant materials that constitute its food. After a survey of the bacterial species composing the gut associated community, its high complexity became obvious (Article III and unpublished results). Further, the presence of certain bacterial species with particular characteristics; i.e. xylanolytic, amylolytic and cellulolytic properties, suggest them as indispensible for insect food digestion. Two clear bacterial communities were defined in the two major sections of the gut - midgut and hindgut (Article III and unpublished results). The most abundant members of the midgut community include γ-, δ-, and β- proteobacteria whereas the hindgut community is composed mainly of Bacteroidia, Clostridia and a large bulk of still unknown bacteria. Some of these groups were abundantly found in eggs and very likely vertical transmission from the mother to offspring could be involved. Furthermore, after surveying the bacterial community associated with the diapausing, unfed, recently molted beetle, the presence of a core community able to overcome these processes and

events was identified (Article III). However, the mechanism underlying this process is unknown. Finally, upon a detailed microscopic analysis of diverse tissues of the *M. hippocastani* hindgut, bacterial cells were found localized to certain specialized organs as well as novel structures, which were yet not known (unpublished results). Because of the particular localization of the bacterial cells in the insect tissue, this might represent an intimate symbiosis, comparable to endosymbionts.

6.2 German

Insekten stellen die artenreichste Gruppe von Tieren auf der Erde dar und interagieren in der Natur mit vielen anderen Organismen. Ein Beispiel für ein solches Zusammenspiel ist das zwischen Insekten und den in ihrem Darmtrakt angesiedelten Bakterien. Dieses Zusammenleben ermöglicht es den Insekten die nahezu unbegrenzten metabolischen Möglichkeiten der Prokaryoten zu nutzen, um verschiedenste ökologische Nischen zu erobern. Ein nicht-spezialisiertes, polyphages Insekt ist *Spodoptera littoralis* (Lepidoptera: Noctuidae), das sich von Pflanzenblättern ernährt. Demgegenüber steht *Melolontha hippocastani* (Coleoptera: Scarabeidae) als ein auf Waldbäume spezialisiertes Insekt. Die Larven von *M. hippocastani* fressen an den Wurzeln der Wirtspflanzen, während sich der adulte Käfer von deren Blättern ernährt. Aufgrund der unterschiedlichen ökologischen Nischen sollten sich die mit den beiden genannten Insekten assoziierten bakteriellen Lebensgemeinschaften deutlich voneinander unterscheiden. Dementsprechend war das übergeordnete Ziel der vorliegenden Arbeit die Identifizierung der wichtigsten Unterschiede bezüglich der Zusammensetzung der Darmbakterien-Konsortien im jeweiligen Insekt.

Bakterielle Darmsymbionten des Generalisten *Spodoptera littoralis*; ein Modellorganismus für die Entwicklung und Anwendung einer neuartigen Methodik (SIP-Pyrosequenzierung)

Arbeiten über die Gemeinschaft von Darmbakterien von Schmetterlingslarven charakterisieren diese generell als eher einfach. Der geringe Umfang und die mangelnde Vielfalt der Gemeinschaft an Darmbakterien von *S. littoralis*-Larven, die in der vorliegenden Studie festgestellt wurden, bestätigen das (Artikel I). Es wurde eine sehr geringe Anzahl an Phylotypen und Bakterienarten entdeckt. Allerdings zeigte sich eine signifikante Veränderung in der Zusammensetzung einer solchen Gemeinschaft in den verschiedenen Entwicklungsstadien der Larven. *S. littoralis* weist eher harsche physiochemische Bedingungen im Darm auf und ist darüber hinaus durch eine schnelle Darmpassage der Nahrung charakterisiert. Dennoch trat in allen Fällen eine Kerngruppe von Bakterienarten auf. Die für eine bakterielle Besiedlung harschen Selektionsfaktoren erklären möglicherweise die vergleichsweise einfache Zusammensetzung der bakteriellen Gemeinschaft im Darm von *S. littoralis.* Da die Ergebnisse des Artikels I die Gesamtheit der Bakterienarten im Insektendarm beschreiben, wurde eine neue Methode zur Identifizierung der jeweils metabolisch aktiven Spezies notwendig, wie das im Artikel II beschriebene Protokoll. Hierbei wurden stabile Isotope (SIP) von ^{13}C-Glucose als universales Substrat *in vivo* verwendet, um so die spezielle Stoffwechselaktivität der mikrobielle Gemeinschaft, in diesem Fall die Assimilation der Glukose, nachzuweisen. Die Identität der beteiligten Spezies wurde nach einer Analyse der angereicherten ^{13}C-DNA der Bakterien-Spezies durch 454-Pyrosequenzierung (Artikel II) bestimmt. Dabei wurden *Enterococcus*, *Clostridium* und *Pantoeae* identifiziert. Die Tatsache, dass viele der Mitglieder der Darmgemeinschaft, die in der ersten Studie identifiziert wurden (Artikel I), sich auch als metabolisch aktiv im Darm von *S. littoralis* erwiesen, unterstreicht deren Bedeutung als wichtige bakterielle Symbionten dieser Insekten.

Die Zusammensetzung der bakteriellen Gemeinschaft im Darm eines spezialisierten Insekts: *Melolontha hippocastani*

Ein gemeinsames Merkmal der Familie der Scarabeidae ist ihre modifizierte Darmanatomie, um bakterielle Symbionten zu beherbergen. In dieser Symbiose sind die Insekten auf ihre bakteriellen Symbionten angewiesen, die ihnen erst den Zugang zu schwer metabolisierbaren Materialien in ihrer Nahrung ermöglichen. Eine Untersuchung der Zusammensetzung der mit dem Darm assoziierten Gemeinschaft an Bakterienarten bestätigte deren hohe Komplexität (Artikel III und unveröffentlichte Ergebnisse). Ferner ist das Vorhandensein bestimmter Bakterienarten mit besonderen Eigenschaften, z.B Xylanase-, Amylase- oder Zellulase-Produzenten, Anzeichen für die Unverzichtbarkeit für den Verdauungsprozess der Insekten (Artikel III und unveröffentlichte Ergebnisse). Zwei eindeutige Bakteriellen-Gemeinschaften wurden in den zwei Hauptabschnitten des Darms - Mitteldarm und Enddarm - bestimmt. Die am häufigsten vorkommenden Mitglieder der Mitteldarm-Gemeinschaft beinhalten γ-, δ-und β-Proteobakterien, während sich die Enddarm-Gemeinschaft größtenteils aus Bacteroidien, Clostridien und einer großen Anzahl unbekannter Bakterien zusammensetzt. Einige dieser Gruppen wurden auch zahlreich in Eiern detektiert, ein Hinweis auf eine mögliche vertikale Übertragung der Bakterien vom Muttertier auf deren Nachkommen. Weiterhin wurde nach Untersuchung der bakteriellen Gemeinschaft im Zusammenhang mit der Diapause bei ungefütterten, gerade gehäuteten Käfern die Anwesenheit einer Kern-Zusammensetzung nachgewiesen, die in der Lage ist, derartig widrige Bedingungen zu überstehen (Artikel III). Der hierbei zugrunde liegende Mechanismus ist aber noch ungeklärt. Schließlich wurden durch eine detaillierte mikroskopische Analyse verschiedener Gewebe des Enddarms von *M. hippocastani* Bakterienzellen in bestimmten, spezialisierten Organen sowie an bisher dafür unbekannten Strukturen entdeckt (unveröffentlichte Ergebnisse). Diese sehr spezielle Lokalisierung der Bakterien im Insektendarm repräsentiert wohlmöglich eine äußerst enge Symbiose, eventuell vergleichbar mit Endosymbiosen

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8. Eigenständigkeitserklärung

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, daß ich die vorliegende Dissertation eigenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Fertigstellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Kapitels genannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht. Weiterhin wurde keine ähnliche oder andere Abhandlung als Dissertation anderswo eingereicht.

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Erika María Arias Cordero

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10. Curriculum vitae

Personal data

Name: Erika Arias Cordero

Date of birth: 01.11.1977

Sex: female

Nationality: costarican

Marital status: married

Education (superior and basic)

Since 04/2009: PhD student in the Max Planck Institute for Chemical Ecology and Friedrich-Schiller Universität Jena, Jena.

Thesis tittle: Comparison of the gut microbiome of a generalist insect, *Spodoptera littoralis* and a specialist, leaf and root feeder one, *Melolontha hippocastani* .

Supervisor: Prof. Dr. Wilhelm Boland

09/2006-09/2008: M.Sc. in International Horticulture, majors: Entomology and Phytopathology, Leibniz Universität Hannover, Hannover.

Thesis title: Effect of different strains of entomopathogenic fungi (EFP) on thrips (*Frankliniella occidentalis*) and selected natural enemies (NE).

Overall performance: magna cum laude, grade 1.7.

Supervisor: Prof. Dr. H-M Poehling

03/1999-03/2003: Licentiate in Agronomic Engineering, phyto-techniques emphasis, University of Costa Rica, San José.

General grade: 8.3 (scale 10 to 5.0).

Practical thesis title: Crop Protection Management of a melon plantation. Graded with honors.

Supervisor: Prof. Dr. Franklin Herrera

03/1995-03-1999: B.Sc. in Agronomic Engineering, phyto-techniques emphasis, University of Costa Rica, San José.

03/1990-12/1994: High School diploma, Liceo Laboratorio, Universidad de Costa Rica, San José.

03/1983-12/1989: Elementary School diploma, Escuela Dulce Nombre de Coronado, San José.

Employment

Since 03/2009: Max Planck Institute for Chemical Ecology, PhD student and scientific worker.

03/2007: Bayer CropScience, Bio-availability of pesticides laboratory Intern.

10/2004-07/2006: Frutas de Parrita, Research Manager of the Guanacaste Division involving administration and direction of the production of microorganisms (entomopathogens and antagonists) in laboratory.

08/1999-06/2004: Frutas de Parrita (melon grower and exporter), Manager of the Crop Protection Department of a 400 ha production unit.

Publications

Arias Cordero, E., Shao, Y., Boland, W. (in press). Identification of metabolically active bacteria in the gut of the generalist *Spodoptera littoralis* via DNA stable isotope probing using 13C-glucose. Journal of Visualized Experiments (in press).

Comparative evaluation of the gut microbiota associated with the below- and above-ground life stages (larvae and beetles) of the forest cockchafer, *Melolontha hippocastani*. PLoS One, 7(12): e51557. doi:10.1371/journal.pone.0051557.

Tang, X., Freitak, D., Vogel, H., Ping, L., Shao, Y., **Arias Cordero, E.**, Andersen, G., Westermann, M., Heckel, D. G., Boland, W. (2012). Complexity and variability of gut commensal microbiota in polyphagous lepidopteran larvae. PLoS One, 7(7), e36978. doi:10.1371/journal.pone.0036978.

Oral Presentations

*Represents the speaker

Arias Cordero E., Ping, L., Reichwald, K., Delb, H., Boland, W. * Persistence of the larval midgut microbiota in the adult of the forest cockchafer (*Melolontha hippocastani*). 8th Symposium, INRA-Rowett on Gut Microbiology, Gut Microbiota, Friend or Foe? Clermont-Ferrand, France, June 2012

Arias Cordero E.* Gut microbiota of the forest cockchafer (*Melolontha hippocastani*). 10th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE, Feb 2011

Ping L.*, Tang X., Shao Y., **Arias Cordero E**., Schönemann L., Vogel H., Boland W. A Survey of Probiotic Bacteria in *Spodoptera littoralis*. 61 Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie (DGHM), Göttingen, DE, Sep 2009

Arias Cordero E*, Obregon M. Effect of *Trichoderma viridae* in the biological control of the 'paper neck stem'(*Mycosphaerella* sp.) disease of a melon plantation, applied through the irrigation system. XXXVIII annual meeting Organization of Nematologists of Tropical America. San José, Costa Rica, June 2006.

Poster Presentations

Arias Cordero E., Ping L., Reichwald K., Platze M., Delb H., Boland W. Comparison of the gut microbiome of larvae and beetle of the Forest Cockchafer. Gordon Research Conference

Frontiers of Science: Applied & Environmental Microbiology, South Hadley MA, USA, Jul 2013.

Arias Cordero E., Shao, Y., Boland, W. Discovering the identity and functional roles of the inhabitants of insect guts. SAB Meeting 2012, MPI for Chemical Ecology, Jena, DE, Oct 2012

Arias Cordero E., Ping L., Reichwald K., Platzer M., Boland W. Gut microbiota of the forest cockchafer (*Melolontha hipposcastani*). Micom 2010, PhD Student Conference, Jena, DE, Oct 2011

Arias Cordero E., Ping L., Reichwald K., Platzer M., Boland W. Gut microbiota of the forest cockchafer (*Melolontha hipposcastani*). ICE Symposium, MPI for Chemical Ecology, Jena, DE, Sep 2011

Arias Cordero E., Ping L, Reichwald K, Platzer M, Boland W. Gut microbiota of the forest cockchafer (*Melolontha hipposcastani*) Essence poster. 13th Congress of the European Society for Evolutionary Biology (ESEB), Tübingen, DE, Aug 2010.

Arias Cordero E. Revealing the bacterial inhabitants of insect guts. SAB Meeting 2010, MPI for Chemical Ecology, Jena, DE, Oct 2010

Arias Cordero E. The transmission mechanism of endosymbionts living in the hindgut lobe of *Melolontha hippocastani*. 9th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE, Feb 2010

Arias Cordero E., Meyhöffer R., Poehling, H-M. Effect of entomopathogenic fungi (EPF) on thrips (*Frankliniella occidentalis*) selected predators. Tropentag 2008. Hohenheim, DE, October 2008.

Skills and Qualifications

2003 Eurep Gap Farm Certification Course Certifications, Latu Sistemas (Montevideo, Paraguay). Company implementation.

Awards

Travel Grant Gordon Research Conference Frontiers of Science: Applied & Environmental Microbiology, South Hadley MA, USA, Jul 2013.

Max Planck Gesellchaft Scholarship for performing PhD studies at the Max Planck Institute for Chemical Ecology, Jena, Germany, 03/2009-03/20012.

DAAD (German Academic Exchange Service) Master Studies Scholarship, Leibniz University of Hannover, Hannover, Germany, 2006-2008.

Honorific mention for the licentiate graduation thesis project ‘Crop Protection Management of a melon plantation’, San José, Costa Rica, 2003.

Languages

Native Spanish speaker.

English, good proficiency.

German (B2 certificate); relatively good knowledge of the language.