From plant metabolites to the active core of the gut microbiota of cotton leafworm, *Spodoptera littoralis*

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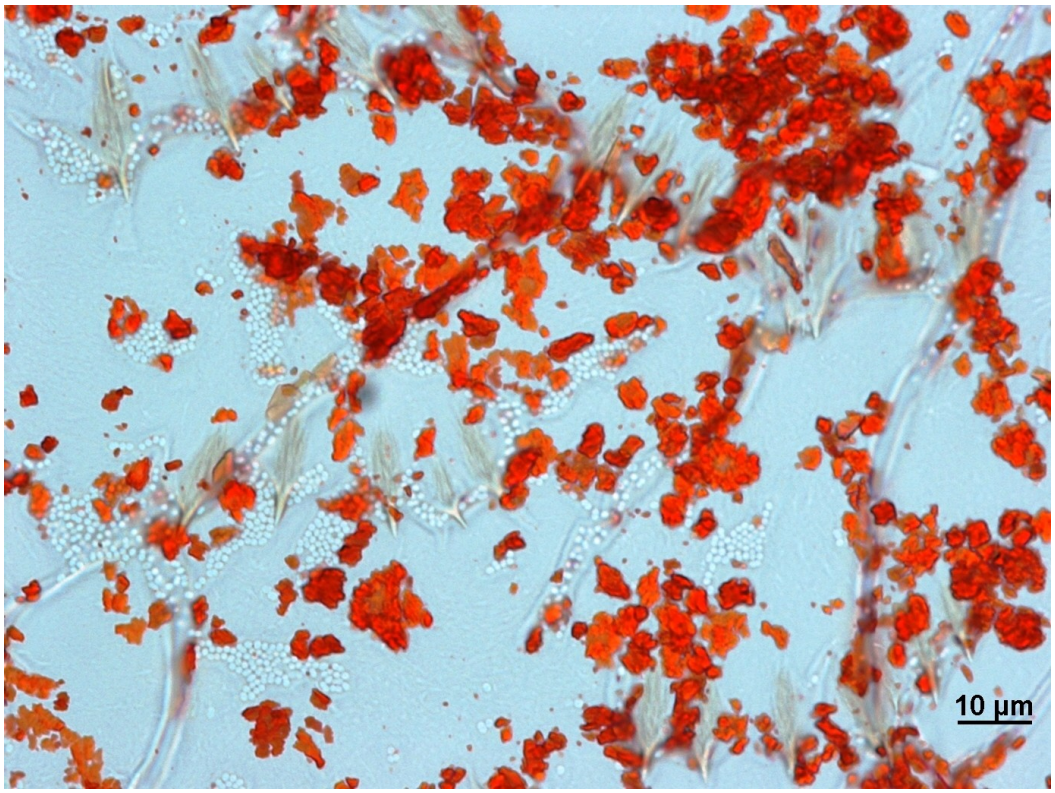

Typical image of the inner surface of a red crop after spreading on a glass slide. In *Spodoptera* larvae, the crop is the food storage organ, belonging to the foregut (Figure 1-8). Red crystals are beta carotene aggregates, derived from food plant *Phaseolus lunatus*, and white spots are *Enterococcus* cells (*Enterococaceae*, Firmicutes).

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Abbreviations and symbols

CBP	carotenoid-binding protein
Cryo-SEM	Cryo-scanning electron microscopy
CR	colonization resistance
DIP	direct insert probe
DAPI	4', 6-diamidino-2-phenylindole
Dps	DNA protection during starvation
EI	electron ionization (impact)
FISH	fluorescence *in situ* hybridization
HPLC	high pressure liquid chromatography
HRMS	high resolution mass spectrometry
IRMS	isotopic ratio mass spectrometry
IPM	integrated pest management
LAB	lactic acid bacterium
NMR	nuclear magnetic resonance
ROS	reactive oxygen species
SIP	stable isotopic probing
TBME	*tert*-butyl methyl ether
TLC	thin layer chromatography

1. Introduction

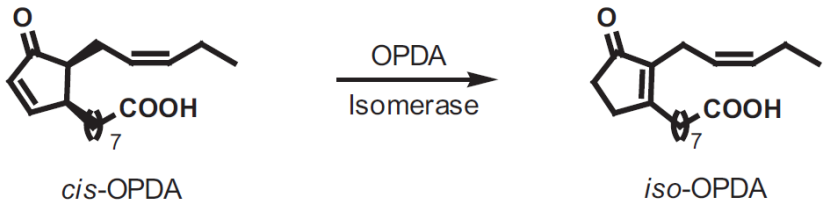
1.1. General introduction: herbivorous insect – host plant interactions

As the largest group of animals on Earth, there are over a million described insect species in nature and nearly half of them feed on plants, being the most significant herbivores (Mithofer and Boland 2012). Generalist herbivorous insects could feed on an extensive range of plant species spanning different families, whereas specialists attack only one or a few plant species within the same family (Wu and Baldwin 2010). On the other hand, plants have deployed multiple defense mechanism to cope with herbivorous insects' attack and microorganisms' challenge (Bednarek et al 2009), which enable them to survive in a mostly hostile and ever-changing environment. During their constant competition over 350 million years between herbivores and host plants, both partners have evolved similarly sophisticated counter-adaptations for the ongoing battle and their coexistence contributes to a major biodiversity of macroscopic organisms on our planet (Kursar et al 2009, Rausher 2001).

The host plants may directly or indirectly influence insect herbivores and their predators (natural enemies of herbivores) through multitrophic interactions. Direct plant defense involves a range of morphological and structural characteristics, including complex surface structures (e.g., trichomes, thorns and wax), multiple layers of armor, and thigmonastic movements, all of which could immediately, constitutively and negatively affect herbivores' performance. Moreover, plants produce a great variety of secondary metabolites, acting as deterrents and toxins, which make them unpalatable to herbivores and lead to a decreased fitness after ingestion, such as the retarded growth, low reproduction or fecundity rate. There are estimated to be more than 200,000 secondary chemicals in plants, which are not involved in the primary metabolism for plant development and reproduction (Mithofer and Boland 2012). As early as 1888, Ernst Stahl (1848–1919, Jena, Germany) already carried out comprehensive feeding experiments with herbivorous snails and slugs that demonstrated the

essential role of secondary metabolites in plant protection against herbivores (Hartmann 2008). Since then the crucial importance of plant secondary metabolites in the interaction between plants and herbivores have been broadly demonstrated and have been better understood nowadays on the genetic, biochemical, physiological, and ecological levels. Additionally, plants also recruit the predator (carnivores) and parasitoid in their defense against feeding herbivores by the production of volatile compounds attracting them or substances nourishing them (for example the extrafloral nectar), which effectively reduce feeding herbivore loads. In recent years, the inducible, indirect plants' defences have received increasing attention (Fatouros et al 2008, Leitner et al 2005, Schuman et al 2012), and the use of indirect defense as part of integrated pest management (IPM) strategies is suggested to reduce chemical pesticide use.

In response to plant defence, herbivorous insects, during the long term of evolutionary adaptation to their food plants, have developed specific mechanisms to tolerate, detoxify or even sequester specific plant defensive compounds and other secondary metabolites for their own use against natural enemies or for attracting mates. For the detoxification, cytochrome P450 monooxygenases are the most common enzymes associated with the catabolism of allelochemicals in the insects (Li et al 2007), other typical sets of detoxificative enzymes include esterases and glutathione-*S*-transferases (GSTs) (Schuler 2012) (Grbic et al 2011). For example, the glutathione-*S*-transferase from the gut of cotton leafworm (*Spodoptera littoralis*) and cotton bollworm (*Helicoverpa armigera*) can rapidly and almost completely isomerize the potentially toxic plant-derived 12-oxophytodienoic acid (*cis*-OPDA) which acts as a reactive electrophile, to *iso*-OPDA (Dabrowska et al 2009) (Figure 1-1).



cis-OPDA *iso*-OPDA

Figure 1-1: Enzyme assisted isomerization of plant-derived *cis*-OPDA to *iso*-OPDA in the insect gut (*Spodoptera littoralis*). Modified from (Dabrowska et al 2009).

While in some cases, insect hosts sequester the plant's toxic compounds into their own tissues or glands without modification as a defense against their predators. For instance, the larvae of Six-spot Burnet moth (*Zygaena filipendulae*) are reported to be able to accumulate cyanogenic glucosides, such as linamarin and lotaustralin, from their food plant (*Lotus corniculatus*) to fend off predators (Zagrobelny et al 2007) (Figure 1-2). Sequestration is considered an important adaptation of herbivorous insects to the plant host's defences and it is reported that the sequestration of plant pyrrolizidine alkaloids in a specialist arctiid moth (*Utetheisa ornatrix*) does not incur a strong cost (Cogni et al 2012).

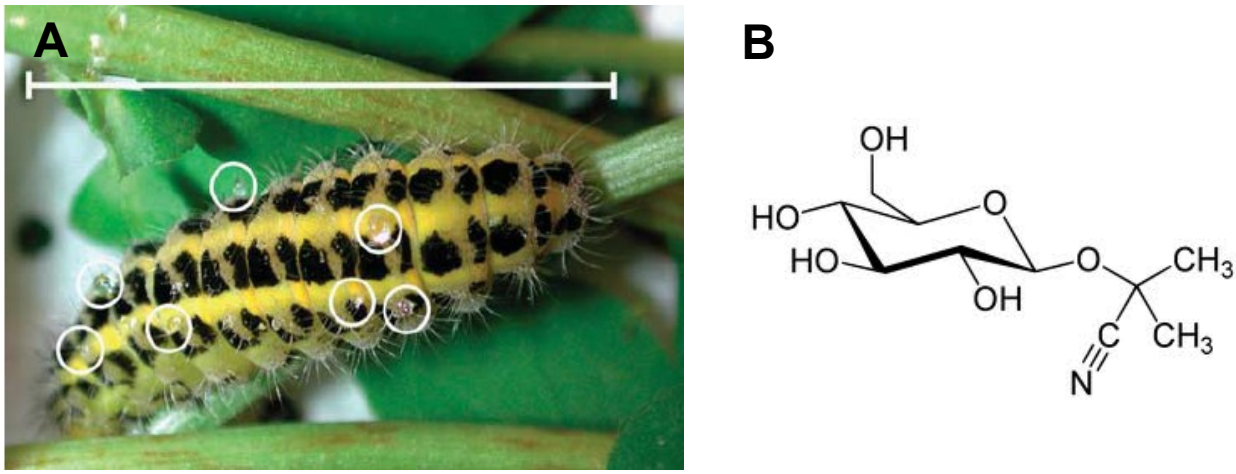


Figure 1-2: (**A**) Six-spot Burnet moth (*Zygaena filipendulae*) larva feeding on its cyanogenic host plant *Lotus corniculatus*. The larva is stimulated to secrete defence droplets (marked by white circles) containing the cyanogenic glucosides (**B**) linamarin. Scale bar, ~ 2 cm. Modified from (Jensen et al 2011).

Not only the toxic phytochemicals, other plant secondary metabolites such as carotenoids are also absorbed from dietary plants by insects, which play diverse and important roles in various organs and generally could not be synthesized by the animal themselves. Those nonpolar pigments could be transferred to proper tissues via passive diffusion and/or lipoproteins (El-Gorab et al 1975, Sakudoh et al 2007), and could accumulate a high concentration there in some insects (Carroll et al 1997, Eichenseer et al 2002, Sakudoh et al 2007). For example in the silkworm *Bombyx mori*, a carotenoid-binding protein (CBP) is responsible for the uptake of lutein into the intestinal mucosa and the silk gland, causing a yellowish coloration of the host haemolymph and cocoon (Figure 1-3).

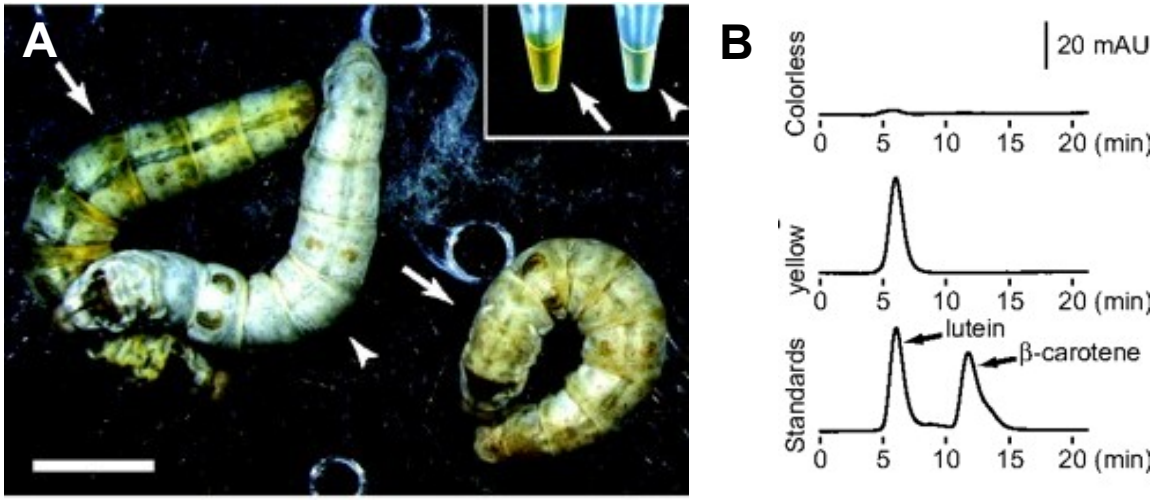


Figure 1-3: Different phenotypes of the silkworm (*Bombyx mori*). (**A**) Yellow haemolymph larvae (arrow) could be visually distinguished from colorless haemolymph larvae (arrowhead) in the rearing container. Inset: Their haemolymph. (**B**) A representative chart of the reverse-phase HPLC analysis of the haemolymph carotenoid composition of the larvae. Detection was at 443 nm. Lutein is the major carotenoid in the yellow haemolymph. Modified from (Sakudoh et al 2007).

In our case, we found that most healthy caterpillars of *Spodoptera littoralis* (cotton leafworm) fed on lima bean plants characteristically possess a remarkable red crop structure visible in the foregut. This red-colored pigmentation is absent in caterpillars raised on artificial diets, indicating that the red color results from the sequestration of a host-plant-derived pigment, most probably the carotenoids, indicated by their specific UV absorption (Figure 1-4). This phenomenon will be discussed in detail in the second section of the introduction, Article I and Unpublished results Part I.

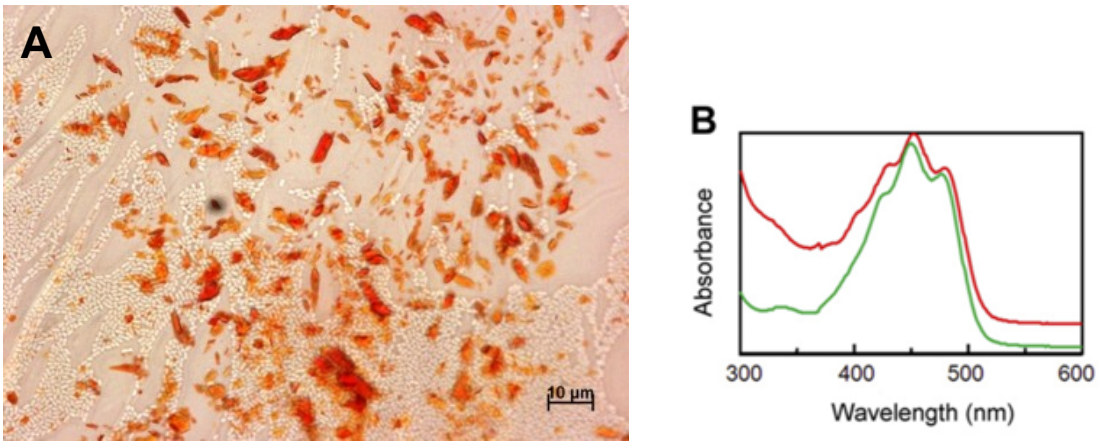


Figure 1-4: (**A**) Typical image of the inner surface of a red-colored crop (belonging to the foregut of cotton leafworm) after spreading on a glass slide. The larvae are reared on lima bean (*Phaseolus lunatus*) plants after hatching in the laboratory. Red crystals are beta carotene derived from food plants and white spots are bacterial cells, *Enterococci* (*Enterococcaceae*, Firmicutes). (**B**) UV absorption spectra of the purified compound (red-colored curve) and authentic beta carotene (green-colored curve) in ethyl acetate. Modified from (Shao et al 2011).

Interestingly, microorganisms are also found to make a substantial contribution to these plant-herbivore interactions, which add another trophic level to this complex network. For example, a bacterium, *Staphylococcus sciuri*, associated with the pea aphid (*Acyrthosiphon pisum*), is reported to produce semiochemicals that effectively attract aphid natural enemies, the hoverfly (*Episyrphus balteatus*) (Leroy et al 2011). Another example of the influence of bacteria is that guaiacol, a key component of the pheromone promoting the aggregation of locusts, is produced by a single indigenous bacterial species, *Pantoea agglomerans*, in the locust (*Schistocerca gregaria*) gut (Dillon et al 2000). And the precursor for guaiacol synthesis is most likely from digested plant material (wheat seedlings). These direct contributions by the herbivore's gut microbiota to its battle against plants or predators were previously underestimated. Given that microorganisms represent a huge metabolic resource

for the transformation of plant secondary compounds (Dillon and Dillon 2004), the involvement of microbes in tritrophic interactions could be a widespread phenomenon in herbivores. However, there is still quite a limited portion of research on the role of gut bacteria in such multitrophic interactions between plants, insects and their predators, probably because of the complexity of gut-microbe symbiosis and the lack of a proper research model system.

The cotton leafworm, *Spodoptera littoralis* (Lepidoptera, Noctuidae), represents one of the most polyphagous and widespread insect herbivores in the temperate regions, causing significant economic losses in crop production (Figure 1-5). As a generalist feeder, cotton leafworm has a very wide host range, feeding on plant species belonging to more than 44 different families, including legumes, crucifers and deciduous fruit trees, all containing species of highly economic importance (http://www.europe-aliens.org/speciesFactsheet.do?speciesId=50890#). Many host plant species are also known to produce high amount of toxic compounds, such as lima bean (containing cyanogenic glucosides), cotton (containing gossypol), and sorghum (containing apigeninidin). *Spodoptera littoralis* is one of the 100 worst alien species in Europe, according to the DAISIE report (Delivering Alien Invasive Species Inventories for Europe, supported from the European Commission under the Sixth Framework). It has been found but not yet established in Western and Northern Europe (Denmark, Finland, Germany, Netherlands and England) (Figure 1-6).

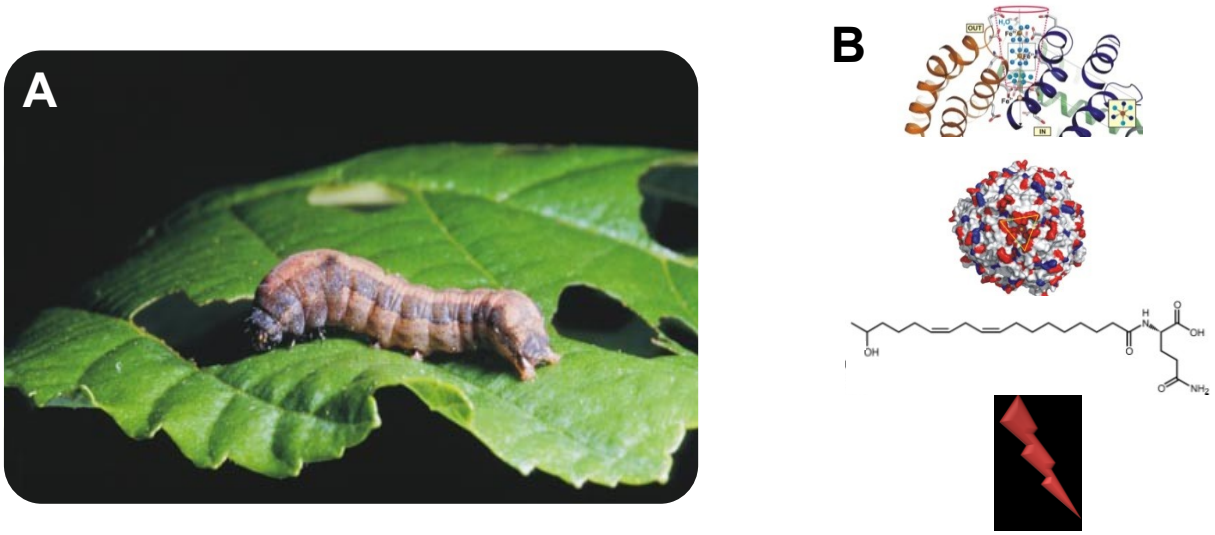


Figure 1-5: (**A**) Typical image of a *Spodoptera* caterpillar on the feeding stage. (**B**) A Dps (DNA protection during starvation) protein from the gut bacterium, *Microbacterium arborescens*, could synthesize and hydrolyze amino acid conjugates (*N*-acyl-glutamine), which trigger the plant's indirect defense. Modified from (Pesek et al 2011).

Like most lepidopterans, cotton leafworms possess a simple alimentary canal, like a tube without any specialized substructures, and the ingested food passes quickly through the digestive tract, making the gut an inhospitable environment for many microorganisms. Gut pH is another important determinant of the microbial flora and cotton leafworms maintain an extremely high pH in the fore- and midgut, about 10 and 9 respectively. In contrast to the simplicity of the alimentary canal and the strongly alkaline milieu inside, a large and stable bacterial population is evident in the gut of cotton leafworm, and the numbers exceed the range of 10^7 mL^{-1} (Funke et al 2008). Previous work in our lab also indicated that the gut bacteria are involved in the herbivore-plant interaction (Funke et al 2008, Pesek et al 2011, Ping et al 2007, Spiteller et al 2000). A Dps (DNA protection during starvation) protein from the gut bacterium, *Microbacterium arborescens*, could synthesize and hydrolyze amino acid conjugates (*N*-acyl-glutamines), which trigger the plant indirect defense such as the emission

of volatiles (Figure 1-5). Therefore, *Spodoptera littoralis* provides an excellent model system for research on the insect-microbe-plant interactions.

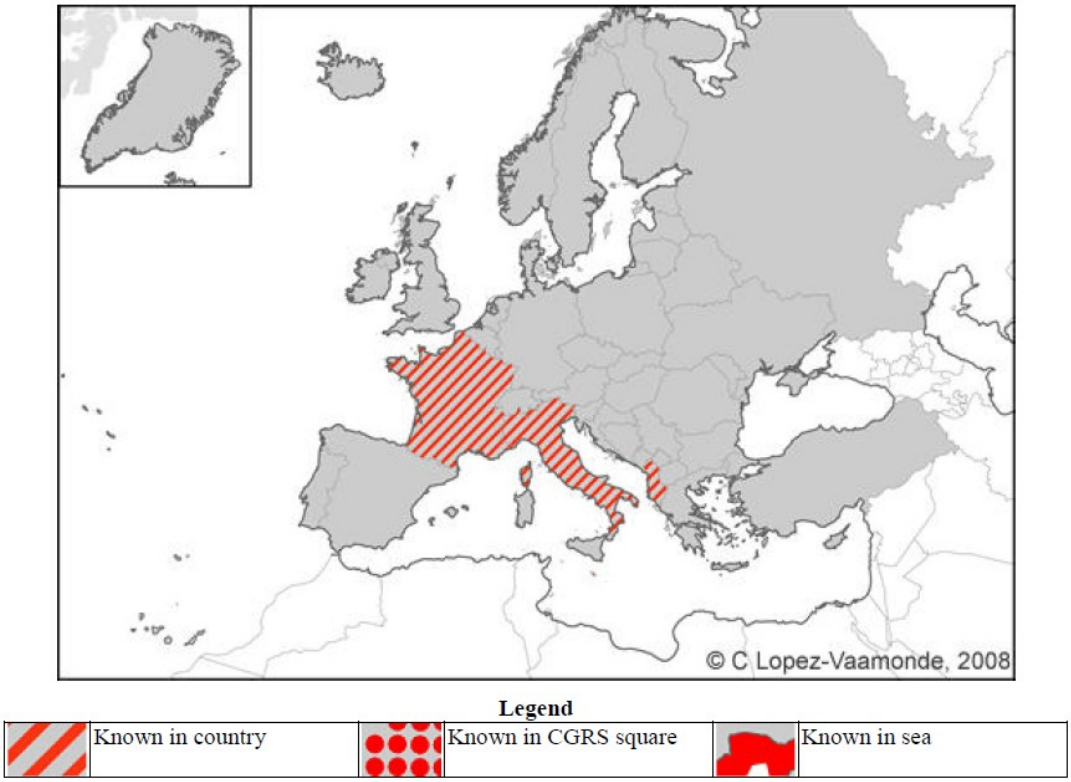


Figure 1-6: European distribution of *Spodoptera littoralis*, according to the DAISIE report (Delivering Alien Invasive Species Inventories for Europe, supported from the European Commission under the Sixth Framework). From: http://www.europealiens.org/speciesFactsheet.do?speciesId=50890#

Our lab has established a rearing protocol and a chemically controlled artificial diet for *Spodoptera littoralis*. Together with the introduction of advanced tools in molecular technology and chemistry analysis, it is available now for detailed studies of the gut microbiota of herbivores, which have the potential to provide new insights and understanding of the impact of microorganisms in that ecology. The gut microbiota of *Spodoptera* will be

discussed later in the third section of this introduction, Article II, Article III and Unpublished results Part II.

Up to now, research in plant-herbivore interactions highly focuses on plant studies and there is a need to pay more attention to other partners involved in this multitrophic interaction, especially to the essential and ubiquitous co-occurring microorganisms, which have been largely ignored before.

1.2. Carotenoids in nature

The carotenoids are the most widespread class of natural pigments comprising over 700 structures with diverse metabolism and various biological functions in all living organisms, including light detection, oxidation control and coloration (Goodwin 1986, Moran and Jarvik 2010, Walter and Strack 2011). Carotenoids are generally synthesized by plants and algae, as well as some microorganisms such as cyanobacteria, red bread mold (*Neurospora crassa*). Carotenoids are split into two classes: xanthophylls, which contain oxygen; and carotenes, which are purely hydrocarbons and contain no oxygen.

As one of the major plant secondary metabolites, carotenoids are constantly synthesized by all photosynthetic plants in plastids (chloroplasts and chromoplasts), as a component of the light harvesting apparatus and a scavenger for highly reactive singlet oxygen (Bartley and Scolnik 1995, Beisel et al 2010). Through absorption of light energy, carotenoids facilitate photosynthesis and provide protection against photo-oxidative damage. Furthermore, a small fraction of non-protein-bound carotenoids serve as antioxidants in the lipid phase of photosynthetic membranes (Beisel et al 2010). A typical carotenoid molecule like lycopene or beta carotene can sustain more than 20 free radical “hits” by lipid radicals before it is completely destroyed (Cantrell et al 2003, Young and Lowe 2001).

Animals also harbour a variety of carotenoids, which account for many of the bright colors of butterflies, fishes, birds and mammals. Although it is a long-standing paradigm that animals are unable to produce their own carotenoids and all carotenoids are sequestered from their diets, recently several cases show that the insects, for example the pea aphid *Acyrthosiphon pisum* (Hemiptera, Aphididae) and the two-spotted spider mite *Tetranychus urticae* (Acari, Tetranychidae), are capable of synthesizing carotenoids themselves via the functional genes horizontally transferred from the fungus to the host own genome during evolution (Altincicek et al 2012, Grbic et al 2011, Moran and Jarvik 2010). Those small sap-sucking insects adapt to feed on the plant juice with their needle like mouthparts; and carotenoids, as lipid-soluble compounds, are not expected to occur in significant quantities there. Thus, they may have limited access to carotenoids from the diet. However, for the chewing herbivores such as cotton leafworm, they can easily get enough carotenoids from the plant leaf. It is reported that cotton leaves contain 275,1 μg lutein and 196,0 μg beta carotene per gram of dry weight, which are a rich source of carotenoids for the chewing insect feeders (Eichenseer et al 2002). Interestingly, endosymbionts are shown to be also involved in the host carotenoids metabolism in some insects. The genome survey of a bacterial endosymbiont (*Portiera*) of whiteflies (*Bemisia tabaci*) found the functional genes for carotenoid biosynthesis, which could serve the host as an alternative source of carotenoids (Sloan and Moran 2012). Therefore, related lineages of sap-feeding insects appear to have convergently acquired the same functional trait by distinct evolutionary mechanisms: bacterial endosymbiosis and fungal lateral gene transfer.

In our study, it is observed that a remarkably enlarged crop structure, which is the insect's food storage organ, develops in the foregut of *Spodoptera littoralis* larvae fed on toxic plants such as lima bean, compared with that of the larvae fed on the artificial diet, and a large amount of red colored crystals accumulates in the crop tissue (Figure 1-4). By chemical

characterization, especially using non-invasive Raman microscopic analysis of the crystal *in situ*, I determined that carotenes, mainly beta carotene, not the most ubiquitous and abundant lutein in the foliage of host plant, are selectively sequestered in the foregut crop tissue and eventually crystallize there. It is known that at high concentrations, carotenoids exhibit a tendency to aggregate or crystallize out of solution (Young and Lowe 2001). Thus, the crystallization in the crop membrane could be the consequence of the high levels of local beta carotene. The formed crystals randomly position in the whole crop membrane, but do not appear in other gut tissues. Notably, the microscopic observation revealed that a large amount of coccus-shaped bacterial cells tightly associated with the red crystals, forming a homogenous lawn on the crop membrane. A single bacterial species, *Enterococcus casseliflavus*, was identified by both the culture-dependent and molecular methods. Interestingly, some *Enterococci* are known to produce carotenoids (Maraccini et al 2012, Taylor et al 1971). Therefore, the huge amount of carotenoids was expected to originate either from the ingested food plant or from the microbial associates located in the larval foregut. The largely higher survival rate of the larvae which successfully develop such a red colored crop when feed on toxic plants, comparing with that of the larvae fail to develop it, emphasizes the importance of this selective accumulation of beta carotene and at the same time leads to a question concerning the biological significance of this sequestration for the herbivore host.

It is well known that plants make both local and systemic defense against the herbivore attack, which would release many toxic compounds. The rapid production of reactive oxygen species (ROS) is an essential part of plant stress responses. There was a significant increase of the superoxide radicals and total peroxide production in the foregut of *S. littoralis* larvae fed on the plants in contrast to those fed the semi-artificial diet (Krishnan and Kodrik 2006). As a polyphagous chewing herbivore, *Spodoptera* larvae typically have a fast food-throughput life style. Therefore, many reactive free radicals arise in the larval digestive tract during foraging,

especially in the crop which is the food (plant pieces) storage organ. These increased oxidative stresses may lead to uncontrolled lipid peroxidation, protein oxidation and other deleterious effects in the host gut. Herbivorous insects generally employ several antioxidant defense mechanisms to control oxidation. They commonly up-regulate their antioxidant enzyme systems to combat oxidative stress imposed upon feeding on plants containing pro-oxidant allelochemicals. The carotenoid pigments in insects are also widely reported to protect the host against oxidative stress (Bi JL 1995, Carroll et al 1997, Eichenseer et al 2002, R.R. AUCOIN 1990, Sloan and Moran 2012). For example, it was suggested that the bright orange-red fat body of the chrysomelid beetle (*Chrysolina hypericin*) contains carotenoids sequestered from the host plant (*Hypericum perforatum*), which protects the beetle larva from phytotoxin (hypericin) associated photooxidative damage (R.R. AUCOIN 1990). In cotton leafworms, the highly sequestered carotenes may serve a similar function against oxidative stress. It is extensively studied that carotenoids act as antioxidants by both a non-destructive physical and chemical quenching process. For instance, the quenching of highly active singlet oxygen ($^1O_2$) by carotenoids (CAR) is known to occur primarily through an energy transfer mechanism, as follows:

$$^1O_2 + CAR \rightarrow {}^3O_2 + {}^*CAR$$

$$^*CAR \rightarrow CAR + h\nu$$

In the reaction, triplet (ground-state) oxygen is generated together with triplet carotene, which dissipates its energy to the environment and returns to its ground state. It is then ready to continue the reaction in cyclic fashion (Goodwin 1986). Beta carotene and lycopene are

reported to exhibit the fastest singlet oxygen quenching rate constants ($2.3–2.5 \times 10^9 M^{-1} s^{-1}$) (Cantrell et al 2003).

Taken together, these studies provide examples of diverse routes of acquiring carotenoids and indicate the importance of carotenoids for the insect in general. Probably there is a strong selective pressure to acquire these compounds among diverse insects. Unlike other carotenoid sequestrations reported in lepidopterans, the crop tissue of *Spodoptera* is extraordinarily selective, concentrating very high levels of just beta carotene. The mechanism of dictating this specific accumulation could be related to the increased oxidative stress during foraging. Our finding extended the carotenoid absorption and metabolism in insect herbivores.

1.3. The gut bacteria of insects

Insects are the most diverse and the largest class of animals on our planet, which occupy in nearly all terrestrial ecological niches and constitute approximately 53% of all living species (Robinson et al 2011). Moreover, insects represent an old group of arthropods; the earliest known insect, *Rhyniognatha hirsti*, appeared almost 400 million years ago in the Silurian period together with the emergence of the Earth's first terrestrial biotas (Engel and Grimaldi 2004). Owing to this great diversity and the long time co-existence, an amazing variety of symbiotic microorganisms have adapted specifically to insects as hosts, which participate in many relationships with the hosts (Bode 2009). Especially, the gut of most insects harbours a rich and complex microbial community, typically 10^7 – 10^9 prokaryotic cells there, outnumbering the host's own cells in most cases (Broderick et al 2004) (Colman et al 2012). Thus, the insect gut is a "hot spot" for diverse microbial activities, representing multiple aspects of microbial relationships, from pathogenesis to obligate mutualism (Dillon and Dillon 2004). It is broadly accepted that the components of the native microbiota (or indigenous microbiota) are true symbionts that promote the host fitness, which range from

enhancing host energy metabolism to shaping immune system (Schneider and Chambers 2008).

The nutritional symbiosis, in which the microbe provides some limiting nutrients in the host's suboptimal diet, has been most often described in insects (Colman et al 2012). Especially for herbivorous insects, the plant material is often low in nitrogen, vitamins and sterols, which poses several challenges to the host. However, many symbiotic microbes possess various metabolic abilities to synthesize these compounds and thereby enable the insects to exploit otherwise inaccessible food sources and diversify in a specific ecological niche. For example, genome analysis revealed that the co-resident bacterial symbionts (*Sulcia*) of the cicada (*Diceroprocta semicincta*) can produce all essential amino acids as well as many vitamins to satisfy their host nutritional needs, which feed exclusively on xylem sap of plant root (McCutcheon et al 2009). In ants, the prevalence of bacterial gut symbionts, *Rhizobiales*, are tightly linked with the evolution of herbivory, which supply host additional nitrogen (Russell et al 2009).

The gut symbiont is also involved in the host detoxification processes. It is thought that food and/or environment derived toxic compounds alter the composition of the gut microbiota and particularly favor those species able to detoxify (Dillon and Dillon 2004). For example, the bean bug (*Riptortus pedestris*) becomes instantly resistant to a common chemical insecticide, the fenitrothion, by swallowing the right bacteria (*Burkholderia*) from environmental soils, which preferentially colonize in the host midgut and could quickly break down this organophosphorus pesticide (Figure 1-7) (Kikuchi et al 2012). The gut microbiota of mealworms (*Tenebrio molitor*) is suggested to have a detoxifying role to the plant glycoside salicin (Genta et al 2006). The ability of gut bacteria to modify or detoxify plant allelochemicals may be extremely important to the generalist herbivores, such as *Spodoptera*

littoralis, which have a very wide host plant range and often ingest a large amount of noxious plant secondary compounds. Surprisingly, this topic has been largely unstudied.

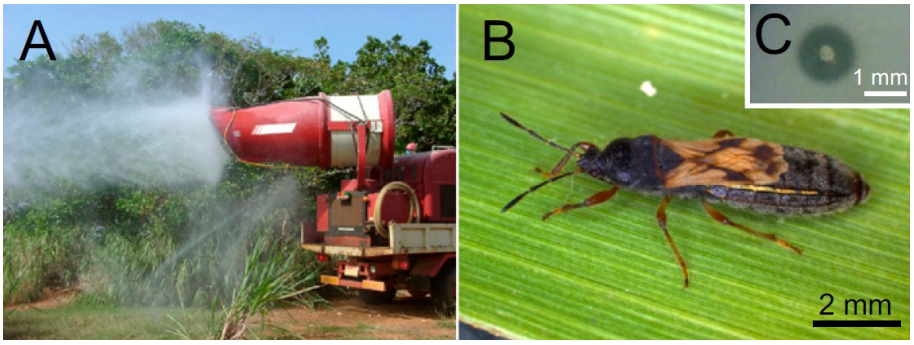


Figure 1-7: Discovery of fenitrothin-degrading *Burkholderia* infection in *C. saccharivorus* at Minami-Daito Island, Japan. (**A**) Fenitrothion spraying in a sugarcane field at Minami-Daito Island. (**B**) An adult female of *C. saccharivorus*. (**C**) Degradation of fenitrothion by a *Burkholderia* strain (MDT2) isolated from *C. saccharivorus*. Modified from (Kikuchi et al 2012).

Although insects have an innate immune system alone, they are rarely succumbed to pathogens and parasites during millions of years of existence. The native gut microbiota is more and more recognized to play as an "extended immune phenotype" for the host against harmful microbes (Koch and Schmid-Hempel 2011). Through their capability to resist the colonization by non-indigenous species, namely colonization resistance (CR), the resident microorganisms actively prevent infections in the digestive tract, which probably is the most important beneficial function of gut microbiota (Dillon and Dillon 2004). Abundant lactic acid bacteria (LAB) maintain in biofilms within the foregut (the crop) of the Western honeybees (*Apis mellifera*) and work in a synergistic matter to inhibit pathogen proliferation in the gut by producing a mixture of antimicrobial agents. This ancient symbiotic relationship between LAB and bees is suggested to be of great benefit to safeguard bee health (Vasquez et al 2012). The gut bacteria of desert locusts (*Schistocerca gregaria*) impart protection against

microbial pathogens mainly by producing antimicrobial phenolic compounds (Dillon et al 2000). A broad range of research indicates microscopic partners to be major modulators of insect health.

In addition to these classic microbial-host relationships, recent years have seen an explosion of novel symbiotic interactions between bacteria and insects. Studies have shown that microbes can mediate thermal tolerance to the host, alter the reproductive mode, and modify the insects' body color, among others (Dunbar et al 2007, Tsuchida et al 2010, Werren et al 2008). Those multiple benefits provided by the host microbiota suggest a closer degree of integration between the host and its microbial community than was previously suspected. Given microbes' much wider range of metabolic properties than do animals and their ability to evolve quickly, mutualistic bacteria probably constitute a key factor for the diversification and enormous success of insects.

However, relatively little is known about the microbiota associated with insects feeding on foliage, where no strict symbiotic interaction has been proposed so far. In fact, most lepidopteran larvae are leaf chewing herbivores and their gut content (food bolus) is not sterile (Dillon and Dillon 2004). Because of their exclusively phytophagous dietary style, lepidopteran insects are consequently expected to have an efficient gut microbial community to enable digestion of the complex lignocellulosic plant material and to protect the host against contaminating microbes from outside (Priya et al 2012). It is proposed that most of digestive enzymes such as cellulase, xylanase and pectinase in silkworms (*Bombyx mori*) are of microbial origin and contribute to larval growth (Anand et al 2010). And, it is reported that the aseptically reared oriental tea tortrix larvae (*Homona magnanima*) larvae supported 20 times greater growth of an entomopathogen (*Bacillus thuringiensis*) than larvae with a normal gut microbiota (Takatsuka and Kunimi 2000).

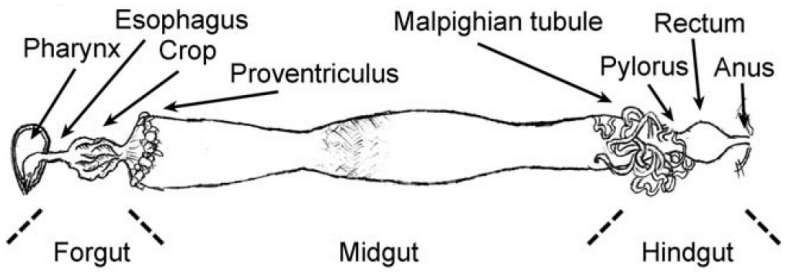


Figure 1-8: The detailed structure of the alimentary canal of *Spodoptera littoralis* larva. The digestive tract is cut into three segments (foregut, midgut, and hindgut) for sampling as indicated by the dotted lines.

Although the cotton leafworm possesses a simple and straight digestive tract, which is the largest part of the body without any substructures (Figure 1-8), a large bacterial population is evident in the gut and the number exceeds 10^7 mL^{-1} (Funke et al 2008). Especially, previous work in our lab indicated that the gut bacteria are involved in the homeostasis of plant defense elicitors (Figure 1-5). In recent years, there is a growing interest about the roles of associated bacteria in the host physiology and in those multitrophic interactions.

As an initial step toward understanding these complex relationships between the host and its associated microbes, I first inventoried the composition of the gut microbiota in detail, including the comparison of community profiles in different larval life stages and after feeding on different diets. The gut microbial community was also compared with that in another highly polyphagous lepidopteran, *Helicoverpa armigera* (cotton bollworm). Those comprehensive analyses of 16S rRNA gene diversity indicated that cotton leafworms do harbor an indigenous microbiota, with several bacterial taxa being qualitatively stable. Furthermore, the metabolic active members were also identified by using stable isotope probing. Clearly, the investigation of gut microbiota is necessary to get a better understanding

of symbiotic relationships between microbes and the insect host, and further to get a more comprehensive understanding of the herbivore-plant interaction, since microorganisms are an important component of this ecosystem.

1.4. Aims and scope of this study

(1) To identify the component of the red colored crystals and characterize the bacteria species which tightly associated with those crystals in the foregut.

It was shown that the red coloration in the crop of *Spodoptera littoralis* larvae probably is based on a carotenoid(s), according to the distinct UV absorption spectrum. Notably, the red crystals are embedded in a homogenous lawn of bacteria. The association between red coloration, carotenoids, and bacteria prompted me to determine the component of the crystals and the bacteria species around them. A combination of several chemical analytic methods (TLC, HPLC/MS, NMR and Raman microscopy) was employed to characterize this compound. The bacteria were identified based on the classic culturing technique and the molecular cloning/sequencing of bacterial 16S rRNA gene.

If carotenoids were indeed the major component of the red crystals, then the next question is where the huge amount of carotenoids comes from. Is it the bacterial symbiont or the host itself producing those reddish crystals? Because the plant leaves contain a large amount of carotenoids too, such as lutein (252.9 μg/g dry weight in soybean) and beta carotene (158.8 μg/g dry weight in soybean), it is also possible that the insect hosts sequester carotenoids from their plant diet (Eichenseer et al 2002). As the second step, I was interested to identify the source of this compound. Due to different metabolic pathways, the stable isotopes of carbon and hydrogen will be selectively enriched or reduced in different organisms, e.g. the ^{13}C value of beta carotene produced by plant is very different from that produced by microalgae (Kroll et al 2008). Thus, the examination of natural stable isotopic composition of carotenoids is a

powerful tool for determining their origin. The isotopic ratio mass spectrometry (IRMS) was used to address this question. On the other hand, the carotenoid profile of the host gut tissue, plant diet and isolated bacteria was analyzed by HPLC/MS.

Further, this phenomenon was also checked in other *Spodoptera* species and the larvae were shifted to different plant diets, such as snap bean, cotton and Arabidopsis. Finally, the significance of this red coloration to the insect host and possible mechanism for the maintenance was tested with a bioassay. It is well known that many oxidative stresses arise in the larval digestive tract during foraging plants, especially in the crop, where a large amount of broken plant pieces was stored. Considering the general antioxidant property of carotenoids, it is reasonable that the hosts may selectively sequester and deposit them in the foregut tissue against increased oxidative stress during foraging. The relationship between the oxidative stress and the carotenoids accumulation was examined in the laboratory-reared larvae by feeding them with the pro-oxidant spiked artificial diet (Unpublished results Part I).

(2) In order to decipher the nonpathogenic interaction between symbiotic gut bacteria and the host, the microbial diversity and composition in the gut flora need to be first studied. The dynamics of microbiota in the course of larval development and the influence of diet on gut microbiota are addressed next.

A standing question about the lepidopteran gut microbiota is whether or not they possess an indigenous community. For a long time, studying insect gut microbiota was mainly performed by classic cultivation and isolation method. These studies formed the basis of our current understanding but often led to a biased description, and the true microbial diversity was often largely overlooked for studying such complex microbial communities, as most microorganisms still are difficult to be cultured in the laboratory. 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 (Broderick

et al 2004). None of the bacteria isolated from the laboratory-bred tobacco hornworm (*Manduca sexta*) belonged to the abundant phylotypes revealed by PCR-single-strand conformation polymorphism of the 16S rRNA genes (van der Hoeven et al 2008).

The development of molecular approaches for the characterization of microbes, such as the construction of clone library, microarray and fluorescence *in situ* hybridization (FISH) based on rRNA or functional genes, has provided valuable insights into the uncultured majority *in situ*. By using several culture-independent methodologies, I tried to comprehensively characterize the composition of gut microbiota and their dynamics in response to the host development and different diets in two generalist feeder, *Spodoptera littoralis* (cotton leafworm) and *Helicoverpa armigera* (cotton bollworm).

Up to now, the extraction and sequencing of 16S rRNA genes are the most comprehensive and least biased enumerations of microbial diversity in the gut flora. The metagenomic DNA was directly extracted from fresh gut tissues of larvae at different instars and several universal PCR primers, which targeted to broad phylogenetic groups including bacteria, Archaea and fungi, were used to profile the microbial community. The amplified PCR products were used to construct several bacterial clone libraries to obtain phylogenetic insights. It is also feasible to compare the communities by using fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE). Additionally, FISH with species-specific rRNA probes provided an approach to visualize particular bacterial populations *in situ*, from which the morphology was observed, number and spatial arrangement of cells in the gut was determined too. It is only recently that the availabilities of new generation sequencing techniques have allowed a high-throughput and high-resolution survey of the complex microbiota. However, this deep analysis was performed in just a few insect species (Sudakaran et al 2012). As the first example in the Lepidoptera, this new technology was applied here to survey the gut microbiota of *Spodoptera littoralis* too.

(3) A further understanding of metabolically active member in the local gut microbiota could potentially provide insights into the proximate and ultimate causes of these associations. In addition, the dynamics of bacterial activity across different larval developmental stages (early-instar vs. late-instar) may shed light on their functional role inside the gut.

While many studies have described the composition of gut microbial communities, as yet, the assessment of metabolically active components in the gut microbiota is surprisingly scarce, particularly *in vivo*. It is recognized that not all microorganisms could colonize the gut although it is a nutrient-rich environment, while some dietary microbes are lysed and some nonindigenous microbes remain dormant during the gut passage. On the other hand, active populations shift in response to host development and environment factors (Nicholson et al 2012). To get a more complete picture about the gut microbiota, it is important to move beyond mainly sequencing based approach towards other advanced tools to identify active fractions, which directly contribute to the current community function.

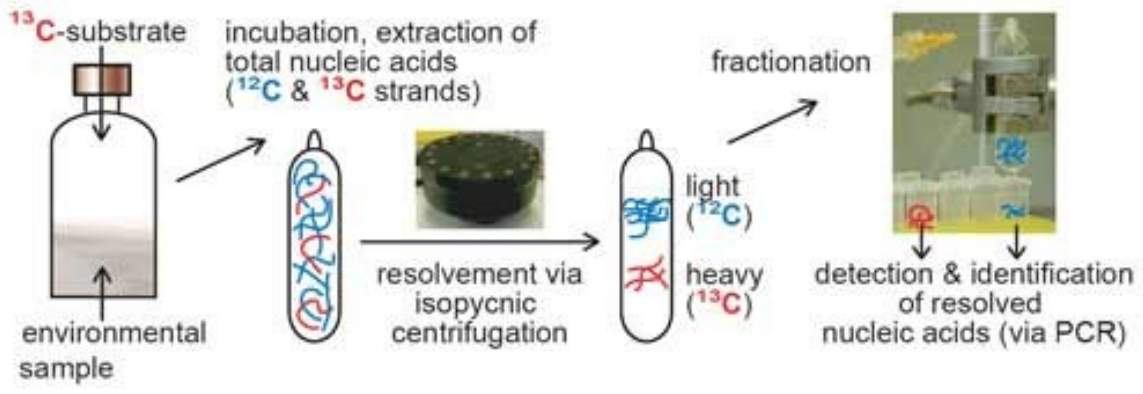


Figure 1-9: A generalized scheme for DNA/RNA stable isotope probing, involving sample incubation, nucleic acids extraction, CsCl density gradient ultracentrifugation and DNA characterization with downstream molecular techniques. http://www.helmholtz-muenchen.de/en/igoe/research/research-groups/molecular-ecology/index.html

Stable isotope probing (SIP) is a promising, culture-free technique that is often used in environmental microbiology to identify active microorganisms involved in various biogeochemical processes (Figure 1-9) (Dumont and Murrell 2005). This methodology relies on the assimilation of a stable isotope (^{13}C)-labeled carbon source into growing microbes and the selective recovery of the isotope-enriched cellular components, such as the most informative nucleic acids, which could provide specific taxonomic information. ^{13}C-enriched "heavy" DNA or RNA can be separated from unlabeled, normal "light" (^{12}C) nucleic acids by density-gradient ultracentrifugation and subsequently retrieved from gradient fractionation procedure for further molecular analysis (Neufeld et al 2007). Stable isotope probing of nucleic acids provides direct evidence of bacterial metabolic activity and has been demonstrated to be more sensitive than an RNA-based approach (Brinkmann et al 2008).

Recently Pilloni *et al.* introduced a combination of DNA-SIP and pyrosequencing, namely Pyro-SIP, in SIP gradient interpretation (Pilloni et al 2011). However, this pioneering research only performed amplicon pyrosequencing on the entire metagenomic DNA without gradient separation and still relied on the low-resolution terminal restriction fragment length polymorphism (T-RFLP) fingerprinting to identify density-resolved DNA fractions and laborious clone library construction to link all data.

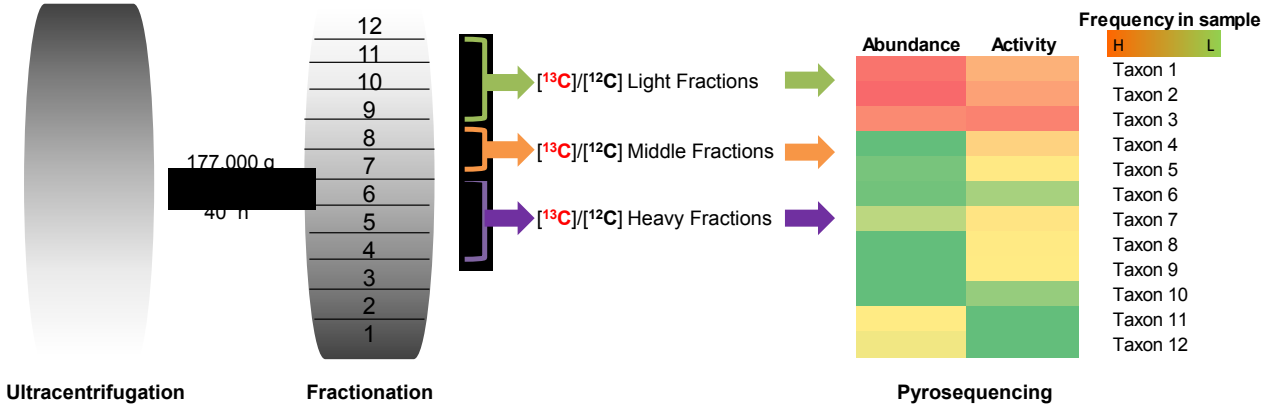


Figure 1-10: Outline of a modified Pyro-SIP experiment to assess microbiota metabolic activity and link it to phylogeny. Isopycnic separated DNA are directly subjected to quantitative pyrosequencing to reveal bacterial lineage and abundance.

Considering all of those drawbacks, here I conduct a fairly straightforward new Pyro-SIP in my study, which directly pursues high resolution pyrosequencing to examine isopycnic separated gradient fractions (labelled and unlabelled) (Figure 1-10). This modified Pyro-SIP effectively merges the microbial diversity investigation with a measurement of metabolic activity of the consortia and is relatively easy to use. As the first attempt, Pyro-SIP was applied to study the gut microbiota in an insect model, *Spodoptera littoralis*. The role of these bacteria in the gut, in terms of their local metabolic activities, was discussed too.

2. Thesis outline – List of articles and manuscripts and author's contribution

Article I

Crystallization of α- and β-carotene in the foregut of *Spodoptera* larvae feeding on a toxic food plant

Yongqi Shao, Dieter Spiteller, Xiaoshu Tang, Liyan Ping, Claudia Colesie,
Ute Münchberg, Stefan Bartram, Bernd Schneider, Burkhard Büdel,
Jürgen Popp, David G. Heckel, Wilhelm Boland

Insect Biochemistry and Molecular Biology 41 (2011) 273-281

This manuscript describes that the larva of *Spodoptera littoralis* (cotton leafworm), a polyphagous lepidopteran pest, acquires a distinct red-colored crop (a crop is the food-storage organ in insects' foregut) when consumes plant diets. By chemical characterization, especially the intact Raman microscopic analysis of the pigment *in situ*, we determined that beta carotene, not the most ubiquitous and abundant lutein in the foliage of host plant, was selectively accumulated in this unique organ and crystallized there. Although crystals were embedded in a homogenous lawn of the bacterium *Enterococcus casseliflavus*, the carotene seems to be selectively taken from the food plant. Caterpillars which failed to develop these carotene crystals exhibited a high mortality or failed to develop to adulthood. The crystallization of carotene and the enlargement of the foregut thus appear to manifest a multiple-step physiological adaptation of the insects to toxic food plants.

I performed the experiments, analyzed the data and wrote the draft of the paper. Dr. Dieter Spiteller and Dr. Liyan Ping helped in experimental design. Prof. Wilhelm Boland designed the research as well, refined the manuscript and supervised the work.

Article II

Complexity and Variability of Gut Commensal Microbiota in Polyphagous Lepidopteran Larvae

Xiaoshu Tang, Dalial Freitak, Heiko Vogel, Liyan Ping, Yongqi Shao, Erika Arias Cordero,

Gary Andersen, Martin Westermann, David G. Heckel, Wilhelm Boland

PLoS ONE 7(7): e36978

This manuscript analyzes the composition of bacterial communities in the gut of phytophagous Lepidoptera *Spodoptera littoralis* (a generalist feeder) and *Helicoverpa armigera* (a specialist feeder). We used a metagenomic approach based on 16S rDNA sequencing and microarray technology (Phylochip), which revealed that a simple gut microbiota consisting of e.g. *Enterococaceae*, *Lactobacilli*, and non-cultivable *Clostridia* was stable in both lepidopteran larvae. Changes occurred in response to larval development and to food plant toxins which allowed low abundant species to become dominant; most likely the gut microbiota serve as an organismic reservoir for adaption to environmental changes.

I performed some experiments in *Spodopter*a and analyzed a large proportion of the data. Prof. Wilhelm Boland, Prof. David G. Heckel, Dr. Heiko Vogel and Dr. Liyan Ping designed the research. Prof. Wilhelm Boland, Dr. Heiko Vogel and Dr. Liyan Ping wrote the manuscript.

Article III

In vivo Pyro-SIP analysis of the active gut microbiota of cotton leafworm, *Spodoptera littoralis*

Yongqi Shao, Huijuan Guo, Stefan Batram & Wilhelm Boland

Submitted

This manuscript characterizes the metabolic activity of the gut microbiota by using a combined pyrosequencing and *in vivo* stable isotope probing approach (Pyro-SIP). With ^{13}C glucose as the trophic link, Pyro-SIP revealed that a relatively simple but distinctive gut microbiota co-develops with the host, both metabolic activity and composition shifting throughout larval (developmental) stages. Three families, including *Enterococcaceae*, *Clostridiaceae* and *Enterobacteriaceae*, are particularly rich and active inside, which are likely the core functional populations linked to nutritional upgrading and pathogen defense. Not only establishes the first in-depth inventory of the gut microbiota of a model organism from the mostly phytophagous Lepidoptera, but this pilot study shows that Pyro-SIP can rapidly gain insight into the microbiota's metabolic activity with high resolution and high precision.

Prof. Wilhelm Boland and I designed the research. I performed experiments, analyzed the data and wrote the manuscript together with Prof. Wilhelm Boland.

3. Article I

Insect Biochemistry and Molecular Biology 41 (2011) 273–281

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Crystallization of α- and β-carotene in the foregut of *Spodoptera* larvae feeding on a toxic food plant

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ABSTRACT

In the animal kingdom, carotenoids are usually absorbed from dietary sources and transported to target tissues. Despite their general importance, the uptake mechanism is still poorly understood. Here we report the "red crop" phenomenon, an accumulation of α- and β-carotene in crystalline inclusions in the enlarged foregut of the polyphagous *Spodoptera* larvae feeding on some potentially toxic plant leaves. The carotene crystals give the insect foregut a distinctive orange-red color. The crystals are embedded in a homogenous lawn of the bacterium *Enterococcus casseliflavus*, but the carotene seems to be selectively taken from the food plant. Caterpillars which fail to develop these carotene crystals exhibit a high mortality or fail to develop to adulthood. The crystallization of carotene and the enlargement of the foregut thus appears to manifest a multiple-step physiological adaptation of the insects to toxic food plants.

1. Introduction

Carotenoids are synthesized by all photosynthetic plants and some microorganisms, as a component of the light harvesting apparatus and oxidative damage protectants. Until recently, it was believed that all animals are incapable of synthesizing them *de novo*, and must acquire them from the diet (Bhosale and Bernstein, 2007; Goodwin, 1986). However, Moran and Jarvik (2010) reported the presence of carotenoid biosynthetic genes in aphids, which appear to have been laterally transferred from fungi. Carotenoids are required by animals because they are the precursor of vitamin A, the light-detecting molecule in vision and developmental hormones (Means and Gudas, 1995). Based on the stereochemical configuration, the two most dominant hydrocarbons are named as α- and β-carotene, respectively. When oxidized, they are termed xanthophylls. The most abundant carotenoid in nature is lutein (α-carotene-3′3-diol), which accounts for more than 50% of the carotenoids in the angiosperm photosynthetic tissue (Pogson et al., 1996).

Carotenoids are also responsible for the pigmentation of many arthropods, where they either occur as free pigments or as carotenoproteins (Goodwin, 1986). The cuticle color of the larva of *Manduca sexta* is influenced by the relative concentration of β-carotene and the xanthophyll violaxanthin (Mummery and Valadon, 1976). The coloration of the silkworm (*Bombyx mori*) cocoons is also due to incorporation of carotenoids (Sakudoh et al., 2007). The camouflaging yellow hair tufts of *Orgyia antiqua* are the result of lutein accumulation (Sandre et al., 2007). When feeding on wild parsnip, the parsnip webworms (*Depressaria pastinacella*) sequester lutein into their fat body and develop a distinct yellow strip on the body (Carroll et al., 1997). In the butterfly *Inachis io*, lutein can be actively transported to the pupal cuticle to control the coloration (Starnecker, 1997). The egg yolk of the stick insect *Anisomorpha buprestoides* is intensely red-colored, because of the presence of a novel xanthophyll, 3,4-didehydro-β,β-carotene-2-one (Davidson et al., 1991), a derivative of β-carotene (Goodwin, 1986).

Abbreviations: cryo-SEM, cryo-scanning electron microscopy; DIP, direct insert probe; EI, electron impact; HPLC, high pressure liquid chromatography; HRMS, high resolution mass spectrometry; TBME, *tert*-butyl methyl ether; TLC, thin layer chromatography.

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Carotenoids are hydrophobic molecules, and can therefore be absorbed by the lipid membrane through passive diffusion (Bhosale and Bernstein, 2007; Goodwin, 1986). This appears to be responsible for the yellow color conferred by accumulation of carotenoids by the mandibular glands of *Helicoverpa zea* larvae feeding on leaves in particular soybean (Eichenseer et al., 2002). Among 38 Lepidoptera species analyzed, the majority tend to store the carotenoids from their food unchanged (Feltwell and Rothschild, 1974). Carotenoid level is high when they are reared on carotenoid-rich diet. On the other hand, some insects have developed active transport systems to accumulate carotenoids (Bhosale and Bernstein, 2007). The *Y* gene that determines the yellowish hemolymph and cocoon of silkworm (*B. mori*) encodes an intracellular carotenoid-binding protein, which enhances carotenoid uptake (Sakudoh et al., 2007). The *ninaD* gene in *Drosophila* encodes a class B scavenger receptor which mediates the uptake of carotenoids (Kiefer et al., 2002). The incorporation of lutein into pupal cuticle of the butterfly *I. io* is subjected to hormonal control (Starnecker, 1997). In vertebrates and invertebrates, carotenoid-binding proteins preferably transfer xanthophylls but not the more hydrophobic carotene (Bhosale and Bernstein, 2007). The only binding protein showing high affinity to β-carotene was purified from ferret liver (Rao et al., 1997).

Here we report a different type of carotenoid uptake phenomenon involving the crystallization of α- and β-carotene in the foregut of *Spodoptera* species. These insects are highly polyphagous and utilize hosts in 40 plant families including some economically important species (Carter, 1984; Daly et al., 1998). When feeding on leaves of lima bean and other potentially toxic hosts, a conspicuous red color developed in their foregut due to the selective sequestration of α- and β-carotene into crystals.

2. Material and methods

2.1. Caterpillar rearing and morphological observation

Eggs of *Spodoptera littoralis*, *Spodoptera exigua*, *Helicoverpa armigera* (Bayer Cropscience, Monheim, Germany), *Spodoptera eridania* (BASF Corporation, Florham Park NJ, USA) were hatched and reared on an artificial diet (Spiteller et al., 2000). Lima bean (*Phaseolus lunatus* strain CV_JWBJ A), cotton (*Gossypium hirsutum* DP 90), sorghum (*Sorghum bicolor*/x/*Sorghum sudanese*), barley (*Hordeum vulgare* subsp. *vulgare* Cultivar: Barke), Arabidopsis (*Arabidopsis thaliana* strain Columbia) and snap bean (*Phaseolus vulgaris* var. Saxa) were cultivated in our greenhouse. Chinese cabbage (*Brassica rapa pekinensis*) was purchased from the local market. Insects were reared on seedlings showing two to six fully developed leaves at 23 °C and 60% humidity with a photo-period of 16 h. Small larvae were reared in a box and supplied with shoots of plant seedlings. To keep the leaves fresh, shoots were submerged in water. Fresh cuttings were supplied every day and the old ones removed. Insects older than 6 days were reared on seedlings growing in pots (1 caterpillar per plant).

To quantify the insect fitness, 50 6-day-old larvae were grown on Lima bean in pots, their number was counted and body weight was measured after 2, 4 and 7 days. After feeding on lima bean for 7 days, insects were dissected to determine the red crop development. Red crystals on the inner surface of the crop were visible under a microscope. The experiment was repeated 3 times, and mean and standard deviation calculated. Other 150 larvae were kept feeding on lima bean until prepupation. As soon as the insects begin to shrink in size, they were dissected to observe the red crop development.

To analyze the anatomy, last-instar larvae were starved for 4 h and washed three times with 70% ethanol and distilled water. The insects were kept at −20 °C for 30 min, and thawed completely on ice before dissection. The cuticle was cut open under a dissecting microscope. To further empty the foregut, a hole was cut immediately behind the proventriculus (Fig. 1A). Due to the contraction of the alimentary tract, the food bolus was squeezed out from the hole. The emptied foregut then shrank to a small size. If the foregut had been folded into the lumen of the midgut, it was drawn out by pulling on the insect head with a pair of tweezers. Images were taken with a Stemi SV 11 stereomicroscope (Carl Zeiss, Jena, Germany) equipped with an AxioCam HRC camera.

2.2. Light microscopy and cryo-electron microscopy

The emptied foregut was washed in water, and placed into a microcentrifuge tube. The tissue was either spread on a SuperFrost Ultra Plus slide (Thermo Scientific) or cut with a cryomicrotome (2800 Frigocut-E; Leica, Wetzlar, Germany) into 10 μm thin sections and examined with a Zeiss Axiophot fluorescence microscope (Carl Zeiss, Jena, Germany) as described by Mankel et al. (2002). To reveal the bacteria, thin sections were stained with 1 μg/ml DAPI solution.

For cryo-scanning electron microscopy (cryo-SEM), fresh larval foregut tissue was fixed onto a specimen-holder with the inner surface upward. The loaded specimen-holder was submerged in slush-nitrogen and then transferred to a high vacuum preparation chamber of a cryo-unit (K1250X Cryogenic Preparation System, Quorum Technologies Ltd, Ashford, Great Britain) at −130 °C. After sublimation at −90 °C for 30 min, the sample was sputter-coated with gold–palladium and transferred to the Low-Temperature-Field-Emission-Scanning-Electron-Microscope (LT-FE-SEM; SUPR A55VP, Carl Zeiss NTS, Oberkochen, Germany) and viewed at a temperature of −130 °C and 5 kV accelerator voltage.

2.3. Purification and identification of the major crystal component

Clean foregut tissue collected from about 100 larvae was kept at −20 °C. The tissue was suspended in 2 ml distilled water and sonicated with a Sonopuls homogenizer (Bandelin, Berlin Germany). The remaining gut tissue was taken out with tweezers. Detached crystals were collected by centrifugation as pellet. The crystals were resuspended in water. The suspension was frozen and thawed 3 times during which the solidified insect lipids on the top of the suspension were taken out with a pipette. After removing the water, the crystals were dissolved in chloroform. The chloroform solution was applied to a silica-gel thin layer chromatography (TLC) plate and developed with a solvent system containing 99% petrol ether and 1% ethyl acetate. The obtained yellow spot from the TLC plates was collected for further analysis.

The yellow compound collected from TLC plate was dissolved in ethyl acetate and measured with a UV/vis-Spectrometer V-550 (Jasco, Maryland, USA). Tandem mass spectrometry (MS/MS) analysis was carried out by direct injection into a Finnigan LTQ machine (Thermo Electron GmbH, Bremen, Germany). The accurate mass of the compound was measured by direct insert probe (DIP) into a MASSPEC masspectrometer (Micromass, Manchester, UK), temperature ramp 0.5 min 50 °C, 200 °C/min to 400 °C, 2 min 400 °C, electron impact (EI) mode (70 eV). The chloroform solution was dried and redissolved in deuterated acetone-d_6 for NMR analysis. ^{1}H NMR spectra (500.13 MHz) were measured on a Bruker Avance 500 NMR spectrometer equipped with a TCI CryoProbe™ (5 mm) (Bruker Biospin, Rheinstetten, Germany). Tetramethylsilane was used as an internal reference. Topspin™ 2.0 software was used to control the NMR spectrometer and for data processing.

Resonance Raman spectra were measured with a micro-Raman setup (HR LabRam inverse, Jobin Yvon, Horiba). The spectrometer

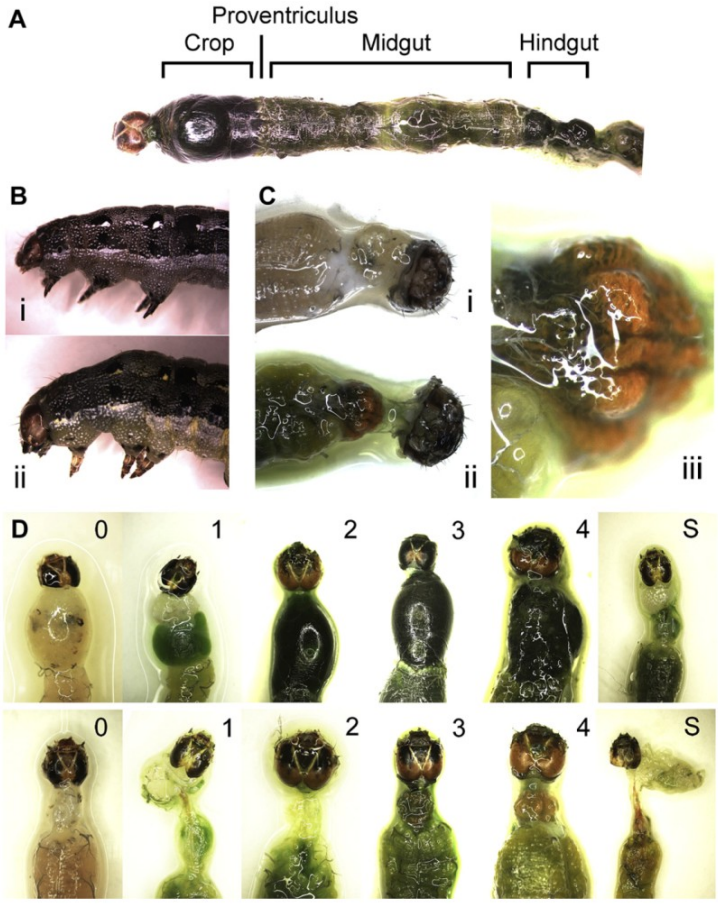

Fig. 1. The development of red crop in *S. littoralis* after feeding on Lima bean. (A) Entire digestive tract of a healthy larva feeding on Lima bean for 8 days. The foregut is full of leaf material. (B) Larval thorax. The upper panel is the thorax of a control insect (i). The lower panel is the thorax of a red crop insect (ii). Note the extended first thoracic section and the shortened second thoracic section. (C) Ventral view of empty foreguts. (i) Foregut of a control larva. (ii) Foregut of a larva feeding on lima bean for 8 days. (iii) Enlarged view of the foregut in (ii). (D) Development of the red crop in 6-day old larvae after switching to lima bean from artificial diet. The upper panels show foreguts full of leaf pieces. The lower panels show empty foreguts. The numbers on the up-right corner of each panel are the number of days after changing the food. The panels labeled "S" show one of the 30% sick larvae that failed to develop the red crop and did not grow in the last 4 days. The white rings behind the heads in 1-day and "S" larvae are enlarged mandibular glands, they were manually displaced in the lower panels to show the brownish gut tissue. Note the midgut tissue of the "S" larva is also brownish due to intoxication.

had a focal length of 800 mm, an entrance slit of 100 μm and was equipped with a 300 lines/mm grating. For excitation, the 532 nm line of a frequency-doubled Nd:YAG laser (Coherent Compass) was used with a power of 1.5 mW incident on the sample. An inverse Olympus microscope BX41 with 100× objective (Leica PLFluotar L100×/0.75) focused the laser light onto the sample with a spot size of less than 1 μm in diameter. Raman scattered light was detected by a Peltier cooled CCD camera operating at 220 K. Samples were measured on fused silica slides with an integration time of 1 s. For measurement in the insect tissue, clean foregut tissues that had been kept in water were spread on a slide and dried at room temperature. Crystal extracts and synthetic β-carotene were dissolved in chloroform, spotted onto slides and measured after evaporation of the solvent. Spectra were baseline corrected and vector normalized.

2.4. Bacterial isolation and identification

The bacteria from control insects and insects with red crop were isolated on Brain Heart Infusion (BHI) plates and identified by PCR and cloning as described previously (Ping et al., 2007). In brief, the almost full length of bacterial 16S rDNA was amplified with a general primer pair 27f and 1492r. The PCR product was cloned with a TOPO TA Cloning Kit (Invitrogen) and sequence. The 16S rDNA sequence was deposited at Genbank with accession number HQ264067. The Neo-Sensitabs and DiaTabs (Rosco Diagnostica A/S, 2630 Taastrup, Denmark) were used for further confirmation. Isolated bacteria were grown in liquid BHI medium at 37 °C for further analysis. In order to screen for carotenoid production, bacteria were precipitated by centrifugation at 16,000*g* for 30 min, and the pellet was extracted and analyzed.

2.5. Carotenoid profiling and isotopic ratio analysis

Total carotenoids were extracted from Lima bean leaves and the foregut and midgut of 11 caterpillars and analyzed with high pressure liquid chromatography (HPLC) as described by Eichenseer et al. (2002). Standard chemicals were purchased from Sigma. Samples were analyzed using high performance liquid chromatography (HPLC) by an Agilent HP1100 system (Böblingen, Germany) equipped with a UV detector. The compounds were separated by gradient elution using a ProntoSIL 200-3-C30 column (3 μm, 250 × 4.6 mm, Bischoff Chromatography, Leonberg, Germany) at a flow rate of 1.0 ml/min with methanol and *tert*-butyl methyl ether (TBME) as solvents. HPLC condition: From 10% TBME to 45% TBME in 35 min followed by 45% TBME to 60% in 15 min, eluting compounds were recorded at 470 nm. The separated compounds were detected with mass spectroscopy, an LCQ (ThermoFinnigan) with atmospheric pressure chemical ionization (APCI).

Carbon isotopic ratio can be a reliable indicator of the origin of the biomolecules; it was used to reveal the origin of the carotene in the crystals. 0.1 mg dried and ground material were put in 0.04 ml tin capsules (3.5 × 5 mm, HEKATech GmbH, Wegberg, Germany). After sealing, the samples were combusted (oxidation at 1020 °C, reduction at 650 °C) in a constant helium stream (80 ml min^{-1}). The samples were completely converted to CO_2, N_2 and H_2O using an elemental analyzer (EuroEA CN2 dual, HEKAtech GmbH). After passing a water trap ($MgClO_4$), the gases were separated chromatographically at 85 °C and transferred via an open split to a coupled isotope ratio mass spectrometer (IsoPrime, Micromass, Manchester, UK). The acetanilide standard (0.1 ± 0.03 mg) was calibrated on the international VPDB scale (Vienna Pee Dee Belemnite) using NBS 22 (IAEA reference material) with a $\delta^{13}C$ value of −30.03‰ (Coplen et al., 2006). A caffeine standard (0.07 mg ± 30%) was analyzed together with the samples as QA reference material (Werner and Brand, 2001). Empty tin capsules were used as blanks. Each sample was measured at least three times. Isotopic ratios are expressed in δ notation versus the VPDB (Vienna Pee Dee Belemnite) standard:

$$\delta^{13}C_{VPDB} = \frac{(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{VPDB}}{(^{13}C/^{12}C)_{VPDB}} \times 1000(‰)$$

3. Results

3.1. Description of the phenomenon

In the digestive tract of lepidopteran larvae, the foregut is composed of an esophagus and an enlarged crop, which holds food prior to its passage into the midgut. The crop is connected to the midgut by a muscular proventriculus (Daly et al., 1998). When larvae of the generalist herbivore *S. littoralis* fed on Lima bean, the crop was significantly enlarged compared to the insects feeding on artificial food [Fig. 1C(i) versus C(ii)]. This enlargement could be easily observed by looking at the intact insects. The first thoracic section became longer and the second and third sections became shorter. When the gut content was emptied during dissection, the crop shrank into a wrinkled red-colored ring. Lima bean is toxic for *S. littoralis*. If the 1st instar larvae were given Lima bean, only 5% larvae survived to adulthood. When the 3rd instars larvae were given Lima bean, there is always a growth retardation at the beginning of the food change. After about 4 days, about 60% recovered and grew as well as, or even faster than the control insects feeding on artificial diet. One third of the larvae stopped to grow, died or were not able to pupate (Fig. 2).

The insect recovery process involves a physiological transition, during which the red crop developed (Fig. 1D). In a third instar larva, after switching to Lima bean, the first physiological change was the enlargement of the mandibular glands. The originally hard-to-detect mandibular glands formed a large white ring surrounding the esophagus and part of the crop in the first day. Underneath the mandibular gland, the foregut inner surface became slightly brownish due to cell necrosis. On the second day, the mandibular glands returned to their normal size. The disappearance of the brownish dead tissue was accompanied by the appearance of a few red crystals on the crop inner surface. Later on the crop became larger and larger, and the red color became deeper and deeper. On the fourth day, a wrinkled light orange crop was detectable in most of the recovered insects. The color became deeper and the size became larger if the insect kept on feeding on Lima bean. In some extreme cases, we have observed the foregut reaching half the length of the insect. The very large red crop folded into the midgut lumen when the insects were starved. In the meantime, those larvae that failed to develop the red crop grew poorly, usually with reduced appetite. When others recovered from the plant toxicity, these insects kept an enlarged mandibular gland, and their foregut and even the midgut showed severe cell necrosis syndrome (Fig. 1D).

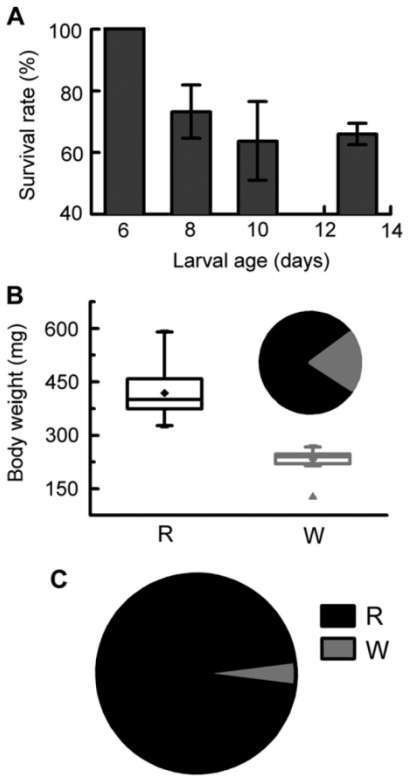

Fig. 2. The larvae with red crop survive better when feeding on Lima bean than those with white crops. (A) Survival rate of 6-day-old *S. littoralis* larvae feeding on Lima bean. (B) Body weight of larvae with a red crop (R) and larvae with a white crop (W) after feeding on lima bean for 7 days. The percentage of larvae with and without red crop is shown as an insert. (C) Percentage of prepupating larvae with a red crop (R) and larvae with a white crop (W).

3.2. The red crop is filled with crystals and bacteria

Under the light microscope, some clear semitransparent orange-red crystals a few micrometers in size were found to be responsible for the red appearance of the crop (Fig. 3). The crystals were embedded in a lawn of a single bacterial species, *Enterococcus casseliflavus*. This bacterium is an indigenous gut inhabitant (data not shown). In the midgut of normal insects, it is one of the major species of the microbiota. Neither on the artificial food nor plant materials could it be detected. It only thrives in the foregut when the insect is shifted to feeding on lima bean. They form large clusters in the red crop. Bacterial cells and the crystals were intimately associated with some extracellular matrix secreted by the bacteria (Fig. 3B). No crystals had been found outside the bacterial clusters under cryo-SEM, where the native state of the gut surface was conserved. Before feeding on Lima bean, not only were the red crystals absent, but bacteria in the foregut were also sparse and heterogeneous (Fig. 3C). In the red crop, the uniform *Enterococci* arose simultaneously with the red crystals. Actually, we could not separate the purified crystals from the associated bacterial cells completely through heating, freezing or sonication.

3.3. Similar phenomena in other species

A similar phenomenon was observed in *S. exigua* larvae feeding on Lima bean, though the size and color of the crop were not as remarkable as in *S. littoralis* (Supplementary data, Fig. S1). We also tested *S. eridania*, a species known to have adapted to Lima bean (Lindroth and Peterson, 1988). We could not detect any red crystals in their foreguts. In another lepidopteran species *H. armigera*, we

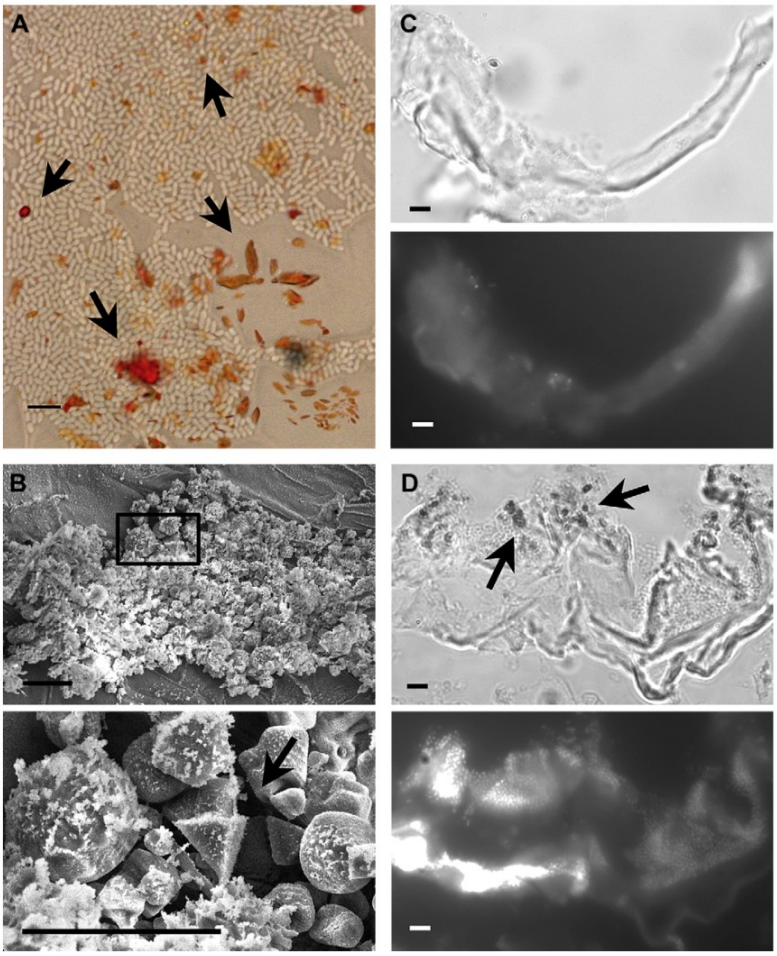

Fig. 3. Microscopic analysis of the red crop. Scale bars equal 5 μm. Arrows indicate crystals. (A) Typical image of the inner surface of a red crop after spreading on a glass slide. (B) Cryo-scanning electron microscopic image of a bacterial cluster on the crop inner surface. The boxed area was enlarged in the lower panel. (C) Thin section of a control crop from larvae feeding on artificial diet. The lower panel displays the fluorescent image of the same area after DAPI staining showing a few bacteria with different morphology. (D) Thin section of a red crop. The lower panel is the fluorescent image after DAPI staining, showing large numbers of bacteria.

could detect some red crystals in the foregut of Lima bean-feeding insects, but the crystals were flat sheets and in deep red color, and the amount was very low (Supplementary data, Fig. S2). The red crop could also be observed in *S. littoralis* feeding on other plant materials such as sorghum and snap bean. It was not detectable when the insect fed on its natural host plant cotton and barley. It is worth to mention that the insects grow healthily on these non-toxic plants, and the survival rate is always 100%. When *S. littoralis* fed on *Arabidopsis*, some smaller crystals, which were slightly red and flat-sheet shaped, were observed, but the amount of crystal was very small. Even this flat-sheet crystal was not detectable when the insect fed on Chinese cabbage.

3.4. The crystal is predominantly composed of β-carotene

The crystals were stable in an aqueous environment, but quickly dissolved in organic solvents such as chloroform. The yellow compounds extracted from the dissolved crystal were separated into 3 bands on silica TLC plates. The largest band migrates quickly with petrol ether containing 1% of ethyl acetate ($R_f = 0.91$) with a faint band following ($R_f = 0.51$). Some yellow compounds formed another band which did not move with the solvent ($R_f = 0$). The amount of the compound, which does not move on the plate, often varied between preparations, but was always small. The MS/MS fragmentation pattern and the UV spectrum of the fast-moving major component were very similar to that of β-carotene (Fig. 4). High resolution mass spectrometry (HRMS) was measured in the EI mode. The compound was evaporated and ionized at 400 °C in a DIP interface. The measured mass of the purified compound was m/z 536.4396, and the calculated mass of β-carotene or α-carotene is m/z 536.4382. Diagnostic signals in the NMR spectra and Raman spectra could be easily identified even within the crude extract by comparison with the authentic standard (Fig. 4C and D). With the micro-Raman setting direct measurement of the crystals in the insect tissue was carried out. The spectra obtained from the crystalline structures, the crude extract, and the synthetic β-carotene showed the same signature signals of C=C stretching, C–C stretching and CH_3 rocking vibrations (Fig. 4D) as reported (Tschirner et al., 2009).

3.5. The β-carotene is taken from food plant

We could confirm that the major component was β-carotene by comparing its retention time by cochromatography with authentic external standards (Fig. 5A). The other two bands on the TLC plates were identified as lutein and α-carotene, respectively, based on their retention time and mass spectra. Lima bean leaves contain all of these carotenoids, but lutein is the dominant component. The ratio of the amount of α-carotene to β-carotene in Lima bean leaves (1:2.23) is very similar to that found in the red crop crystal (1:3.16). The isolated bacteria, however, did not produce any carotenoids. After feeding on plants, whether the red crystal formed or not, the foregut and midgut tissues of the insects became greenish yellow. When the midgut wall was extracted, the relative ratios of the carotenoids adsorbed on the gut wall are similar to those in the plant. Other xanthophylls were not detectable in either plant leaves or insect tissues. The $\delta^{13}C$ value of the carotene in crystals is statistically indistinguishable from that of

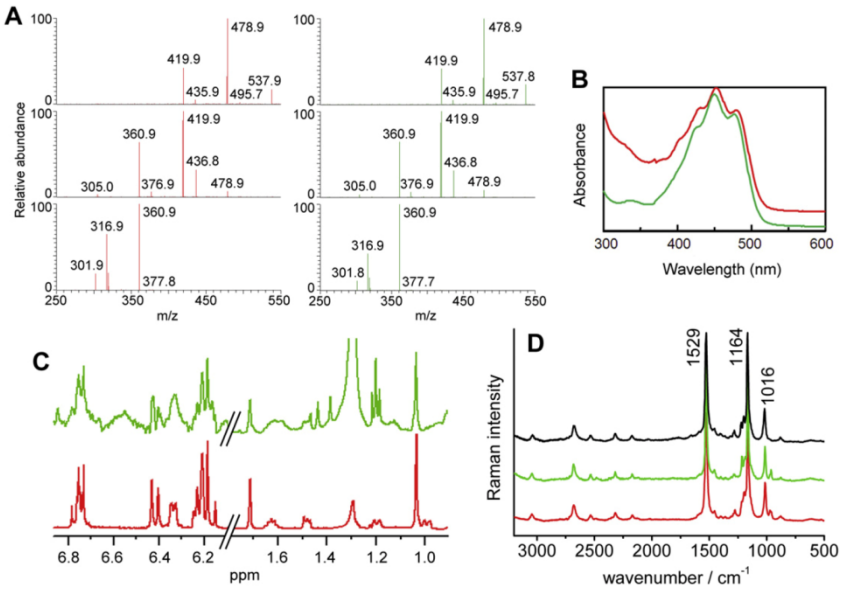

Fig. 4. Identification of the major component of the crystals. (A) Comparison of the MS/MS fragmentation between the purified (right) and carotene standard (left). The MS/MS of $m/z = 537.8$ was first fragmented by collision with He^{2+} ions into smaller ions (upper panels). The middle panel shows the fragments derived from $m/z = 478.9$. The bottom panel show the fragments derived from $m/z = 419.9$. (B) UV absorption spectra of the purified compound (red) and synthetic β-carotene (green) in ethyl acetate. (C) ^1H NMR spectra (500 MHz) in acetone-d_6. Upper panel: signals of purified compounds (α/β-carotene). Lower panel: authentic β-carotene. The signal between 6.15 ppm and 6.80 ppm correspond to double bond signals; the signal at 1.72 ppm corresponds to allylic methyl group; the signal at 1.03 ppm corresponds to geminal dimethyl group. (D) Resonance Raman spectra. Upper trace: purified compound from the insects' crop. Middle trace: direct measurement of the crystals in the insect crop. Lower trace: authentic β-carotene in red. The main signals at 1527 cm^{-1} and 1167 cm^{-1} originate from stretching vibrations of the C=C and C–C bonds of the polyene chain respectively, while the peak at 1016 cm^{-1} is due to rocking vibrations of the methyl groups. (For interpretation of reference to color in this figure, please refer to the web version of this article).

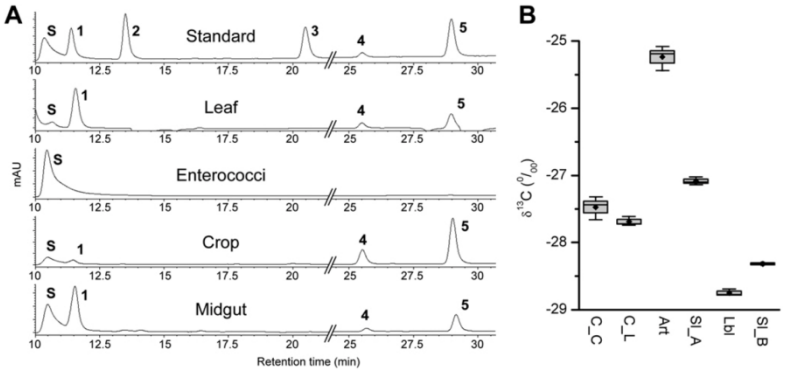

Fig. 5. α- and β-carotene were selectively taken up from food plant. (A) Liquid chromatographic analysis of carotenoids from different sources. The chromatogram of standards is shown on the top: 1, lutein; 2, zeaxanthin; 3, cryptoxanthin; 4, α-carotene; 5, β-carotene; S, astaxanthine. Other chromatograms represent: Leaf, lima bean extract; *Enterococci*, cell-free Enterococcal culture; Crop, red crop extract; Midgut, yellow midgut tissue extract. (B) Isotopic ratio ($d^{13}C$) of different samples. C_C, carotene purified from crystal; C_L, carotene purified from lima bean leaf; Art, dried artificial food; Sl_A, larvae of *S. littoralis* feeding on artificial food; Lml, dried lima bean leaves; Sl_B, insect feeding on lima bean leaves.

the carotenoids from leaf material, which is very different from the artificial diet and the insect itself (Fig. 5B).

4. Discussion

Carotenoids are highly abundant in plant leaves (Pogson et al., 1996). Because of their high lipophilicity, they are easily absorbed by the insect cellular membrane through passive diffusion. They can be found in the gut, fat body, epidermis and hemolymph of lepidopteran larvae (Starnecker, 1997). It has already been noticed that the midgut epithelium and mandibular glands of *H. zea* became yellow after feeding on plant materials, and the relative ratio of lutein and β-carotene absorbed into the gut is similar to that in plant leaves (Eichenseer et al., 2002). In all the lepidopteran species we tested, the color of their gut wall changed from white to greenish yellow after feeding on plant leaves, due to passive absorption of carotenoids and possibly some chlorophyll degradation products, which are green and lipophilic (Mangos and Berger, 1997). Our results suggest that at least the uptake of carotenoids by the gut tissue was passive, because the relative amount of the carotenoids is similar to that found in Lima bean leaves (Fig. 5A). It has been noticed that in general high carotenoids in the diet enhance the yellow coloration of Lepidoperan larvae, pupae and adults (Feltwell and Rothschild, 1974). Lutein, the dominant plant carotenoid, is present probably in the body of all species of Lepidoptera, and often the most abundant one.

However, the crystallization of α- and β-carotene in the crop is most likely associated with the physiological adaptations of insects to a toxic plant diet. When the insects were switched to natural host plant such as cotton and barley, they do not develop the red crop. The red crop inducing plants, such as lima bean and sorghum are known as cyanogenic (Ballhorn et al., 2008; Panasiuk and Bills, 1984). But the non-cyanogenic snap bean (Ballhorn and Lieberei, 2006) induces the crystal formation as well as the cyanogenic plants. It is interesting to note that the glucosinolate producing *Arabidopsis* (Kliebenstein et al., 2001), but not the less toxic Chinese cabbage, induces the accumulation of carotenoid crystal to a lesser extent, and possibly with slightly different chemical composition, which awaits further investigation. Interestingly, in the crop of *S. eridania*, a species adapted to lima bean, we could not detect any crystals. In an earlier report, lutein was the only detectable carotenoid in the body of *S. eridania* and β-carotene was not detected (Ahmad and Pardini, 1990). However, which part of the insect was sampled in this study was not specified.

What is also of interest is the change in insect body during the physiological adaptation. The enlargement of the mandibular gland is the first step (Fig. 1D). It appeared at the time when insect tissue damage caused by the suboptimal food plant was obvious, and disappeared quickly after the appearance of carotene crystals. Unlike the mandibular glands of *H. zea* which were stained yellow by carotenoids (Eichenseer et al., 2002), the enlarged glands of *S. littoralis* were not colored. During the crystallization of carotene, the size of the crop is continuously enlarged, which may result in a prolonged storage time of the food prior to digestion.

Without carotene crystal, or the physiological process buoyed by the crystals, the insect would be poisoned by certain plant-derived material(s), as indicated by the brownish necrotic gut tissue of the insect feeding on Lima bean within a day and of those failed to develop the red crop (Figs. 1 and 2). Herbivore attack caused significant increase of the oxidative radical formation, which might cause damage to both the plant and the herbivore insect (Bi and Felton, 1995). In plants and protozoa, carotenoids in soluble state are known to scavenge singlet oxygen ($^1O_2$) and free radicals (Goodwin, 1986). However, crystallization is often associated with a reduction of chemical reactivity, e.g. in blood-feeding insects free heme is crystallized to suppress the generation of free radicals (Oliveira et al., 2007). Carotenoids are also known to help some insects defend against phototoxicity (Aucoin et al., 1995). However, in the body of the lepidopterans that consume phototoxic plants, lutein, which is more effective on scavenging free radicals than the hydrocarbons, is always the dominant carotenoid and the concentration level mirrors the lutein content of their diets (Ahmad and Pardini, 1990).

Carotenoids are known to form crystals at high concentration. When the phytoene synthase gene was overexpressed in *A. thaliana*, carotene formed small crystals in the plastid of the root (Maass et al., 2009). The β-carotene concentration in fresh cotton leaves has been reported at 1621 μg/g (Ratnayaka et al., 2003). The concentration in fresh lima bean leaves has been determined to be 209 μg/g (Wall and Kelley, 1943). Even considering different methodologies employed, the natural host plant cotton does not contain less carotenoids than the toxic lima bean. There must be a factor controlling the crystallization in the red crop. The relative ratio of α- and β-carotene seems not change much in crystals compared to the plant leaves, but the oxidized derivative lutein was

excluded from the crystals (Fig. 5A). The trace amount of lutein detected in dissolved crystals probably came from the attached bacterial or insect cellular membrane. Due to their different metabolic pathways, the stable isotopes of carbon and hydrogen will be selectively enriched or reduced in different organisms, e.g. the $\delta^{13}C$ value of β-carotene produced by plant is very different from that produced by microalgae (Kroll et al., 2008). The $\delta^{13}C$ value of the carotenoid crystallized in the crop of the insects is statistically indistinguishable from those purified from Lima bean, indicating that it was taken from the food plant.

The carotene cannot be a bacterial product. Although *Enterococci* were topically closely associated with the crystals, we could not detect any β-carotene in the bacterial cell culture (Fig. 5A). The thrive of *Enterococci* in the red crop might be the consequence of cyanide selection because they are cyanide tolerant due to the homolactic fermentative respiration pathway (Leblanc, 2006). Some *Enterococci* are known to produce carotenoids (Taylor et al., 1971). However, the *E. casseliflavus* strain isolated from the insect gut is not yellow-pigmented like the known carotenoid-producing strains. On the other hand, the bacterial carotenoids are not C_{40} molecules, but likely to be a C_{32} acyclic carotenoid aldehydes (Taylor et al., 1971). Although the selective sequestration of carotene can be partially explained by the crystallization procedure, which excludes structurally different molecules, the main question still remains, namely the nucleation factor must be highly selective. It should only bind carotenes, not xanthophylls. Whether this nucleation factor is of insect origin or bacterial origin is another interesting question to be addressed.

5. Conclusion

We observed the crystallization of α- and β-carotene in the foregut of *Spodoptera* larvae, when feeding on certain toxic plants such as *P. lunatus*. The insect crop was simultaneously enlarged while its inner surface was covered with carotene crystals. A single bacterial species, *E. casseliflavus* was found tightly associated with the red crystal. Sequestration of carotenoids can protect caterpillars from UV radiation or free radicals, but the chemical reactivity of slowly dissolving crystals is reduced. However, only those insects which develop the red crop can recover from intoxication by the food plant. Most individuals that fail to develop the red crop, do not recover and finally die. Thus the presence of carotene crystals and/or the presence of *E. casseliflavus* in the foregut should represent a yet unknown mechanism in insect adaptation to toxic food.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ibmb.2011.01.004.

References

Ahmad, S., Pardini, R.S., 1990. Mechanisms for regulating oxygen toxicity in phytophagous insects. Free Radic. Biol. Med. 8, 401–413.
Aucoin, R., Guillet, G., Murray, C., Philogène, B.J.R., Arnason, J.T., 1995. How do insect herbivores cope with the extreme oxidative stress of phototoxic host plants? Arch. Insect Biochem. Physiol. 29, 211–226.
Ballhorn, D., Lieberei, R., 2006. Oviposition choice of Mexican bean beetle (*Epilachna varivestis*) depends on host plants cyanogenic capacity. J. Chem. Ecol. 32, 1861–1865.
Ballhorn, D.J., Kautz, S., Lion, U., Heil, M., 2008. Trade-offs between direct and indirect defences of lima bean (*Phaseolus lunatus*). J. Ecol. 96, 971–980.
Bhosale, P., Bernstein, P.S., 2007. Vertebrate and invertebrate carotenoid-binding proteins. Arch. Biochem. Biophys. 458, 121–127.
Bi, J., Felton, G., 1995. Foliar oxidative stress and insect herbivory: primary compounds, secondary metabolites, and reactive oxygen species as components of induced resistance. J. Chem. Ecol. 21, 1511–1530.
Carroll, M., Hanlon, A., Hanlon, T., Zangerl, A.R., Berenbaum, M.R., 1997. Behavioral effects of carotenoid sequestration by the parsnip webworm, *Depressaria pastinacella*. J. Chem. Ecol. 23, 2707–2719.
Carter, D.J., 1984. Pest Lepidoptera of Europe: With Special Reference to the British Isles. Dr. W. Junk Publishers, Dordrecht, and the Trustees of the British Museum (Natural History), London.
Coplen, T.B., Brand, W.A., Gehre, M., Gröning, M., Meijer, H.A.J., Toman, B., Verkouteren, R.M., 2006. New guidelines for $\delta^{13}C$ Measurements. Anal. Chem. 78, 2439–2441.
Daly, H.V., Doyen III, J.T., Purcell, A.H., 1998. Introduction to Insect Biology and Diversity, second ed. Oxford University Press, Oxford.
Davidson, B.S., Eisner, T., Meinwald, J., 1991. 3,4-Didehydro-beta, beta-caroten-2-one, a new carotenoid from the eggs of the stick insect *Anisomorpha buprestoides*. Tetrahedron Lett. 32, 5651–5654.
Eichenseer, H., Murphy, J.B., Felton, G.W., 2002. Sequestration of host plant carotenoids in the larval tissues of *Helicoverpa zea*. J. Insect Physiol. 48, 311–318.
Feltwell, J., Rothschild, M., 1974. Carotenoids in thirty-eight species of Lepidoptera. J. Zool. 174, 441–465.
Goodwin, T.W., 1986. Metabolism, nutrition, and function of carotenoids. Annu. Rev. Nutr. 6, 273–297.
Kiefer, C., Sumser, E., Wernet, M.F., von Lintig, J., 2002. A class B scavenger receptor mediates the cellular uptake of carotenoids in *Drosophila*. Proc. Natl. Acad. Sci. U.S.A. 99, 10581–10586.
Kliebenstein, D.J., Kroymann, J., Brown, P., Figuth, A., Pedersen, D., Gershenzon, J., Mitchell-Olds, T., 2001. Genetic control of natural variation in *Arabidopsis* glucosinolate accumulation. Plant Physiol. 126, 811–825.
Kroll, H., Friedrich, J., Menzel, M., Schreier, P., 2008. Carbon and hydrogen stable isotope ratios of carotenoids and β-carotene-based dietary supplements. J. Agric. Food Chem. 56, 4198–4204.
Leblanc, D., 2006. Enterococcus. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E. (Eds.), The Prokaryotes. Springer, New York, pp. 175–204.
Lindroth, R.L., Peterson, S.S., 1988. Effects of plant phenols on performance of southern armyworm larvae. Oecologia 75, 185–189.
Maass, D., Arango, J., Wüst, F., Beyer, P., Welsch, R., 2009. Carotenoid crystal formation in *Arabidopsis* and carrot roots caused by increased phytoene synthase protein levels. PLoS One 4, e6373.
Mangos, T.J., Berger, R.G., 1997. Determination of major chlorophyll degradation products. Z. Lebensm. Unters. F. A 204, 345–350.
Mankel, A., Krause, K., Kothe, E., 2002. Identification of a hydrophobin gene that is developmentally regulated in the ectomycorrhizal fungus *Tricholoma terreum*. Appl. Environ. Microbiol. 68, 1408–1413.
Means, A.L., Gudas, L.J., 1995. The roles of retinoids in vertebrate development. Annu. Rev. Biochem. 64, 201–233.
Moran, N.A., Jarvik, T., 2010. Lateral transfer of genes from fungi underlies carotenoid production in aphids. Science 328, 624–627.
Mummery, R., Valadon, G., 1976. The carotenoids of a blach variety of the larva of the moth, *Manduca sexta*. J. Insect Physiol. 22, 1219–1221.
Oliveira, M.F., Gandara, A.C.P., Braga, C.M.S., Silva, J.R., Mury, F.B., Dansa-Petretski, M., Menezes, D., Vannier-Santos, M.A., Oliveira, P.L., 2007. Heme crystallization in the midgut of triatomine insects. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 146, 168–174.
Panasiuk, O., Bills, D.D., 1984. Cyanide content of sorghum sprouts. J. Food Sci. 49, 791–793.
Ping, L., Büchler, R., Mithöfer, A., Svatoš, A., Spiteller, D., Dettner, K., Gmeiner, S., Piel, J., Schlott, B., Boland, W., 2007. A novel Dps-type protein from insect gut bacteria catalyses hydrolysis and synthesis of *N*-acyl amino acids. Environ. Microbiol. 9, 1572–1583.
Pogson, B., McDonald, K.A., Truong, M., Britton, G., DellaPenna, D., 1996. Arabidopsis carotenoid mutants demonstrate that lutein is not essential for photosynthesis in higher plants. Plant Cell 8, 1627–1639.
Rao, M.N., Ghosh, P., Lakshman, M.R., 1997. Purification and partial characterization of a cellular carotenoid-binding protein from ferret liver. J. Biol. Chem. 272, 24455–24460.
Ratnayaka, H.H., Molin, W.T., Sterling, T.M., 2003. Physiological and antioxidant responses of cotton and spurred anoda under interference and mild drought. J. Exp. Bot. 54, 2293–2305.
Sakudoh, T., Sezutsu, H., Nakashima, T., Kobayashi, I., Fujimoto, H., Uchino, K., Banno, Y., Iwano, H., Maekawa, H., Tamura, T., Kataoka, H., Tsuchida, K., 2007. Carotenoid silk coloration is controlled by a carotenoid-binding protein, a product of the yellow blood gene. Proc. Natl. Acad. Sci. U.S.A. 104, 8941–8946.
Sandre, S.L., Tammaru, T., Esperk, T., Julkunen-Tiitto, R., Mappes, J., 2007. Carotenoid-based colour polyphenism in a moth species: search for fitness correlates. Entomol. Exp. Appl. 124, 269–277.

Spiteller, D., Dettner, K., Boland, W., 2000. Gut bacteria may be involved in interactions between plants, herbivores and their predators: microbial biosynthesis of *N*-acylglutamine surfactants as elicitors of plant volatiles. Biol. Chem. 381, 755–762.
Starnecker, G., 1997. Hormonal control of lutein incorporation into pupal cuticle of the butterfly *Inachis io* and the pupal melanization reducing factor. Physiol. Entomol. 22, 65–72.
Taylor, R.F., Ikawa, M., Chesbro, W., 1971. Carotenoids in yellow-pigmented enterococci. J. Bacteriol. 105, 676–678.
Tschirner, N., Schenderlein, M., Brose, K., Schlodder, E., Mroginski, M.A., Thomsen, C., Hildebrandt, P., 2009. Resonance Raman spectra of beta-carotene in solution and in photosystems revisited: an experimental and theoretical study. Phys. Chem. Chem. Phys. 11, 11471–11478.
Wall, M., Kelley, E., 1943. Determination of pure carotene in plant tissue – a rapid chromatographic method. Ind. Eng. Chem. 15, 18–20.
Werner, R.A., Brand, W.A., 2001. Referencing strategies and techniques in stable isotope ratio analysis. Rapid Commun. Mass Spectrom. 15, 501–519.

4. Article II

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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.

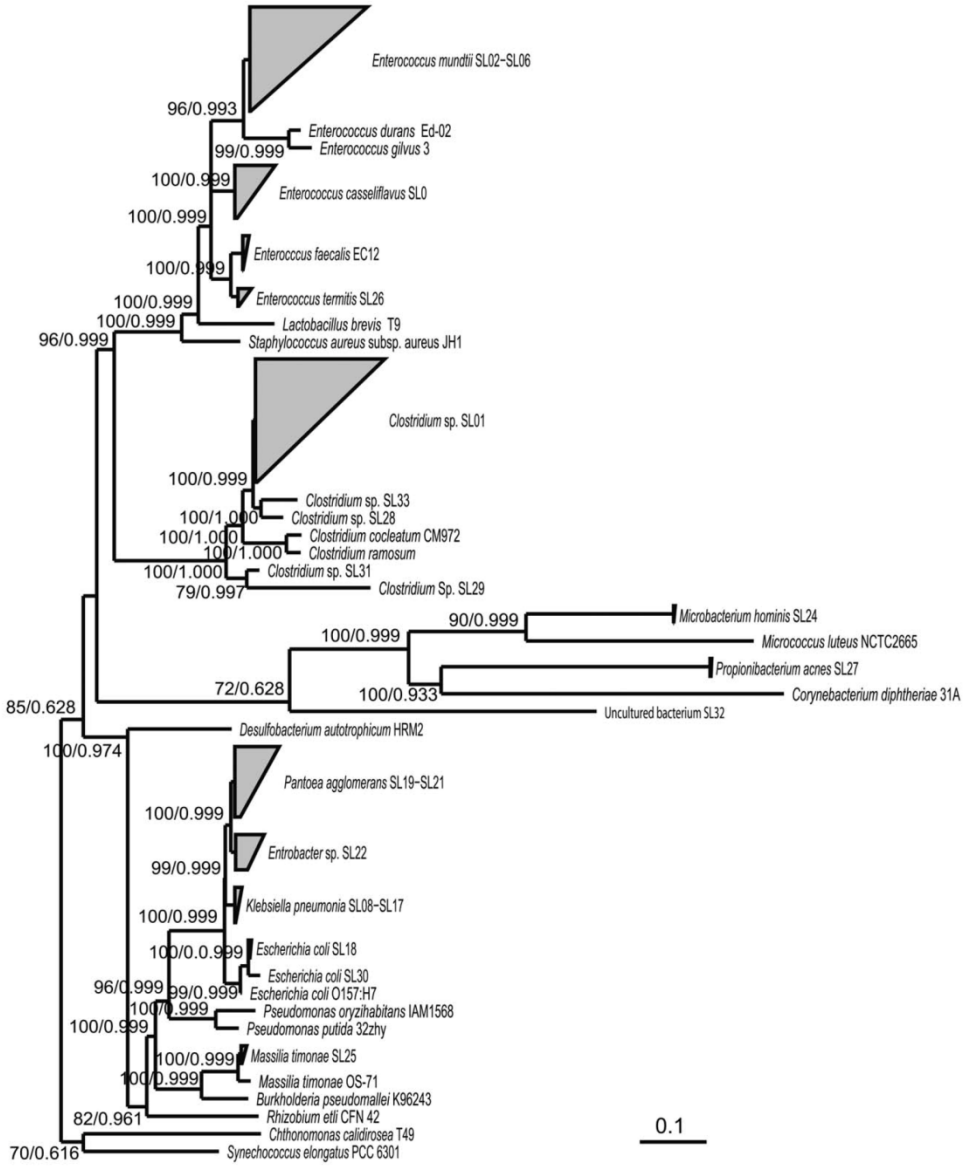

Figure 1. Phylogenetic tree of bacterial divisions retrieved from *S. littoralis* larval gut based on sequence similarity. The 16S rRNA gene sequence of the cyanobacterium *Synechococcus elongatus* PCC 6301 (NC_006576.1) and the Armatimonadetes *Chthonomonas calidirosea* T49 (AM749780.1) were used as 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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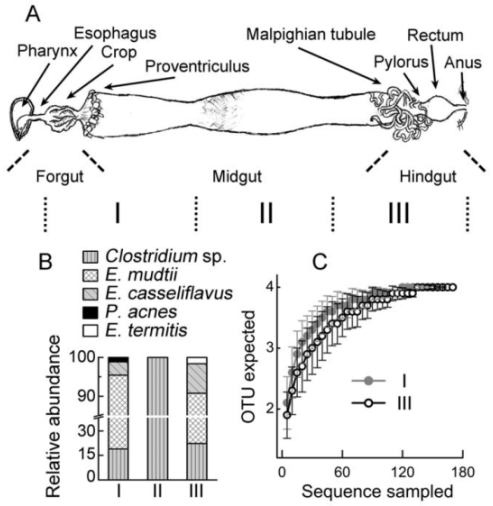

Figure 2. Change of bacterial composition along the digestive tract of 5th-instar larvae of artificial food-feeding *S. littoralis*. (a), The structure of the alimentary canal. The digestive tract was cut into three segments (I, II, and III) for sampling as indicated by the dotted lines. (b), Relative 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

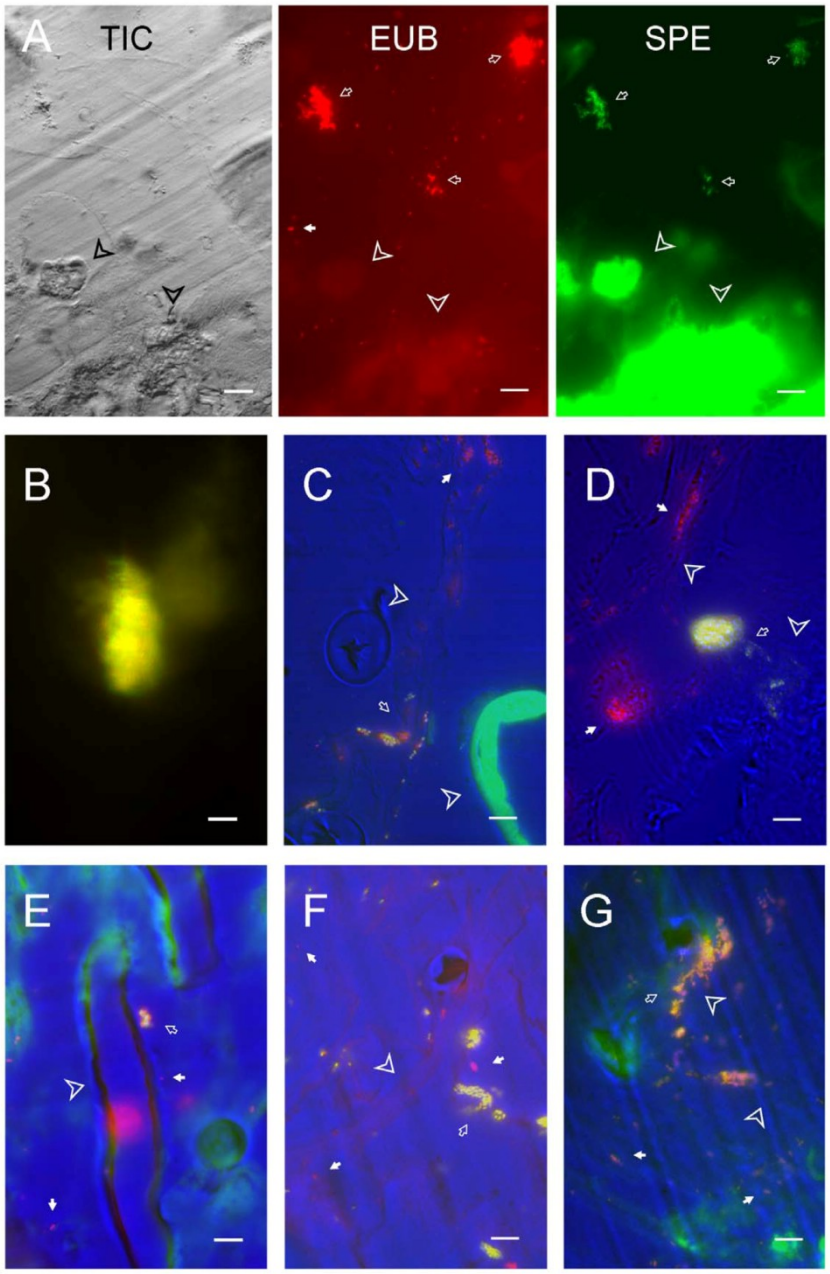

Figure 3. Bacterial localization in the gut of S. littoralis larvae with Fluorescent In Situ Hybridization. Scale bar equals 10 μm. A, Detection of *Clostridium* sp. In the midgut. The three images shown are TIC image, fluorescent image of universal probe (EUB, red) and of specific probe (SPE, green). 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-

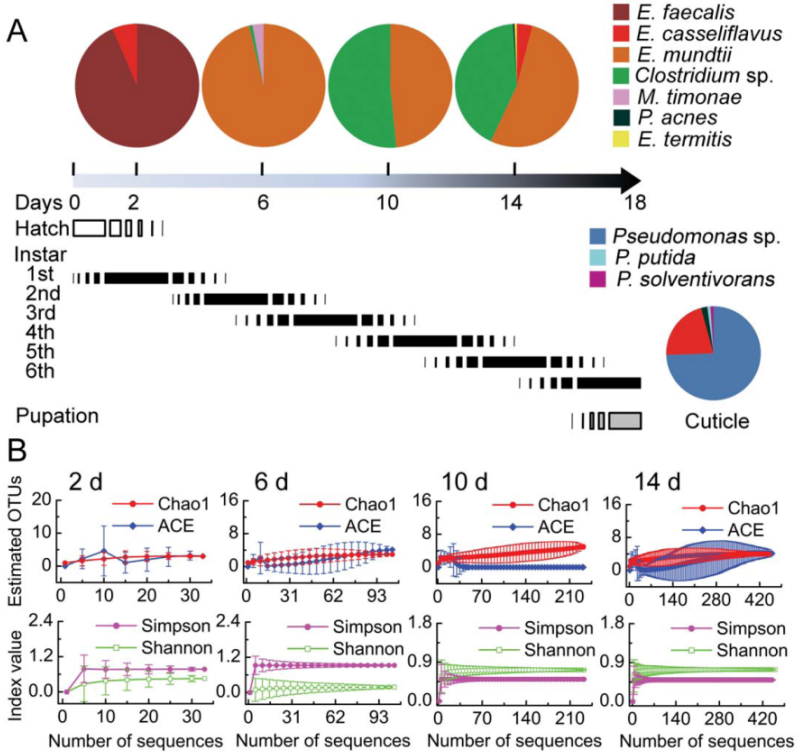

Figure 4. Different gut bacterial community structures in *S. littoralis* larvae of different instars feeding on artificial diet. A, The bacterial community compositions detected by cloning and sequencing from insects that are 2-days (n = 33), 6-days (n = 104), 10-days (n = 232), and 14-days (n = 490). 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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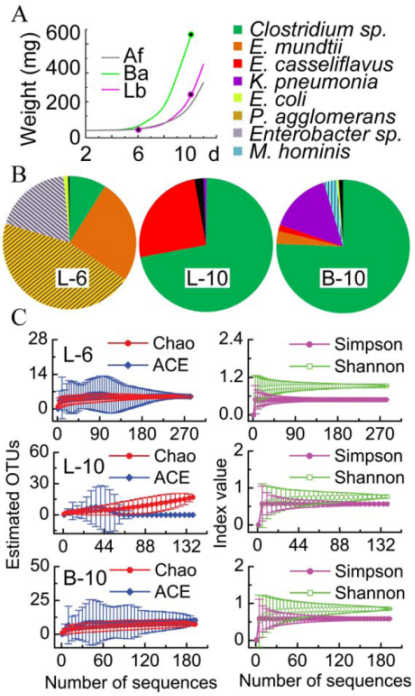

Figure 5. The impact of diet on the gut community in *S. littoralis* larvae revealed by cloning and sequencing. A, Growth curve of the insects. Black dots indicate where the insect gut was sampled. Af, artificial food; Ba, barley; Lb, Lima bean. B, Gut bacterial composition of 6-day-old larvae feeding 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*.
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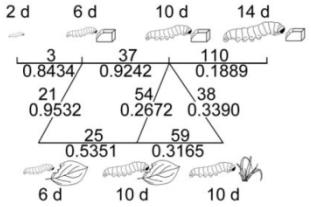

Figure 6. The phylogeny-based β-diversity values between bacterial communities detected in the gut of *S. littoralis* larvae at different instars and after feeding on different diets by cloning and sequencing. The upper values are the parsimony scores and the lower values are the weighted UniFrac scores. 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.
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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.

References

1. Gil R, Latorre A, Moya A (2004) Bacterial endosymbionts of insects: insights from comparative genomics. Environ Microbiol 6: 1109–1122.
2. Wernegreen JJ (2002) Genome evolution in bacterial endosymbionts of insects. Nat Rev Genet 3: 850–861.
3. Brauman A, Kane MD, Labat M, Breznak JA (1992) Genesis of acetate and methane by gut bacteria of nutritionally diverse termites. Science 257: 1384–1387.
4. Chen DQ, Purcell AH (1997) Occurrence and transmission of facultative endosymbionts in aphids. Curr Microbiol 34: 220–225.
5. Warnecke F, Luginbuhl P, Ivanova N, Ghassemian M, Richardson TH, et al. (2007) Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. Nature 450: 560–565.
6. Carter DJ (1984) Pest Lepidoptera of Europe: with special reference to the British Isles; Spencer KA, editor. London: Dr. W. Junk Publishers, Dordrecht, and the Trustees of the British Museum (Natural History).
7. Daly HV, Doyen JT, III AHP (1998) Introduction to insect biology and diversity. Oxford: Oxford University Press.
8. Dillon RJ, Dillon VM (2004) The gut bacteria of insects: Nonpathogenic Interactions. Annu Rev Entomol 49: 71–92.
9. Morrison M, Pope PB, Denman SE, McSweeney CS (2009) Plant biomass degradation by gut microbiomes: more of the same or something new? Curr Opin Biotech 20: 358–363.
10. Dillon RJ, Vennard CT, Charnley AK (2000) Pheromones: Exploitation of gut bacteria in the locust. Nature 403: 851–851.
11. Freitak D, Heckel DG, Vogel H (2009) Dietary-dependent trans-generational immune priming in an insect herbivore. Proc R Soc Lond [Biol] 276: 2617–2624.
12. Freitak D, Wheat C, Heckel D, Vogel H (2007) Immune system responses and fitness costs associated with consumption of bacteria in larvae of Trichoplusia ni. BMC Biol 5: 56.
13. Ping L, Büchler R, Mithöfer A, Svato A, Spiteller D, et al. (2007) A novel Dps-type protein from insect gut bacteria catalyses hydrolysis and synthesis of *N*-acyl amino acids. Environ Microbiol 9: 1572–1583.
14. Matthias F, Rita B, Vertica M, Alexander S, Michael R, et al. (2008) Rapid hydrolysis of quorum-sensing molecules in the gut of lepidopteran larvae. ChemBioChem 9: 1953–1959.
15. Spiteller D, Dettner K, Boland W (2005) Gut bacteria may be involved in interactions between plants, herbivores and their predators: microbial biosynthesis of *N*-acylglutamine surfactants as elicitors of plant volatiles. Biol Chem 381: 755–762.
16. Broderick NA, Raffa KF, Goodman RM, Handelsman J (2004) Census of the bacterial community of the gypsy moth larval midgut by using culturing and culture-independent methods. Appl Environ Microbiol 70: 293–300.
17. van der Hoeven R, Betrabet G, Forst S (2008) Characterization of the gut bacterial community in *Manduca sexta* and effect of antibiotics on bacterial diversity and nematode reproduction. FEMS Microbiol Lett 286: 249–256.
18. Brinkmann N, Martens R, Tebbe CC (2008) Origin and diversity of metabolically active gut bacteria from laboratory-bred larvae of *Manduca sexta* (Sphingidae, Lepidoptera, Insecta). Appl Environ Microbiol 74: 7189–7196.
19. Xiang H, Wei G-F, Jia S, Huang J, Miao X-X, et al. (2006) Microbial communities in the larval midgut of laboratory and field populations of cotton bollworm (*Helicoverpa armigera*). Can J Microbiol 52: 1085–1092.
20. Ballhorn DJ, Kautz S, Lion U, Heil M (2008) Trade-offs between direct and indirect defences of lima bean (*Phaseolus lunatus*). J Ecol 96: 971–980.
21. Friedman M (2002) Tomato glycoalkaloids: Role in the plant and in the diet. J Agr Food Chem 50: 5751–5780.
22. Wilson KH, Wilson WJ, Radosevich JL, DeSantis TZ, Viswanathan VS, et al. (2002) High-density microarray of small-subunit ribosomal DNA probes. Appl Environ Microbiol 68: 2535–2541.
23. Brodie EL, DeSantis TZ, Joyner DC, Baek SM, Larsen JT, et al. (2006) Application of a high-density oligonucleotide microarray approach to study bacterial population dynamics during uranium reduction and reoxidation. Appl Environ Microbiol 72: 6288–6298.
24. Douglas AE, Wong CNA, Ng P (2011) Low-diversity bacterial community in the gut of the fruitfly *Drosophila melanogaster*. Environ Microbiol 13: 1889–1900.
25. Robinson C, Schloss P, Ramos Y, Raffa K, Handelsman J (2010) Robustness of the bacterial community in the cabbage white butterfly larval midgut. Microb Ecol 59: 199–211.
26. Cox CR, Gilmore MS (2007) Native microbial colonization of *Drosophila melanogaster* and its use as a model of *Enterococcus faecalis* pathogenesis. Infect Immun 75: 1565–1576.
27. Lehman R, Lundgren J, Petzke L (2009) Bacterial communities associated with the digestive tract of the predatory ground beetle, *Poecilus chalcites*, and their modification by laboratory rearing and antibiotic treatment. Microb Ecol 57: 349–358.
28. Hunt J, Charnley AK (1981) Abundance and distribution of the gut flora of the desert locust, *Schistocerca gregaria*. J Invertebr Pathol 38: 378–385.
29. Zaspel JM, Hoy MA (2008) Microbial diversity associated with the fruit-piercing and blood-feeding moth *Calyptra thalictri* (Lepidoptera: Noctuidae). Ann Entomol Soc Am 101: 1050–1055.
30. Ren C, Webster P, Finkel SE, Tower J (2007) Increased internal and external bacterial load during *Drosophila* aging without life-span trade-off. Cell Metab 6: 144–152.
31. Pinto-Tomás A, Uribe-Lorío L, Blanco J, Fontecha G, Rodríguez C, et al. (2007) Actividades enzimáticas en aislamientos bacterianos de tractos digestivos de larvas y del contenido de pupas de *Automeris zugana* y *Rothschildia lebeau* (Lepidoptera: Saturniidae). Rev Biol Trop 55: 401–415.
32. Svec P, Vancanneyt M, Sedlacek I, Naser SM, Snauwaert C, et al. (2006) *Enterococcus silesiacus* sp. nov. and *Enterococcus termitis* sp. nov. Int J Syst Evol Microbiol 56: 577–581.
33. Egert M, Stingl U, Dyhrberg Bruun L, Pommerenke B, Brune A, et al. (2005) Structure and topology of microbial communities in the major gut compartments of *Melolontha melolontha* Larvae (Coleoptera: Scarabaeidae). Appl Environ Microbiol 71: 4556–4566.
34. Garcia ES, Castro DP, Figueiredo MB, Azambuja P (2010) Immune homeostasis to microorganisms in the guts of triatomines (Reduviidae): a review. Mem Inst Oswaldo Cruz 105: 605–610.
35. Vallet-Gely I, Lemaitre B, Boccard F (2008) Bacterial strategies to overcome insect defences. Nat Rev Micro 6: 302–313.
36. Santo Domingo JW, Kaufman MG, Klug MJ, Holben WE, Harris D, et al. (1998) Influence of diet on the structure and function of the bacterial hindgut community of crickets. Mol Ecol 7: 761–767.
37. Shao Y, Spiteller D, Tang X, Ping L, Colesie C, et al. (2011) Crystallization of α- and β-carotene in the foregut of *Spodoptera* larvae feeding on a toxic food plant. Insect Biochem Mol Biol 41: 273–281.
38. Sleator RD, Shortall C, Hill C (2008) Metagenomics. Lett Appl Microbiol 47: 361–366.
39. Ryu J-H, Kim S-H, Lee H-Y, Bai JY, Nam Y-D, et al. (2008) Innate immune homeostasis by the homeobox gene *Caudal* and commensal-gut mutualism in *Drosophila*. Science 319: 777–782.
40. Pauchet Y, Muck A, Svatoš A, Heckel DG, Preiss S (2008) Mapping the larval midgut lumen proteome of *Helicoverpa armigera*, a generalist herbivorous insect. J Proteome Res 7: 1629–1639.
41. Ping L, Vogel H, Boland W (2008) Cloning of prokaryotic genes by a universal degenerate primer PCR. FEMS Microbiol Lett 287: 192–198.
42. Ludwig W, Strunk O, Westram R, Richter L, Meier H, et al. (2004) ARB: a software environment for sequence data. Nucl Acids Res 32: 1363–1371.
43. Drummond A, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol 7: 214.
44. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, et al. (2009) Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75: 7537–7541.
45. Flanagan JL, Brodie EL, Weng L, Lynch SV, Garcia O, et al. (2007) Loss of bacterial diversity during antibiotic treatment of intubated patients colonized with *Pseudomonas aeruginosa*. J Clin Microbiol 45: 1954–1962.

5. Article III

In vivo Pyro-SIP assessing active gut microbiota of cotton leafworm, *Spodoptera littoralis*

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Running title

Active bacteria in the cotton leafworm gut

Abstract

The gut microbiota is of crucial importance for the host with considerable metabolic activity. Although great efforts have been made toward characterizing microbial diversity, measuring components' metabolic activity surprisingly hasn't kept pace. Here we used a combined pyrosequencing of amplified 16S rRNA genes and *in vivo* stable isotope probing approach (Pyro-SIP) to unmask metabolically active bacteria in the gut of cotton leafworm (*Spodoptera littoralis*), a polyphagous insect herbivore that consumes large amounts of plant material in a short time, liberating abundant glucose in the alimentary canal as a most important carbon and energy source for both host and active gut bacteria. With ^{13}C glucose as the trophic link, Pyro-SIP revealed that a relatively simple but distinctive gut microbiota co-developed with the host, both metabolic activity and composition shifting throughout larval stages. *Pantoea*, *Citrobacter* and *Clostridium* were particularly active in early-instar, likely the core functional populations linked to nutritional upgrading. *Enterococcus* was the single predominant genus in the community, and it was essentially stable and metabolically active in the larval lifespan. Based on that *Enterococci* formed biofilm-like layers on the gut epithelium and that the

isolated strains showed antimicrobial properties, *Enterococcus* may be able to establish a colonization resistance effect in the gut against potentially harmful microbes from outside. Not only establishes the first in-depth inventory of the gut microbiota of a model organism from the mostly phytophagous Lepidoptera, but this pilot study shows that Pyro-SIP can rapidly gain insight into the microbiota's metabolic activity with high resolution and high precision.

Keywords: DNA-SIP/ gut bacteria/ insect microbiology/ lactic acid bacterium/ metabolic activity/ *Spodoptera littoralis*

Subject Category: Microbe-microbe and microbe-host interactions

Introduction

The gut microbiota, largely composed of bacteria, is a complex ecosystem, and forms close symbiotic associations with the host. Not only thriving in the gut, these inner microbial residents also contribute to host metabolism through nutrient release, xenobiotic detoxification and immune regulation, all of which greatly boost host fitness (Clemente et al 2012, Dillon and Dillon 2004). With the rapid development of new generation sequencing technologies, a growing number of studies from humans to ants, have demonstrated the fascinating microbial diversity and composition within the gut (2012, Poulsen and Sapountzis 2012). However, the assessment of metabolically active components in the gut microbiota to date is surprisingly scarce, particularly under the host physiology. Clearly, not all microorganisms are able to colonize the gut, even though it is a nutrient-rich habitat, as some dietary microbes are lysed and some transients remain dormant during gut passage (Dillon and Dillon 2004). On the other hand, active populations constantly shift in response to host development and environmental effects, and hence metabolic potentials delineated by pure

metagenomic analysis have to be verified *in vivo* (Reichardt et al 2011). To get a more complete picture, it is important to move beyond a mainly sequencing-based approach towards other advanced tools to identify active fractions of the community that directly contribute to the current function of the microbiota.

Stable isotope probing (SIP) is a promising, culture-free technique that is often used in environmental microbiology to identify active microorganisms involved in various biogeochemical processes (Dumont and Murrell 2005). This methodology relies on the assimilation of a stable isotope (^{13}C)-labeled carbon source into growing microbes and the selective recovery of the isotope-enriched cellular components, such as the most informative nucleic acids, which could provide specific taxonomic information. ^{13}C-enriched "heavy" DNA or RNA can be separated from unlabeled, normal "light" (^{12}C) nucleic acids by density-gradient ultracentrifugation and subsequently retrieved from gradient fractionation procedure for further molecular analysis (Neufeld et al 2007). Stable isotope probing of nucleic acids provides direct evidence of bacterial metabolic activity and has been demonstrated to be more sensitive than an RNA-based approach (Brinkmann et al 2008). Recently Pilloni *et al*. introduced a combination of DNA-SIP and pyrosequencing, namely Pyro-SIP, in SIP gradient interpretation (Pilloni et al 2011). However, this study only performed pyrosequencing on the entire metagenomic DNA without gradient separation and still relied on the low-resolution gel electrophoresis-based fingerprinting (T-RFLP) and laborious clone library construction to identify active bacteria. Considering all of those drawbacks, here we directly pursue pyrosequencing to examine separated gradient fractions to get more resolution (Figure 2a), which is relatively straightforward and effectively merges the microbiota structure investigation with a measurement of local metabolic activity.

As a first attempt, we successfully conducted this refined Pyro-SIP to unravel the metabolically active bacterial community in the gut of an insect model organism, *Spodoptera*

littoralis (Lepidoptera, Noctuidae), a highly polyphagous pest found worldwide that causes considerable yield losses of many economically important crops. The devastating larval stage of *S. littoralis* (cotton leafworm) consumes large amounts of plant material in a short time, liberating abundant plant-derived saccharides mainly glucose in the alimentary canal to be a most important carbon and energy source for both host and active gut bacteria. More recently, the microbiology of this important insect has received increasing attention, a complex and variable commensal microbiota is found in the larval gut (Tang et al 2012) and gut bacteria are suggested to be involved in multitrophic interactions between plant, herbivore and predator, which is previously underestimated (Pesek et al 2011, Ping et al 2007, Shao et al 2011).

Using ^{13}C glucose as the trophic link, Pyro-SIP revealed that a relatively simple but distinctive gut microbiota co-develops with the host, both metabolic activity and composition shifting throughout larval stages. Three families, including *Enterococcaceae*, *Clostridiaceae* and *Enterobacteriaceae*, are particularly rich and active inside, which likely represent the core functional populations in the gut linked to nutritional upgrading and pathogen defense for improving larval fitness. This study also establishes the first in-depth inventory of the gut microbiota of a model organism from mostly phytophagous Lepidoptera. Knowledge of the gut bacteria of such a major insect herbivore could lead to new targets for pest control.

Materials and methods

Insect and plant rearing and sample collection

Spodoptera littoralis (eggs purchased from Bayer Cropscience, Monheim, Germany) were hatched and reared on a sterile artificial diet (Tang et al 2012). All cotton plants (*Gossypium hirsutum* DP90) were cultivated in our greenhouse under standard conditions (23±2℃; 50±5% humidity; 16 h light photo-period). To analyze the sugar composition in the gut, larvae were

grown on cotton seedlings in pots or on fresh artificial diet in boxes, respectively. After 7 days of feeding, larvae were washed, sedated on ice and dissected to collect gut contents under a dissecting microscope. The alimentary canal was divided into three regions (foregut, midgut and hindgut) with sterile scissors (Figure 1c), and gut contents from each section were released into Eppendorf tubes. Material from the same section of five to eight specimens was pooled to obtain approximately 0.6 g per sample for sugar extraction. To analyze active gut bacteria, stable isotope probing was conducted on early-instar larvae (from 1^{st} to 2^{nd}) or late-instar larvae (5^{th}) for 24 and 48 h using glucose-amended artificial diet. The artificial diet was frequently changed to avoid any contamination. The whole guts of early-instar larvae or midguts of late-instar larvae were collected as above for DNA extraction. For each analysis, the experiment was performed in triplicate.

Sugar composition analysis

For the characterization of soluble organic compounds in the alimentary canal, freshly collected gut content was extracted according to the published literature (Drake et al 2011, Horn et al 2003). Briefly, samples were extracted with 2 ml double-distilled water in the thermomixer (Comfort, Eppendorf, Hamburg, Germany) at 60°C, 1400 rpm for approximately 2 min and subsequently cooled on ice, and homogenized with a Rotator Mixer (RM-Multi 1, STARLAB, Hamburg, Germany) for 6 h at 4°C. Supernatant fluids (extracts) were separated by centrifugation (22,000 × g, 5 min at 4°C) and were filtered (0.22 µm pore size) in order to analyze the soluble sugar compounds. Because saccharides are non-volatile, a derivatization step was then conducted on the extract before GC-MS analysis as previously described (Price 2004). After the derivativization reaction, 1 µl supernatant was subjected to GC-EIMS analysis (Finnigan Trace GC-MS 2000, ThermoQuest, Egelsbach, Germany). A Phenomenex ZB-5 column (15 m × 0.25 mm, film thickness 0.25 µm) was equipped to separate sugar derivatives. Helium was used as a carrier gas at a flow rate of 1.5 ml min^{-1}. The temperature

program was set as follows: 80°C (2 min), then at 15°C min^{-1} to 300°C (6 min). Data were acquired and processed using the software Xcalibur (Thermo Scientific, Sunnyvale, CA, USA). Mass spectra were taken in the selected ion monitoring (SIM) mode at 70 eV. All compounds were identified by comparing their retention times and MS data with authentic references. An aliquot of the extract was also hydrolyzed with 2 M trifluoroacetic acid (TFA; Sigma-Aldrich, Saint Louis, MO, USA) at 100°C for 2 h, solubilizing the matrix polysaccharides into their monosaccharides, and subsequently derivatized to corresponding aldononitrile-acetates. Further GC-MS analysis was identical to those of non-hydrolyzed extracts. Glucose concentration was verified using a glucose assay kit based on the specific and sensitive enzymatic method according to the protocol supplied by the manufacturer (GAHK-20, Sigma-Aldrich).

DNA extraction and amplification

All freshly collected gut samples were dried at 45℃ in a Speedvac (Concentrator 5301, Eppendorf) and crushed in a 1.5 ml Eppendorf tube with a sterile plastic pestle. Genomic DNA was extracted using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocol. An additional heating step at 65 ℃ for 10 min was included just prior to bead-beating. After purification, DNA concentrations were quantified with the NanoVue spectrophotometer (GE Healthcare Europe GmbH, Freiburg, Germany). The successful extraction of microbial metagenomic DNA from the gut was verified by using PCR assays with general bacterial 16S rRNA primers (27f and 1492r) (Egert et al 2005). The PCR reaction was described previously (Tang et al 2012). Archaea- and fungus-specific primers were used to amplify archaeal 16S or fungus ITS genes (Supplementary Table S1) (Anderson and Cairney 2004). Subsequently, the extracted DNA was used for IRMS measurement and density-gradient ultracentrifugation.

Measurement of ^{13}C enrichment in DNA by IRMS

Carbon isotopic ratio can be a reliable indicator of the ^{13}C enrichment in extracted nucleic acids. The ^{13}C composition was determined by a coupled elemental analyzer/isotope ratio mass spectrometry (EA/IRMS). Purified DNA samples were placed into 0.04 ml tin capsules (3.5 × 5 mm, HEKAtech GmbH, Wegberg, Germany) and dried overnight. Samples were completely converted to CO_2, N_2 and H_2O after combustion (oxidation at 1020℃, reduction at 650℃) in a constant helium flow (80 ml min^{-1}) by using an Elemental Analyzer (EuroEA CN2 dual, HEKAtech GmbH). After passing a water trap ($MgClO_4$), the gases were separated chromatographically at 85℃ and transferred via the split valve to a coupled isotope ratio mass spectrometer (IRMS) (IsoPrime, Micromass, Manchester, UK). The acetanilide standard was calibrated against the international standard Vienna Pee Dee Belemnite (VPDB) using NBS 22 (IAEA reference material) with a $\delta^{13}C$ value of -30.03‰ (Coplen et al 2006). Empty tin capsules were used as blanks. Each sample was analyzed in triplicate. Isotopic ratios are expressed in delta notation as follows (Lenhart et al 2012):

$$\delta^{13}C_{VPDB} = \frac{(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{VPDB}}{(^{13}C/^{12}C)_{VPDB}} \times 1000(‰)$$

Separation of ^{13}C-labeled DNA by density

The density-dependent resolution of the extracted DNA was conducted in cesium chloride (CsCl, Sigma-Aldrich) solution with an average density of 1.725 g ml^{-1} using a published protocol (Neufeld et al 2007). In short, approximately 1500 ng purified DNA was loaded in the CsCl centrifugation medium and filled into a 5.1 ml Quick-Seal tube (Beckmann, Fullerton, CA, USA) and sealed. All samples were set up with the same batch of CsCl medium and run in parallel to minimize potential variations. ^{13}C-labeled DNA was separated by isopycnic ultracentrifugation under 50,000 r.p.m. in a NVT90 rotor in an Optima L-90K ultracentrifuge (Beckmann) at 20℃ for 40 h with vacuum and subsequently the formed

density gradients were fractionated from bottom to top into 12 equal fractions (425 µl each) by displacement from above with water using a HPLC pump at a flow rate of 850 µl min^{-1} (Agilent HP1100 system, Waldbronn, Germany) (Figure 2a). Fraction density was determined by weighing an aliquot of each fraction and fractions containing water (usually the last one) were discarded. Afterwards, DNA was precipitated from every fraction, redissolved in nuclease-free water and quantified as above for the following microbial community analysis. The distribution of DNA within a given sample was calculated as the amount of the respective fraction divided by the total amount of DNA of all fractions within the sample to facilitate comparison between the gradients.

Fingerprinting of 16S rRNA gene in gradient fractions by DGGE

Separated gradient fractions were screened for differences in bacterial community composition by denaturing gradient gel electrophoresis (DGGE) analysis. Bacterial 16S rRNA gene was amplified from all fractions in two steps as a nested PCR with the primer set 27f/1492r and 968F/1401R (Supplementary Table S1). DGGE was performed with the DCode system (Bio-Rad, Munich, Germany). The roughly equal amounts of PCR products (300 ng) were loaded onto the 8% polyacrylamide gel with a 20 to 80% denaturant gradient (100% denaturant was 7 M urea and 40% (v/v) deionized formamide). Electrophoresis was carried out in 1×TAE buffer at 100 V for 16 h at 60°C and the gels were stained for 30 min in 0.5×TAE buffer with SYBR-Gold nucleic acid gel stain (Invitrogen, Karlsruhe, Germany) for photographing. The DGGE patterns were compared to assess the variability of the bacterial community structure between the labeled treatment and the control. As DGGE profiles showed the difference in gradients of the labeled treatment, the respective fractions were combined to obtain one heavy (4-5), one middle (7) and one light (9-11) DNA density fraction from every sample.

Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) and molecular phylogenetic analysis

Representative gradients were submitted to pyrosequencing as described previously (Ishak et al 2011, Sudakaran et al 2012). Basically, the hypervariable V1-V3 portion in the 16S rDNA was amplified using a Gray28F/519r primer pair and sequenced using the Roche 454 FLX Titanium based strategy (Supplementary Table S1). The software package Quantitative Insight into Microbial Ecology (QIIME) was used to process sequencing data and to calculate diversity (Caporaso et al 2010). Sequences were first passed through the quality-control to remove potential artifacts and errors (denoising) and trimmed of the part with low quality. Later chimera and low abundance reads were removed from analysis. The high-quality reads were clustered into operational taxonomic units (OTUs) with 97% similarity cut-offs. For each OTU, one representative sequence was extracted and the Ribosomal Database Project (RDP) classifier was used to determine the highest resolution of taxonomy. Finally, an OTU table was generated describing the occurrence of bacterial phylotypes within the sample. Representative sequences were aligned to reference sequences obtained from the NCBI nucleotide database using the ClustalW algorithm. Phylogenetic trees were calculated using the Maximum Likelihood method (Tamura-Nei model) and the Neighbor-Joining method with 1000 bootstrap replicates in MEGA5 (Tamura et al 2011).

Fluorescence *in situ* hybridization (FISH)

To localize the dominant gut symbionts, FISH was performed on 5 μm thin cross sections of the cold polymerizing resin (Technovit 8100, Heraeus Kulzer GmbH, Wehrheim, Germany) embedded gut tissue. The specificity of probes was tested and hybridization condition was achieved as described (Tang et al 2012). Shortly, the slide was hybridized with 1.5 mM of each probe (Supplementary Table S1) in hybridization buffer containing 900 mM NaCl, 20 mM Tris-HCl (pH 8.0), 20% formamide, 1% SDS. FITC-labeled general eubacteria probe and

Cy3-labeled *Enterococcus*-specific probe was used for detection and images were taken with an Axio Imager Z1 microscope (Carl Zeiss, Jena, Germany) (Amann et al 1990, Behr et al 2000, Manero and Blanch 2002).

Antibiotic bioassays

Enterococci were isolated from cotton leafworm gut by *Enterococcus*-selective agar plate (Fluka, Munich, Germany) and 16S rRNA gene was amplified and sequenced. The antimicrobial activity of isolated strains against different indicator bacteria was evaluated using an agar diffusion assay. The overnight culture supernatant of an *E. mundtii* strain in MRS broth (Roth, Karlsruhe, Germany) at 30℃ was adjusted to pH 7.0 and filtered through 0.22 µm PVDF membrane. Holes (diameter, 6 mm) were cut out from the BHI agar plate (Roth) inoculated with indicator strains (*Micrococcus luteus* or *Leuconostoc mesenteroides*) and filled with 60 µl of *E. mundtii* culture filtrates. The agar plates were incubated at 30℃ for 24 h, and antimicrobial activity was detected by the formation of clearance zones around loading holes.

Nucleotide sequence accession numbers

The pyrosequencing data have been deposited at the NCBI GenBank Short Read Archive under accession no. SRA057979.

Results

1. Identification and quantification of saccharides in the gut content

Sugar composition was analyzed in different gut regions, namely the foregut, midgut and hindgut (Figure 1c). Gut contents collected from larvae fed on their native diet (cotton) or defined artificial diet were analyzed by gas chromatography-mass spectrometry (GC-MS)

after water extraction and aldononitrile acetate derivatization, which is a rapid and sensitive derivatization method for non-volatile saccharides and each sugar forms a single chromatographic peak after GC separation.

The GC-MS profile indicated that several pentose and hexose sugars were present in the gut, including ribose, arabinose, mannose, glucose and galactose (Figures 1a and b). However, glucose was dominant in all portions of the gut and other monomers at much lower concentrations. The similar pattern of sugar monomer composition in a given larval section was detected in every replicate. Sugar profiles of larvae fed on artificial diet were nearly identical with those of larvae fed on cotton, except with a somewhat higher amount of galactose and an absence of mannose. After acid hydrolysis, the number and concentration of detectable monosaccharides increased, with glucose and arabinose being dominant, reflecting various soluble polysaccharides in the gut (Supplementary Figures S1 and S2).

As the major sugar, glucose was quantified based on a specific enzymatic assay, which revealed that maximal glucose concentrations occurred in the foregut and then dropped from the anterior to posterior end of the gut. In cotton-feeding larvae, the average concentration of glucose in the aqueous phase of the foregut was approximately 17.7 mM, significantly higher than that in the hindgut, which was around 9.8 mM (Figure 1d). The rapid decrease of glucose from the foregut to the hindgut along the short alimentary canal indicated that glucose was remarkably consumed during gut passage. The same trend was obtained with larvae fed on artificial diet. However, the mean values of glucose concentration in all gut regions of larvae fed on artificial diet were lower than those of larvae fed on cotton (Figure 1d).

2. *In vivo* labeling strategy with ^{13}C glucose as the trophic link

As the most effective energy and carbon source available, nutritive glucose in the gut provided a good chance to carry out *in vivo* SIP by performing ^{13}C-glucose amendment. Initial concentrations of glucose in the foregut of larvae fed on cotton reached 20 mM, but only

about 10 mM in larvae fed on artificial diet (Figure 1d). Thus, the artificial diet was amended with 10 mM exogenous glucose to mimic the *in situ* concentration of glucose in larvae fed on cotton. In order to track metabolically active bacteria, fully ^{13}C-labeled glucose was supplemented as the labeling treatment, whereas the same amount of native-glucose (^{12}C) was used in the control group. We tested the labeling process by feeding early-instar larvae (from 1st to 2nd) on glucose-amended artificial diets for 24 and 48 hours, respectively. For revealing the difference during larval development, the same process was also employed in the study of late-instar larvae (5th).

Larvae taken from both the ^{13}C treatment and unlabeled control group were dissected at each time point, and total DNA was extracted from fresh gut tissues. Isotopic ratio mass spectrometry (IRMS) measurements of $\delta^{13}C$ in the extracted DNA showed substantial isotope enrichment in both 24 h ($\delta^{13}C$ = 33.6±5.3‰) and 48 h ($\delta^{13}C$ = 141.2±33.1‰) labeling samples, compared to natural stable isotope abundance in the control ($\delta^{13}C$ = −30.7±1.3‰) (Figure 2b). Feeding for 48 h enhanced the labeling process with the isotopic shift exceeding 100‰ (P = 0.0008), indicating that abundant newly-divided bacterial cells had already replaced old ones. Considering that a large amount of insect DNA was co-extracted from the gut tissue, the real proportion of ^{13}C in pure bacterial DNA will be even higher, which is suitable for density gradient centrifugation to separate labeled nucleic acids and identify active community members.

3. Density gradient ultracentrifugation and recovery of separated DNA

Both the labeled and control samples were conducted in the same batch of isopycnic ultracentrifugation to minimize potential variation during this process. After 40 h ultracentrifugation, formed gradients were fractionated into 12 equal aliquots, and the abundance of DNA present in each individual fraction was quantified, allowing us to compare

DNA template distribution in the formed gradients (Figure 2a). The density of all fractions from the control and labeled samples was checked by weighing, covering an average gradient from 1.688g ml^{-1} to 1.761g ml^{-1}, which was in the expected range according to previous reports (Neufeld et al 2007). Density determination revealed a linear trend from the bottom to the top, indicating proper gradient formation (Figure 2d).

After 24 h feeding, labeled DNA from metabolically active bacteria was measurable in the ^{13}C treatment, which had a new peak of abundant DNA at the buoyant density (BD) of 1.730g ml^{-1} (fraction 5). After 48 h, the peak shifted to an even heavier fraction at BD of 1.735g ml^{-1} (fraction 4), indicating more ^{13}C incorporation into DNA, which confirmed the IRMS data. By contrast, most of DNA from the unlabeled control was still distributed over light fractions (BD was <1.718g ml^{-1}) and no peak shifted towards the high BD (Figure 2c). Notably, a low background of unspecific nucleic acids was detected in all gradient fractions, which is common in the environmental SIP experiment (Lueders et al 2004). Since the 48 h-feeding sample generated a significantly higher level of enrichment and a larger shift in BD ensured an efficient separation of isotopically labeled DNA from the unlabeled DNA, samples from this treatment were selected for the downstream molecular characterization of microbial community.

16S rRNA gene was amplified with a general bacterial primer from each gradient fraction. The amplification of control gradients yielded apparent PCR products from fractions 8 to 12, which is in line with the DNA smear formed after ultracentrifugation, and some weak bands in the heavy fractions, which were expected from unspecific background DNA. In contrast, the labeled sample displayed increased band intensity in the related heavy fractions (3-6), which was caused by the increased amount of ^{13}C-DNA template. Next, 16S rDNA amplicons detected in gradient fractions were analyzed by denaturing gradient gel electrophoresis (DGGE) in order to fingerprint the community. Changes became visible when

the DGGE profile derived from the ^{13}C treatment was compared with that from the unlabeled control. Several strong bands were clearly detected from heavy fractions (3-6) of the labeled sample, whereas almost no pattern changed over all gradients of the control (Supplementary Figure S3). Those unique patterns associated with heavy fractions from the stable isotope-amended sample but not from the native substrate-amended control provided strong evidence linking certain organisms with the *in situ* gut metabolic activity. Taken together, the data corroborated the successful incorporation of ^{13}C into the DNA of active gut bacteria.

Based on the fingerprinting analysis, we combined fractions with BDs ranging from 1.730 to 1.735g ml^{-1} into a compiled "heavy" fraction. BDs ranged around 1.718g ml^{-1} in a "middle" fraction and from 1.688 to 1.705g ml^{-1} in a "light" fraction for subsequent pyrosequencing.

4. General microbial structure in the gutflora and phylogenetic analysis

Quantitative pyrosequencing was performed directly on representative SIP gradients to reveal species lineage and relative abundance. PCR amplification using archaea- or fungus-specific primers failed to amplify any archaeal 16S rRNA or fungal ITS sequences. Cultivation-based attempts to recover fungi from the gut with three general fungus-growing agar plates were also unsuccessful (Supplementary Figure S4).

Bacteria-specific primer for pyrosequencing amplified the most informative V1-V3 region of 16S rRNA gene for taxonomic classification. After denoising and removing chimeric sequences, 120,045 high quality reads were generated, with an average length of 404 nucleotides. There was a low incidence of sequences unclassified at the phylum level (maximum 3.82%). For all samples, the rarefaction curve tended towards saturation at similar numbers of sequences at the species level, indicating the sampling was comprehensive (Figures 3a and 4a). Various methods were employed to accurately estimate the diversity of individual samples (Table 1). The gut microbiota of cotton leafworm was taxonomically

restricted, which contained 22–42 operational taxonomic units (OTUs) detected at 97% sequence similarity, with Proteobacteria and Firmicutes being co-dominant phyla. The Shannon index of diversity at 1.46-2.48 was the low boundary of the diversity in soils (2.4-3.6) (Fierer and Jackson 2006).

The "light" fraction collected from unlabeled controls, where most metagenomic DNA still distributed in, served for profiling the majority of the bacterial community. Due to fewer DNA templates from dominant groups being translocated to the heavy fraction of the control, some less-abundant bacteria, such as *Comamonas* and *Stella*, had more chance to be amplified and showed an increased proportion in the profile of [^{12}C]-Heavy fraction (Figure 3b). Therefore, comprehensive analysis of all fractions from the unlabeled SIP control provided more detailed information about the overall community diversity. The clearly decreased abundance of dominant groups, like *Pantoea* and *Citrobacter*, in the middle and heavy fraction, caused by fewer target sequences, furthermore reflected some quantitative properties of pyrosequencing (Benson et al 2010). In total, eleven phyla were identified by the RDP classifier from the dataset, but only five displaying a relative abundance larger than 0.1% in at least one of the analyzed samples (Table 2). Firmicutes apparently dominated both larval developmental stages, which represented 59.2% of the number of sequences of early-instar larvae and 97.2% of late-instar.

Proteobacteria was another major phylum in early-instar larvae, accounting for 38.9% of total sequences. Bacteria in this phylum are especially wide-spread in herbivore microbiotas and often affiliated with insect symbionts. Most dominant OTUs corresponded to *Pantoea citrea* and *Citrobacter farmeri*, which represented 17.2% and 16.4% of the reads, respectively, and belonged to the *Enterobacteriaceae* family of the Gammaproteobacteria class ([^{12}C] light, Figure 3b). In addition to *Enterobacteriaceae*, other Gammaproteobacteria from the family *Xanthomonadaceae*, *Pseudomonadaceae* and *Moraxellaceaee* were detected, including populations related to *Stenotrophomonas*, *Pseudomonas* and *Acinetobacter* (Figures

3b and 5b). Other classes of Proteobacteria, such as *Paracoccus* from Alphaproteobacteria and *Geobacter* from Deltaproteobacteria, were also identified. Organisms from the phylum Actinobacteria, including the genera *Micrococcus*, *Solirubrobacter* and *Propionibacterium*, and a novel group, GP2, from the phylum Acidobacteria, made up a small fraction of reads. However, those rare phylotypes contributed to the richness of the community.

Firmicutes were represented by the family *Enterococcaceae*, comprising >50% of all sequences. The major OTU was closely associated with *Enterococcus mundtii* as determined by Mega BLAST with the representative sequence. Other *Enterococcus* species with a low identification score, such as *E. casseliflavus*, were found too, but these accounted only for a small fraction. A low number of *Clostridia* were also retrieved from early-instar larvae.

Gut microbiota underwent a drastic change in late-instar, principally characterized by decreased species richness and diversity (Table 1). The rarefaction curve was significantly lower than that obtained in early-instar larvae (Figure 4a). Firmicutes absolutely dominated the entire community, comprising >97% of all reads; simultaneously, sequences from other phyla decreased ([^{12}C]-light, Figure 4b). Pyrosequencing reads belonging to the *Clostridiaceae* family strongly increased: 21% of total sequences were affiliated with *Clostridium* species. *Enterococcus* still predominated, making up 75% of reads. Further bacteria of Firmicutes, such as members from the genera *Lactobacillus*, *Lactococcus*, *Anoxybacillus* and *Staphylococcus*, were detected too (Figure 5a).

However, in the context of Proteobacteria, late-instar larvae mainly reduced the proportion of Gammaproteobacteria. *Pantoea* was detected at a small proportion with only 1.8% of total sequences ([^{12}C]-light, Figure 4b). *Citrobacter* quickly decreased together. In addition to common *Paracoccus*, more Alphaproteobacteria, such as members from the genera *Sphingomonas*, *Caulobacter* and *Rhizobium*, were detected. But at the same time *Stella* and *Methylobacterium* were removed. Betaproteobacteria disappeared from late-instar larvae, except for *Vogesella*. Other phylogenetic groups affiliating with the genera *Prevotella*

(Bacteroidetes), *Micrococcus* and *Propionibacterium* (Actinobacteria) appeared in a small number of sequences.

Phylogenetic analysis showed that some pyrosequencing reads clustered together with the near full length of 16S sequences retrieved from other clone-library-based studies, suggesting common taxa present in *S. littoralis* (Figure 5). Notably, the gut microbiota composition in larva fed on this artificial diet spiked with physiological dose of sugar was similar with that fed on its native plant diet (Tang et al 2012). Many low-abundant taxa were uncovered from our large-scale pyrosequencing, which more accurately represented the overall microbial community and also supplied sufficient taxonomic resolution.

5. Characterization of metabolically active bacteria in the community

The "heavy" fractions collected from the labeled sample containing significant quantities of ^{13}C-enriched DNA, and those collected from the unlabeled control served for profiling active populations in the community. Based on previous research, the metabolic activity of bacteria was assessed by calculating the difference between the relative abundance of individual taxa in the heavy fraction of the labeled sample and that of the control (Figure 2a) (Drake et al 2011, Lu and Conrad 2005). Moreover, involving a proper control in the SIP analysis subtracted the impact of the background ^{12}C-DNA contamination in the heavy fraction, which ensured that bacterial diversity and abundance appearing or disappearing was not artifacts of the method itself. The rarefaction curve of the "heavy" fraction sample was below that of the "light" fraction because the active bacteria group was a subset of the total community (Figure 3a).

In early-instar, the pyrosequencing profile of the heavy fraction from the labeled sample ([^{13}C]-Heavy, Figure 3b) showed a largely increased abundance of certain species including *Pantoea*, *Citrobacter* and *Clostridium*, while negligible portions of these sequences were detected from the heavy fraction of the control ([^{12}C]-Heavy, Figure 3b). Therefore these bacteria's DNA was significantly labeled and they were considered to be metabolically active.

The highest activity was assessed for *Pantoea* and *Citrobacter*, with a stimulation factor above 10% (Figure 6). A diverse array of other Proteobacteria from the genera *Acinetobacter*, *Stenotrophomonas*, *Delftia*, *Achromobacter* and *Vogesella* also showed some activity according to their slightly increased intensity in the heavy fraction. *Clostridium* was another group which showed high metabolic activity although it was less abundant at this stage. The closely related phylotypes of these active species have been commonly identified from herbivore guts and are well-known plant biomass degraders. *Enterococcus* was abundantly present in all SIP fractions independent of the condition, but the number of sequences peaked in the middle fraction of the labeled sample compared with that of the control, indicating that it was metabolically less active, DNA from which was less incorporated with ^{13}C and could not completely migrate to the heavy fraction. Members from genera such as *Paracoccus*, *Solirubrobacter* and *Propionibacterium* were neither enriched nor detected in the ^{13}C-DNA fraction, and thus were considered metabolically inactive bacteria.

In late-instar, *Enterococcus* was more abundant in the heavy fraction of the labeled sample than in that of the control, being the most active member within the community. Fluorescence *in situ* hybridization (FISH) revealed a large amount of *Enterococci* closely adhered to the mucosal layer of gut epithelium, where they formed a thick biofilm-like structure (Supplementary Figures S5a and b). Interestingly, *Enterococcus* isolates can produce novel antimicrobial compounds against other bacteria (Supplementary Figures S5c and d). *Clostridium* sp. stayed at a high population level, which was the consequence of its high activity in early-instar; however, there was little or no metabolic activity at this stage. *Pantoea* and another Proteobacteria, *Vogesella*, were actively persistent in both developmental stages. In contrast, *Citrobacter* was not detected. But the genus *Legionella* was particularly active. Bacteroidetes, including *Prevotella* and *Hymenobacter*, showed some metabolic activity. Other populations were detected to be active, including *Tetrasphaera*, *Microbacterium* and *Corynebacterium* associated with the phylum Actinobacteria, and *Anoxybacillus* of Firmicutes.

Collectively, *in situ* SIP denoted different patterns of metabolic activity found in the gut microbiota during larval development. A consortium of *Enterobacteriaceae* (especially *Pantoea*, *Citrobacter*) and *Clostridium* apparently were more active in early-instar larvae, while *Enterococcus* became strongly active in fully grown late-instar larvae (Figure 6). Those changes may directly associate with their functional roles inside the gut.

Discussion

Although SIP has been frequently used in environmental microbiology, very few researches have applied this valuable technique to the study of gut microbiota. Those pioneering researches have almost exclusively pursued *in vitro* experiment systems to mimic the gut environment, which obviously could not fully duplicate true physiological conditions in the intact gut, especially host factors shaping the microbial community, and hence may be limited or biased (Drake et al 2011, Reichardt et al 2011).

In contrast, we directly studied the gut microbiota of cotton leafworm *in vivo* by coupling pyrosequencing and SIP approaches. Cotton leafworms possess a simple tube-like alimentary tract, which is the largest part of the whole body and lacks any specialized substructures (Figure 1c). Despite its simplicity, a large amount of bacteria, exceeding 10^7 mL^{-1} (Funke et al 2008), has been found occupying the gut and a high concentration of nutritive glucose is liberated there, making the gut an ideal environment for diverse microbial activities, including fermentation. These properties make this organism an ideal naturally-occurring model in which to study digestive-tract microbial symbiosis. As an effective and ubiquitous energy and carbon source, glucose provides a useful trophic link to identify metabolically active gut bacteria by tracing their assimilation of amended ^{13}C-glucose in the artificial diet. The gut contains other non-labeled carbon sources, such as amino acids, which could be utilized by microbes. However those metabolically active bacteria should

simultaneously consume the dominant common glucose too and thus are being labeled. The near *in situ* glucose concentration and relatively short labeling time ensured the original gut community would not be influenced by the SIP approach, as explained in detail elsewhere (Drake et al 2011). Cross-feeding is usually a major constraint in interpreting SIP experiments, which is not a problem in the present study since we aimed to capture the entire active community and active bacteria most likely are involved in the trophic network *in situ* (Kovatcheva-Datchary et al 2009). DNA obtained from ^{13}C-glucose treatments became enriched with ^{13}C on the basis of a discernible DNA shift toward higher BDs; in contrast, no shift was observed with native-glucose amended controls. The substantial labeling of DNA was also validated by complementary methods, including isotopic ratio mass spectrometry measurement and fingerprint analysis. *In vivo* SIP provides key information about which groups are currently involved in gut metabolism, and these active bacterial associates potentially contribute to host fitness. The large sampling depth of pyrosequencing warranted the effective diversity survey, and rarefaction analysis indicated that our dataset afforded a sufficient degree of coverage for all samples.

Comprehensive analysis of the recovered DNA revealed a relatively simple but distinctive gut microbiota that co-develops with the host; both the composition and metabolic activity sustainably shift throughout larval stages. Overall, pyrosequencing reads were dominated by taxa from Proteobacteria and Firmicutes. A similar pattern has recently been described in fruitfly larvae (Wong et al 2011). Notably bacteria from these phyla are widespread in the gut of turtle ants, bees, moths and butterflies, suggesting that they may represent the chiefly phyla in insect herbivore gut microbiota (Martinson et al 2011, Robinson et al 2010, Russell et al 2009). The simplicity of this community, comprised of 22-42 OTUs, is especially apparent compared to the gut microbiota of insects from orders such as termites of Isoptera, or vertebrates, which often harbor hundreds of phylotypes (Perkins et al 2012, Warnecke et al 2007). Strong alkalinity in the gut, considered an important determinant of

community structure in beetle larvae, could also be the case in *Spodoptera*, which has a midgut pH >10 (Egert et al 2005, Funke et al 2008). Other key factors, including a fast food throughput and immune system function, may also account for this taxonomically restricted gutflora (Pauchet et al 2010). However, three families, including *Enterococcaceae*, *Clostridiaceae* and *Enterobacteriaceae*, are particularly rich and active, and these are likely the core functional populations living inside the gut. Furthermore a clear developmental change (early-instar vs. late-instar) is found. Not only do they consistently occur throughout the whole larval lifespan, these bacteria are also persistently identified from different sampling batches of normal larvae, indicating they are the indigenous gut residents and may be the true symbionts of *S. littoralis*.

In early-instar, a diverse assembly of Proteobacteria, especially Gammaproteobacteria dominated by *Pantoea* and *Citrobacter* from the family *Enterobacteriaceae*, was the most active group. Bacteria in this phylum closely associate with insect herbivores and possess broad polysaccharide-degrading abilities (Adams et al 2011, Anand et al 2010, Engel et al 2012). For example, cultivation-based studies reported *Enterobacteriaceae* isolated from silkworm (*Bombyx mori*) gut had the ability to secrete enzymes important in the digestion of complex dietary plant biopolymers such as cellulose, xylan, and pectin (Anand et al 2010). Genomic analysis of a *Pantoea* strain from a woodwasp (*Sirex noctilio*) detected genes putatively encoding for carbohydrate-active enzymes, with the majority predicted to be active on hemicellulose and more simple sugars (Adams et al 2011). Those *in vitro* growth assays and genomic investigation provide an initial picture of potential metabolic capabilities but do not offer information on bacterial activity *in situ*. Our Pyro-SIP results are the direct evidence of microbial metabolic activity *in vivo*, supporting the previous hypothesis that Gammaproteobacterial symbionts are involved in carbohydrate degradation. In spite of its low titer, *Clostridium* was another highly active group in early-instar larvae; these obligate anaerobes are capable of forming endospores. The colonization of *Clostridia* has commonly

been linked to its highly efficient cellulose digestion and its ability to ferment a variety of sugars (Watanabe and Tokuda 2010). It can be assumed that the bacteria associated with cotton leafworm play a similar role. In addition, *Clostridia* may enhance host immunity (Atarashi et al 2011). Taking together, we suggest that the most active *Enterobacteriaceae* and *Clostridiaceae* synergistically participate in the digestive process in the gut, facilitating the breakdown of organic substrates in the foliage and making them more suitable for the host's digestion, absorption and metabolism. Given that sufficient nutrient supply is important in the early life, the released simple sugars would directly benefit early larval development, which further could be fermented to various other nutrients such as short-chain fat acids, greatly promoting the gut ecological environment. These bacteria, widely distributed in nature, could be easily acquired when larvae forage. The large sampling depth of this study revealed a number of rare taxa in the gut. Although this variety diminished over time, it is possible that rare members perform some of the microbiota's functions or have a role in special situations, as suggested by previous studies (Jones and Lennon 2010). Further work need to be done to decipher their potential biological functions in the gut.

In late-instar, the gut microbiota became even simpler with a large decrease of microbial diversity and richness. More than 97% of community members belonged to Firmicutes, partially because of a significant *Clostridium* proliferation. *Enterococcus* predominated and was detected as the most active group. The successful expansion of Firmicutes over time probably in turn suppressed the growth of bacteria from other phyla in the same habitat, particularly Proteobacteria. Linked with larval development, changes in insect physiology such as dropped redox potential and enhanced host immunological response could be potential driving factors for observed changes. As larvae grow bigger, less oxygen can penetrate into the gut lumen over the thicker gut wall and elongated alimentary tract, which cause consistently low oxygen tension in the gut compartment (Tang et al 2012). This

largely anoxic condition probably promotes the development of obligate anaerobic *Clostridium* and facultative anaerobic *Enterococcus*.

Enterococcus, a Gram-positive lactic acid bacterium (LAB), is the single predominant genus in the community. LAB are well-recognized beneficial organisms of the gut microbiota of many animals, including insects (Vasquez et al 2012). *Enterococci* are essentially stable throughout *Spodoptera*'s lifespan and are vertically transmitted from mother to offspring via egg, as shown for *Manduca* (Brinkmann et al 2008). Therefore, they are the first to gain access to the gut and can immediately dominate there after hatching. As the founder species, *Enterococcus* likely controls the whole microbiota together with the host, which may be crucial for establishing a distinctive and reoccurring gut community. Besides in cotton leafworm, *Enterococcus* is present in diverse Lepidoptera, such as the larva of gypsy moth (*Lymantria dispar*) and cotton bollworm (*Helicoverpa armigera*) from both field and laboratory-reared samples (Broderick et al 2004, Priya et al 2012). Notably, *Enterococcus* was found to be already active on the eggs of tobacco hornworm (*Manduca sexta*) based on rRNA detection (Brinkmann et al 2008). These intimate associations indicate that *Enterococcus* may have important functional implications for Lepidoptera in general. FISH indicated that a high number of *Enterococci* closely attach to the mucus layer of gut epithelium to form a biofilm-like structure, which may be the reason for its high stability in the gut of healthy larvae and, furthermore, may prevent the gut from being invaded by harmful pathogenic microbes (Engel et al 2012, Koch and Schmid-Hempel 2011). Besides providing a physical barrier, isolated strains showed strong inhibition against other bacteria. A novel antimicrobial peptide was identified from the broth culture (Shao, unpublished). Collectively, these findings indicate that *Enterococcus* is likely a defensive symbiont for the health of the host. Considering cotton leafworm is a generalist feeder in the field, its digestive tract is constantly challenged by the potentially harmful bacteria and fungal endophytes that it ingests. *Enterococci*, maintained in

biofilm-like structure and showing potent antimicrobial properties, may be able to establish a colonization resistance effect in the gut, which protects the host against pathogens and a wide range of noncommensal competing microbes from outside (Dillon and Dillon 2004, Silverman and Paquette 2008). However, the well established *Enterococci* do not need fully turn on their metabolic machinery in a semisterile laboratory rearing environment, which may explain their low metabolic activity in early-instar. Not only are they related to nutritional upgrading and pathogen defense, active bacteria may also contribute to other gut metabolism, such as detoxifying plant-derived noxious allelochemicals and regulating the host's immune homeostasis. Metagenomic analysis of the labeled DNA from this study will increase our knowledge of other functions supplied by the active microbiota.

During coevolution, indigenous gut bacteria have adapted to work together in this distinct ecological niche and supply their metabolic benefits to the host. On the other hand, different physicochemical conditions with respect to host development, such as gut alkalinity, oxygen tension, might impact the community's activity, which in turn influences its composition and consequently metabolic functions. In that manner, both the host and true symbionts elaborately regulate their own metabolic activity and efficiency to preserve the host-microbial mutualism. *In vivo* activity measurement offered by SIP helps to better understand the change and maintenance of the microbial community and gives further insights into the role of active members in host fitness. Our data also established the first in-depth inventory of the gut microbiota of a model organism from mostly phytophagous Lepidoptera. Knowledge of the gut bacteria in such a major herbivore insect may also provide new targets for agricultural pest control.

This pilot study shows Pyro-SIP could rapidly gain insight into not only the structure of the community but, more important, its components' local metabolic activity with high resolution and high precision, which provides a starting point for research on more complex

ecosystems, such as termite or human microbiota. Although our work has focused on the discrimination of general active gut bacteria by tracking the ubiquitous glucose, similar analysis can be performed on more specific carbon sources, for instance, labeling plant defense compounds to assess active bacteria involved in the host detoxification process. Therefore, Pyro-SIP also provides another way to understand the complex gut metabolism by breaking it down. With the development of such new approaches, a revolution in understanding of the inner world of life may come into reach soon.

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Author contributions

Y.S. and W.B. designed the study. H.G. performed sugar derivatization and GC analysis, S.B. carried out IRMS measurement, Y.S., and W.B. interpreted data, H.G., and S.B. contributed to the discussion. Y.S. and W.B. wrote the paper.

Additional information

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References

(2012). Structure, function and diversity of the healthy human microbiome. *Nature* **486:** 207-214.

Adams AS, Jordan MS, Adams SM, Suen G, Goodwin LA, Davenport KW *et al* (2011). Cellulose-degrading bacteria associated with the invasive woodwasp Sirex noctilio. *ISME J* **5:** 1323-1331.

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56:** 1919-1925.

Anand AAP, Vennison SJ, Sankar SG, Prabhu DIG, Vasan PT, Raghuraman T *et al* (2010). Isolation and characterization of bacteria from the gut of Bombyx mori that degrade cellulose, xylan, pectin and starch and their impact on digestion. *J Insect Sci* **10**.

Anderson IC, Cairney JWG (2004). Diversity and ecology of soil fungal communities: increased understanding through the application of molecular techniques. *Environmental Microbiology* **6:** 769-779.

Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y *et al* (2011). Induction of colonic regulatory T cells by indigenous Clostridium species. *Science* **331:** 337-341.

Behr T, Koob C, Schedl M, Mehlen A, Meier H, Knopp D *et al* (2000). A nested array of rRNA targeted probes for the detection and identification of enterococci by reverse hybridization. *Systematic and applied microbiology* **23:** 563-572.

Benson AK, Kelly SA, Legge R, Ma F, Low SJ, Kim J *et al* (2010). Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. *Proc Natl Acad Sci U S A* **107:** 18933-18938.

Brinkmann N, Martens R, Tebbe CC (2008). Origin and diversity of metabolically active gut bacteria from laboratory-bred larvae of Manduca sexta (Sphingidae, Lepidoptera, Insecta). *Appl Environ Microbiol* **74:** 7189-7196.

Broderick NA, Raffa KF, Goodman RM, Handelsman J (2004). Census of the bacterial community of the gypsy moth larval midgut by using culturing and culture-independent methods. *Appl Environ Microbiol* **70:** 293-300.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK *et al* (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7:** 335-336.

Clemente JC, Ursell LK, Parfrey LW, Knight R (2012). The impact of the gut microbiota on human health: an integrative view. *Cell* **148:** 1258-1270.

Coplen TB, Brand WA, Gehre M, Groning M, Meijer HAJ, Toman B *et al* (2006). New guidelines for delta C-13 measurements. *Analytical chemistry* **78:** 2439-2441.

Dillon RJ, Dillon VM (2004). The gut bacteria of insects: nonpathogenic interactions. *Annu Rev Entomol* **49:** 71-92.

Drake HL, Wust PK, Horn MA (2011). Clostridiaceae and Enterobacteriaceae as active fermenters in earthworm gut content. *Isme Journal* **5:** 92-106.

Dumont MG, Murrell JC (2005). Stable isotope probing - linking microbial identity to function. *Nat Rev Microbiol* **3:** 499-504.

Egert M, Stingl U, Bruun LD, Pommerenke B, Brune A, Friedrich MW (2005). Structure and topology of microbial communities in the major gut compartments of Melolontha melolontha larvae (Coleoptera: Scarabaeidae). *Appl Environ Microbiol* **71:** 4556-4566.

Engel P, Martinson VG, Moran NA (2012). Functional diversity within the simple gut microbiota of the honey bee. *Proc Natl Acad Sci U S A* **109:** 11002-11007.

Fierer N, Jackson RB (2006). The diversity and biogeography of soil bacterial communities. *P Natl Acad Sci USA* **103:** 626-631.

Funke M, Buchler R, Mahobia V, Schneeberg A, Ramm M, Boland W (2008). Rapid hydrolysis of quorum-sensing molecules in the gut of lepidopteran larvae. *Chembiochem* **9:** 1953-1959.

Horn MA, Schramm A, Drake HL (2003). The earthworm gut: an ideal habitat for ingested N2O-producing microorganisms. *Appl Environ Microbiol* **69:** 1662-1669.

Ishak HD, Plowes R, Sen R, Kellner K, Meyer E, Estrada DA *et al* (2011). Bacterial diversity in Solenopsis invicta and Solenopsis geminata ant colonies characterized by 16S amplicon 454 pyrosequencing. *Microb Ecol* **61:** 821-831.

Jones SE, Lennon JT (2010). Dormancy contributes to the maintenance of microbial diversity. *Proceedings of the National Academy of Sciences of the United States of America* **107:** 5881-5886.

Koch H, Schmid-Hempel P (2011). Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. *Proc Natl Acad Sci U S A* **108:** 19288-19292.

Kovatcheva-Datchary P, Egert M, Maathuis A, Rajilic-Stojanovic M, de Graaf AA, Smidt H *et al* (2009). Linking phylogenetic identities of bacteria to starch fermentation in an in vitro model of the large intestine by RNA-based stable isotope probing. *Environ Microbiol* **11:** 914-926.

Lenhart K, Bunge M, Ratering S, Neu TR, Schuttmann I, Greule M *et al* (2012). Evidence for methane production by saprotrophic fungi. *Nature communications* **3:** 1046.

Lu Y, Conrad R (2005). In situ stable isotope probing of methanogenic archaea in the rice rhizosphere. *Science* **309:** 1088-1090.

Lueders T, Manefield M, Friedrich MW (2004). Enhanced sensitivity of DNA- and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. *Environ Microbiol* **6:** 73-78.

Manero A, Blanch AR (2002). Identification of Enterococcus spp. based on specific hybridisation with 16S rDNA probes. *Journal of microbiological methods* **50:** 115-121.

Martinson VG, Danforth BN, Minckley RL, Rueppell O, Tingek S, Moran NA (2011). A simple and distinctive microbiota associated with honey bees and bumble bees. *Mol Ecol* **20:** 619-628.

Neufeld JD, Vohra J, Dumont MG, Lueders T, Manefield M, Friedrich MW *et al* (2007). DNA stable-isotope probing. *Nat Protoc* **2:** 860-866.

Pauchet Y, Wilkinson P, Vogel H, Nelson DR, Reynolds SE, Heckel DG *et al* (2010). Pyrosequencing the Manduca sexta larval midgut transcriptome: messages for digestion, detoxification and defence. *Insect molecular biology* **19:** 61-75.

Perkins GA, den Bakker HC, Burton AJ, Erb HN, McDonough SP, McDonough PL *et al* (2012). Equine stomachs harbor an abundant and diverse mucosal microbiota. *Appl Environ Microbiol* **78:** 2522-2532.

Pesek J, Buchler R, Albrecht R, Boland W, Zeth K (2011). Structure and Mechanism of Iron Translocation by a Dps Protein from Microbacterium arborescens. *J Biol Chem* **286:** 34872-34882.

Pilloni G, von Netzer F, Engel M, Lueders T (2011). Electron acceptor-dependent identification of key anaerobic toluene degraders at a tar-oil-contaminated aquifer by Pyro-SIP. *FEMS Microbiol Ecol*.

Ping L, Buchler R, Mithofer A, Svatos A, Spiteller D, Dettner K *et al* (2007). A novel Dps-type protein from insect gut bacteria catalyses hydrolysis and synthesis of N-acyl amino acids. *Environ Microbiol* **9:** 1572-1583.

Poulsen M, Sapountzis P (2012). Behind every great ant, there is a great gut. *Molecular ecology* **21:** 2054-2057.

Price NP (2004). Acylic sugar derivatives for GC/MS analysis of 13C-enrichment during carbohydrate metabolism. *Analytical chemistry* **76:** 6566-6574.

Priya NG, Ojha A, Kajla MK, Raj A, Rajagopal R (2012). Host Plant Induced Variation in Gut Bacteria of Helicoverpa armigera. *PLoS One* **7**.

Reichardt N, Barclay AR, Weaver LT, Morrison DJ (2011). Use of Stable Isotopes To Measure the Metabolic Activity of the Human Intestinal Microbiota. *Appl Environ Microb* **77:** 8009-8014.

Robinson CJ, Schloss P, Ramos Y, Raffa K, Handelsman J (2010). Robustness of the bacterial community in the cabbage white butterfly larval midgut. *Microb Ecol* **59:** 199-211.

Russell JA, Moreau CS, Goldman-Huertas B, Fujiwara M, Lohman DJ, Pierce NE (2009). Bacterial gut symbionts are tightly linked with the evolution of herbivory in ants. *Proc Natl Acad Sci U S A* **106:** 21236-21241.

Shao Y, Spiteller D, Tang X, Ping L, Colesie C, Munchberg U *et al* (2011). Crystallization of alpha- and beta-carotene in the foregut of Spodoptera larvae feeding on a toxic food plant. *Insect Biochem Mol Biol* **41:** 273-281.

Silverman N, Paquette N (2008). Immunology. The right resident bugs. *Science* **319:** 734-735.

Sudakaran S, Salem H, Kost C, Kaltenpoth M (2012). Geographical and ecological stability of the symbiotic mid-gut microbiota in European firebugs, Pyrrhocoris apterus (Hemiptera, Pyrrhocoridae). *Mol Ecol*.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011). MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular biology and evolution* **28:** 2731-2739.

Tang X, Freitak D, Vogel H, Ping L, Shao Y, Cordero EA *et al* (2012). Complexity and variability of gut commensal microbiota in polyphagous lepidopteran larvae. *PLoS One* **7:** e36978.

Vasquez A, Forsgren E, Fries I, Paxton RJ, Flaberg E, Szekely L *et al* (2012). Symbionts as major modulators of insect health: lactic Acid bacteria and honeybees. *PLoS One* **7:** e33188.

Warnecke F, Luginbuhl P, Ivanova N, Ghassemian M, Richardson TH, Stege JT *et al* (2007). Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. *Nature* **450:** 560-565.

Watanabe H, Tokuda G (2010). Cellulolytic systems in insects. *Annu Rev Entomol* **55:** 609-632.

Wong CN, Ng P, Douglas AE (2011). Low-diversity bacterial community in the gut of the fruitfly Drosophila melanogaster. *Environ Microbiol* **13:** 1889-1900.

Figures and tables

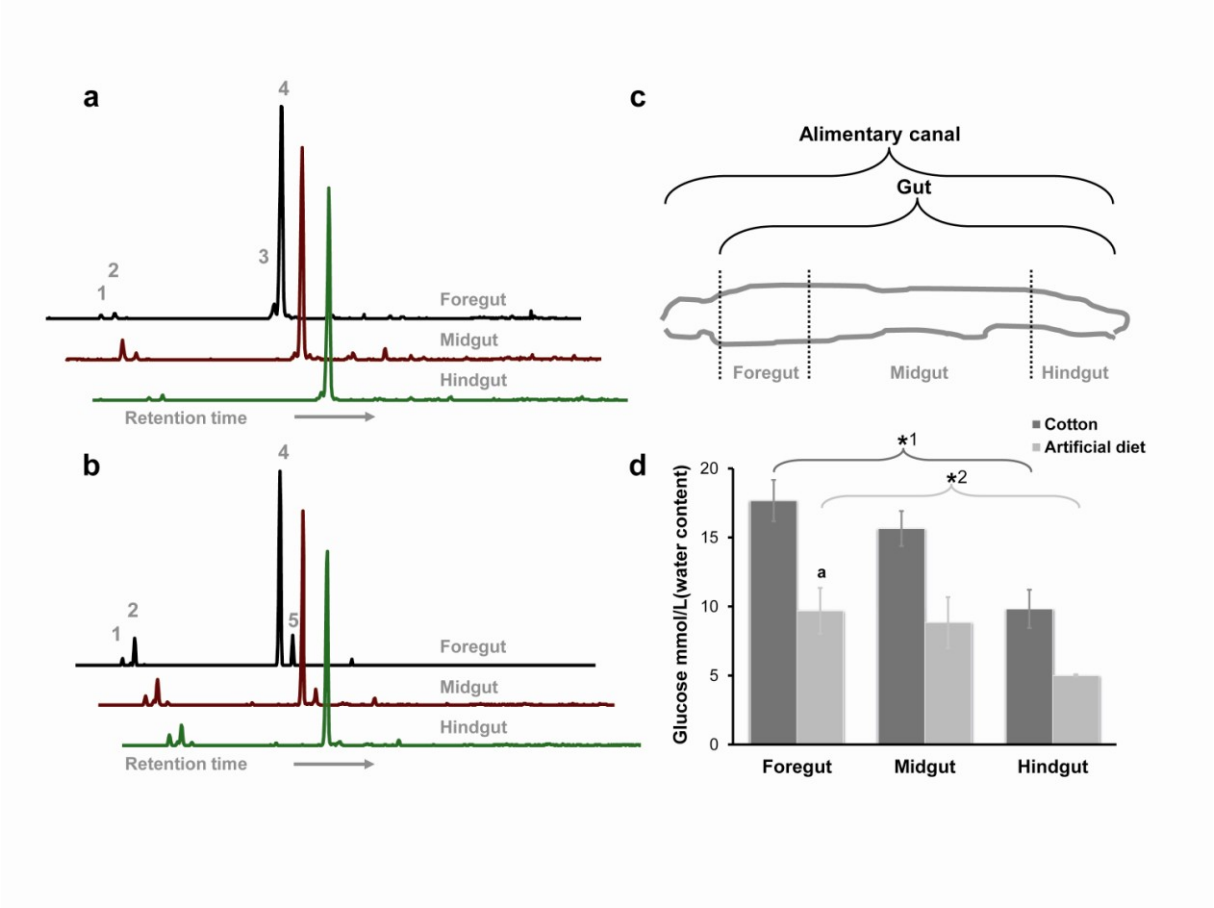

Figure 1 | Sugar composition in the gut content of cotton leafworm. (**a**) Sugars in the gut content of larvae fed on cotton or (**b**) artificial diet by GC-MS characterization after aldononitrile acetate derivatization**.** The larval alimentary canal is divided into three regions: foregut, midgut and hindgut, shown in diagram (**c**). (**d**) Quantification of dominant glucose reveals a significant decrease in average content along the gut. However, cotton-feeding larvae exhibit higher amount of glucose in all gut regions. * and ^{a} indicate significant difference: P ($*^{1}$) = 0.0020, P ($*^{2}$) = 0.0366, P (^{a}) = 0.0017). Error bars indicate standard errors. 1, Ribose; 2, Arabinose; 3, Mannose; 4, Glucose; 5, Galactose.

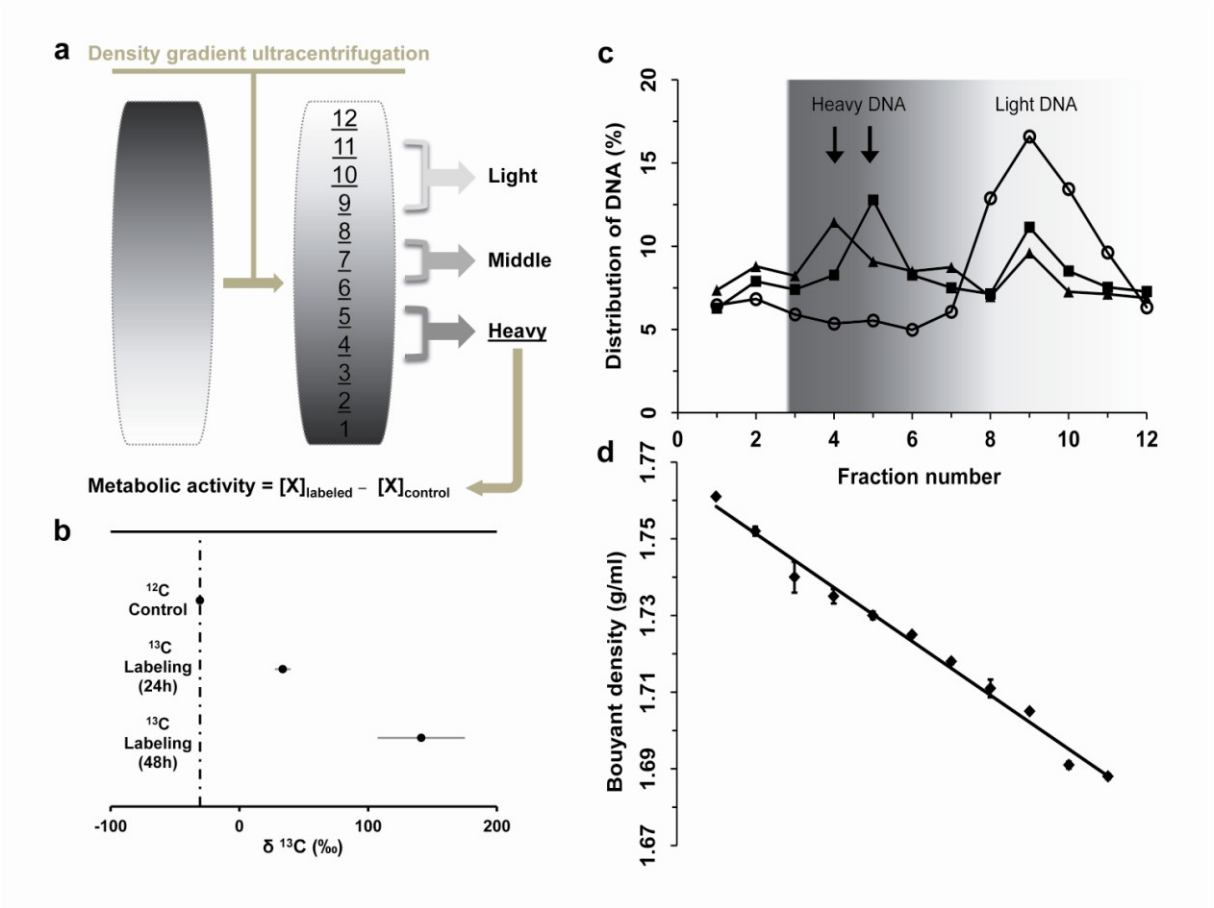

Figure 2 | Profiling of active bacteria. (**a**) For determining active bacteria, ^{13}C-labeled DNA is separated by density-gradient ultracentrifugation and subsequently retrieved from representative fractions, with darker color indicating labeled heavy DNA. All separated DNA samples (light, middle and heavy) were directly subjected to quantitative pyrosequencing for revealing species lineage and relative abundance. For each taxon **X**, metabolic activity is calculated as the difference in relative abundance between heavy fraction of the labeled sample and that of the control. (**b**) Isotopic ratio (δ^{13}C) of DNA samples. (**c**) Distribution of DNA content in gradient fractions of glucose treatments. Symbols: **O**, DNA extracted from the [^{12}C]-glucose control; ■, DNA extracted from the [^{13}C]-glucose treatment for 24 h; ▲, DNA extracted from the [^{13}C]-glucose treatment for 48 h. Arrows indicate that considerable ^{13}C-labeled DNA shifted to heavy gradients, compared with the control. (**d**) Gradient

fraction analysis by density after 40 h centrifugation of a 1.725 g ml^{-1} starting CsCl solution. Error bars indicate standard errors.

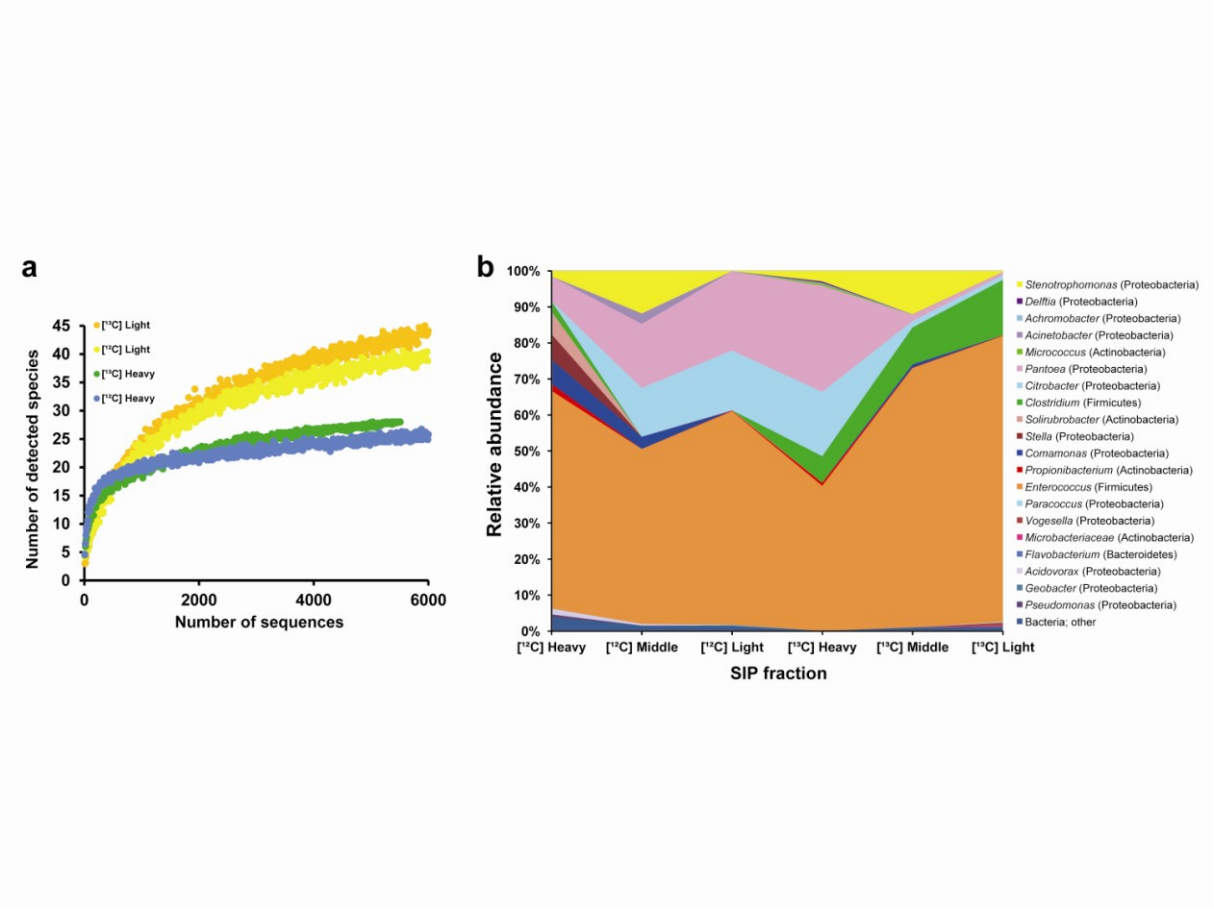

Figure 3 | Bacterial diversity and relative abundance in the gut microbiota of early-instar larvae. (**a**) Rarefaction curves of 16S rDNA sequences were obtained from representative SIP fractions of the control ([^{12}C]) and labeling treatment ([^{13}C]). (**b**) Relative abundance of bacterial taxa in different SIP fractions, represented in a relative area graph as revealed by pyrosequencing. Abbreviations: [^{12}C] Light, light fractions (fractions 9-11, Figure 2a) of native-glucose amendment; [^{12}C] Middle, middle fraction (fraction 7) of that; [^{12}C] Heavy, heavy fractions (fractions 4-5) of that; [^{13}C] Light, light fractions of ^{13}C-glucose amendment; [^{13}C] Middle, middle fraction of that; [^{13}C] Heavy, heavy fractions of that.

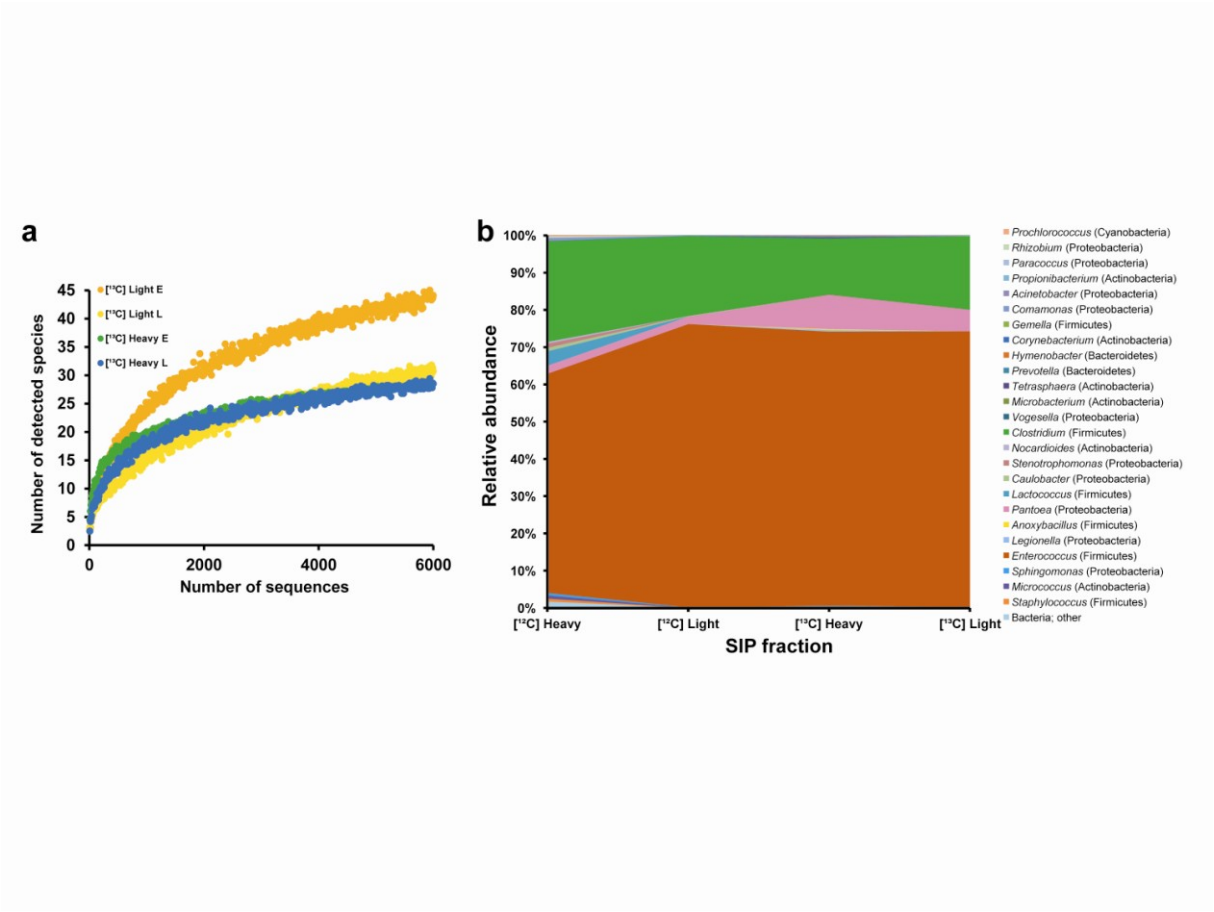

Figure 4 | Bacterial diversity and relative abundance in the gut microbiota of late-instar larvae. (**a**) Rarefaction curves of 16S rDNA sequences were obtained from representative SIP fractions of early-instar and late-instar larvae. Abbreviations: E, representative fractions from early-instar larvae fed on ^{13}C-glucose; L, fractions from late-instar larvae fed on ^{13}C-glucose. (**b**) Relative abundance of bacterial taxa in different SIP fractions, represented in a relative area graph as revealed by pyrosequencing. Abbreviations are the same as in Figure 3.

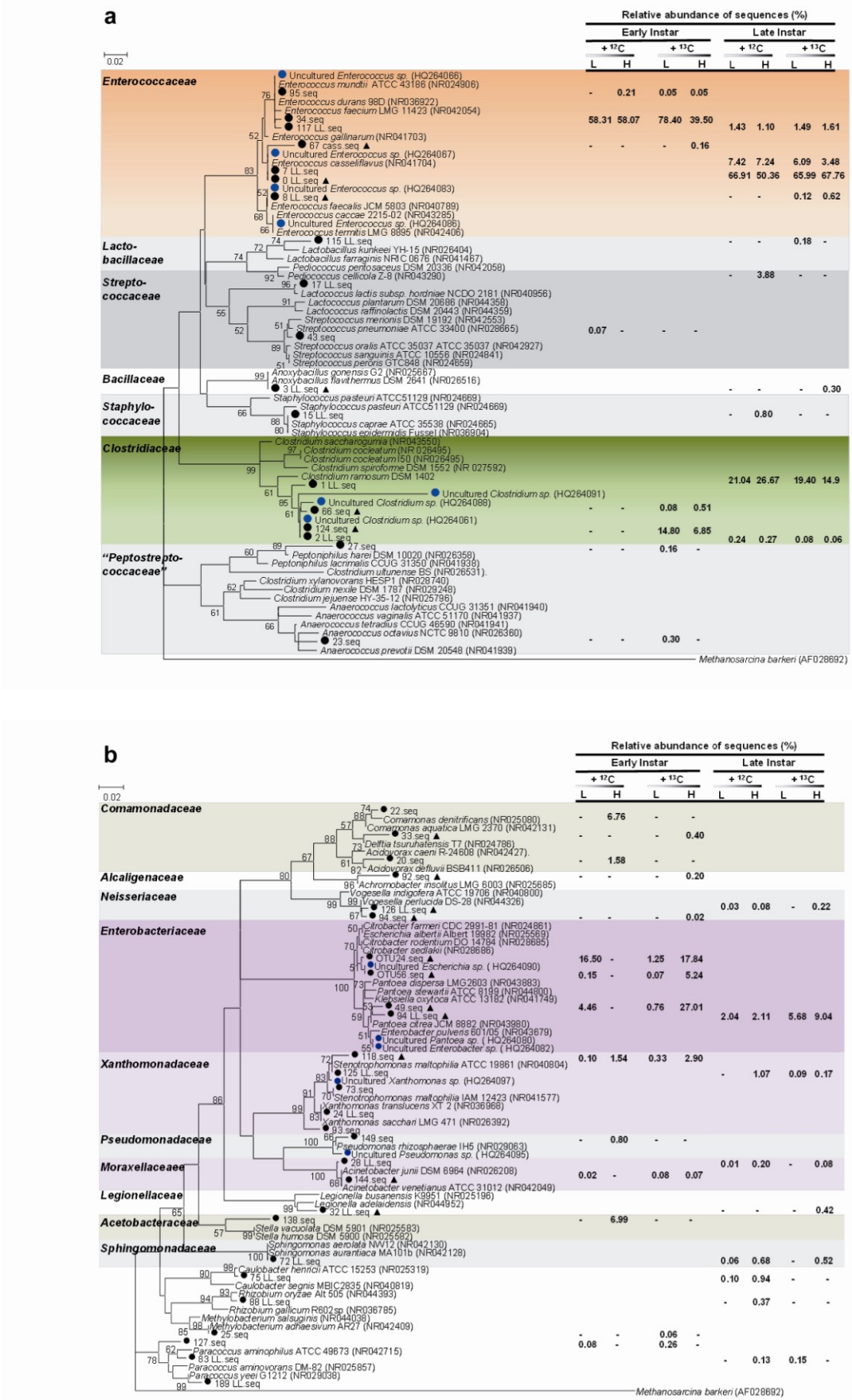
a
Relative abundance of sequences (%)
Early Instar
Late Instar
+ 12C
+ 13C
L
H
0.02
Enterococcaceae
Lacto-bacillaceae
Strepto-coccaceae
Bacillaceae
Staphylo-coccaceae
Clostridiaceae
"Peptostrepto-coccaceae"
Uncultured Enterococcus sp. (HQ264066)
Enterococcus mundtii ATCC 43186 (NR024906)
95.seq
Enterococcus durans 98D (NR036922)
Enterococcus faecium LMG 11423 (NR042054)
94.seq
117 LL.seq
Enterococcus gallinarum (NR041703)
67 cass.seq
Uncultured Enterococcus sp. (HQ264067)
Enterococcus casseliflavus (NR041704)
7 LL.seq
0 LL.seq
Uncultured Enterococcus sp. (HQ264083)
8 LL.seq
Enterococcus faecalis JCM 5803 (NR040789)
Enterococcus caccae 2215-02 (NR043285)
Uncultured Enterococcus sp. (HQ264086)
Enterococcus termitis LMG 8895 (NR042406)
115 LL.seq
Lactobacillus kunkeei YH-15 (NR026404)
Lactobacillus farraginis NRIC 0676 (NR041467)
Pediococcus pentosaceus DSM 20336 (NR042058)
Pediococcus cellicola Z-8 (NR043290)
17 LL.seq
Lactococcus lactis subsp. hordniae NCDO 2181 (NR040956)
Lactococcus plantarum DSM 20686 (NR044358)
Lactococcus raffinolactis DSM 20443 (NR044359)
Streptococcus merionis DSM 19192 (NR042553)
Streptococcus pneumoniae ATCC 33400 (NR026665)
43.seq
Streptococcus oralis ATCC 35037 ATCC 35037 (NR042927)
Streptococcus sanguinis ATCC 10556 (NR024841)
Streptococcus peroris GTC848 (NR024659)
Anoxybacillus gonensis G2 (NR025667)
Anoxybacillus flavithermus DSM 2641 (NR026516)
3 LL.seq
Staphylococcus pasteuri ATCC51129 (NR024669)
Staphylococcus pasteuri ATCC51129 (NR024669)
15 LL.seq
Staphylococcus caprae ATCC 35538 (NR024665)
Staphylococcus epidermidis Fussel (NR036904)
Clostridium saccharogumia (NR043550)
Clostridium cocleatum (NR 026495)
Clostridium cocleatum 150 (NR026495)
Clostridium spiroforme DSM 1552 (NR 027592)
Clostridium ramosum DSM 1402
1 LL.seq
Uncultured Clostridium sp. (HQ264091)
Uncultured Clostridium sp. (HQ264088)
66.seq
Uncultured Clostridium sp. (HQ264061)
124.seq
2 LL.seq
27.seq
Peptoniphilus harei DSM 10020 (NR026358)
Peptoniphilus lacrimalis CCUG 31350 (NR041938)
Clostridium ultunense BS (NR026531).
Clostridium xylanovorans HESP1 (NR028740)
Clostridium nexile DSM 1787 (NR029248)
Clostridium jejuense HY-35-12 (NR025786)
Anaerococcus lactolyticus CCUG 31351 (NR041940)
Anaerococcus vaginalis ATCC 51170 (NR041937)
Anaerococcus tetradius CCUG 46590 (NR041941)
Anaerococcus octavius NCTC 9810 (NR026360)
23.seq
Anaerococcus prevotii DSM 20548 (NR041939)
Methanosarcina barkeri (AF028692)
0.21 0.05 0.05
58.31 58.07 78.40 39.50
1.43 1.10 1.49 1.61
0.16
7.42 7.24 6.09 3.48
66.91 50.36 65.99 67.76
0.12 0.62
0.18
3.88
0.07
0.30
0.80
21.04 26.67 19.40 14.9
0.08 0.51
14.80 6.85
0.24 0.27 0.08 0.06
0.16
0.30
b
Relative abundance of sequences (%)
Early Instar
Late Instar
+ 12C
+ 13C
L
H
0.02
Comamonadaceae
Alcaligenaceae
Neisseriaceae
Enterobacteriaceae
Xanthomonadaceae
Pseudomonadaceae
Moraxellaceaee
Legionellaceae
Acetobacteraceae
Sphingomonadaceae
22.seq
Comamonas denitrificans (NR025080)
Comamonas aquatica LMG 2370 (NR042131)
33.seq
Delftia tsuruhatensis T7 (NR024786)
Acidovorax caeni R-24608 (NR042427).
20.seq
Acidovorax defluvii BSB411 (NR026506)
92.seq
Achromobacter insolitus LMG 6003 (NR025685)
Vogesella indigofera ATCC 19706 (NR040800)
Vogesella perlucida DS-28 (NR044326)
126 LL.seq
94.seq
Citrobacter farmeri CDC 2991-81 (NR024861)
Escherichia albertii Albert 18982 (NR025689)
Citrobacter rodentium DO 14784 (NR028685)
Citrobacter sedlakii (NR028686)
OTU24.seq
Uncultured Escherichia sp. (HQ264090)
OTU56.seq
Pantoea dispersa LMG2603 (NR043883)
Pantoea stewartii ATCC 8199 (NR044800)
Klepsiella oxytoca ATCC 13182 (NR041749)
49.seq
94 LL.seq
Pantoea citrea JCM 8882 (NR043980)
Enterobacter pulvens 601/05 (NR043679)
Uncultured Pantoea sp. (HQ264080)
Uncultured Enterobacter sp. (HQ264082)
118.seq
Stenotrophomonas maltophilia ATCC 19861 (NR040804)
125 LL.seq
Uncultured Xanthomonas sp. (HQ264097)
73.seq
Stenotrophomonas maltophilia IAM 12423 (NR041577)
Xanthomonas translucens XT 2 (NR036966)
24 LL.seq
Xanthomonas sacchari LMG 471 (NR026392)
93.seq
149.seq
Pseudomonas rhizosphaerae IH5 (NR029083)
Uncultured Pseudomonas sp. (HQ264095)
28 LL.seq
Acinetobacter junii DSM 6964 (NR026208)
144.seq
Acinetobacter venetianus ATCC 31012 (NR042049)
Legionella busanensis K9951 (NR025196)
Legionella adelaidensis (NR044952)
32 LL.seq
138.seq
Stella vacuolata DSM 5901 (NR025583)
Stella humosa DSM 5900 (NR025582)
Sphingomonas aerolata NW12 (NR042130)
Sphingomonas aurantiaca MA101b (NR042128)
72 LL.seq
Caulobacter henrici ATCC 15253 (NR025319)
75 LL.seq
Caulobacter segnis MBIC2835 (NR040819)
Rhizobium oryzae Alt 505 (NR044393)
88 LL.seq
Rhizobium gallicum R602sp (NR036785)
Methylobacterium salsuginis (NR044038)
Methylobacterium adhaesivum AR27 (NR042409)
25.seq
127.seq
Paracoccus aminophilus ATCC 49673 (NR042715)
83 LL.seq
Paracoccus aminovorans DM-82 (NR025857)
Paracoccus yeei G1212 (NR029038)
189 LL.seq
Methanosarcina barkeri (AF028692)
0.10 1.54 0.33 2.90
0.02 0.08 0.07
0.06 0.26
0.08
6.76
0.40
1.58
0.20
0.03 0.08 0.22
0.02
16.50 1.25 17.84
0.15 0.07 5.24
4.46 0.76 27.01
2.04 2.11 5.68 9.04
1.07 0.09 0.17
0.80
0.01 0.20 0.08
6.99
0.06 0.68 0.52
0.10 0.94
0.37
0.13 0.15

Figure 5 | Phylogenetic analysis of (a) Firmicutes and (b) Proteobacteria identified from the gut microbiota of cotton leafworm. (**a**) Maximum Likelihood tree was derived from partial 16S rDNA sequence data for members of Firmicutes. (**b**) Neighbor-Joining tree was derived from partial 16S rDNA sequence data for members of Proteobacteria. Representative pyrosequences from this work and near full-length 16S rDNA sequences retrieved from previous clone-library-based studies are indicated by black circles (●) and blue circles, respectively. Labeled taxa are marked with triangles (▲). Reference sequences are downloaded from GenBank (accession numbers are in parentheses.). *Methanosarcina barkeri* (AF028692) is used as an outgroup. Family-level clusters are indicated by different colors. Bootstrap values (in percent) are based on 1000 replications. Bar represents 2% sequence divergence. Right section denotes percentage of representative bacterial 16S rRNA sequences in the total dataset of each sample. Abbreviations: + ^{12}C, native-glucose amendment; + ^{13}C, ^{13}C-glucose amendment; L, light fractions; H, heavy fractions.

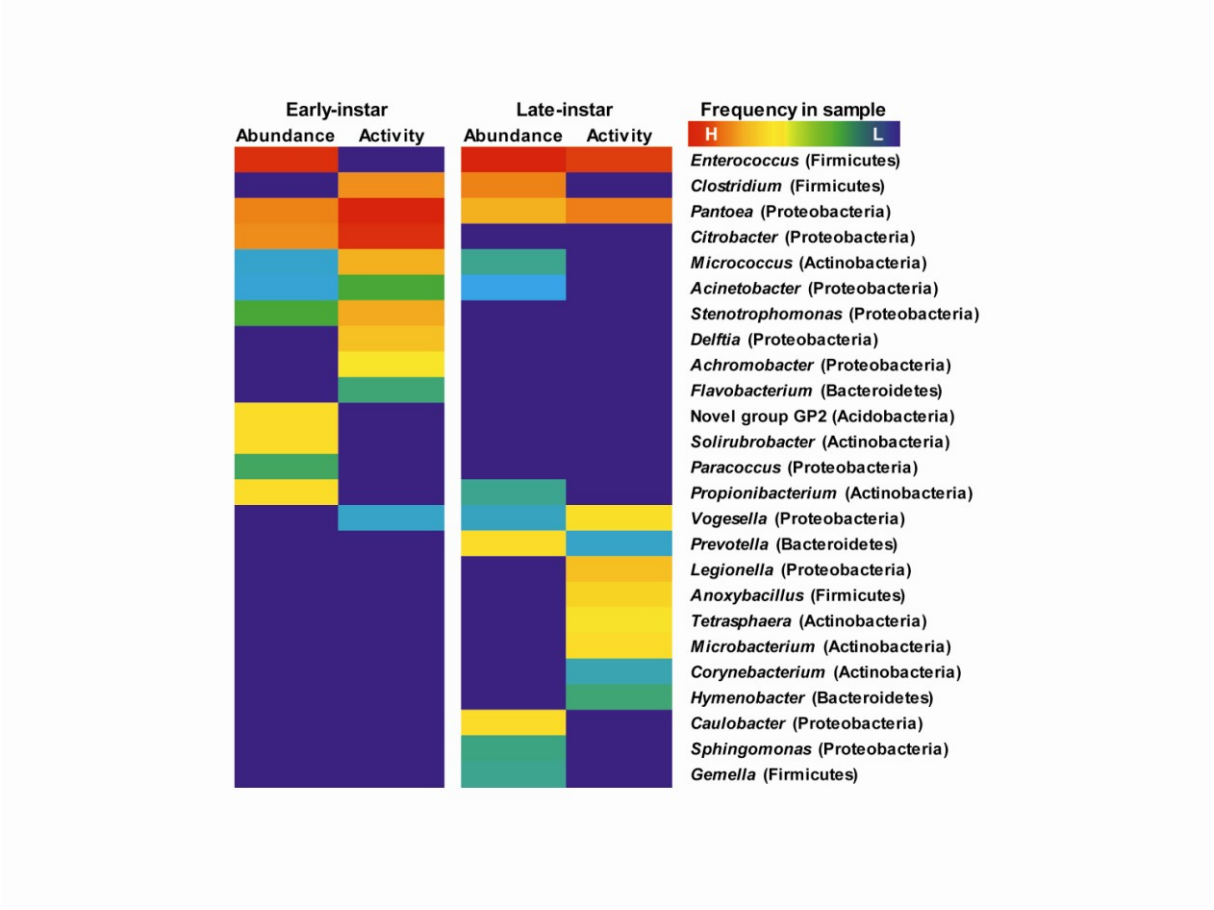

Figure 6 | Frequency of 16S rRNA sequences in the microbiota obtained from the native-glucose control (bacterial relative abundance) and [^{13}C]-glucose treatment (bacterial metabolic activity), represented as a heatmap. Left panel displays dynamic changes of taxa in early-instar larvae and right panel for late-instar. Warm colors indicate higher and cold colors lower abundance, calculated according to the formula in Figure 2a.

Sample	Number of reads	Species richness indices			Species diversity indices	
		Chao1	Observed	PD tree	Shannon	Simpson
Early-instar control						
Light fraction	17856	42	34	3	1.85	0.60
Middle fraction	3534	27	26	2	2.48	0.72
Heavy fraction	11701	28	23	3	2.43	0.64
Early-instar labeled						
Light fraction	23042	51	38	3	1.24	0.36
Middle fraction	4479	29	22	2	0.16	0.47
Heavy fraction	5517	30	27	2	2.57	0.75
Late-instar control						
Light fraction	10872	29	23	3	1.46	0.50
Heavy fraction	22891	47	42	4	2.36	0.67
Late-instar labeled						
Light fraction	9254	32	24	2	1.57	0.52
Heavy fraction	10899	28	25	3	1.66	0.51

Table 1 | Richness and diversity estimate of the 16S rRNA gene from the pyrosequencing analysis.

% of total sequence reads in each developmental stage		
Phylum	**Early-instar larvae**	**Late-instar larvae**
Actinobacteria	1.28	0.11
Acidobacteria	0.18	0.00
Bacteroidetes	0.00	0.11
Firmicutes	59.24	97.21
Proteobacteria	38.88	2.21
Other	0.43	0.36

Table 2 | Abundance of the 16S rRNA gene in each larval stage at phylum level.

6. Unpublished results Part I

Introduction

Abundant carotenes are sequestered in the crop of healthy cotton leafworms (*Spodoptera littoralis*), suggesting that unknown physiological roles of these pigments. Moreover, this phenomenon also appears in other *Spodoptera* species, such as *S. exigua* (Beet Armyworm), and in another lepidopteran, *Helicoverpa armigera*. I was prompted to investigate the nature of this accumulation. This part described experimental data that argue for the ROS as a factor controlling the accumulation and a role of carotenes as part of the anti-oxidant system in herbivores.

During the herbivore attack, it is known that plants respond with both local defenses and systemic defenses, which may release many toxic compounds. And many plant secondary compounds are capable of acting as photosensitizers and can cause biological damage to insects. It is well recognized that the rapid production of reactive oxygen species (ROS) is an essential part of plant stress responses, including herbivory (Wu and Baldwin 2010). Superoxide anion (O_2-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hydroxyl radical (·OH) are collectively called ROS. A large body of evidence has demonstrated that herbivory attacks rapidly induce a localized oxidative burst in plants (Bi and Felton 1995, Leitner et al 2005, Maffei et al 2006, Mithofer et al 2004). Especially in legume plants such as lima bean, the herbivory causes a significant increase of hydroxyl radical (·OH) and hydrogen peroxide (H_2O_2) (Bi and Felton 1995, Maffei et al 2006). H_2O_2 levels remained elevated as long as the attacks persist (Maffei et al 2006). Oxidative responses in the plants corresponded with oxidative damage in the midguts of insects (*Helicoverpa armigera*) feeding on previously wounded soybean (*Glycine max*) (Bi and Felton 1995). The same situation could also happen in *Spodoptera* species. A feeding larva of *Spodoptera* easily consumes several grams of leaves per hour, thus its digestive tract is exposed to a consistent attack of ROS

during foraging. In particular, the crop, as a food storage organ in the foregut, would suffer most diverse and the highest concentration of ROS. On the other hand, herbivores should have some strategies to counterattack those direct oxidative injuries during the competition with plants. It is reported that beta carotene, at a dietary concentration of 0.1%, could effectively protect *Manduca sexta* against the deleterious singlet oxygen and free radicals formed by the photo-activated plant secondary compounds (R.R. AUCOIN 1990). Therefore, carotenes, among the nature's best antioxidant, could be selectively sequestered by *Spodoptera* as a defense against toxic ROS.

Results

The red colored crop phenomenon is not unique to *Spodoptera littoralis* feeding on lima bean plants. I also observed the formation of red colored crop in the larvae fed on other plants, such as snap bean, cotton and Arabidopsis. Although the formation of carotene aggregates (crystals) was somehow different in the larvae fed on different plant diets, the principle behind this selective accumulation should be similar (Figure 6-1 E-G). Furthermore, same structure was found in different caterpillar species, for example in *Spodoptera exigua* (Figure 6-1 A-D).

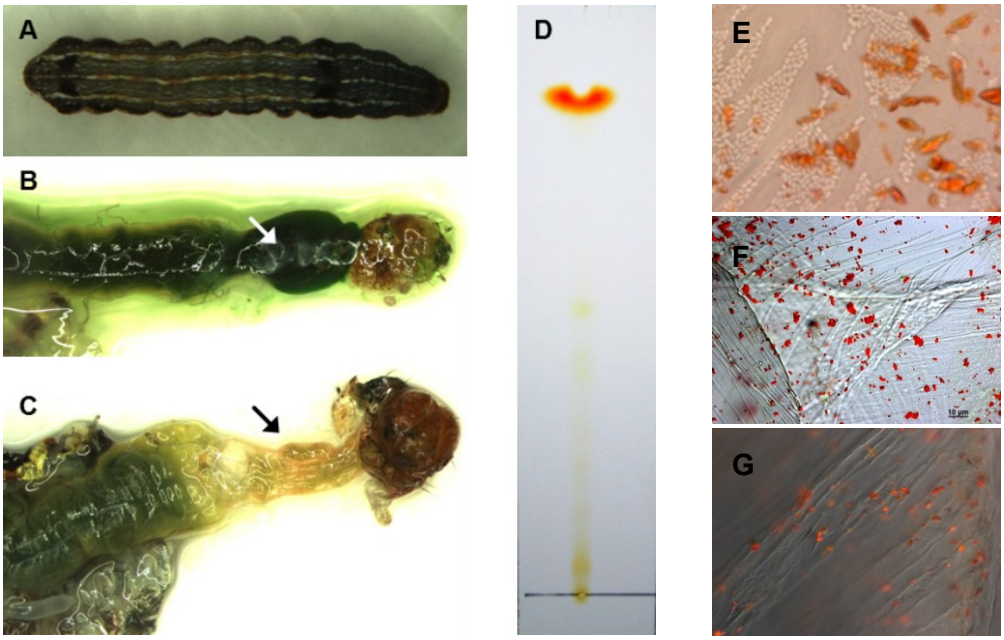

Figure 6-1: The development of red crop in *Spodoptera exigua* after feeding on lima bean plants. (**A**) Larvae of *S. exigua* feeding on lima bean until 3rd - 4th instar. (**B**) Ventral view of enlarged foregut (white arrow), full of ingested plant leaf pieces. (**C**) Ventral view of empty foreguts, showing a red colored crop (black arrow). (**D**) Thin layer chromatography (TLC) of the red crop extraction with acetone, indicating major carotenoid components. The orange spot on the top of the TLC plate is beta carotene. (**E**) - (**G**) Different shape of carotenoid crystals from larvae fed on (E) lima bean, (F) Arabidopsis and (G) cotton for 4 days.

The panel of carotenoid molecules found in the red colored crop had been extensively analyzed by mass spectrometry after extraction and chromatographic isolation. Raman imaging technology was directly preformed on the gut tissue to reveal the beta carotene signature (Figure 6-2). The red colored crop contained mostly beta carotene. Other carotenoids comprised a trace amount of alpha carotene and lutein. IRMS indicated that the $\delta^{13}C$ value of the carotenoid crystallized in the crop of *Spodoptera* was statistically

indistinguishable from those purified from lima bean leaves, indicating that it was taken from the food plant.

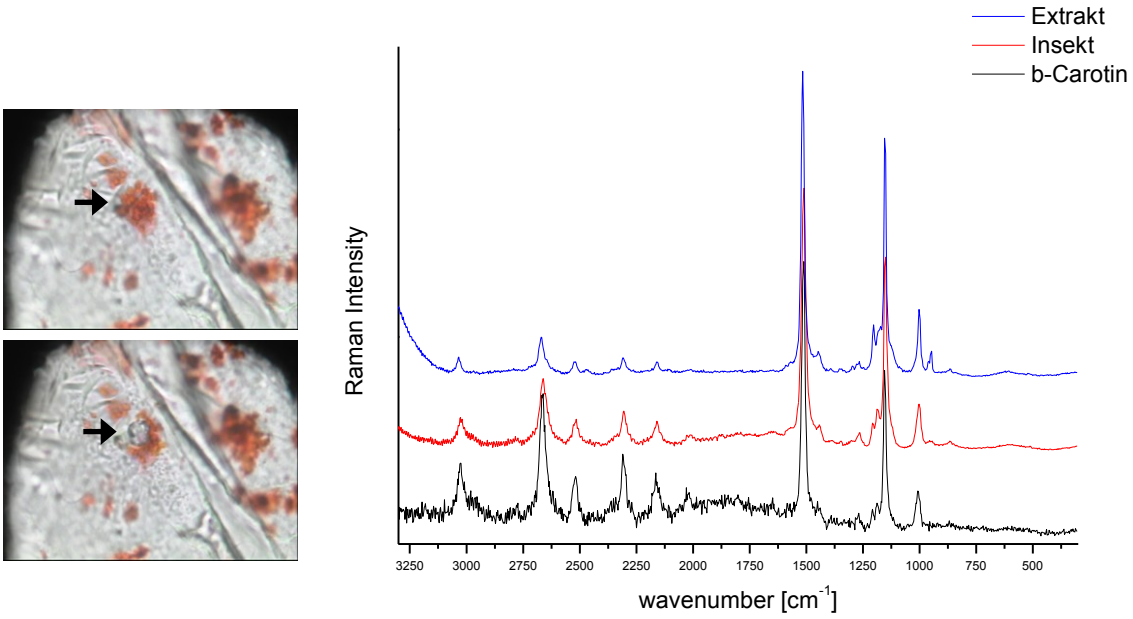

Figure 6-2: Carotene signature of the native crystal in the red crop tissue of the health larvae by Raman imaging. A 488 nm laser excitation of the Raman spectrometry was used. The microscope laser was directly focused on a crystal (black arrows in the left panel) in the insect crop tissue (red-colored spectrum) and on spontaneous crystals obtained after extraction of the red crop tissue (blue-colored spectrum). A control with pure beta carotene was shown (black-colored spectrum).

However, this phenomenon was complicated by the fact that when beta carotene was amended with artificial diet to feed larvae, we did not observe the formation of red crop (Figure 6-3 A; B). Therefore, besides the carotenoids, some other factors from the host plant were needed to induce this sequestration, probably the ROS. It is known that lima bean leaves generated higher production of ROS at the wounding site after the attack by *Spodoptera*, which may reach intracellular concentrations of up to 1 M H_2O_2 in about 13 min (Maffei et al

2006). The elevated ROS potentially causes damage in the insect gut tissue. And the idea that carotenoids especially beta carotene, both in polar and in nonpolar environments, may be protective antioxidants in all kinds of organisms and tissues has been widely accepted. Here, I investigated whether the carotene accumulation in the crop was due to the elevated ROS or not. The larvae fed on toxic artificial diet, which contained ROS generators (pro-oxidants, such as *tert*-Butyl hydroperoxide), exhibited symptoms of severe oxidative stress: slowly growing, high mortality. But in the larvae, which were transferred to the carotene fortified artificial diet soon after one hour oxidative stress treatment on the toxic diet, these symptoms were largely ameliorated. After dissection, the formation of red crystals in intact membrane of the crop was confirmed under microscopy (Figure 6-3 D), which was different with previous carotene addition alone (Figure 6-3 C). Thus, the high level of ROS could trigger the sequestration of carotenes from the diet, although this artificial oxidative stress only partially restored the red crop, which was not as obviously as the larvae fed on native plants.

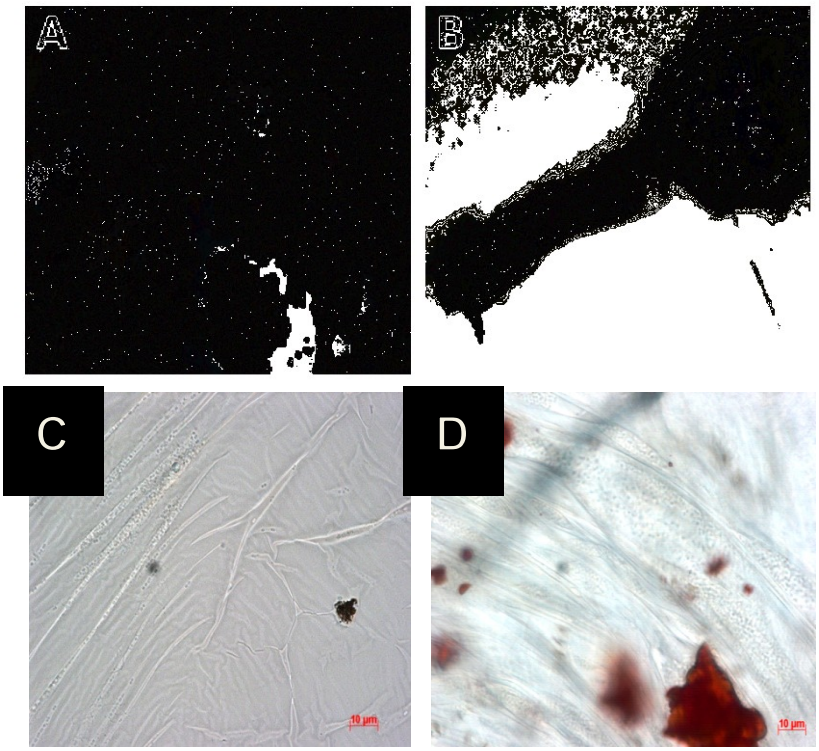

Figure 6-3: (**A**) *Spodoptera* larvae were reared on artificial diet mixed with 2.0 μM g^{-1} beta carotene (Calbiochem), a concentration frequently found in plants in nature (R.R. AUCOIN 1990). (**B**) No carotene accumulation in the larval crop after one week's feeding on carotene fortified diet. (**C**) Microphotography showed a clear colorless crop tissue from (B) under the phase contrast microscopy. (**D**) Native crystals formed in the larval crop tissue after oxidative stress treatment.

In conclusion, since the host plant releases high concentrations of ROS during herbivore foraging and on the other hand carotene pigments may protect the cell membrane from oxidative damage (Taylor et al 1971), it is reasonable to suppose that in the phytophagous *Spodoptera*, those highly sequestered carotenes may act as an effective antioxidant and have survival value to those herbivores, which most often have a very fast food-throughput life style under sunlit conditions in the wild. Thus, the facilitated absorption of carotenes could be one of the strategies of successful herbivore defense against direct oxidative injury during feeding plants.

7. Unpublished results Part II

Introduction

Previous studies showed that several *Enterococcus* species are consistently present and dominate in the digestive tract of cotton leafworm but are not derived from their food plants. It is necessary to elucidate the transmission route of this core symbiont since transmission from generation to generation is a key factor for the success of the symbiotic microorganisms. Although the environmental uptake of symbionts in every host generation has been reported in a stinkbug (Kikuchi et al 2007), vertical transmission from mother to offspring via egg appears to be the predominant mode of symbiont transfer in insects. However, the routes of vertical transmission are quite diverse among different symbiotic systems (Bright and Bulgheresi 2010). Here I investigated the transmission route of *Enterococcus*, the core bacteria in the gut microbiota of *Spodoptera littoralis*. Since it was difficult to identify different *Enterococcus* species simply by morphology, several biochemical tests and the molecular technique were combined to fast screen bacteria isolated from the egg mass. All *Enterococcus* species, which also dominated in the gut late, were directly identified from the egg, suggesting a typical vertical transmission mode. Clone library analysis of the bacterial 16s rRNA gene also supported this conclusion. Successful cultivation of those symbionts was convenient for the later functional study too.

This vertical transmission strongly suggests important roles of *Enterococci* in the host. Generally, supplying the nutrient to the host or protecting the host against harmful microbes is proposed for those symbionts' function. Here I tested those hypotheses. A low concentration of erythromycin could effectively remove or at least decrease the *Enterococcus* population in the gut but did not obviously impact other bacteria. After oral administration of antibiotics, the survival rate and growth rate of larvae did not significantly decrease. It appears that those *Enterococci* do not play a role in the nutrition of the host. However the cultured *Enterococcus*

strain quickly and efficiently produces some antibiotic substances which inhibit several tested gram positive and negative bacteria. The initial purification and characterization of antibiotic compounds were reported too. Therefore, *Enterococcus* could be involved in the host defense against harmful bacteria from outside.

Results

1. Transmission route of *Enterococcus* species

Three *Enterococcus* species, including *E. faecalis*, *E. casseliflavus* and *E. mundtii*, were regularly detected on crushed eggs based on biochemical tests (Table 7-1) (Manero and Blanch 1999). And the near full length 16S rRNA gene sequences were obtained by colony-PCR for the isolates, which confirmed the correct identification (Identity > 99.5%).

Enterococcus sp.	Fermentation pattern		Pigment	16s rDNA identity
	L-arabinose	Methyl-α-D-glucopyranoside		
E. faecalis	+	-	-	100%
E. mundtii	+	-	+	99.78%
E. casseliflavus	+	+	+	100%

Table 7-1: Biochemical key to identify three *Enterococcus* spp. associated with *S. littoralis* eggs. The identification was also confirmed by bacterial 16s rRNA gene sequencing.

Interestingly by scanning electron microscopy, some bacteria were also found to locate inside the eggshell, which morphology resembled that of the cultured *Enterococcus* (Figure 7-1, C; D). Examination of the insect oocyte with FISH proved that some *Enterococcus* cells located

on the pole of the oocyte (Figure 7-1, E; F). Notably, *Enterococcus* was also found in eggs of tobacco hornworm (*Manduca sexta*) and those bacteria were already metabolically active there based on rRNA detection (Brinkmann et al 2008). It is know that the endosymbionts of diverse insects tend to localize at the pole of host eggs (Matsuura et al 2011).

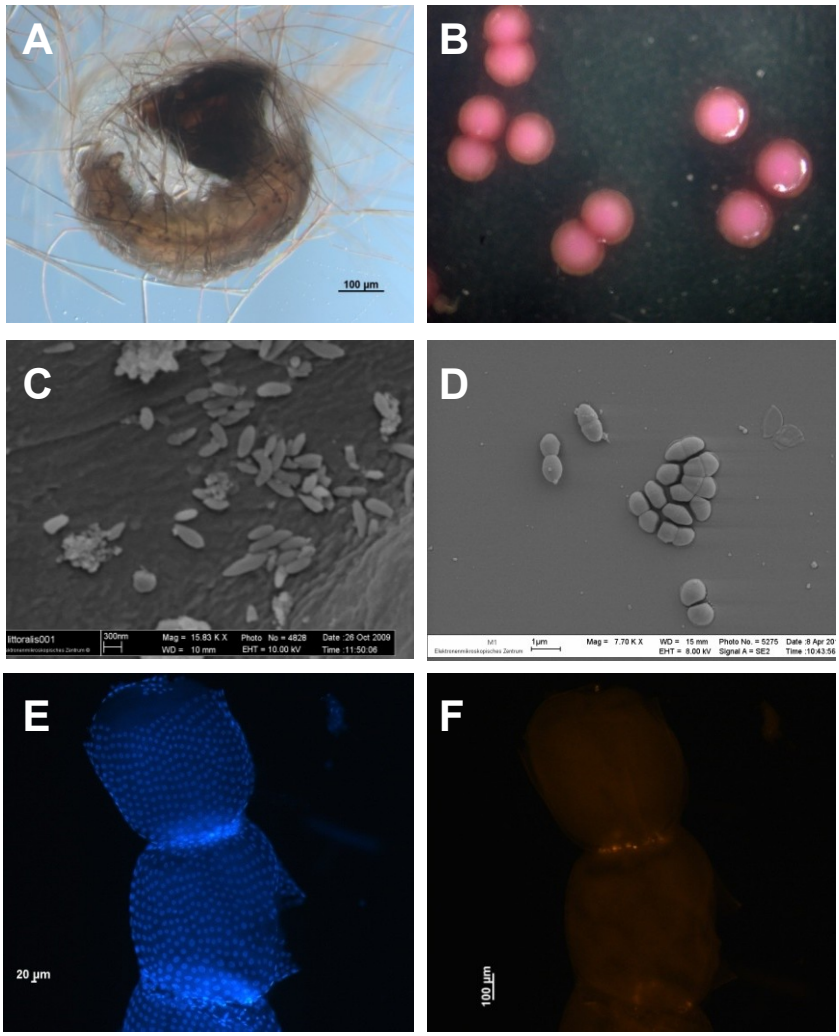

Figure 7-1: (**A**) a typical egg showing the larva under hatching. (**B**) *Enterococcus* is regularly isolated from the egg mass, which produces smooth, red-colored colony on agar plate. (**C**) Scanning electron micrograph (SEM) of inner surface of egg shell. (**D**) SEM indicates similar morphology of *Enterococcus* cultured *in vitro*. (**E**) DAPI staining showing the host oocyte nuclei and a smear of bacteria. (**F**) FISH with an *Enterococcus*-specific probe show some bacteria located on the pole of oocytes.

Representative *Enterococcus* 16S rDNA sequences from both the egg and the gut were most similar in a phylogenetic study, which strongly suggested that the bacteria in the gut originated from the egg (Figure 7-2). It is observed that before the larvae hatch out, they already start feeding and have to bite enough eggshell material to make a hole and then could successfully escape from the egg case. Symbionts must be transmitted from the egg to the gut during this process. Most of the hatched larvae immediately eat the plant leaves not the eggshell anymore.

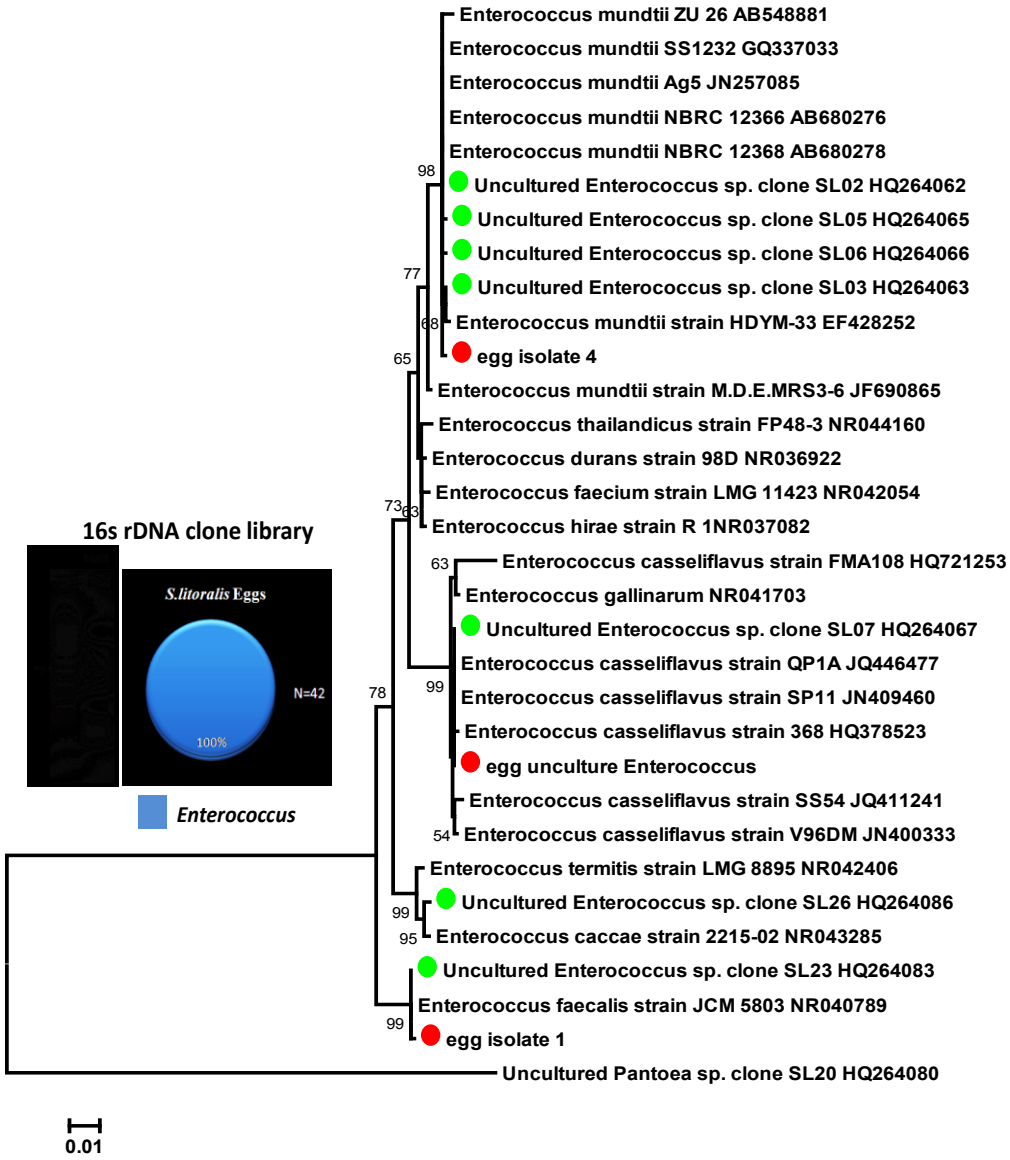

Figure 7-2: Phylogenetic position of *Enterococcus* species isolated from the egg and the gut of *Spodoptera littoralis*. Neighbor-joining tree constructed on the basis of 16S rRNA gene sequences. Bootstrap values (%) were obtained from a search with 100 replicates. Red color indicates bacterial sequences got from the egg sample and green from the gut sample. A clone library analysis detected only *Enterococcus* spp. from eggs.

2. Possible functions of *Enterococcus*

By using an effective antibiotic (erythromycin), *Enterococci* were successfully cured from the larval gut. But there was no difference in growth rate and survival between the *Enterococcus*-cured larvae and the non-treated control fed on different food sources (Figure 7-3). This targeted manipulation indicated that predominate *Enterococci* likely did not contribute to the host nutrition.

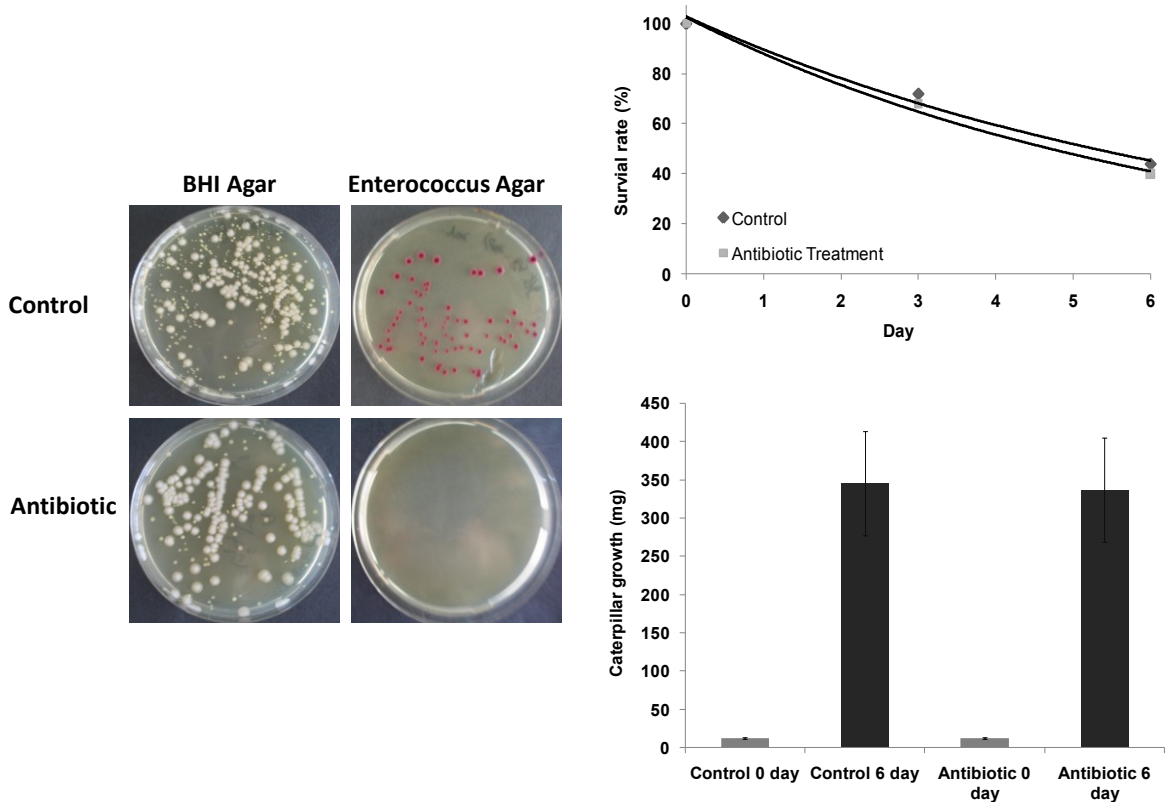

Figure 7-3: Erythromycin curing of *Enterococcus* from larval gut. After a low concentration of erythromycin feeding, no *Enterococcus* was detected on agar plate and this treatment did not obviously alter other gut bacteria. Survival rate and caterpillar growth did not significantly change between *Enterococcus*-curing group and the control.

However, the isolated *Enterococcus* strains could quickly and efficiently produce some antibiotic substances which inhibit several tested gram positive and negative bacteria (Table 7-2). A mixture of antimicrobial agents, including common organic acids and antimicrobial peptides, was characterized from the bacteria fermentation product. By comparing with the authentic standard substance, benzoic acid was identified from the bacterial broth culture (for HPLC/MS and NMR spectra see Supplementary material). Bacterially derived phenolics have a central role against a wide range of pathogens in the locust (*Schistocerca gregaria*) (Dillon and Charnley 2002, Dillon et al 2000). Some antimicrobial peptides, which presumably represented novel enterocin, were purified too, although their exact structures were not determined (too less amount for NMR, see Supplementary material). Further investigation on possible defensive function with entomopathogen challenging of the *Enterococcus*-cured larvae could be of particular interest.

Species	Strain designation(s)	Origin	Source[a]	Inhibition in the agar diffusion assay[b]
Micrococcus luteus	DSM 20030		DSMZ	+
Pseudomonas syrigae				+
Lysinibacillus sphaericus	DSM 28		DSMZ	-
Xenorhabdus nematophila	DSMZ 3370		DSMZ	+
Serratia entomophila	DSM 12358		DSMZ	-
Bacillus thuringiensis	DSM 2046		DSMZ	-
Streptococcus thermophilus Sfi39			DSMZ	+
Lactococcus lactis subsp. lactis	DSM 20481		DSMZ	+
Leuconostoc mesenteroides subsp. mesenteroides	DSM 20343		DSMZ	+
Lactobacillus plantarum	*DSM 20174*		DSMZ	+
Lactobacillus brevis	*DSM 1267*			+
Lactobacillus gasseri	*DSM20243*			+

Table 7-2: Indicator strains used in this study and the inhibition of their growth by the *Enterococcus* strain isolated from the larval gut.

From this study, I concluded that *Enterococcus*, as the core component in gut community of cotton leafworm, is vertically transmitted from mother to offspring via the egg. The isolated *Enterococcus* strain could efficiently produce a mixture of antibiotic compounds including phenolic acids and antimicrobial peptides, which may contribute to the host health in the wild. The vertical transmission of beneficial gut bacteria therefore may represent an important benefit to the host.

8. General discussion

1) Carotenoids in the gut

Plants and insect herbivores have continually co-evolved based on a chemical arms race, including deployment of refined chemical defense systems by each player. Plants produce diverse toxic secondary metabolites against herbivores and/or other competitors, for example the pro-oxidant allelochemicals which may lead to uncontrolled lipid peroxidation, DNA and protein oxidation and other deleterious effects in the host gut. In turn, insect herbivores have evolved counter-measures to detoxify, sequester, or secrete those harmful compounds. Various studies have focused on those defensive secondary metabolites, which represent a major barrier to herbivory (Mithofer and Boland 2012).

Carotenoids, not just another large group of secondary metabolites synthesized by plants, perform critical functions in photosynthesis and photoprotection, which are fundamental to the survival of plants. Carotenoids are highly abundant in plant leaves, which provide a rich source for the phytophagous chewing insects. Because of their high lipophilicity, carotenoids are easily absorbed by the insect cellular membrane through passive diffusion (Bhosale and Bernstein 2007). Carotenoids can be found in various tissues of the lepidopteran larvae, such as the gut, fat body, epidermis and haemolymph. The relative ratio of two predominant carotenoids, lutein and beta carotene, in the gut epithelium of cotton bollworm (*Helicoverpa zea*) is similar to that in plant leaves. Whereas only lutein accumulates in the mandibular glands of *Helicoverpa zea* and confers a yellow color of the gland (Eichenseer et al 2002).

In our study, while dissecting *Spodoptera littoralis* (Lepidoptera, Noctuidae) larvae reared on different plants, in particular on lima bean, a conspicuous red crop structure is most often observed in the foregut of health larvae. This coloration appears to be important for the larvae surviving on potentially toxic plants since the larvae with red-crop grew much better and had

lower mortality than those with colorless crop. After continuously feeding on lima bean leaves, a high proportion (more than 95%) of matured sixth-instar larvae successfully developed the red crop and could undergo the pupation process. Under microscopy, it was found that huge amounts of red crystals incorporated into the crop tissue and abundant coccus-shaped bacteria located there too. There was almost no or only a low number of bacteria attaching on the colorless crop of larvae fed on the same plant diet, which could indicate a toxic environment in the crop lumen. This coloration depended on the diet that caterpillars had been raised on: a green plant diet, such as lima bean, snap bean, cotton and Arabidopsis, causes obvious accumulation of red crystals; while on a carotenoid-free artificial diet, this red color lacks. However, some plant species in our survey, such as Chinese cabbage, did not generate this crop coloration. The reason for the lack of response is not clear, but it may be related to some characteristics of different plant ingredients or their physicochemical properties. Not only *S. littoralis*, other *Spodoptera* species, for example *S. exigua*, and another lepidopteran, *Helicoverpa zea*, also generate similar red coloration in the crop tissue in response to plants feeding. Therefore, this phenomenon may be more widespread in such lepidopteran herbivores living in the wild. And this also suggests important roles of this pigmentation in the host biology.

HPLC/MS analysis showed that beta carotene was the major component in the red crop extraction, accompanied by a tiny amount of its isomer alpha carotene. But lutein, which is the most common carotenoid partitioning into various tissues of the lepidopteran, appeared in a trace amount in the crop. The direct Raman microscopy measurement of the crystals in the intact insect tissue was carried out and the Raman spectra clearly verified that beta carotene was the component of red crystals. The crystallization procedure generally excludes structurally different molecules. The trace amount of lutein detected in dissolved crystals probably came from the attached plant pieces or insect cellular membrane. On the other hand,

diverse carotenoids were detected in the midgut tissue of the red-crop larvae, with highest amount of lutein followed by carotene. This carotenoid profile of the midgut was similar to the lima bean leaves that larvae ingested. Like most flowering plants, lutein is the most abundant carotenoid in lima bean and next beta carotene. Thus, carotenoids absorption in the gut tissue of *Spodoptera* larva is distinctively different: the midgut is most likely to nonselectively accumulate various carotenoids via passive diffusion from its plant diet, notably the lutein and beta carotene; but the crop (the anterior part of the foregut) significantly sequesters only beta carotene into the tissue at a high level, which leads to the overload and crystallization of carotenes in the crop. It is known that at high concentrations, carotenoids exhibit a tendency to aggregate or crystallize out of solution (Young and Lowe 2001). For example in *Arabidopsis thaliana*, when the phytoene synthase gene was overexpressed, which caused enough beta carotene enriching at the site of formation, the carotene left the lipophilic phase of the membrane and formed small crystals in the plastid of the root (Maass et al 2009). Similarly, the observed crystallization in the crop membrane could be due to the very large amount of local accumulating carotenes. Generally, the uneven distribution of carotenoids in the tissue or body part of animals suggests specialized physiological functions (Eichenseer et al 2002). There may be some kind of facilitated carotene utilization in the crop. It is also interested to know where the huge amount of beta carotene comes from.

Although *Enterococci* were topically closely associated with the crystals and some *Enterococcus* species were reported to produce carotenoids (Maraccini et al 2012), the carotene cannot be a bacterial product. The isolated *E. casseliflavus* strain from the foregut was not yellow-pigmented like the known carotenoid producing strains. And I could not detect any beta carotene in the bacterial cell culture. On the other hand, the reported bacterial carotenoids were not C_{40} molecules, but likely to be a C_{32} acyclic carotenoid aldehydes (Taylor et al 1971). Evidence supporting this conclusion was also from the antibiotic-cured

caterpillars. During feeding, the lima bean leaves were sprayed with erythromycin solution, which could effectively clear *Enterococci*. This low dose antibiotic treatment did not cause obvious side effect to the larvae. After larvae dissection, I still found the red colored crop structure without difference with the control, but no bacteria in the tissue. Therefore, those data indicated that the abundant *Enterococci* in the crop did not contribute to the development of red coloration, but they may benefit from this phenomenon since they could successfully multiply there.

Generally, animals lack the enzymatic machinery required for the carotenoid biosynthesis. Thus they could not make their own carotenoids. Until recently, Nancy A. Moran reported the presence and expression of carotenoid biosynthetic genes in aphids (Insecta, Hemiptera) for the first time in animal kingdom, which appear to have been laterally transferred from fungi (Moran and Jarvik 2010). In that case, pea aphids live on phloem sap and carotenoids, as lipid soluble compounds, are not expected to occur in significant quantities there. However, cotton leafworm, as a chewing herbivore, ingests the whole plant leaves, which normally contain abundant carotenoids. It is not reasonable that *S. littoralis* would spend high energy cost to synthesize carotenoids *de novo* since it could easily obtain these compounds from food.

Because the carbon isotopic ratio (^{13}C) can be a reliable indicator of the origin of the biomolecules, it was finally used to reveal the source of beta carotene in the crystals. Carotenoids were purified from both the red crop tissue and lima bean leaves and were analyzed with an accurate gas chromatography-combustion-isotope ratio mass spectrometer (GC/C/IRMS). The ^{13}C isotopic ratio mass spectrometry showed that the ^{13}C value of the carotenoid crystallized in the crop of *Spodoptera* is indistinguishable from those purified from lima bean leaves, with both the mean enrichment value around -27.50‰. In general, the ^{13}C isotopic carbon content of whole plant leaves is near -28‰ in C3 plants. The mean enrichment in the whole larvae fed on lima bean plant was relatively lower than this value

because animals are more preferable to ^{12}C than ^{13}C. Collectively, those data indicated that the selectively accumulated beta carotene in the *Spodoptera* crop most probably came from its food: the lima bean leaves.

However, the mechanism of this high level selective sequestration of only beta carotene over all other plant carotenoids is not clear. The physicochemical conditions in the foregut lumen and/or some specific carotenoid binding proteins are likely to play a role in the absorption and transport processes. It was reported that the transport and absorption of lipid and oxidative stress may be some reasons for the differential carotenoid accumulation in *Helicoverpa zea* (Eichenseer et al 2002). Generally, the lepidopteran larvae have a bias to store oxygenated xanthophylls in various tissues, such as fat body and glands. And lutein-binding proteins are found to play an important role in dictating this tissue specific accumulation (Bhosale and Bernstein 2007). The red crop in *Spodoptera*, with the sequestration of sole carotene in its tissue, is unique to other lepidopterans. But relatively little information has been available about the high-affinity carotene-binding protein in animals. To my knowledge, only one beta carotene-binding protein was reported until now from the ferret (*Mustela putoris furo*) liver (Rao et al 1997). In the mammalian, carotenes are often absorbed by mucosal cells in the digestive tract and subsequently appear unchanged in the circulation and peripheral tissues (Bhosale and Bernstein 2007). Notably, the regurgitant (oral secretion in the foregut) of cotton leafworm showed some nonpolar properties. A diverse and high concentration of amphiphilic fatty acid amino acid conjugates (FACs) commonly present in the gut lumen of lepidopteran caterpillars, which are known to serve as biosurfactants for emulsification of lipids during ingestion (Spiteller et al 2000). Total FACs came up to 1.3-3.0 nmol/μl in the gut content of *Spodoptera litura* larvae (Mori et al 2003, Yoshinaga et al 2008). Those emulsifying agents could alter carotenoid solubility, which may facilitate absorption into the gut tissues (Eichenseer et al 2002).

Since the carotenes accumulated in the crop came from its plant diet, a carotene-fortified artificial diet (0.1% carotene, a concentration frequently found in plants) was prepared to feed the larvae with the expect of same coloration occurring in the crop. Surprisingly, the addition of carotene did not restore any red coloration in the larvae continuously fed on such artificial diet. Then the carotene concentration in the artificial diet was serially increased or decreased and the carotene was dissolved with several different solvents. The feeding time on such diet was elongated too. But there was still no any detectable carotenoid in the crop tissue. Thus, there should be some other factors from the host plant which stimulate the beta carotene incorporation into the crop tissue.

During the herbivore attack, plants activate both local and systemic defense mechanisms. Especially, the production of ROS in plants is enhanced upon exposure to many stresses, which is highly toxic and is capable to cause unrestricted oxidation of various cellular components, and even leads to the destruction of the cell. It is known that lima bean plants generate a strong H_2O_2 response to the herbivore (*S. littoralis*) attack, and H_2O_2 levels are likely to be elevated as long as the attacks persist (Maffei et al 2006). Not only high concentrations of H_2O_2, ROS may originate from other sources, for example, oxygen radicals produced by photoactivated phytotoxins (furanocoumarins and thiophenes). Such radicals also arise in normal metabolic processes and during immune challenge. ROS have been associated with plant-herbivore interactions, and oxidative changes in the plants correspond with oxidative damage in the gut of herbivores feeding on previously wounded plants (Bi and Felton 1995). Especially in *Spodoptera littoralis*, the larvae have an immoderate feeding style and consume plant leaves very fast. The chewed plant particles quickly accumulate inside the crop until they can be processed through the remaining sections of the alimentary canal, which could cause an oxidative burst in the crop tissue.

In turn, herbivores have developed some strategies to counterattack this direct oxidative injury during feeding plants. The major defense mechanism in insects against oxidative stress includes a group of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and a glutathione S-transferase with peroxidase-like activity (GSTpx) (Krishnan and Kodrik 2006). Commonly, herbivorous insects up-regulate their antioxidant enzyme systems to combat oxidative stress imposed upon feeding on plants containing pro-oxidant allelochemicals. However, the presence of antioxidant enzymes in the digestive lumen, with the exception of catalase, is largely unknown (Felton and Summers 1995). Additionally, insects possess a suite of small molecular water-soluble and lipid-soluble antioxidants, such as ascorbate, glutathione, and carotenoid, which form a concatenated response to an onslaught of dietary and endogenously produced oxidants (Felton and Summers 1995). The role of carotenoids as antioxidants in insects is apparent (Ahmad 1992). Especially studies with lepidopterans have shown that dietary beta carotene can decrease the toxicity of some phytotoxins. For example, the toxicity of a phototoxin (α-terthienyl) to the tobacco hornworm (*Manduca sexta*) was reduced in the presence of dietary beta carotene, with a significant decrease in larval mortality (R.R. AUCOIN 1990).

Is it possible that the accumulation of antioxidant carotenes in the crop is simply stimulated by the toxic ROS from the larva's plant diet? I checked this possibility with a bioassay. The caterpillars of *S. littoralis* were first fed on a toxic artificial diet containing a pro-oxidant and then were transferred to the carotene-fortified artificial diet. The growth and survival rate were compared and the restoration of red coloration was examined too. The experiment revealed that oxidative stress in the diet decreased the growth of larvae. But the larvae fed on the carotene fortified diet grew better than those fed on only the toxic diet. This result was consistent with a previous study, in which beta carotene effectively protected *M. sexta* at a dietary concentration (R.R. AUCOIN 1990). After dissection, some red crystals were

observed in the crop membrane under microscopy, which was different with previous the sole carotene-fortified treatment. But the abundance of crystals was not as high as the larvae fed on native plants. Thus, the enhancement of carotene uptake and coloration of the crop was partially achieved by the oxidative stimulation.

The idea that carotenoids may be protective antioxidants in cells, tissues and organisms, has been widely accepted (Goto et al 2001, Young and Lowe 2001). The antioxidant activities of beta carotene, as a representative carotenoid, in various biological membranes and model membrane systems have been extensively studied. Carotenoids may act as antioxidants by physical or chemical quenching of free radicals. The benefit of physical quenching is that carotenoids may act as antioxidants without losing its own structure. Quenching of singlet oxygen by carotenoids is known to occur primarily through an energy transfer mechanism and finally leads to energy dissipation to the environment as heat (Cantrell et al 2003). And in nature, beta carotene has the highest quenching rate known for any quencher of singlet oxygen (Foote 1987). Whereas reactions of peroxyl radicals with carotene, both in polar and nonpolar environments, are proceeded mainly by adduct formation process (Galano and Francisco-Marquez 2009). Although in our case, abundant overloaded beta carotenes crystallize in the crop tissue, quenching of reactive radicals by the left high concentration of free carotenoid monomers in the membrane still can be efficient and fast. Even the formation of small carotene aggregates is also proposed to be of significance in their ability to quench singlet oxygen (Cantrell et al 2003). Specifically, beta carotene exhibits good radical-trapping antioxidant behavior at low oxygen partial pressure, which is expected to happen in the gut lumen (Burton and Ingold 1984, Young and Lowe 2001). Thus, it is reasonable to suppose that cotton leafworm selectively sequesters carotene pigments to protect the foregut cell membrane from oxidative damage, which enhances herbivore behavior and survival on a host plant.

In view of the widespread dependence of animals on carotenoids, the randomly distributed carotenes in the crop lipid membrane may also have some other yet unknown functions in the host biology. It is known that carotenoids are able to change the nature of the lipid membrane depending on the carotenoid incorporated. Nonpolar carotene has been shown to increase the motional freedom of lipids in the headgroup region in contrast to the polar carotenoids (lutein and zeaxanthin), which restrict molecular motion of the membrane as the role of 'molecular rivet'. And this effect of carotenes to decrease rigidity of the membrane may be important to the efficiently expanding of the crop for food storage during larvae forage. Moreover, carotenes could increase the penetration of small molecules and ions into the polar zone of the membrane (Britton 2008). However, there is still little direct evidence for the real physiological significance of carotenes *in vivo*.

In conclusion, an extraordinarily selective sequestration of carotenes, mainly beta carotene, was observed in the foregut of *Spodoptera* larvae when feeding on certain toxic plants such as *P. lunatus*. After accumulating a high enough level in the tine crop layer, overloaded carotenes left the lipophilic phase of the membrane and crystallized there. The insect crop was simultaneously enlarged while its inner surface was covered with carotene crystals. This carotene pigmentation had survival value to the host. Only those insects which developed the red crop can recover from intoxication by the food plant. Most individuals that failed to develop the red crop did not recover and finally died. Cotton leafworms have an immoderate feeding style, which consume large amounts of plant in short time and most often are exposed to high levels of light in the wild. Thus, they may experience a greater oxidative stress during foraging and need for the protective effects of antioxidants. Sequestration of carotenoids, especially the beta carotene, can efficiently protect caterpillars from UV radiation or free radical. Therefore, this facilitated absorption of beta carotene in the foregut may represent one of the strategies of successful herbivore defense against its host plant. However, little is

known about the regulation of carotene uptake from the diet and transmission to specific tissues in insects. The physicochemical conditions such as the consistent oxidative stress and high concentrations of amphiphilic compounds in the foregut lumen and/or some specific carotenoid binding proteins are likely to play a role in the absorption and transport processes. Since the absorbed carotenoids have various physiological functions in animals, other effects of this selective accumulation of beta carotenes should be considered as well.

Although *Enterococcus casseliflavus* is the single bacterial species thriving in the crop and those bacteria were found tightly associated with the red crystals, clearly they were not involved in this carotene accumulation. Previous work suggests that the carotenoid pigments are able to quench reactive oxygen species and thus protect *Enterococcus* from photoactive damages (Maraccini et al 2012, Taylor et al 1971, Ziegelhoffer and Donohue 2009). Thus the predominant *Enterococci* in the host crop may also benefit from this carotene accumulation.

2) Gut microbiota

Abundant and diverse microorganisms, including microeukaryotes, archaea, bacteria and viruses coexist and continuously coevolve within the animal gut. Not only thriving in the gut, where the host provides a nutrient-rich and relatively stable environment needed for the microbial growth, this inner microbial ecosystem essentially provides many services to the host, including digesting the food, releasing nutrients, detoxifying xenobiotics, preventing colonization by potentially harmful microbes, among others (Gill et al 2006). The presence of microbes and their metabolites products, such as short chain fat acids (SCFA), also stimulate the gut development and the mature of the host immune system. Recent years, with the development of next-generation DNA sequencing technology, an explosion of discoveries have revealed intimate microbial–host relationships in vertebrates, particularly in the human gut microbiome (2012). There are millions of unique protein-coding genes existing in the human microbiome, which is 360 times more than humans contributing. Thus in most

situations, the gut microbiota greatly extends the host's own metabolism and promotes the host fitness through microbial components' metabolic activities. The gut of most insects harbours nonpathogenic microorganisms. The essential role of the insect microbiota echoes that of the higher vertebrates, which ranges from nutritional upgrading to colonization resistance (Dillon and Dillon 2004). In addition, some novel microbe–host interactions are revealed recently in insects. Studies have shown that microbes can mediate thermal tolerance to the host, alter the reproductive mode, and modify the insects' body color, which is an ecologically important trait. These physiologic processes maintaining inside the host have a mutual nature and may contribute to the global success of insects.

In contrast to the importance of the Lepidoptera as a large group of herbivory pests and the evidence that the gut bacteria are involved in the host interaction with their food plants (Pesek et al 2011, Ping et al 2007, Shao et al 2011, Spiteller et al 2000), surprisingly, little is known about microbes associated with lepidopterans, even though it is also one of the largest insect orders, containing more than 150,000 species (Broderick et al 2004). In the present work, a combination of several cultivation-independent techniques, mainly based on 16S rRNA gene, was applied to comprehensively determine the composition of the gut microbiota in two lepidopteran organisms, *Spodoptera littoralis* and *Helicoverpa armigera*, which are widely used as experimental models in ecological and physiological studies. Furthermore, the metabolically active bacteria were identified from *Spodoptera littoralis* by using an *in vivo* stable isotope probing approach. The transmission route and functional relationships of those gut bacteria with the host were discussed too.

a. Composition and stability of the gut microbiota

Empirical research has described the gut microbiota in insects, which often indicates a complex microbial diversity, especially those of the insects collected from the field. However most studies did not distinguish transient bacteria from indigenous populations, which should

be always found in normal individuals, maintain stable populations and persist in the gut (Dillon and Dillon 2004). It is already known that the gut of most lepidopteran is not sterile, but it is not clear whether indigenous bacteria exist in these herbivorous insect guts or not. As an initial step towards understanding these complex relationships, the composition of the gut microbiota was inventoried in detail, including the comparison of community profiles in different larval life stages and after feeding on different diets. Those comprehensive analyses of 16S rRNA gene diversity indicate that cotton leafworms do harbour an indigenous microbiota, with several bacterial taxa being qualitatively and quantitatively stable. *Enterococcus* spp., *Clostridium* spp., as well as *Pantoea* spp. are consistently found in all samples and predominate in both clone library and pyrosequencing datasets. In addition, the similar microbial profile was obtained from the larvae reared on the sterile artificial diet, suggesting potential vertical transmission mode of those bacteria. Furthermore, the microbiota associated with the skin of the larvae and their native diets (plant leaf) were largely different with the gut flora.

Central to the design of this study is to compare the community profiles in response to host development. Overall, the clone library and pyrosequencing analyses concur that the bacterial composition changed from early instar to late instar but the indigenous bacteria always persisted throughout the larval lift span. *Pantoea*, *Citrobacter* and *Enterococcus* species dominated in the early instar larvae. *Clostridium* species were only present as minor community components, but it strongly increased in numbers in the late instar larvae. A higher diversity of bacterial taxa was detected in the early instar larvae compared with that in the late instar, probably because the indigenous microbiota was not well established in the early instar. Gut physiology such as the largely anaerobic condition in the late instar larvae and the host immune system are likely to play a role to the microbiota changes in respond to the host development.

Recent data in some insects, obtained from the large screenings and the deep sequencing analysis, also reveal a specialized and stable symbiotic gut microbiota associated with the distinct host. For example, several independent studies resolve that the same set of core bacterial community, composed of eight distinctive phylotypes, dominates gut communities of honey bees (*Apis mellifera*) (Moran et al 2012). In European firebugs (*Pyrrhocoris apterus*), a very stable midgut microbiota is revealed in populations collected from various geographical locations and reared on different diets, with six predominant taxa being consistently abundant (Sudakaran et al 2012). It is broadly accepted that native microbes (indigenous microbiota) are true symbionts that promote the host fitness, which range from enhancing host energy metabolism to shaping the immune system regulation (Schneider and Chambers 2008). However, different feeding habits in insects result in different structure and function of the alimentary tracts, which promote the establishment of a specialized community.

The microbial richness in lepidopteran gut is often low. In the gut of the gypsy moth and cabbage white butterfly were found 23 and 15 OTUs, respectively. In the case of *S. littoralis*, 22-42 dominant OTUs are detected in the larval gut. By using both culturing and molecular methods, I did not detect any Archaea or fungi there, in good agreement with the observation on other lepidopteran species. The simplicity is especially apparent compared to the gut microbiota of insects from other orders such as those wood-decaying xylophagous termites of Isoptera, or vertebrates, which often harbor hundreds of phylotypes. The strong alkalinity, a fast food throughput, reactive oxygen species produced by cells of the gut membrane along with the ionic strength in insect gut may account for this taxonomically restricted gutflora.

A comprehensive understanding of the biology of gut microbiota requires that they be studied in an ecological context. Thus, different native plant diets were supplied to show how the herbivore responds to a changing host environment. The shift in diet from artificial diet to

lima bean or barley plants influenced the gut microbiota, with an increase in the diversity and the bloom in populations of some low abundant bacterial species. However, the indigenous residents (*Enterococcus* spp., *Clostridium* spp., and *Pantoea* spp.) were still consistently present in the gut; especially the *Clostridium* had a significant proliferation, implying its functional importance in the plant digestion. Furthermore, the metabolic activity of gut microbiota was measured *in vivo* by using a stable isotope probing approach.

b. Metabolic activity and putative function of the gut microbiota

In this study, I made attempts to distinguish active members from the gut microbiota with a stable isotope probing (SIP) approach. The measured metabolic activity could reflect symbionts' functional roles inside the gut. Although SIP has been frequently used in environmental microbiology, very few researches have applied this valuable technique to the study of gut microbiota, probably because of the complexity of the gut. Those pioneering researches have almost exclusively pursued *in vitro* experiment systems to mimic the gut environment, which obviously could not fully duplicate true physiological conditions in the intact gut, especially host factors shaping the microbial community, and hence may be limited or biased. In contrast, I studied the gut microbiota of cotton leafworm directly *in vivo* by coupling pyrosequencing and SIP approaches.

The glucose is the most important energy source in the gut of cotton leafworm feeding on either artificial diet or native plant, which fuels diverse metabolic activities inside and also supplies a good chance to carry out *in situ* SIP to identify metabolically active bacteria. The concentration of glucose was significantly low in larvae fed on artificial diet, comparing with that fed on its preferred host plant, cotton. Thus, exogenous glucose was amended with artificial diet to mimic the *in vivo* concentration and perform the labeling experiment for tracking metabolically active gut bacteria. Bacteria from three families, including

Enterococcaceae, *Clostridiaceae* and *Enterobacteriaceae*, were particularly active and likely the core functional populations linked to nutritional upgrading and pathogen defense.

A diverse assembly of Proteobacteria, especially Gammaproteobacteria dominated by *Pantoea* and *Citrobacte*r from the family *Enterobacteriaceae*, was the most active group in the early instar larvae. Bacteria in this phylum closely associate with insect herbivores and possess broad polysaccharide-degrading abilities. Genomic analysis of a *Pantoea* strain from a woodwasp (*Sirex noctilio*) detected genes putatively encoding for carbohydrate-active enzymes, with the majority predicted to be active on hemicellulose and more simple sugars (Adams et al 2011). In spite of its low titer, *Clostridium* was another highly active group in the early instar larvae and the colonization of *Clostridia* has commonly been linked to its highly efficient cellulose digestion and its ability to ferment a variety of sugars. FISH revealed that *Clostridium* spp. formed large aggregates in the deep anoxic area of the food bolus, which may facilitate the digestion of plant lignocellulose. Furthermore, after the larvae were shifted from artificial diet to fresh plants (lima bean or barley), there was a significant proliferation of *Clostridium* in the gut, which also supported this conclusion. It is proposed that most of digestive enzymes such as cellulose, xylanase and pectinase in silkworms (*Bombyx mori*) were produced from microbial origin and contributed to larval growth (Anand et al 2010). By the induction of their rich and diverse digestive enzymes, those active bacteria work in a synergistic matter and largely enhance the efficacy of the utilization of complex plant polysaccharides in the gut, supplying enough soluble carbon and energy sources for the insect host. The released simple sugars would directly benefit early larval development, which further could be fermented to various other nutrients such as short-chain fat acids, greatly promoting the gut ecological environment.

Gut microbiota is more and more recognized to be an “extended immune phenotype” to help the host cope with harmful microbes. For example, it was reported that aseptically-reared

oriental tea tortrix larvae (*Homona magnanima*) supported 20 times greater growth of an entomopathogen (*Bacillus thuringiensis*) than larvae with a normal gut microbiota (Takatsuka and Kunimi 2000). *Enterococcus* was essentially active during the whole larval life span, which probably played a key role in the insect defense against potentially harmful microorganisms. FISH showed that a large amount of *Enterococci* intimately associated with the gut mucus layer and formed a biofilm-like structure. The biofilm-like structure could block the direct interaction between pathogens and the host epithelium and also prevent the non-indigenous bacteria competing for nutrients and living space in the gut, an effect called colonization resistance. Attachment on the gut epithelium helps oppose washout from the gut, which may be the reason for the stability of *Enterococcus* in the gut community. In mammals, colonization resistance plays a vital role to prevent colonization of the gut by pathogens. There is also some evidence of colonization resistance in insects. *Schistocerca gregaria* locusts harboring a normal gut bacterial flora was shown to be usually resistant to infection by fungal entomopathogens, but became susceptible to such fungal infections in gut germ-free individuals (Dillon and Charnley 2002). The antimicrobial property of the isolated *Enterococcus* strain by producing some phenolic compounds and antimicrobial peptides added further support. Three phenolics were identified from the locust gut, which have a central role to help the host defend itself against microbial pathogens (Dillon et al 2000). Considering cotton leafworm is a generalist feeder in the field, its digestive tract is continually challenged by potentially harmful bacteria and fungal endophytes that it ingests. Thus, the predominant *Enterococci*, maintained in biofilm-like structure and showing potent antimicrobial properties, may be of great importance for the insect host's health, which lacks an adaptive immune system.

Not only are they related to nutritional upgrading and pathogen defense, active bacteria may also contribute to other gut metabolism, such as detoxifying noxious allelochemicals and

regulating the host's immune homeostasis. *Enterococcus faecium* strains isolated from the human intestine could efficiently transform the toxic heterocyclic aromatic amine (HAA), such as 2-Amino-1-methyl-6-phenylimidazo [4, 5-*b*] pyridine (PhIP) (Vanhaecke et al 2008). Given the general detoxification ability of microbes and their ability to evolve quickly, the indigenous gut microbes could be an extremely important contributor for the generalist feeder to overcome the toxicity of their various plant diets (Werren 2012). However surprisingly, to date little is known about the exact contribution of insect symbionts to the detoxification of plant secondary metabolites in the gut. Based on the well established system here, our lab is putting more effect to answer this question by amending pure plant toxins into the defined artificial diet and comparing the gut microbial community change accordingly.

c. Transmission route of core members of the gut microbiota

Enterococcus and *Clostridium* spp. are the most abundant members in the gut microbiota of cotton leafworm and are consistently found in the gut of healthy individuals independent of food source, indicating stable transmission routes for those bacteria.

Several *Enterococcus* species were frequently isolated from eggs, strongly suggesting vertical transmission of these symbionts via the egg. A clone library study identified only *Enterococci* from the egg. But it sequenced only 42 clones, it was possible that low abundant bacterial species, such as some *Clostridium* spores, will be detected from the egg mass with a deep sequencing approach, which needs further studies. Interestingly, FISH and scanning electron microscopy studies have already shown that some *Enterococci* locate on the inner surface of the eggshell. However, it is not clear how these bacteria get into female's reproductive organ and survive the metamorphosis process (transmit from the larva to the adult). Future studies by using the fluorescent protein-labeled *Enterococcus* strain to track their transmission route would shed light on those questions. Notably, *Enterococcus* is widely distributed in related lepidopterans, such as in the larva of gypsy moth (*Lymantria dispar*) and cotton bollworm

(*Helicoverpa armigera*) from both field-caught and laboratory-reared samples. An *Enterococcus* spp. had been detected in all gypsy moth (*Lymantria dispar*) larvae independent of the plant diet (Broderick et al 2004). *Enterococcus* was the major and the only metabolically active bacterium in the gut and eggs of tobacco hornworm (*Manduca sexta*) (Brinkmann et al 2008). These intimate associations indicate that *Enterococcus* may have important functional implications for the lepidopteran herbivores in general. Several lines of evidence indicating *Enterococcus* as the host defensive symbiont have already been showed in this study.

Clostridium is another frequently detected and highly active group in the gut, which particularly dominates in the late instar larvae. *Clostridia* are obligate anaerobes and capable of forming endospores, which are widely dispersed in nature. Those bacteria could be acquired by newly hatched larvae from the surrounding environment such as leaf surface, soils during foraging.

Therefore, these indigenous gut residents are either initially derived from the egg via vertical transmission or from the surrounding environment during larvae forage. A reliable transmission route ensures the expansion of those native species late in the gut of offspring.

Collectively, these data suggest the existence of an indigenous gut microbiota in *Spodoptera*, which mainly consists of bacteria from the phyla Firmicutes and Proteobacteria. The high metabolic activity of core components *in vivo*, including *Enterococcaceae*, *Enterobacteriaceae* and *Clostridiaceae*, indicates that they are functionally important symbionts for the host fitness, including nutritional upgrading and colonization resistance. Owing to the short generation cycle and the ease of adaptation, gut bacteria may also have other active roles in the host physiology and in the herbivore-plant interaction via their huge reservoir of metabolic pathways. The microbial transformation of toxic plant secondary compounds in herbivore guts need further study and more unexpected benefits supplied by

microbes could also be identified from detailed studies. With a growing interest in the use of natural microorganisms to control herbivory pests (Colman et al 2012) (Leroy et al 2011), the increased knowledge about gut bacteria and the benefits they are providing to the insect host have wide implications for the generation of new methods in the biological control of agricultural pest.

9. Summary

The herbivorous insect gut is a unique biochemical environment, which provides constant and controlled nutrient fluxes, principally abundant polysaccharides, amino acids and a diverse array of plant secondary metabolites (phytotoxins, carotenoids etc.) for the host and microbial metabolism. Through efficient digestion, the insect host gains carbon and energy. But phytotoxins commonly make plants unpalatable to herbivores and lead to a decreased fitness after ingestion. Carotenoids, not just another group of plant secondary metabolites, have a wide distribution in insects with various and fundamental functions. Microorganisms are actively involved in all those metabolic processes due to their fast generation cycle and the ease of adaptation.

In the present work, I first investigate an unusual carotenoid uptake phenomenon in *Spodoptera*, which involves the crystallization of carotenes in the foregut and the accumulation of a single bacterial species. Next, the indigenous gut microbiota is characterized, which probably plays an important role in the host physiology and in the multitrophic interaction between insects and plants.

a. Carotenoids in the gut

Carotenoids are currently being intensely investigated regarding their very wide distribution in nature and critical function in all living organisms for light detection, oxidation control or coloration. In the leaf chewing herbivores such as cotton leafworm, carotenoids are usually absorbed from their host plants which contain a rich source of those organic pigments; and the ingested carotenoids are often accumulated in various host organs, either unaltered or with some metabolic modifications. Despite carotenoids' general importance, the uptake mechanism is still poorly understood. Here, I investigated the "red crop" phenomenon, an accumulation of carotenes in crystalline inclusions in the enlarged foregut of the polyphagous

Spodoptera larvae fed on some toxic plants. This pigmentation has survival value to the host. Caterpillars which fail to develop this "red crop" structure exhibit a high mortality.

A combination of chemical characterization methods, especially the Raman microscopic analysis of the crystals *in situ*, revealed that beta carotene, not the most abundant and ubiquitous lutein in the foliage of host plant, is selectively sequestered in the crop and finally crystallizes there due to the exceptionally high concentrations. The carotene crystals give the insect foregut a distinctive orange-red color. Notably, the crystals are embedded in a homogenous lawn of the bacterium *Enterococcus casseliflavus*. However, ^{13}C-IRMS data clearly indicated that carotenes are selectively taken from the food plant. The physicochemical conditions inside the foregut lumen (e.g. the consistent oxidative stress and high concentrations of amphiphilic compounds) and/or some specific carotene binding proteins are likely to play a role in the carotene absorption and transport process. Further investigations, such as the gene expression analysis in the crop tissue, are needed to characterize candidates responsible for this extraordinary beta carotene selectivity.

Considering that cotton leafworms experience a greater oxidative stress during foraging, it has been hypothesized that high concentrations of beta carotene can efficiently prevent oxidative damage in this tissue by enhancing the non-enzymatic detoxification. Bioassay with the pro-oxidant partially restored the red crop phenomenon in larvae fed on carotene-fortified artificial diet, suggesting that ROS, but may be not the sole one, could stimulate the carotene sequestration in the crop. Since carotenoids have various functions in animals, other effects of this selective accumulation of beta carotene should be considered as well. Although huge amounts of *Enterococci* thrive in the crop, no evidence indicated their contribution to the formation of carotene crystals but they may benefit from this phenomenon by using carotenes as their own antioxidant.

This red crop is not specific to *Spodoptera littoralis*; I also observed the formation of pigments in other lepidopteran species, such as *Spodoptera exigua* and *Helicoverpa armigera*. Therefore, this phenomenon may be ubiquitous in such lepidopteran herbivores living in the wild. And this also suggests an important role of carotenoids in the host biology, probably as the frontline of defense against oxidative stress during foraging.

b. Bacteria in the gut

The gut microbiota is of crucial importance for the host with considerable metabolic activity. Although Lepidoptera is one of the largest insect orders and a primary group of phytophagous agricultural pests, little is known about the microbes associated with them. This study has made efforts to comprehensively characterize the gut microbiota in different lepidopteran model organisms and provide some light into the potential metabolic functions of the core components inside the host.

The gut microbiota profile of two lepidopteran species, *Spodoptera littoralis* and *Helicoverpa armigera*, is very similar regarding high abundant bacterial families that are dominated by Firmicutes and Proteobacteria. Different bacteria colonize specialized niches within the gut. A core community, consisting of *Pantoea*, *Enterococci*, *Lactobacilli* and *Clostridia*, is revealed in the insect larvae. These bacteria are constantly present in the digestion tract at relatively high frequency despite that the developmental stage and the diet have some impacts 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 as the indigenous community, which may contribute to the host fitness.

Not only examined the composition and diversity of the gut microbiota, the components' metabolic activity was also measured in *Spodoptera littoralis* by using a refined Pyro-SIP

approach. With ^{13}C glucose as the trophic link, Pyro-SIP revealed that the gut microbiota co-develops with the host, both metabolic activity and composition shifting throughout larval stages. Bacteria from the *Clostridiaceae* and *Enterobacteriaceae* families are particularly active in the early instar, which are well-known plant biomass degraders and likely the core functional populations linked to nutritional upgrading. *Enterococcaceae* is highly active in the late instar. On the grounds that *Enterococci* are maintained in a biofilm-like structure on the gut epithelium and that the isolated strains efficiently produce a mixture of antibiotic regents, *Enterococcus* is suggested to be a defensive mutualist which helps the host resist potentially harmful bacteria from outside. Notably, *Enterococcus* is vertically transmitted from eggs. This pilot study shows that Pyro-SIP can rapidly gain insight into the microbiota's metabolic activity with high resolution and high precision, which sets the stage for future studies on the targeted metabolic pathway, for instance, labeling plant defense compounds to assess active bacteria involved in the host detoxification process. With the development of such new approaches, the role of lepidopteran gut microbiota will become more apparent than currently.

Because of the simplicity of gut and the well-defined gut microbiota, cotton leafworm provides an excellent naturally-occurring model in which to study the complex digestive-tract microbial symbiosis and furthermore the multitrophic herbivore-microbe-plant interaction. A better understanding of the insect microbiology and transforming this knowledge to manage herbivorous pests in general will secure our food supply and economics.

10. Zusammenfassung

Der Darm herbivorer Insekten bildet eine einzigartige biochemische Umwelt, die geprägt ist durch weitgehend konstante und kontrollierte Nährstoffzufuhr, darunter Polysaccharide, Aminosäuren, und einer Vielzahl an pflanzlichen Sekundärmetaboliten (Phytotoxine, Karotinoide etc.); beide dienendem Wirt- als auch dem Mikrobenmetabolismus.

Durch möglichst effiziente Verdauung gewinnt das Wirtsinsekt Kohlenstoff und Energie. Allerdings machen Phytotoxine die pflanzliche Nahrung häufig schwer verdaulich und führen zu verringerter Fitness nach der Aufnahme. Karotinoide, die eine weitere Gruppe pflanzlicher Sekundärmetabolite darstellen, sind weit verbreitet, auch innerhalb der Insekten und erfüllen sehr verschiedene und wichtige Aufgaben.

Aufgrund ihrer kurzen Generationszeit und einfachen Anpassungsmechanismen sind Mikroorganismen aktiv in viele metabolischen Prozesse involviert.

a. Karotinoide im Darm

Obwohl die allgemeine Bedeutung von Karotinoiden anerkannt ist, ist der Aufnahmemechanismus bis jetzt nur schlecht verstanden. Ich untersuchte das „red crop“ - Phänomen, welches gekennzeichnet ist durch die Akkumulation von kristallinem Karotin im vergrößerten Vorderdarm von Larven des Generalisten *Spodoptera*, die mit toxischen Pflanzen gefüttert wurden. Diese Einschlüsse sind wertvoll für das Überleben. Raupen, die diese Struktur des „red crop“ nicht bilden können, weisen eine erhöhte Mortalität auf.

Die Kombination verschiedener Methoden zur chemischen Charakterisierung, insbesondere die *in situ* Ramanmikroskopie der Kristalle, hat gezeigt dass β-Karotin und nicht das in den Blättern der Wirtspflanzen häufigere Karotinoid Lutein, sich selektiv im Kropf anreichert und dort anschliessend wegen seiner hohen Konzentration kristallisiert. Die Karotinkristalle geben dem Vorderdarm der Raupen eine charakteristische orange-rote Färbung. Besonders

bemerkenswert ist, dass die Kristalle eingeschlossen in einem Bakterienrasen von *Enterococcus casseliflavus* eingebettet sind. Allerdings zeigen ^{13}C-IRMS Messungen eindeutig, dass das Karotin selektiv von der Futterpflanze stammt. Die physiologischen Bedingungen im Lumen des Vorderdarms (z.B. der beständige oxidative Stress und die hohen Konzentrationen an amphiphilen Substanzen) und/oder einige spezifische karotinbindende Proteine spielen sehr wahrscheinlich eine Rolle bei der Karotinaufnahme und seinem Transport. Weitere Untersuchungen, wie zum Beispiel Genexpressionanalysen im Vorderdarmgewebe (z.B. von Transportproteinen für Karotin), sind notwendig, um Kandidaten, die für die außergewöhnliche β-Karotinanreicherung verantwortlich sind, zu charakterisieren.

Unter Beachtung, dass *Spodoptera*-Larven besonders während der Futteraufnahme großem oxidativen Stress ausgesetzt sind , wurde vermutet, dass hohe Konzentrationen von β-Karotin die oxidative Zerstörung von Zellen und Geweben effektiv verhindern kann, in dem es die nicht-enzymatische Entgiftung von ROS erhöht. In einem Bioassay mit einen Prooxidant konnte der „red crop“ Phänotyp in Larven, die mit karotinangereichertem Kunstfutter gefüttert worden, teilweise erzeugt werden, was zeigt, dass reaktive Sauerstoffspezies (ROS), wenn auch vielleicht nicht alleine, die Karotinsequestrierung im Kropf stimulieren können. Da Karotinoide in Tieren verschiedenartigste Funktionen übernehmen, müssen auch andere Effekte dieser selektiven Akkumulierung von β-Karotin in Betracht gezogen werden. Obwohl eine große Anzahl an *Enterococci* im Vorderdarm vorkommt, gibt es keinen Hinweis darauf, dass sie an der Bildung der Karotinkristalle beteiligt sind; vielmehr profitieren sie wahrscheinlich von diesem Phänomen, indem sie das β-Karotin als eigenes Antioxidant benutzen.

Das „red crop“ Phänomen ist nicht artspezifisch für *Spodoptera littoralis*; wir fanden diese Kristallbildung auch in anderen Schmetterlingsarten wie zum Beispiel *Spodoptera exigua* und

Helicoverpa exigua. Daraus kann man vielleicht schließen, dass dieses Phänomen in wildlebenden Schmetterlingsarten häufiger verbreitet sein könnte. Desweiteren legen diese Ergebnisse eine wichtige Rolle von Karotinoiden in der Wirtsbiologie nahe, sehr wahrscheinlich als vorderste Verteidigungslinie gegen oxidativen Stress während der Nahrungsaufnahme.

b. Bakterien im Insektendarm

Die mikrobielle Flora des Darms ist aufgrund ihrer beträchtlichen metabolischen Aktivität. von entscheidender Bedeutung für den Wirt Obwohl die Ordnung der Lepidoptera eine der größten Ordnungen innerhalb der Insekten und eine Gruppe mit wichtigen phytophagen Schädlingen für die Landwirtschaft ist, ist bislang nur wenig über die mit ihnen assoziierten Mikroorganismen bekannt. In der vorliegenden Arbeit wurde die Darmflora verschiedener Arten aus der Ordnung Lepidoptera charakterisiert und gibt erste Einblicke in potentielle metabolische Funktionen innerhalb des Wirtes der Hauptmitglieder der mikrobiellen Lebensgemeinschaft.

Das Profil der Darmbakterien der beiden Falterarten, *Spodoptera littoralis* und *Helicoverpa armigera*, ist sehr ähnlich im Hinblick auf die am häufigsten vorhandenen Bakterienfamilien; diese werden von Firmicutes und Proteobakterien dominiert. Verschiedene Bakterienarten besiedeln spezialisierte Nischen innerhalb des Darms. Es konnte eine Kernlebensgemeinschaft bestehend aus *Pantoea*, *Enterococci*, *Lactobacilli* und *Clostridia*, in Insektenlarven identifiziert werden. Diese Bakterien sind in relativer hoher Zahl vorhanden im Verdauungstrakt, obwohl natürlich das jeweilige Entwicklungsstadium und die Nahrungsquelle Einfluss auf die Zusammensetzung der bakteriellen Lebensgemeinschaft haben. Arten, die nur in geringer Zellzahl vorkommen, können durch externe Einflüsse in der Zahl zunehmen; die Kerngemeinschaft jedoch veränderte sich nicht signifikant. Es ist klar, dass der Insektendarm spezielle bakterielle Taxa selektiert, welche zur Wirtsfitness beitragen.

Wir untersuchten in *Spodoptera littoralis* nicht nur die Zusammensetzung und Diversität der Darm Mikroflora, sondern auch die metabolische Aktivität der einzelnen Mitglieder mit Hilfe eines modifizierten Pyro-SIP Ansatzes. Mit ^{13}C-Glukose als trophischem Marker zeigte Pyro-SIP, dass sich die Darmflora parallel zum Wirtsorganismus entwickelt; metabolische Aktivität und Zusammensetzung verändern sich im Laufe der Larvalentwicklung. Bakterien aus den Familien Clostridiaceae und Enterobacteriaceae, die bekannt sind für den Abbau pflanzlicher Biomasse und damit sehr wahrscheinlich die funktionelle Kerngemeinschaft für die Aufwertung der pflanzlichen Nahrung bilden, sind besonders in den frühen Larvenstadien aktiv. Mitglieder der Familie der *Enterococcaceae* sind dagegen hoch aktiv in den späten Larvenstadien. Auf der Grundlage, dass *Enterococci* in einer biofilmähnlichen Struktur auf dem Darmepithel vorkommen und dass die isolierten Stämme einen effektiven Antibiotikamix produzieren, ist anzunehmen, dass *Enterococcus* ein Mutualist ist, der dem Wirtsinsekt hilft, sich gegen potentiell gefährliche Bakterien von der Außenwelt zu verteidigen. Bemerkenswert ist, dass *Enterococcus* vertikal über die Eier von einer Generation zur nächsten übertragen wird. Diese Pilotarbeit zeigt, dass man mittels Pyro-SIP schnell Einblicke mit hoher Auflösung und Präzision in die metabolische Aktivität der Darmflora gewinnen kann. Die Daten bilden die Grundlage für zukünftige gezielte Studien an metabolischen Reaktionswegen, zum Beispiel der Markierung von pflanzlichen Verteidigungsstoffen, um die aktiv an der Entgiftung beteiligten Bakterien zu ermitteln. Mit der Entwicklung neuer Methoden wird die Rolle der Darmbakterien in Schmetterlingslarven offensichtlicher werden als sie es im Moment ist.

Aufgrund der Einfachheit des Darmes und der wohldefinierten Darmflora ist *Spodoptera littoralis* ein ausgezeichnetes natürliches Modellsystem, um die komplexe Symbiose zwischen Mikroorganismen und Verdauungstrakt als auch die multitrophische Interaktion zwischen Pflanzenfresser, Pflanze und Mikroorganismen zu studieren. Ein besseres Verständnis der

Mikrobiologie in Insekten und die Anwendung dieses Wissens um Pflanzenschädlinge zu kontrollieren wird allgemein helfen, unsere Ernährung zu sichern.

11. References

(2012). A framework for human microbiome research. *Nature* **486:** 215-221.

Adams AS, Jordan MS, Adams SM, Suen G, Goodwin LA, Davenport KW *et al* (2011). Cellulose-degrading bacteria associated with the invasive woodwasp Sirex noctilio. *ISME J* **5:** 1323-1331.

Ahmad S (1992). Biochemical Defense of Prooxidant Plant Allelochemicals by Herbivorous Insects. *Biochem Syst Ecol* **20:** 269-296.

Altincicek B, Kovacs JL, Gerardo NM (2012). Horizontally transferred fungal carotenoid genes in the two-spotted spider mite Tetranychus urticae. *Biology letters* **8:** 253-257.

Anand AAP, Vennison SJ, Sankar SG, Prabhu DIG, Vasan PT, Raghuraman T *et al* (2010). Isolation and characterization of bacteria from the gut of Bombyx mori that degrade cellulose, xylan, pectin and starch and their impact on digestion. *J Insect Sci* **10**.

Bartley GE, Scolnik PA (1995). Plant carotenoids: pigments for photoprotection, visual attraction, and human health. *Plant Cell* **7:** 1027-1038.

Bednarek P, Pislewska-Bednarek M, Svatos A, Schneider B, Doubsky J, Mansurova M *et al* (2009). A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. *Science* **323:** 101-106.

Beisel KG, Jahnke S, Hofmann D, Koppchen S, Schurr U, Matsubara S (2010). Continuous turnover of carotenes and chlorophyll a in mature leaves of Arabidopsis revealed by 14CO2 pulse-chase labeling. *Plant Physiol* **152:** 2188-2199.

Bhosale P, Bernstein PS (2007). Vertebrate and invertebrate carotenoid-binding proteins. *Archives of biochemistry and biophysics* **458:** 121-127.

Bi JL, Felton GW (1995). Foliar Oxidative Stress and Insect Herbivory - Primary Compounds, Secondary Metabolites, and Reactive Oxygen Species as Components of Induced Resistance. *J Chem Ecol* **21:** 1511-1530.

Bi JL FG (1995). Foliar oxidative stress and insect herbivory: primary compounds, secondary metabolites, and reactive oxygen species as components of induced resistance. *J Chem Ecol* **21:** 1511–1530.

Bode HB (2009). Entomopathogenic bacteria as a source of secondary metabolites. *Current opinion in chemical biology* **13:** 224-230.

Bright M, Bulgheresi S (2010). A complex journey: transmission of microbial symbionts. *Nat Rev Microbiol* **8:** 218-230.

Brinkmann N, Martens R, Tebbe CC (2008). Origin and diversity of metabolically active gut bacteria from laboratory-bred larvae of Manduca sexta (Sphingidae, Lepidoptera, Insecta). *Appl Environ Microbiol* **74:** 7189-7196.

Britton G (2008). Functions of Intact Carotenoids. **Vol 4.**

Broderick NA, Raffa KF, Goodman RM, Handelsman J (2004). Census of the bacterial community of the gypsy moth larval midgut by using culturing and culture-independent methods. *Appl Environ Microbiol* **70:** 293-300.

Burton GW, Ingold KU (1984). Beta-Carotene - an Unusual Type of Lipid Antioxidant. *Science* **224:** 569-573.

Cantrell A, McGarvey DJ, Truscott TG, Rancan F, Bohm F (2003). Singlet oxygen quenching by dietary carotenoids in a model membrane environment. *Archives of biochemistry and biophysics* **412:** 47-54.

Carroll M, Hanlon A, Hanlon T, Zangerl AR, Berenbaum MR (1997). Behavioral effects of carotenoid sequestration by the parsnip webworm, Depressaria pastinacella. *J Chem Ecol* **23:** 2707-2719.

Cogni R, Trigo JR, Futuyma DJ (2012). A free lunch? No cost for acquiring defensive plant pyrrolizidine alkaloids in a specialist arctiid moth (Utetheisa ornatrix). *Molecular ecology*.

Colman DR, Toolson EC, Takacs-Vesbach CD (2012). Do diet and taxonomy influence insect gut bacterial communities? *Mol Ecol* **21:** 5124-5137.

Dabrowska P, Freitak D, Vogel H, Heckel DG, Boland W (2009). The phytohormone precursor OPDA is isomerized in the insect gut by a single, specific glutathione transferase. *Proceedings of the National Academy of Sciences of the United States of America* **106:** 16304-16309.

Dillon R, Charnley K (2002). Mutualism between the desert locust Schistocerca gregaria and its gut microbiota. *Res Microbiol* **153:** 503-509.

Dillon RJ, Vennard CT, Charnley AK (2000). Exploitation of gut bacteria in the locust. *Nature* **403:** 851.

Dillon RJ, Dillon VM (2004). The gut bacteria of insects: nonpathogenic interactions. *Annu Rev Entomol* **49:** 71-92.

Dumont MG, Murrell JC (2005). Stable isotope probing - linking microbial identity to function. *Nat Rev Microbiol* **3:** 499-504.

Dunbar HE, Wilson AC, Ferguson NR, Moran NA (2007). Aphid thermal tolerance is governed by a point mutation in bacterial symbionts. *PLoS Biol* **5:** e96.

Eichenseer H, Murphy JB, Felton GW (2002). Sequestration of host plant carotenoids in the larval tissues of Helicoverpa zea. *J Insect Physiol* **48:** 311-318.

El-Gorab MI, Underwood BA, Loerch JD (1975). The roles of bile salts in the uptake of beta-carotene and retinol by rat everted gut sacs. *Biochimica et biophysica acta* **401:** 265-277.

Engel MS, Grimaldi DA (2004). New light shed on the oldest insect. *Nature* **427:** 627-630.

Fatouros NE, Broekgaarden C, Bukovinszkine'Kiss G, van Loon JJA, Mumm R, Huigens ME *et al* (2008). Male-derived butterfly anti-aphrodisiac mediates induced indirect plant defense. *Proceedings of the National Academy of Sciences of the United States of America* **105:** 10033-10038.

Felton GW, Summers CB (1995). Antioxidant systems in insects. *Archives of insect biochemistry and physiology* **29:** 187-197.

Foote CS (1987). *Type I and type II mechanisms of photodynamic action* ACS Symposium Series No. 339, American Chemical Society: Washington, D.C.

Funke M, Buchler R, Mahobia V, Schneeberg A, Ramm M, Boland W (2008). Rapid hydrolysis of quorum-sensing molecules in the gut of lepidopteran larvae. *Chembiochem* **9:** 1953-1959.

Galano A, Francisco-Marquez M (2009). Reactions of OOH radical with beta-carotene, lycopene, and torulene: hydrogen atom transfer and adduct formation mechanisms. *J Phys Chem B* **113:** 11338-11345.

Genta FA, Dillon RJ, Terra WR, Ferreira C (2006). Potential role for gut microbiota in cell wall digestion and glucoside detoxification in Tenebrio molitor larvae. *J Insect Physiol* **52:** 593-601.

Gill SR, Pop M, DeBoy RT, Eckburg PB, Turnbaugh PJ, Samuel BS *et al* (2006). Metagenomic analysis of the human distal gut microbiome. *Science* **312:** 1355-1359.

Goodwin TW (1986). Metabolism, nutrition, and function of carotenoids. *Annual review of nutrition* **6:** 273-297.

Goto S, Kogure K, Abe K, Kimata Y, Kitahama K, Yamashita E *et al* (2001). Efficient radical trapping at the surface and inside the phospholipid membrane is responsible for highly potent antiperoxidative activity of the carotenoid astaxanthin. *Bba-Biomembranes* **1512:** 251-258.

Grbic M, Van Leeuwen T, Clark RM, Rombauts S, Rouze P, Grbic V *et al* (2011). The genome of Tetranychus urticae reveals herbivorous pest adaptations. *Nature* **479:** 487-492.

Hartmann T (2008). The lost origin of chemical ecology in the late 19th century. *Proceedings of the National Academy of Sciences of the United States of America* **105:** 4541-4546.

Jensen NB, Zagrobelny M, Hjerno K, Olsen CE, Houghton-Larsen J, Borch J *et al* (2011). Convergent evolution in biosynthesis of cyanogenic defence compounds in plants and insects. *Nature communications* **2:** 273.

Kikuchi Y, Hosokawa T, Fukatsu T (2007). Insect-microbe mutualism without vertical transmission: a stinkbug acquires a beneficial gut symbiont from the environment every generation. *Appl Environ Microb* **73:** 4308-4316.

Kikuchi Y, Hayatsu M, Hosokawa T, Nagayama A, Tago K, Fukatsu T (2012). Symbiont-mediated insecticide resistance. *Proc Natl Acad Sci U S A* **109:** 8618-8622.

Koch H, Schmid-Hempel P (2011). Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. *Proc Natl Acad Sci U S A* **108:** 19288-19292.

Krishnan N, Kodrik D (2006). Antioxidant enzymes in Spodoptera littoralis (Boisduval): Are they enhanced to protect gut tissues during oxidative stress? *J Insect Physiol* **52:** 11-20.

Kroll H, Friedrich J, Menzel M, Schreier P (2008). Carbon and hydrogen stable isotope ratios of carotenoids and beta-carotene-based dietary supplements. *Journal of agricultural and food chemistry* **56:** 4198-4204.

Kursar TA, Dexter KG, Lokvam J, Pennington RT, Richardson JE, Weber MG *et al* (2009). The evolution of antiherbivore defenses and their contribution to species coexistence in the tropical tree genus Inga. *Proceedings of the National Academy of Sciences of the United States of America* **106:** 18073-18078.

Leitner M, Boland W, Mithofer A (2005). Direct and indirect defences induced by piercing-sucking and chewing herbivores in Medicago truncatula. *New Phytol* **167:** 597-606.

Leroy PD, Sabri A, Heuskin S, Thonart P, Lognay G, Verheggen FJ *et al* (2011). Microorganisms from aphid honeydew attract and enhance the efficacy of natural enemies. *Nature communications* **2:** 348.

Li X, Schuler MA, Berenbaum MR (2007). Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Annu Rev Entomol* **52:** 231-253.

Maass D, Arango J, Wust F, Beyer P, Welsch R (2009). Carotenoid crystal formation in Arabidopsis and carrot roots caused by increased phytoene synthase protein levels. *PloS one* **4:** e6373.

Maffei ME, Mithofer A, Arimura G, Uchtenhagen H, Bossi S, Bertea CM *et al* (2006). Effects of feeding Spodoptera littoralis on lima bean leaves. III. Membrane depolarization and involvement of hydrogen peroxide. *Plant Physiol* **140:** 1022-1035.

Manero A, Blanch AR (1999). Identification of Enterococcus spp. with a biochemical key. *Appl Environ Microbiol* **65:** 4425-4430.

Maraccini PA, Ferguson DM, Boehm AB (2012). Diurnal variation in Enterococcus species composition in polluted ocean water and a potential role for the enterococcal carotenoid in protection against photoinactivation. *Appl Environ Microbiol* **78:** 305-310.

Matsuura Y, Kikuchi Y, Hosokawa T, Koga R, Meng XY, Kamagata Y *et al* (2011). Evolution of symbiotic organs and endosymbionts in lygaeid stinkbugs. *ISME J*.

McCutcheon JP, McDonald BR, Moran NA (2009). Convergent evolution of metabolic roles in bacterial co-symbionts of insects. *Proc Natl Acad Sci U S A* **106:** 15394-15399.

Mithofer A, Schulze B, Boland W (2004). Biotic and heavy metal stress response in plants: evidence for common signals. *FEBS letters* **566:** 1-5.

Mithofer A, Boland W (2012). Plant defense against herbivores: chemical aspects. *Annual review of plant biology* **63:** 431-450.

Moran NA, Jarvik T (2010). Lateral transfer of genes from fungi underlies carotenoid production in aphids. *Science* **328:** 624-627.

Moran NA, Hansen AK, Powell JE, Sabree ZL (2012). Distinctive Gut Microbiota of Honey Bees Assessed Using Deep Sampling from Individual Worker Bees. *Plos One* **7**.

Mori N, Yoshinaga N, Sawada Y, Fukui M, Shimoda M, Fujisaki K *et al* (2003). Identification of volicitin-related compounds from the regurgitant of lepidopteran caterpillars. *Bioscience, biotechnology, and biochemistry* **67:** 1168-1171.

Neufeld JD, Vohra J, Dumont MG, Lueders T, Manefield M, Friedrich MW *et al* (2007). DNA stable-isotope probing. *Nat Protoc* **2:** 860-866.

Nicholson JK, Holmes E, Kinross J, Burcelin R, Gibson G, Jia W *et al* (2012). Host-gut microbiota metabolic interactions. *Science* **336:** 1262-1267.

Pesek J, Buchler R, Albrecht R, Boland W, Zeth K (2011). Structure and Mechanism of Iron Translocation by a Dps Protein from Microbacterium arborescens. *J Biol Chem* **286:** 34872-34882.

Pilloni G, von Netzer F, Engel M, Lueders T (2011). Electron acceptor-dependent identification of key anaerobic toluene degraders at a tar-oil-contaminated aquifer by Pyro-SIP. *FEMS Microbiol Ecol*.

Ping L, Buchler R, Mithofer A, Svatos A, Spiteller D, Dettner K *et al* (2007). A novel Dps-type protein from insect gut bacteria catalyses hydrolysis and synthesis of N-acyl amino acids. *Environ Microbiol* **9:** 1572-1583.

Priya NG, Ojha A, Kajla MK, Raj A, Rajagopal R (2012). Host Plant Induced Variation in Gut Bacteria of Helicoverpa armigera. *PLoS One* **7**.

R.R. AUCOIN (1990). THE PROTECTIVE EFFECT OF ANTIOXIDANTS TO PHOTOTOXIN-SENSITIVE INSECT HERBIVORE, Manduca sexta.

Rao MN, Ghosh P, Lakshman MR (1997). Purification and partial characterization of a cellular carotenoid-binding protein from ferret liver. *J Biol Chem* **272:** 24455-24460.

Rausher MD (2001). Co-evolution and plant resistance to natural enemies. *Nature* **411:** 857-864.

Robinson GE, Hackett KJ, Purcell-Miramontes M, Brown SJ, Evans JD, Goldsmith MR *et al* (2011). Creating a Buzz About Insect Genomes. *Science* **331:** 1386-1386.

Russell JA, Moreau CS, Goldman-Huertas B, Fujiwara M, Lohman DJ, Pierce NE (2009). Bacterial gut symbionts are tightly linked with the evolution of herbivory in ants. *Proc Natl Acad Sci U S A* **106:** 21236-21241.

Sakudoh T, Sezutsu H, Nakashima T, Kobayashi I, Fujimoto H, Uchino K *et al* (2007). Carotenoid silk coloration is controlled by a carotenoid-binding protein, a product of the Yellow blood gene. *Proceedings of the National Academy of Sciences of the United States of America* **104:** 8941-8946.

Schneider DS, Chambers MC (2008). MICROBIOLOGY Rogue Insect Immunity. *Science* **322:** 1199-1200.

Schuler MA (2012). Insect P450s: mounted for battle in their war against toxins. *Molecular ecology* **21:** 4157-4159.

Schuman MC, Barthel K, Baldwin IT (2012). Herbivory-induced volatiles function as defenses increasing fitness of the native plant Nicotiana attenuata in nature. *eLife* **1:** e00007.

Shao Y, Spiteller D, Tang X, Ping L, Colesie C, Munchberg U *et al* (2011). Crystallization of alpha- and beta-carotene in the foregut of Spodoptera larvae feeding on a toxic food plant. *Insect biochemistry and molecular biology* **41:** 273-281.

Sloan DB, Moran NA (2012). Endosymbiotic bacteria as a source of carotenoids in whiteflies. *Biol Lett*.

Spiteller D, Dettner K, Boland W (2000). Gut bacteria may be involved in interactions between plants, herbivores and their predators: microbial biosynthesis of N-acylglutamine surfactants as elicitors of plant volatiles. *Biol Chem* **381:** 755-762.

Sudakaran S, Salem H, Kost C, Kaltenpoth M (2012). Geographical and ecological stability of the symbiotic mid-gut microbiota in European firebugs, Pyrrhocoris apterus (Hemiptera, Pyrrhocoridae). *Mol Ecol*.

Takatsuka J, Kunimi Y (2000). Intestinal bacteria affect growth of Bacillus thuringiensis in larvae of the oriental tea tortrix, Homona magnanima diakonoff (Lepidoptera: tortricidae). *J Invertebr Pathol* **76:** 222-226.

Taylor RF, Ikawa M, Chesbro W (1971). Carotenoids in yellow-pigmented enterococci. *J Bacteriol* **105:** 676-678.

Tsuchida T, Koga R, Horikawa M, Tsunoda T, Maoka T, Matsumoto S *et al* (2010). Symbiotic bacterium modifies aphid body color. *Science* **330:** 1102-1104.

van der Hoeven R, Betrabet G, Forst S (2008). Characterization of the gut bacterial community in Manduca sexta and effect of antibiotics on bacterial diversity and nematode reproduction. *FEMS Microbiol Lett* **286:** 249-256.

Vanhaecke L, Vercruysse F, Boon N, Verstraete W, Cleenwerck I, De Wachter M *et al* (2008). Isolation and characterization of human intestinal bacteria capable of transforming the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. *Appl Environ Microbiol* **74:** 1469-1477.

Vasquez A, Forsgren E, Fries I, Paxton RJ, Flaberg E, Szekely L *et al* (2012). Symbionts as Major Modulators of Insect Health: Lactic Acid Bacteria and Honeybees. *PLoS One* **7**.

Walter MH, Strack D (2011). Carotenoids and their cleavage products: Biosynthesis and functions. *Nat Prod Rep* **28:** 663-692.

Werren JH, Baldo L, Clark ME (2008). Wolbachia: master manipulators of invertebrate biology. *Nat Rev Microbiol* **6:** 741-751.

Werren JH (2012). Symbionts provide pesticide detoxification. *Proc Natl Acad Sci U S A* **109:** 8364-8365.

Wu JQ, Baldwin IT (2010). New Insights into Plant Responses to the Attack from Insect Herbivores. *Annu Rev Genet* **44:** 1-24.

Yoshinaga N, Aboshi T, Abe H, Nishida R, Alborn HT, Tumlinson JH *et al* (2008). Active role of fatty acid amino acid conjugates in nitrogen metabolism in Spodoptera litura larvae. *Proc Natl Acad Sci U S A* **105:** 18058-18063.

Young AJ, Lowe GM (2001). Antioxidant and prooxidant properties of carotenoids. *Archives of biochemistry and biophysics* **385:** 20-27.

Zagrobelny M, Bak S, Ekstrom CT, Olsen CE, Moller BL (2007). The cyanogenic glucoside composition of Zygaena filipendulae (Lepidoptera: Zygaenidae) as effected by feeding on wild-type and transgenic lotus populations with variable cyanogenic glucoside profiles. *Insect Biochem Mol Biol* **37:** 10-18.

Ziegelhoffer EC, Donohue TJ (2009). Bacterial responses to photo-oxidative stress. *Nat Rev Microbiol* **7:** 856-863.

12. Selbständigkeitserklärung

Hiermit erkläre ich, entsprechend § 5 Absatz 3 der Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich Schiller Universität Jena, das mir die geltende Promotionsordnung bekannt ist. Die vorliegende Dissertation habe ich eigenständig und nur unter Verwendung angegebener Quellen und Hilfsmittel angefertigt, wobei von Dritten übernommene Textabschnitte entsprechend gekennzeichnet wurden. Alle Personen, die einen entscheidenden Beitrag zu den Manuskripten geleistet haben, sind in Kapitel 2 aufgeführt beziehungsweise in der Danksagung erwähnt. Die Hilfe eines Promotionsberaters wurde nicht in Anspruch genommen noch haben Dritte geldwerte Leistungen für Arbeiten im Zusammenhang mit der vorliegenden Dissertation erhalten. Gemäß Anlage 5 zum § 8 Absatz 3 der Promotionsordnung wurde die Beschreibung des von mir geleisteten Eigenanteils von dem Betreuer der Dissertation, Prof. Dr. Wilhelm Boland, mit Unterschrift bestätigt und der Fakultät vorgelegt. Zu keinem früheren Zeitpunkt wurde diese Dissertation, eine in wesentlichen Teilen ähnliche Arbeit oder eine andere Abhandlung bei einer Hochschule als Dissertation eingereicht.

Yongqi Shao

Jena, den 18.12.2012

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14. Curriculum vitae

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Publications

Shao Y., Guo H., Batram S., and Boland W. *In vivo* Pyro-SIP assessing active gut microbiota of cotton leafworm, *Spodoptera littoralis*. Submitted.

Shao Y., Arias-Cordero E., and Boland W. Identification of the metabolically active bacteria in the gut of the generalist *Spodoptera littoralis* via DNA stable isotope probing using ^{13}C-glucose. **Co-first author**, JoVE.

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

Shao, Y., Spiteller, D., Tang, X., Ping, L., Colesie, C., Münchberg, U., Bartram, S., Schneider, B., Büdel, B., Popp, J., Heckel, D.G., and Boland, W. (2011). Crystallization of alpha- and beta-carotene in the foregut of *Spodoptera* larvae feeding on a toxic food plant. Insect Biochem Mol Biol 41, 273-281.

Lee, S., Jia, B., Pham, B.P., **Shao, Y.**, Kwak, J.M., and Cheong, G.W. (2011). Architecture and characterization of sarcosine oxidase from *Thermococcus kodakarensis* KOD1. Extremophiles: life under extreme conditions. **Co-first author**.

Shao, Y., Chung, B.S., Lee, S.S., Park, W., and Jeon, C.O. (2009). *Zoogloea caeni* sp. nov., a floc-forming bacterium isolated from activated sludge. Int J Syst Evol Microbiol 59, 526-530.

Wang, Q., **Shao, Y.**, Huong, V.T.T., Park, W.J., Park, J.M., and Jeon, C.O. (2008). Fine-scale population structure of *Accumulibacter phosphatis* in enhanced biological phosphorus removal sludge. J Microbiol Biotechn 18, 1290-1297.

Shao Y. (2007). The Functional Mechanism of Sarcosine Oxidase from *Thermococcus kodakaraensis* KOD1. Master Thesis, Gyeongsang National University.

Park, S.C., Jia, B.L., Yang, J.K., Le Van, D., **Shao, Y.**, Han, S.W., Jeon, Y.J., Chung, C.H., and Cheong, G.W. (2006). Oligomeric structure of the ATP-dependent protease La (Lon) of *Escherichia coli*. Mol Cells 21, 129-134.

Oral presentations

Shao Y. (2012). The microbiology of cotton leafworm, *Spodoptera littoralis*. 44th Doktorandenworkshop: Naturstoffe: Chemie, Biologie und Ökologie. Jena, Germany.

Shao Y. (2012). *In vivo* Pyro-SIP analysis of active gut microbiota of cotton leafworm, *Spodoptera littoralis*. JSPS Core-to-Core Program: Studies on ecological interaction networks that promote biodiversity -From gene to ecosystem. Abbe Centre, Beutenberg (lecture hall), Hans-Knöll-Str. 1, Jena, Germany.

Shao Y. (2010). A novel carotenoid-based coloration in *Spodoptera*: the smart insect defense or science fiction? The First European Student Conference on Microbial Communication. Jena, Germany.

Poster presentations

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

Shao Y. (2011). Functional analysis of a herbivore's gut microbiota. JSMC Excellence Initiative Defense. Bonn, Germany.

Shao Y. (2011). Purification and characterization of a novel bacteriocin produced by *Enterococcus* from insect gut. 13th Congress of the European Society of Evolutionary Biology. Tübingen, Germany.

Shao Y. (2010). Survey of functional bacteria from laboratory-bred larvae of *Spodoptera littoralis*. 2nd Workshop on Symbiotic Interactions. Würzburg, Germany.

Tang X., **Shao Y.** (2009). A Survey of Probiotic Bacteria in *Spodoptera littoralis*. 61. Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie (DGHM). Goettingen, Germany.

Shao Y. (2009). What Makes Some Insect Herbivore Generalists is the Specialistic Gut Biota. 109th General meeting of the American society for Microbiology, Philadelphia, Pennsylvania, USA.

Awards

2009-2012 Stipend from Jena School of Microbial Communication (JSMC, funded by DFG)
2005-2007 Scholarship for National Core Research Centre, South Korea
2005-2007 Brain Korea 21 Students' Scholarship of Gyeongsang National University
2004 Student Scholarship of Soochow University
2003 Student Scholarship of Soochow University
2002 Student Scholarship of Soochow University
2001 Student Scholarship of Soochow University (Honor for top 10% students)

15. Supplementary material

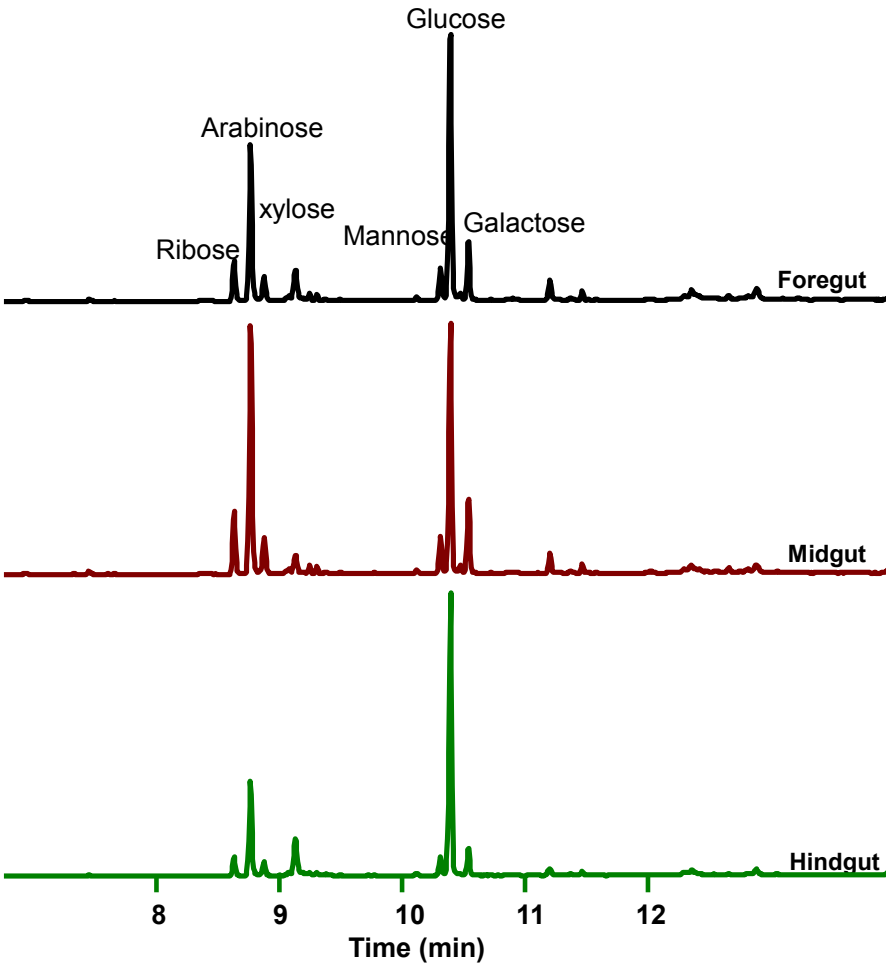
a. Article III

Supplementary table

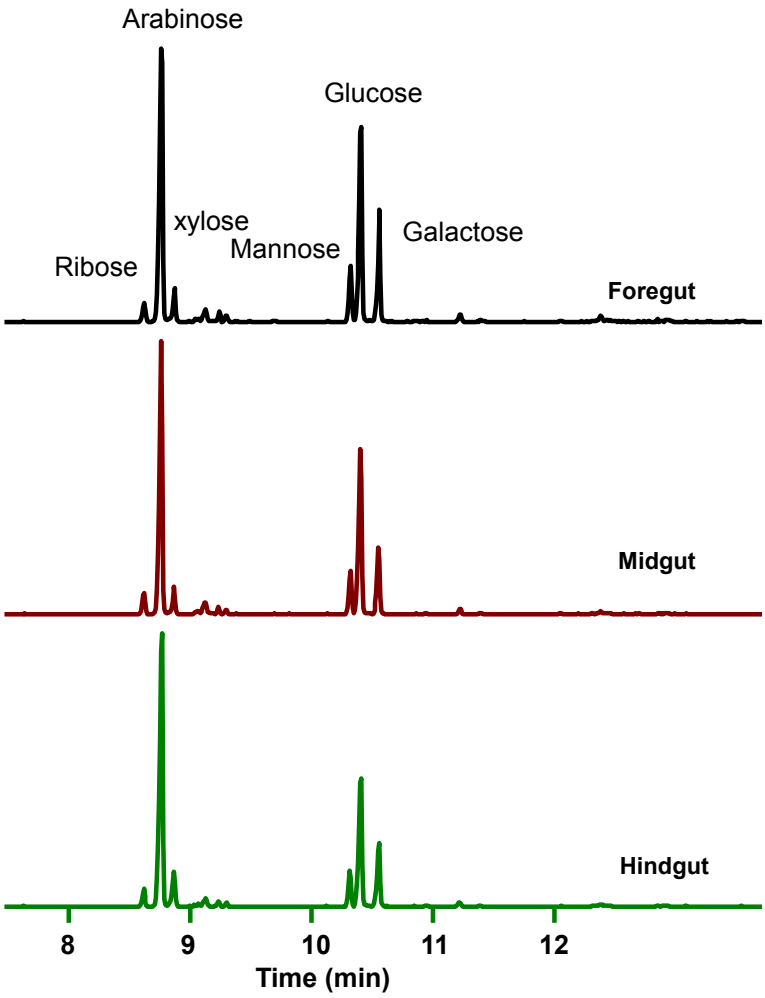
Primer/Probe	Primer sequence (5'-3')	Target	Use	References
27f	AGAGTTTGATCCTGGCTCAG	Eubacteria	General amplification	Egert *et al.* 2005
4fa	TCCGGTTGATCCTGCCRG	Archaea	General amplification	
1492r	GGTTACCTTGTTACGACTT	Eubacteria	General amplification	
ITS1	TCCGTAGGTGAACCTGCGG	Fungi	General amplification	Anderson *et al.* 2004
ITS4	TCCTCCGCTTATTGATATGC	Fungi	General amplification	
M13F	GTAAAACGACGGCCAG	Eubacteria	Sequencing	
M13R	CAGGAAACAGCTATGAC	Eubacteria	Sequencing	
968F-GC-Clamp	CGCCCGGGGCGCGCCCCGGGCG GGGCGGGGGCACGGGGGGAACG CGAAGAACCTTAC	Eubacteria	DGGE	van Ems *et al.* 2008
1401Ra	CGGTGTGTACAAGGCCCGGGAACG	Eubacteria	DGGE/Sequencing	
1401Rb	CGGTGTGTACAAGACCCGGGAACG	Eubacteria	DGGE/Sequencing	
968F	AACGCGAAGAACCTTAC	Eubacteria	Sequencing	
Gray28F	GAGTTTGATCNTGGCTCAG	Eubacteria	454 sequencing	Ishak *et al.* 2011
Gray519r	GTNTTACNGCGGCKGCTG	Eubacteria	454 sequencing	
EUB338-Cy5	GCTGCCTCCCGTAGGAGT	Eubacteria	FISH	Amann *et al.* 1990
CGF -Cys3	GCGGAAAATAGTGTTATACGG	*Enterococccus spp.*	FISH	Manero *et al.* 2002
Ecf459-Cy3	GGGATGAACATTTTACTC	*Enterococccus spp.*	FISH	Behr *et al.* 2000

Supplementary Table S1. Primers and probes used for the characterization and localization of bacterial taxa in the gut of *Spodoptera littoralis*.

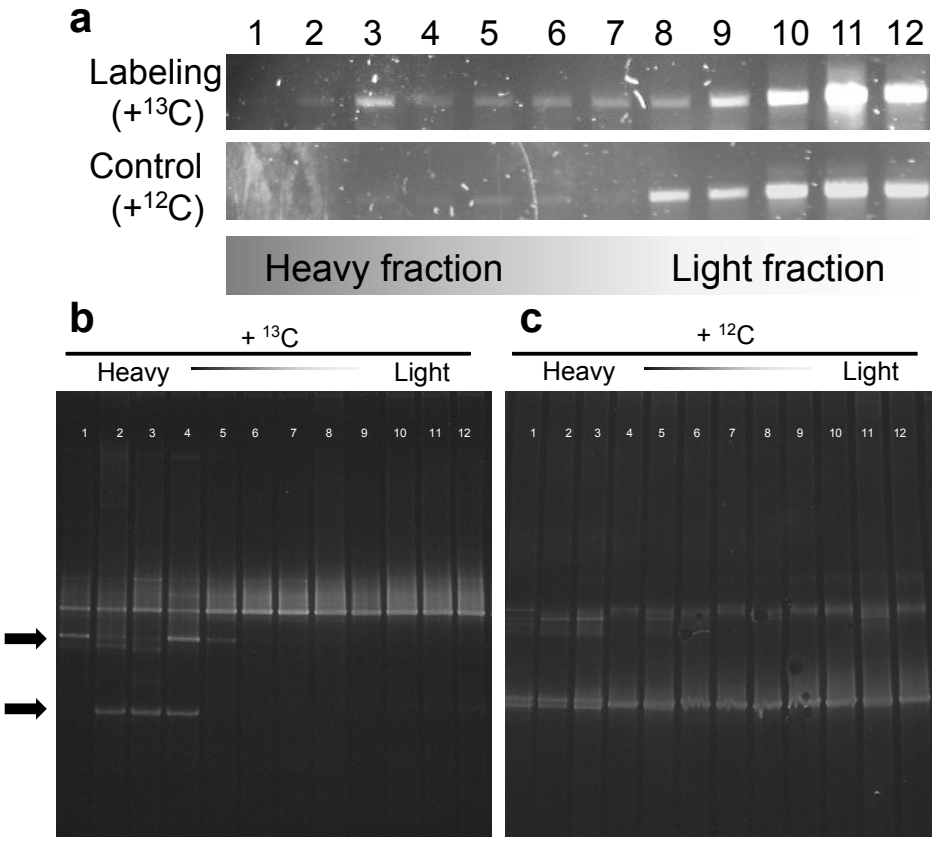
Supplementary figures



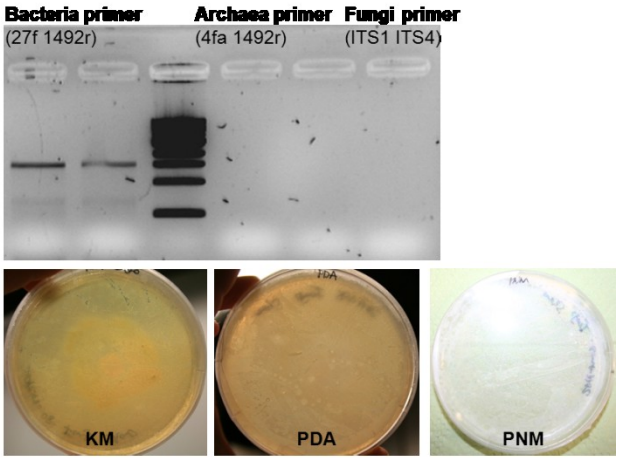
Supplementary Figure S1. Sugar composition of the gut content after acid hydrolysis. Larvae fed on cotton plants.



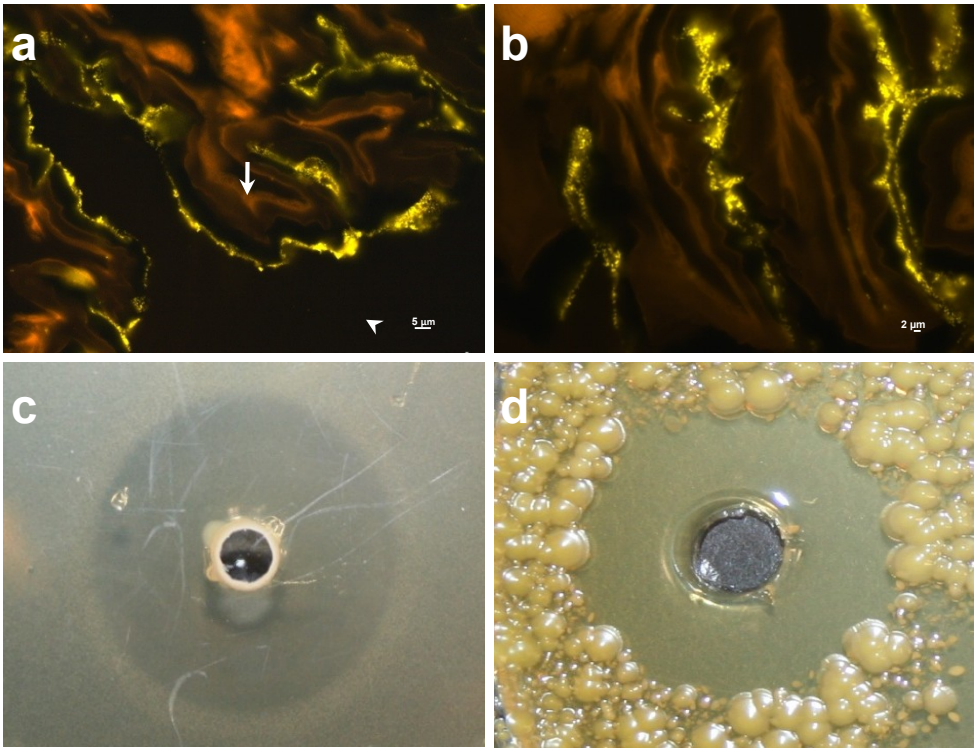
Supplementary Figure S2. Sugar composition of the gut content after acid hydrolysis. Larvae fed on artificial diet.



Supplementary Figure S3. The DGGE fingerprinting of density-revealed gradient fractions from the ^{13}C-glucose labeling and the native glucose control (^{12}C-glucose). (**a**) Fraction-dependent PCR assay of amplifying bacterial 16S rRNA gene. Fractions (1 - 12) were obtained from density-revealed gradients of the labeling treatment (+ ^{13}C) or the control (+ ^{12}C). Increased band intensity was observed in heavy fractions of the labeling treatment. (**b**) The DGGE profile of bacterial 16S rRNA genes in gradients of the labeling treatment and (**c**) the control. Arrows indicate noticeable changes in community composition of the labeling treatment.



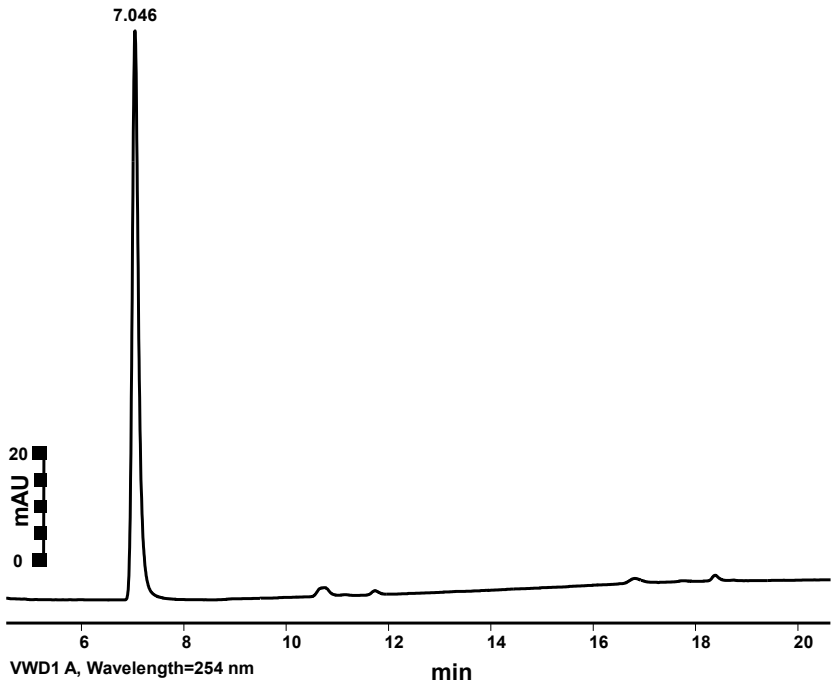
Supplementary Figure S4. Fungi and archaea detection in the gut of *Spodoptera littoralis*. Gel electrophoresis shows the absence of amplification products with fungus- and archaea-specific primers. Three kinds of common fungi-growing agar plates (KM, Kempler-McKay agar; PDA, potato dextrose agar; PNM, plant nutrient medium) were used in the fungal cultivation attempt and no fungus was recovered.



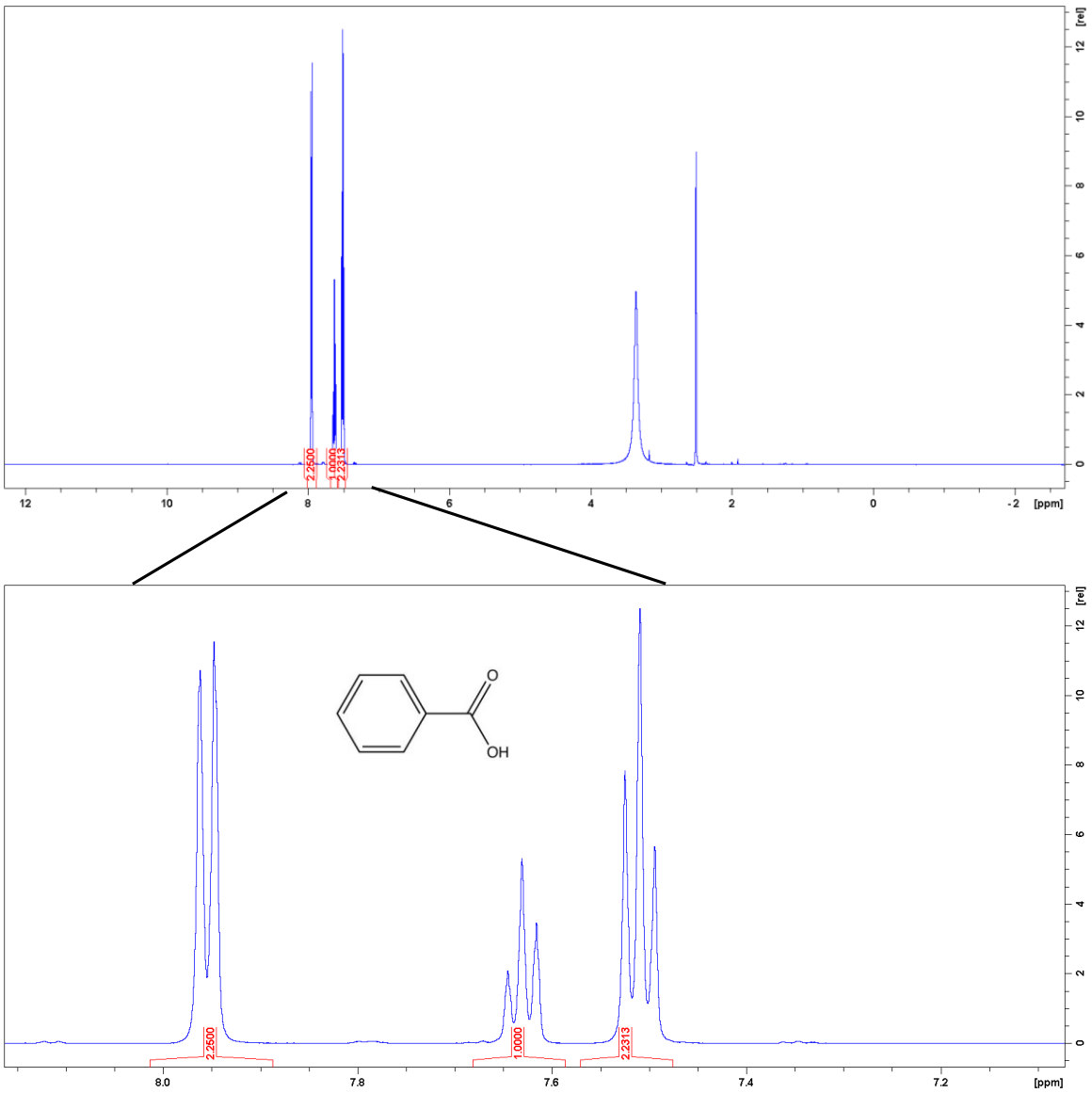
Supplementary Figure S5. Images of *Enterococcus* sp. from *S. littoralis* reveal bacterial gut localization and antimicrobial activity. (**a**) FISH with a Cy3-labeled *Enterococcus*-specific probe (yellow) show a high density of bacterial cells adhere on the mucus layer lining the gut epithelium, and that under higher magnification (63 × 10) (**b**). White arrow indicates the gut epithelium tissue. White arrowhead indicates the gut lumen. (**c**) Agar diffusion assays show *Enterococcus* culture filtrates against *Micrococcus luteus* and (**d**) *Leuconostoc mesenteroides*. Antimicrobial activity is detected by the formation of clearance zone around the loading hole.

b. HPLC profile and NMR spectra of the purified benzoic acid from *Enterococcus* culture

HPLC trace:

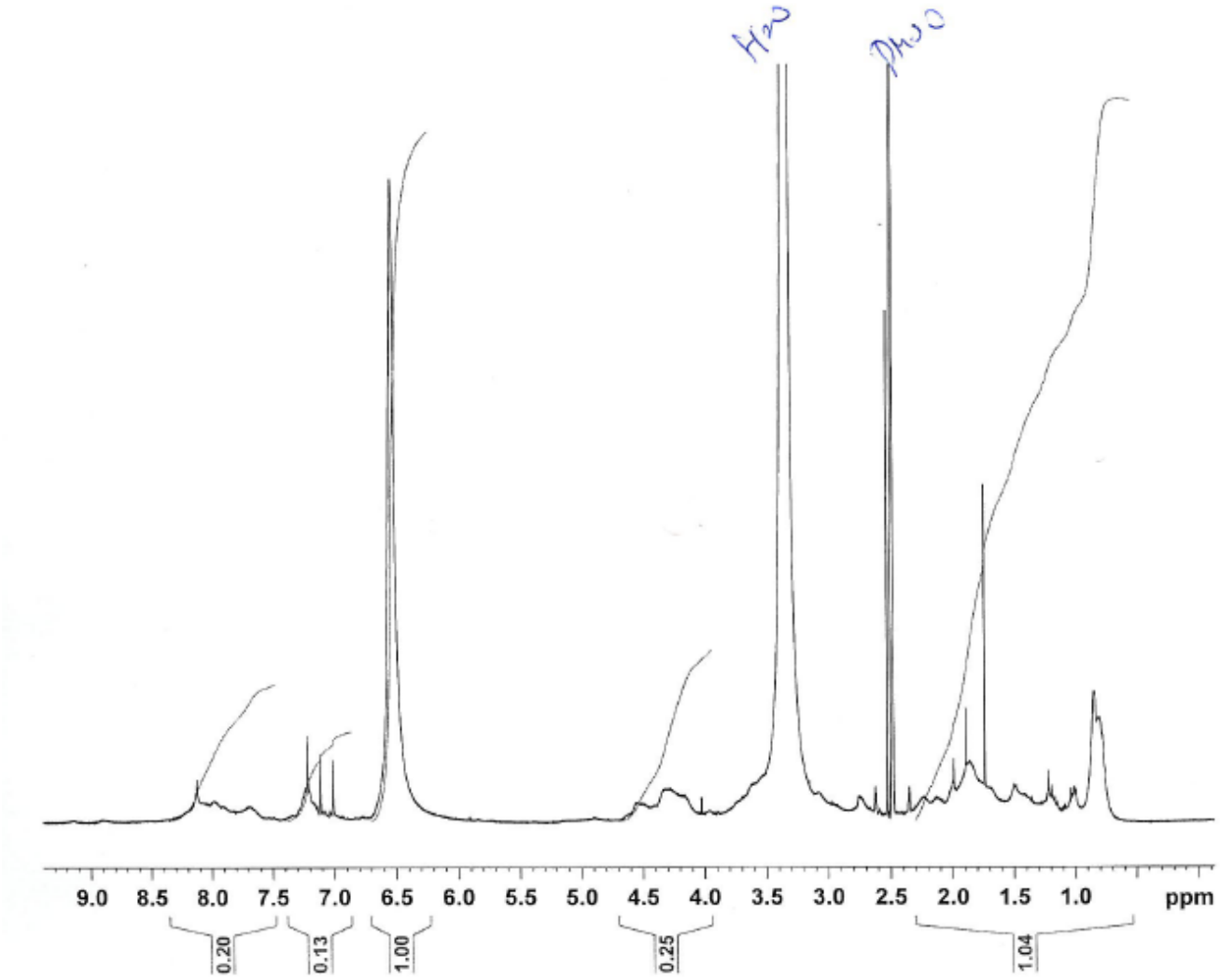


NMR spectra:



b. NMR spectra of the purified <u>peptide</u> from *Enterococcus* culture and amino acid composition profile after acid hydrolysis

NMR spectra:



Amino acid analysis by GC-MS (acid hydrolysis MSTFA):

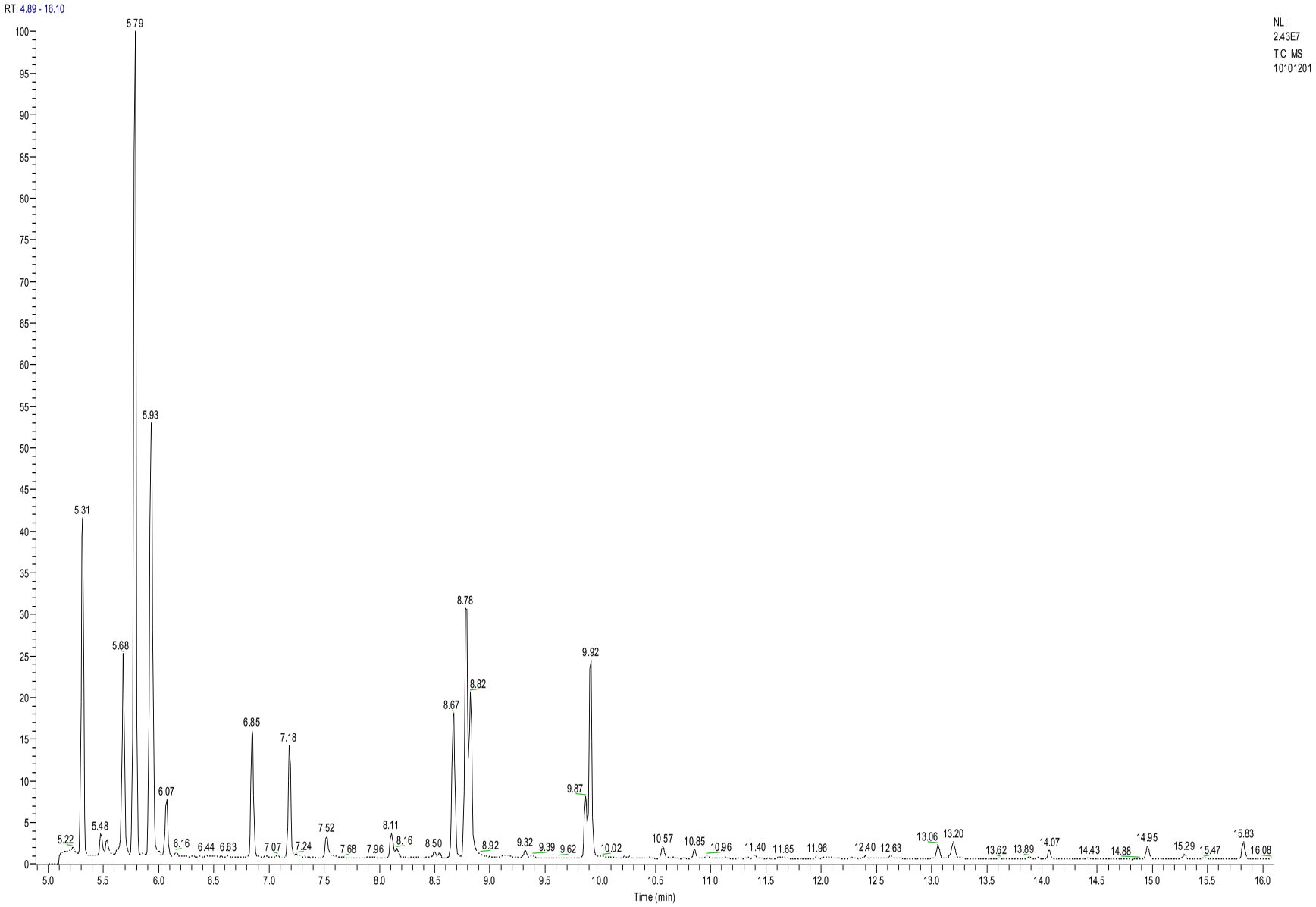
Strong ions: Asp (Asn), Glu (Gln), Pro, Val, Leu (Ile), Lys

Middle ions: Gly, Ala, Ser, Thr, Phe, Tyr

Weak ions: His, Lys, Arg

No ions: Cys, Met, lanthionine, methyllanthionine

GC trace: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NL:
2.43E7
TIC MS
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5.79
5.93
5.31
5.68
8.78
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9.92
8.67
6.85
7.18
6.07
9.87
5.48
5.22
7.52
8.11
6.16
6.44
6.63
7.07
7.24
7.68
7.96
8.16
8.50
8.92
9.32
9.39
9.62
10.02
10.57
10.85
10.96
11.40
11.65
11.96
12.40
12.63
13.06
13.20
13.62
13.89
14.07
14.43
14.88
14.95
15.29
15.47
15.83
16.08
Time (min)

150