Development and role of the indigenous gut microbiota of *Spodoptera littoralis*

Dissertation

To Fulfill the

Requirements for the Degree of

„doctor rerum naturalium“ (Dr. rer. nat.)

Submitted to the Council of the Faculty

of Biology and Pharmacy

of the Friedrich Schiller University Jena

by Beng-Soon Teh (M.Sc)

born on 12.02.1985 in Penang, Malaysia

Gutachter:

1. Prof. Dr. Wilhelm Boland

   Abteilung für Bioorganische Chemie, Max-Planck-Institut für Chemische Ökologie, Jena

2. Prof. Dr. Erika Kothe

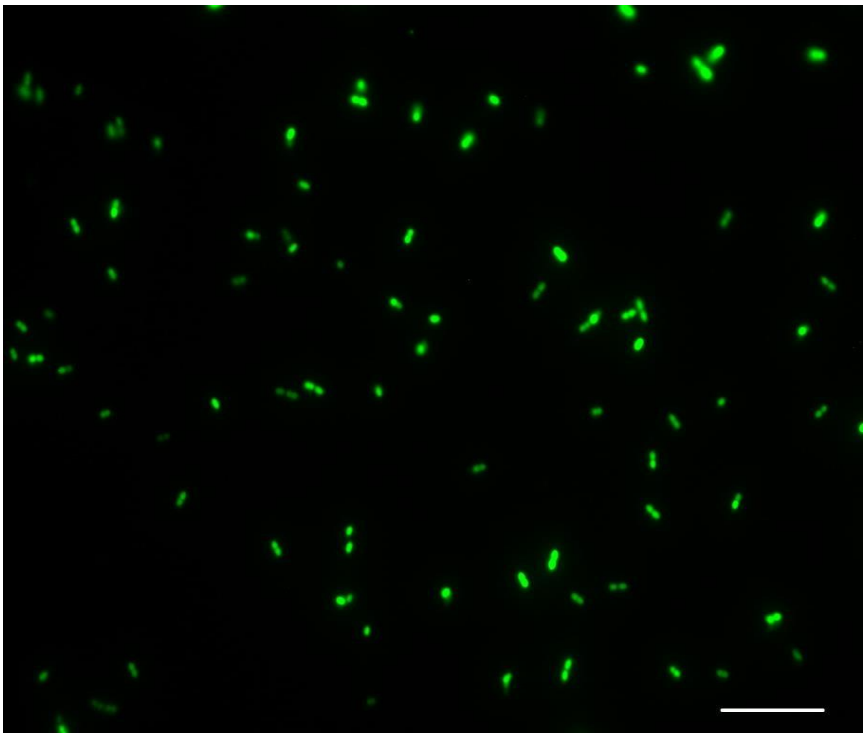
   Institut für Mikrobiologie, Biologisch-Pharmazeutische Fakultät, Friedrich Schiller Universität, Jena

3. Prof. Dr. Joern Piel

   Institute of Microbiology, ETH Zurich, Wolfgang-Pauli-Strasse 10, CH-8093 Zurich, Switzerland

Tag der öffentlichen Verteidigung:

10.07.2018



Fluorescent GFP-tagged *Enterococcus mundtii*

TABLE of CONTENTS

Abbreviations and symbols

AMP	antimicrobial peptide
ROS	reactive oxygen species
OTU	operational taxonomic unit
LAB	lactic acid bacteria
FAO	Food and Agricultural Organization
WHO	World Health Organization
GAS	group A streptococci
GBS	group B streptococci
GI	gastrointestinal tract
GFP	green fluorescent protein
FACS	fluorescence-activated cell sorting
Em	erythromycin
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
CDS	coding sequence
MCS	multiple cloning site
RBS	ribosome binding site
COG	clusters of orthologous groups of protein
KEGG	Kyoto Encyclopedia of Genes and Genomes
FISH	fluorescence *in situ* hybridization
DUOX	dual oxidase
NADPH	nicotinamide adenine dinucleotide phosphate

NADH	nicotinamide adenine dinucleotide
CFU	colony forming unit
PGN	peptidoglycan
PGRP	peptidoglycan recognition protein
IMD	immune deficiency
SCFA	short chain fatty acid
PTS	phosphotransferase system
CAZy	carbohydrate active enzyme
EPS	extracellular polymeric substance
8-HQA	8-hydroxyquinoline-2-carboxylic acid

1. Introduction

1.1 Host-microbiota symbiosis interactions

Symbiosis is a common phenomenon that occurs in most animals. The term ‘symbiosis’ was initially defined by Anton de Bary in 1879 as "the living together of two unlike organisms" in which the interaction will benefit at least one of the partners (De Bary, 1879). Microbial symbioses can be categorized as parasitism, commensalism, and mutualism (Moya et al., 2008). Parasitism occurs when one partner gains the advantage while the other is negatively affected by the association. Commensalism occurs when the microbe is not harmful, but instead reaps the benefits from the host, and produces nothing in return (Dillon and Dillon, 2004).

Humans and animals harbor microbiomes either on or within the body surfaces such as the gastrointestinal tracts and the skin. Vertebrates and invertebrates are ideal model organisms to study host-microbiome interplay through the identification of microbial-associated and host-derived molecules that enable symbiosis. The popular host-microbe symbiosis is the relationships between the squid host *Euprymna scolopes* and its marine bacterium *Vibrio fischeri*. The squid normally feed at night in the presence of moonlight which can reveal its shadow, and thus alerts the predators. To prevent from predator detection, the squid camouflages by counter-illumination from light generated by its symbiont *V. fischeri* during colonization of the light organ. In return, the squid provides nutrients for its bacterial symbiont in the light organ (Nyholm and McFall-Ngai, 2003). The research on microbial pathogenesis and innate immunity has gained attention lately in the fruit fly *Drosophila melanogaster* (Dionne and Schneider, 2008, O'Callaghan and Vergunst, 2010). The interaction between *Drosophila* and its gut symbiotic microbiota focuses primarily on mate selection. It was reported that *Drosophila* prefers to mate with flies possessing similar gut microbes (Sharon et al., 2010). To test this

hypothesis, the flies were treated with antibiotics and recolonized with specific microbiota. It was found that the symbiotic bacterium *Lactobacillus plantarum* contributes to the mating preference of *Drosophila* (Sharon et al., 2010).

1.1.1 Insect-bacteria symbiosis interactions

Insects are arguably the most diverse group of animals due to their abundance and adaptability to a wide range of habitat. They are associated with many microorganisms, including viruses, bacteria, fungi, and parasites (Dillon and Dillon, 2004). Early studies have focused mostly on insect defensive strategies against pathogens by the production of antimicrobial peptides or mechanisms to overcome bacterial infection (Brown and Hancock, 2006, Lemaitre and Hoffmann, 2007, Lazzaro, 2008). In recent years, studies on symbiotic interaction between insect and bacteria have been growing at large scale. Symbiotic bacteria are found within the gut, tissues, and cells of the insects (Buchner, 1965). Some commensal microbes could be mutualists, for example, the insect gut microbiota that aid the host in food digestion, provide vitamins, and protect against harmful pathogens. Due to the changes in environmental conditions, pathogenic microbes such as chlamydia which initially act as pathogens but over time have evolved mechanisms to enable their own survival as well as the host (Horn et al., 2004).

Bacterial symbionts are typically acquired by the host either horizontally from the environment or vertically from the parents or combination of both mechanisms (Bright and Bulgheresi, 2010). Symbiotic bacteria are acquired from the environment such as in squid-*vibrio* and plant legume-*Rhizobium* relationships (Nyholm and McFall-Ngai, 2004, Gage, 2004). Symbionts can be either horizontally transferred from food into the human gut (Ley et al., 2006)

or via "egg smearing", a phenomenon which female stinkbugs covering the surface of her eggs with symbiotic bacteria during oviposition (Funkhouser and Bordenstein, 2013). Apart from horizontal transfer, symbionts can be transmitted vertically through reproductive cells and larvae, for example in insects (Muller and Nebe-von-Caron, 2010), sponges (Webster et al., 2010) and ascidians (Kojima and Hirose, 2012). Another example of vertical transmission involves symbiosis between the pea aphid *Acrythosiphon pisum* and its endosymbiont *Buchnera aphidicola* (Baumann et al., 1995).

1.2 Physiological conditions and stresses in the gut environment of insects

Several factors, including the pH, redox potential, oxygen availability, nutrient, and immune system can shape the microbial composition of the gut of insects (Engel and Moran, 2013). Many insects have the intestinal pH in the range of 6-8, and some lepidopteran larvae have higher pH of 11-12 in their midguts (Wieczorek et al., 2009, Funke et al., 2008).

However, diet is one of the main determinants of the microbial gut composition in vertebrates and invertebrates (Lozupone et al., 2012). It has been shown that diet composition contributes to the microbial composition in the midgut of insects such as larval cotton bollworms (Xiang et al., 2006) and gypsy moths (Broderick et al., 2004).

Host tissues are the source of complex stress environments that shape the composition of its microbiota. The host tissues produce chemical or physical stress (Cullen et al., 2015), nutrient (Pickard and Chervonsky, 2015), and various signaling molecules (Mullard, 2009). Animals secrete various stress products, such as urea, mucus, bile (Begley et al., 2005, Russell and Rychlik, 2001), and gastric acid that restrict microbial colonization. For example, the animal-associated bacterium, *Helicobacter pylori* can overcome the highly acidic stomach by secreting

urease and coordinates its movement towards less acidic niche (Huang et al., 2015). In addition, the presence of microbes is easily detected by the host immune system (Figure 1.1). For example, the bacterial symbiont, *Vibrio fischeri* produces extracellular polymeric substances (EPS) as a strategy to escape from the immune response created by the squid's light organ (Shibata et al., 2012, Brooks et al., 2014). In other case, the gut symbiont *Bacteroides thetaiotataomicron* survives from the antimicrobial peptide (AMP) secreted by the mouse immune system (Cullen et al., 2015).

Apart from the host-associated stress, gut microbes produce bacteriocins (Kommineni et al., 2015), reactive oxygen species (Liu et al., 2012), antimicrobials (Schoenian et al., 2011), and bacteriophages (Barr et al., 2015) that can induce nutrient competition among them (Figure 1.1). The production of coproporphyrin III, a small molecule by *Propionibacterium* sp. stimulates EPS secretion by *Staphylococcus aureus*. This stimulation leads to the adherence of these two-bacterial species together, which form biofilm (Wollenberg et al., 2014). It has been shown that the ability of *E. mundtii* to produce bacteriocin might be one of the mechanisms to control the pathogenic bacteria (Parekh et al., 2016). This antibiotic-killing ability supports the successful dominance of *E. mundtii* in the gut of *S. littoralis*.

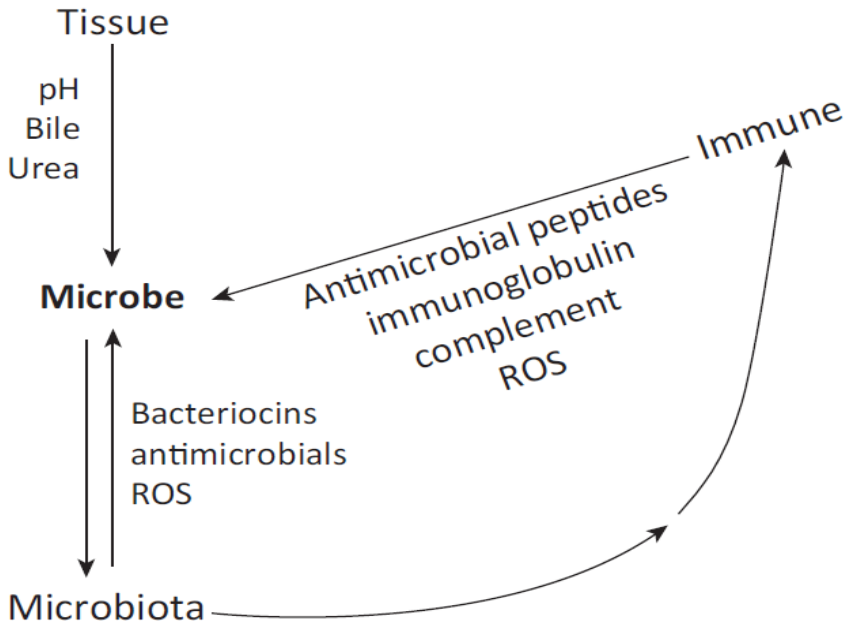


Figure 1.1. Flow diagram shows different chemical and physical stresses of the host tissues. Stresses may derive from host-microbe or through microbe-microbe interactions. Adapted from (Schwartzman and Ruby, 2016).

1.3 Contributions of the gut microbiome

The gut microorganisms play important roles in enhancing the lifestyles of the host insects in many aspects by providing necessary nutrients (Moran et al., 2008), aid in food digestion, and protection against harmful pathogen (Douglas, 2011, Shao et al., 2017). However, our knowledge on microbial functions in the gut of insects is still limited due to enormous diversity of insect species. The endosymbionts harbor genes encode for the synthesis of essential amino acids (Baumann, 2005), chemosynthesis (Dubilier et al., 2008) or photosynthesis (Venn et al., 2008) clearly provide many metabolic benefits to the host insects by enabling them to adapt and live in certain environments. For example, the gut bacteria of some insects produce

compounds in the form of pheromones and kairomones which involve in intraspecific and interspecific communication. The gut bacterium, *Pantoea agglomerans* and other similar bacteria are known to convert the dietary components to the aggregation pheromone in the desert locus, *Schistocerca gregaria* (Dillon et al., 2002). Another example involves the production of kairomones by a gut inhabitant of aphids, *Staphylococcus sciuri* that attract the aphid predator, the hover fly females, *Syrphidae* (Leroy et al., 2011). Some gut bacteria of termites, ants, cockroaches, and beetles can convert nitrogenous waste products of the host into essential nutrients (Hongoh et al., 2008, Russell et al., 2009, Sabree et al., 2009, Alonso-Pernas et al., 2017). It has been shown that the gut bacteria can breakdown toxins from the diet (Kikuchi et al., 2012). The gut microbiota in the hindgut of termites can degrade high cellulolytic fibres into non-complex oligosaccharides (Warnecke et al., 2007). The commensal gut bacteria of *D. melanogaster* are involved in the renewal of gut epithelial cell and growth enhancement (Buchon et al., 2009, Storelli et al., 2011).

1.4 Diversity of the gut microbiota in insects

Most insects, including mosquitoes, *Drosophila melanogaster*, *Galleria mellonella*, and *Spodoptera littoralis* all display complete metamorphosis from larval to adult stages, which leads to the disturbance of exoskeletal lining of the gut (Truman and Riddiford, 1999). It has been reported that insects from the order Lepidoptera and Diptera maintain their gut bacteria during metamorphosis (Wong et al., 2011, Hammer et al., 2014) and the same phenomenon also occurs in Coleoptera (Delalibera et al., 2007) and Hymenoptera (Brucker and Bordenstein, 2012) as well.

It is known that herbivorous insects harbor microbial communities of relatively few taxa (Jones et al., 2013) compared with the mammalian guts, which are colonized by bacteria of about 500-1000 taxa (Nemergut et al., 2011). For example, *Drosophila* species only possess about 30 operational taxonomic units (OTUs) on average (Chandler et al., 2011). In terms of the number of bacteria, the human microbiome possesses approximately 10^{14} bacteria compared with 10^{5} bacterial cells in *Drosophila* (Ley et al., 2006). The different feeding habits of insects dictate the diversity of its gut microbiome. The xylophagous insects harbor among the highest bacterial communities (103 OTUs per sample), followed by leaf-feeders (38 OTUs per sample), and sap-feeders (3-7 OTUs per sample) (Colman et al., 2012, Russell et al., 2013, Jing et al., 2014).

The gut microbiota of insects differs among taxa, mostly dominated by Proteobacteria, Firmicutes, and Protists, whereas the bacteria in most mammals are mainly Bacteroidetes and Firmicutes (Dillon and Dillon, 2004, Morales-Jimenez et al., 2009). Several factors that can shape the composition of the gut microbiota, including diet, sex, physiological conditions, developmental stage, and genotype (Sharon et al., 2010). It has been shown that the gut microbiome composition of laboratory-reared larvae of the lepidopteran *S. littoralis* and *Helicoverpa armigera* is influenced by diet, but displayed a rather stable microbial community, largely consists of Enterococci, Clostridia, and Lactobacilli (Figure 1.2) (Tang et al., 2012). A core microbiome of honeybees was found to be conserved regardless of regions worldwide (Hamdi et al., 2011). In contrast, a highly variable gut bacterial community was recorded from *H. armigera* obtained from different field locations and host plants (Priya et al., 2012). The dominant bacterial taxa of *Drosophila* also differ among laboratories and are influenced by diet. For example, a laboratory study showed that the bacterial communities of *D. melanogaster* are largely composed of *Enterococcus* (Firmicutes) and *Acetobacteraceae* or of *Enterobacter*

(*Gammaproteobacteria*), but less *Lactobacillus* (Cox and Gilmore, 2007). While another study showed that members of *Enterobacteriaceae* and *Acetobacteraceae* dominate, whereas low numbers of *Lactobacillus* or no *Enterococcus* could be detected (Chandler et al., 2011).

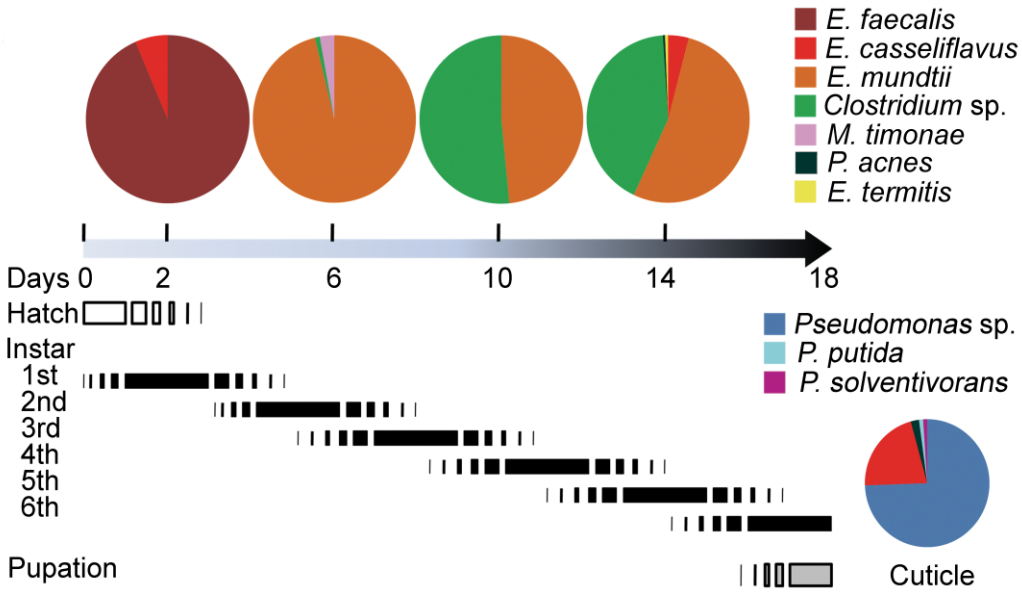


Figure 1.2. Different gut bacterial community compositions in *S. littoralis* larvae feeding on artificial diet. The chart shows the *Clostridium* sp. (green) and *E. mundtii* (orange) are the predominant bacteria towards the late instar larvae. Adapted from (Tang et al., 2012).

The intracellular gut symbionts of insects choose to live in specialized cells called bacteriocytes. Some of the insects that have these cells, include cockroaches, lice, hemipterans, some beetles, ants, and flies (Douglas, 2007).

1.5 Model organism: *Spodoptera littoralis*

The polyphagous cotton leaf worm, *S. littoralis* is one of the most important pests of vegetables and fruits including the cotton plant, tobacco, soybean, and corn. Each year, the agricultural industry suffers major economic losses due to the attack of the larvae of the insect on important crops. All my works reported in this PhD thesis are based on the model insect, *S. littoralis*.

The *S. littoralis* (Lepidoptera: Noctuidae) is a native to Africa and can be found worldwide especially in Middle East, Asia and the Mediterranean Europe. The life cycle of this insect is divided into several stages, including the eggs, six larval instars, pupae, and adults (Figure 1.3). In recent years, extensive works have focused on characterizing the gut microbial compositions of this polyphagous insect. Unfortunately, nothing much has been known about the co-evolution of the core bacterial symbiont with each of the development stage of the insect host. Therefore, in this thesis, I have reported the key findings with specific focus on *E. mundtii*, a bacterium that co-developed together with *S. littoralis* (Article I).

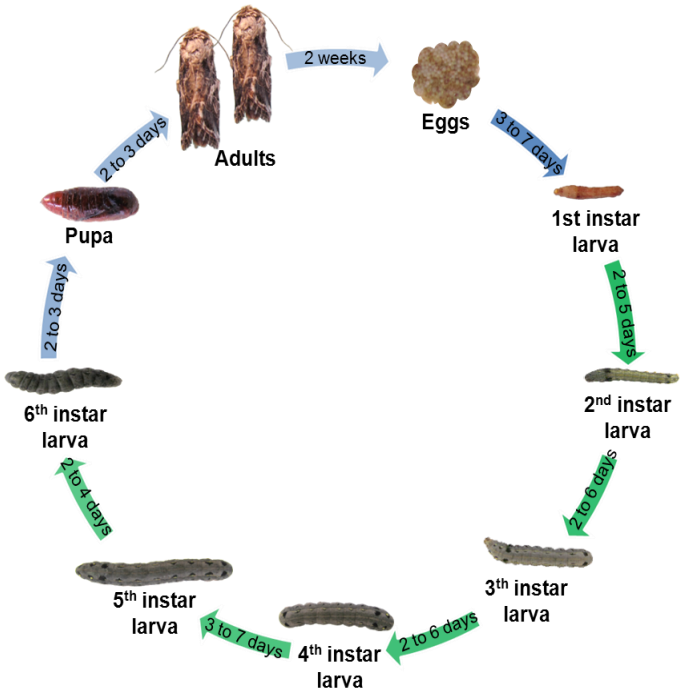


Figure 1.3. Life cycle of *S. littoralis*. A complete developmental cycle of this generalist insect requires between four and five weeks.

1.6 The physiology of lactic acid bacteria

Humans, insects, and animals harbor lactic acid bacteria (LAB) as part of their microbiomes. Most of the food fermentation depends on lactic acid fermentation performed by LAB or ethanol fermentation performed by the yeast *Saccharomyces cerevisiae* (Papadimitriou et al., 2015). Orla-Jansen defined LAB as Gram positive, non-sporulating, and non-motile cocci or bacilli that were capable to catabolize sugars into lactic acid. Among the core LAB genera including *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Streptococcus*, *Leuconostoc*, *Pediococcus*, *Weissella*, *Carnobacterium*, *Oenococcus*, and *Tetragenococcus*. All the LAB genera form the order *Lactobacillales* in the class *Bacilli* of the phylum *Firmicutes* (Makarova et al., 2006). In

recent years, stress physiology of LAB has been studied intensively due to its applications in the food industry.

LAB help to maintain the microbiomes by acidifying the environment which can be important in human health and diseases (Borges et al., 2014). In addition, LAB are known to produce antimicrobial substances in the form of hydrogen peroxide, organic acids, and antimicrobial peptides (De Vuyst and Vandamme, 1994). Several LAB are used as probiotic bacteria to improve human health (FAO/WHO, 2002). In contrast, LAB can turn into pathogens such as Group A streptococci (GAS), group B streptococci (GBS), and *Streptococcus pneumonia* which can cause severe infections, while *Enterococcus faecalis* and *Enterococcus faecium* are agents of nosocomial infections (Fischetti et al., 2006). While LAB are well studied in human, however, little is known about its association with invertebrates.

1.6.1 General characteristics of enterococci

Enterococci are common inhabitants of the gastrointestinal tracts of human and animals and in the guts of insects. They can also be found in the environments, including soil, water, and plants. The Enterococcaceae family consists of genus *Enterococcus*, *Catellicoccus*, *Melissococcus*, *Bavariicoccus*, *Pilibacter*, *Vagococcus*, and *Tetragenococcus* (Ludwig et al., 2009). Members of Enterococci belong to a low GC content of Gram positive bacteria. In the 1940's, Enterococci were placed in group D streptococci (Sherman, 1937). In 1984, the names of *Streptococcus faecalis* and *Streptococcus faecium* were changed to *E. faecalis* and *E. faecium*, respectively (Schleifer and Kilpper-Balz, 1984).

According to Schleifer and Kilpper-Bälz (1984), members of the genus *Enterococcus* are Gram positive, ovoid, and occurs mostly as single, pairs or short chains cells (Figure 1.4). The

cells may be motile and contain no endospores. They are facultative anaerobic chemo-organotrophs, capable of fermenting glucose into lactic acid, without production of gas (Klein, 2003). All species of Enterococci possess the lysine-D-asparagine of peptidoglycan, except in *E. faecalis*, which has a lysine-alanine$_{2-3}$ type. They grow at a temperature ranging from 10 to 45ºC and reach optimum growth temperature at 35ºC (Sherman, 1937).

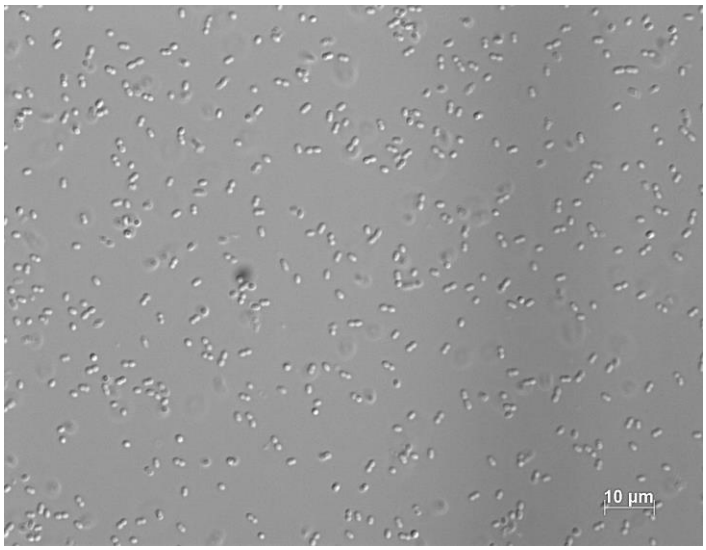


Figure 1.4. Morphology of *E. mundtii* showing cocci structure either in the form of chains or in pairs (diplococci).

More than 30 species of enterococci have been identified to date (Figure 1.5). Among them, *E. sanguinicola*, *E. pallens*, *E. gilvus*, *E. canintestini*, *E. faecalis*, and *E. faecium* have been known to cause human infection (Carvalho Mda et al., 2008, Tan et al., 2010, Tyrrell et al., 2002). Enterococci have been known to cause the spread of antibiotic resistance and virulence

genes in the environment through transfer of plasmids and transposons (Nallapareddy et al., 2005, Mundy et al., 2000).

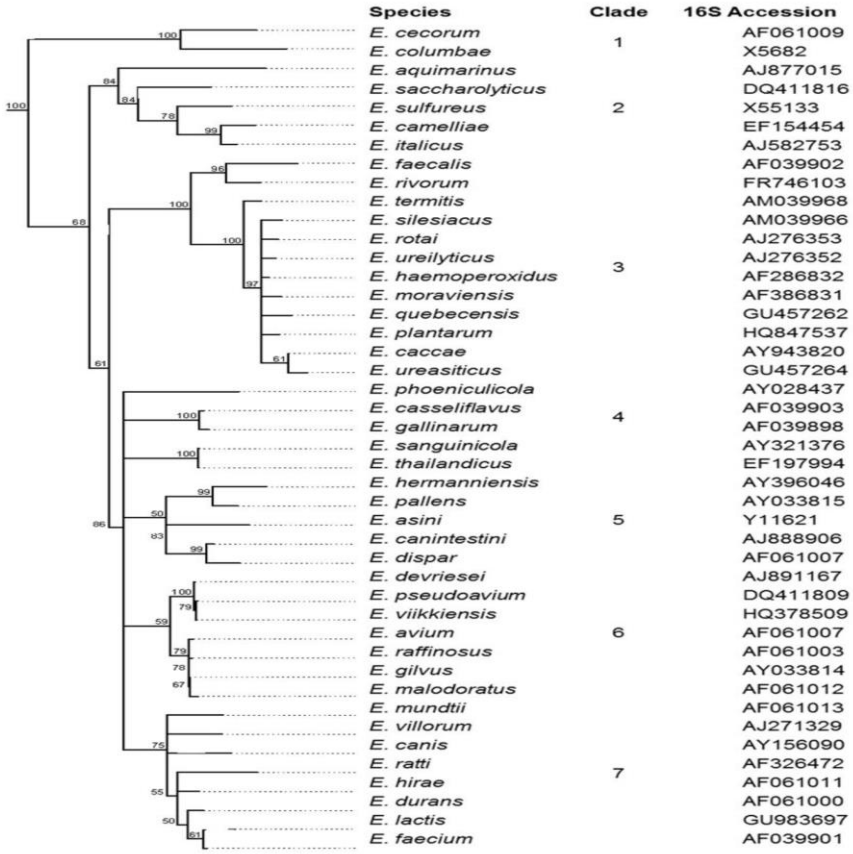


Figure 1.5. Dendrogram of the genus *Enterococcus*. The dendrogram was constructed based on the 16S rRNA gene sequences for members of the *Enterococcus* genus using the Geneious software (Biomatters Ltd) using the neighbor- joining algorithm. The 16S sequence of *Tetragenococcus solitarius* was used as outgroup. Adapted from (Gilmore et al., 2013).

1.7 Colonization of enterococci in insects

Insects for examples, beetles, termites, ants, flies and bees have been known to harbor enterococci as their gut microbiota. The most common bacteria, including *E. faecalis*, *E. faecium*, and *E. casseliflavus* were found in a wide range of insect orders, especially wild insects from 37 different taxa (Martin and Mundt, 1972). Enterococci are found in the Lepidopteran insects, such as the larva of gypsy moth (*Lymantria dispar*) and cotton bollworm (*H. armigera*) (Broderick et al., 2004, Priya et al., 2012). The GI tract of *Drosophila* has almost parallel anatomy to that of humans, such that both systems carry an alimentary canal that connects the esophagus to a ventriculus (stomach), extends to the intestine, reaches the rectum, and ends at the anus (Figure 1.6). The similar GI structure also can be found in the larva of *S. littoralis* (Figure 1.7). Enterococci live in a wide range of pH, a characteristic that allows their survival in the intestinal tract of insects, such as *Drosophila*, which consists of acidic crops, alkaline midguts, and acidic to neutral hindguts (Clark, 1999).

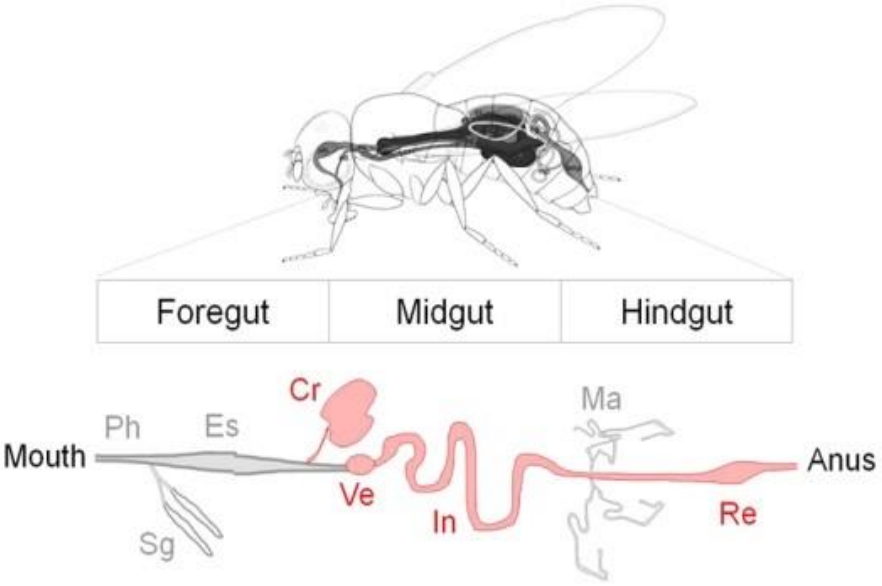


Figure 1.6. Schematic diagram of the enterococcal colonization of *Drosophila* gastrointestinal tract (GI). Red indicates the gut region colonized by *Enterococcus*. Ph, pharynx; Sg, salivary gland; Es, esophagus; Cr, crop; Ve, proventriculus; In, intestine; Mal, Malpighian tubule; Re, rectum. Adapted from (Hartenstein, 1993).

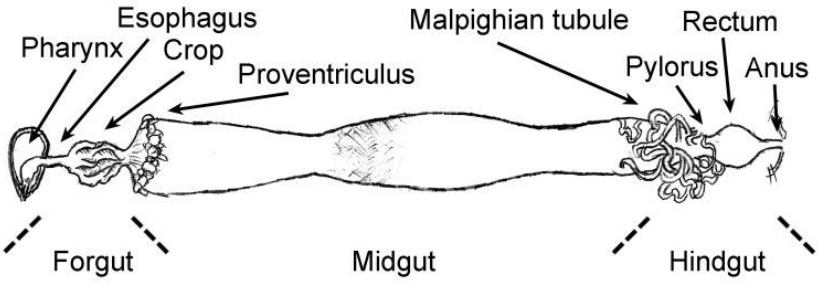


Figure 1.7. Schematic diagram of the alimentary canal of the larva of *S. littoralis*. The digestive tract is divided into foregut, midgut, and hindgut. Adapted from (Tang et al., 2012).

1.8 Applications of symbiotic bacteria in insect control

Due to the devastating effects on crops, robust and effective pest management is a prerequisite to control *S. littoralis*. In Turkey, chemical insecticides, including lufenuron, fenpropathrin, mephospholan, and cyfluthrin are used intensively in pest control. Humans, plants, and animals (predators of pests) are under threat from the negative side effects of these pesticides. The emergence of resistance populations of *S. littoralis* towards insecticides have been reported (Mosallanejad and Smagghe, 2009, El-Guindy et al., 1983). Therefore, other biological safe method involved microbial-based approach has been introduced in pest control. Some viruses, nematodes, fungi, and entomopathogenic bacteria (*Bacillus thuringiensis*) have been used as agent to control *S. littoralis*.

One of the ways to control insect pests is by manipulating its bacterial symbionts. It has been suggested to treat the insects with antibiotics to reduce its endosymbionts *in vitro*, however, this approach is not suitable to control pest in natural environments. Therefore, other bioactive compounds are necessary for pest management (Douglas, 2007). A new strategy through para-transgenesis has been used to genetically modify the symbionts to alter the insect fitness. This approach is successful to control the spread of parasite in insect vectors. For example, the parasitic protozoan *Trypanosoma cruzi* causing the Chagas disease is spread by the blood-sucking insect *Rhodnius prolixus*. Researchers modify its endosymbiont *Rhodococcus rhodnii* to express the antitrypanosomal gene in the insect gut, thus producing the insect incapable of transmitting the disease (Beard et al., 2002).

Wolbachia has been used to introduce transposable elements through germline transformation to disrupt parasite transmission in mosquitoes, for example, *Aedes aegypti* (Coates et al., 1998) and *Anopheles gambiae* (Grossman et al., 2001). We believe that a thorough

study of the core microbiome (*E. mundtii*) of *S. littoralis* will enhance our understanding into its ability in controlling the insect, and thus the bacterium could serve as a potential therapeutic target for pest management in the future.

1.9 Aims of this study

The gut microbiota of *S. littoralis* is well studied lately. Research has been carried out to investigate the diversity and composition of the microbial composition, which lead to the identification of the so-called 'core microbiome', which present constantly and abundantly in different parts of the intestinal tract of the host insect. Although the 16S rRNA gene marker identification have identified a plethora of gut bacteria in *S. littoralis*, yet how well and stable this core microbiome colonize the gut environment remains poorly understood, and requires further investigations to gain more insights into its survival mechanisms. It has been repeatedly shown in several papers that bacteria of the genus *Enterococcus* have co-evolved with several host insects, e.g. *S. littoralis*, *D. melanogaster*, and human (Tang et al., 2012, Liu et al., 2017, Franz et al., 1999). Many of the gut bacterial symbionts are commensal and exert benefits on the insects. It is therefore beneficial for us to further investigate this bacterial genus to better understand its mechanisms to control the gut microbial community as well as the insect host. Recent data show that *E. mundtii*, part of the core microbiome of *Spodoptera* can control other gut inhabitants with its antimicrobial toxic peptide. Together with several unresolved questions in mind, we would like to investigate the development, control, and adaptation of the gut microbiome to the insect host. To this end, this study has the following aims:

Specific aim 1: How does an indigenous bacterium colonize the gut?

Presently, it is unknown whether *E. mundtii* could survive in the stressful alkaline gut environment of *S. littoralis*. Where are the locations in the gut that the bacterium colonizes? The gut microbiome changes according to the insect's developmental stage. A GFP-tagged *E. mundtii* is an ideal reporting organism to monitor the developmentally changes in the microbiome of *S. littoralis*. In this study, GFP plasmids were transformed into the bacterial cells by electroporation. The transmission of fluorescent *E. mundtii* was monitored in the foregut, midgut, and hindgut, and bacterial CFU counts were performed. The fluorescent *E. mundtii* was integrated into the gut community and their presence can be monitored in all gut areas of the larvae and at developmental stages such as the pupa and the adult. Labeling bacteria with green fluorescent protein (GFP) is a powerful method to be used for live cell imaging, and beneficial for downstream process, for example sorting live cells with fluorescence-activated cell sorting (FACS). The concept of using a fluorescent reporter organism will provide insights into the adaptation strategies used by the microbes to survive harsh gut conditions. The patterns of the colonization of the fluorescent bacteria will be reported in the article I of this thesis.

Specific aim 2: Which gut microbes are metabolically active and how active are they?

Information on the gut microbiota and their role in *S. littoralis* is scarce. Therefore, the microbial composition at full extent across the entire life cycle of *S. littoralis* was profiled using 16S rRNA amplicon sequencing approach. Previous studies only focused on characterizing the microbial diversity at the larval stage without other developmental stages, such as pupa and adult. Most of the metagenomics works are based on characterizing the bacterial communities at the 16S rDNA level, which may a caveat as DNA may represent either live or dead cells. Considering this

limitation, the 16S rRNA was reverse-transcribed into cDNA to characterize the metabolically active microbial communities across life stages. The 16S rRNA sequences will tell us about the diversity and activities of the gut microbes, all together serve as a platform to understand the cross-talk between the insect and microbes. This work will be reported in the article II.

Specific aim 3: How does the indigenous gut bacterium survive the adverse gut conditions?

Several species of *E. mundtii* have been discovered to live in the intestinal tract of *S. littoralis*. One of the species was found to display the characteristic of suppressing the growth of other bacteria especially *E. faecalis*. An antimicrobial toxin is produced by the isolate, *E. mundtii* SL-16. To further explore its other metabolic capabilities, therefore the genome of this bacterium was sequenced to better decode its strategies in colonizing the host gut. The genome findings will be reported in the article III. The data from the genome will be supported with the *in vivo* genome wide gene expression analysis of *E. mundtii* in response to the gut environment. The transcriptome data will be reported in the unpublished result II.

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

Article I

Colonization of the Intestinal Tract of the Polyphagous Pest *Spodoptera littoralis* with the GFP-tagged Indigenous Gut Bacterium *Enterococcus mundtii*

Beng-Soon Teh, Johanna Apel, Yongqi Shao, Wilhelm Boland

Frontiers in Microbiology 7:928 (2016)

This manuscript describes about the ability of an indigenous gut bacterium *E. mundtii* in colonizing the extreme alkaline environment of the intestinal tract of the insect pest *S. littoralis*. Genetic engineering technique by labeling the bacterium with plasmid-carried GFP under the control of three different promoters with different strengths was employed in this study. Further methods, including flow cytometry and Western Blot have identified the *ermB* as the strongest promoter in regulating GFP expression, therefore was used to label the bacterium. We observed that the integrated fluorescent *E. mundtii* could thrive in different parts of the gut tissues (foregut, midgut, and hindgut) and different stages of development of the insect (larvae, pupae, adults, and eggs), indicating its pivotal role as commensal gut symbiont. This interesting result serves as a platform for us to further identify the unknown mechanisms of successful bacterial gut colonization through transcriptomics approach.

Contributions: Conceived and designed the experiments: BT (80%), YS (10%) and WB (10%). Performed the experiments: BT (95%) and JA (5%). Analyzed the data: BT (100%). Wrote the paper: BT (90%) and WB (10%).

Article II

Biodiversity and Activity of the Gut Microbiota across the Life History of the Insect Herbivore *Spodoptera littoralis*

Bosheng Chen, Beng-Soon Teh, Chao Sun, Sirui Hu, Xingmeng Lu,

Wilhelm Boland, Yongqi Shao

Scientific reports 6:29505 (2016)

This manuscript involves the study on profiling the 16S rRNA genes of the gut microbial communities across all developmental stages of *S. littoralis*. Although the bacterial communities in the larval stages have been well characterized years ago, yet microbial studies of other stages, particularly during pupation remain uncharacterized. Therefore, using the 16S rRNA derived from DNA and RNA through pyrosequencing, we have identified Proteobacteria and Firmicutes as the dominant phylum across the life cycle of the insect. Strikingly, enterococci were dominantly persisting through the pupal stage. Using PICRUSt software, we could predict the metabolic functions of microbes from phylogeny. Through the analysis, we found that gut microbes during the early-instar larval tend to be metabolically active in using carbohydrate as carbon source. In contrast, during the late-instar larval, gut bacteria change their behavior by investing in vitamin and amino acid metabolism. Genes involved in energy and nucleotide metabolism were abundant in pupae. The gut bacteria in female adult need more energy, while microbial genes associated with replication and repair pathway were enriched in male adult.

Contributions: YS and WB contributed to the initial design of the research. BC (40%) and BT (10%) conducted the experimental and CS (30%) performed bioinformatics analyses with guidance from YS, SH (10%) and XL (10%) collected field samples. All authors contributed to the subsequent stages of manuscript preparation.

Article III

Draft Genome Sequence of *Enterococcus mundtii* SL 16, an Indigenous Gut Bacterium of the Polyphagous Pest *Spodoptera littoralis*

Bosheng Chen, Chao Sun, Xili Liang, Xingmeng Lu, Qikang Gao, Pol Alonso-Pernas,

Beng-Soon Teh, Alexey L. Novoselov, Wilhelm Boland, Yongqi Shao

Frontiers in Microbiology 7:1676 (2016)

This manuscript describes the isolation, characterization, assembly, and sequencing of the genome of the gut bacterium *Enterococcus mundtii* SL 16. The availability of *Enterococcus* genome is important to gain insights into the mechanisms of its colonization in the gut of host insect. The size of the bacterial genome was approximately 3.3 Mbp with 2939 protein-coding genes (CDS), and 59 tRNA. Further analyses of the genome have identified genes involved in carbohydrate transport and metabolism. Some carbohydrate hydrolysis enzymes, for examples glycosyl hydrolases could be identified in the genome. The ability of *E. mundtii* to produce carbohydrate-active enzymes, implying the strategy to digest complex carbon sources (cellobiose, xylose, and sucrose) in the gut. Genes involved in the production of L-lactate, formate, and acetate as fermentation products were also found.

Contributions: Designed experiments: YS (40%), WB (20%), BC (20%), and CS (20%); Isolation of bacterium: XLi (50%) and BT (50%); Bioinformatics analyses: AN (50%) and PA (50%); DNA sequencing: QG (50%) and XLu (50%).

3. Article I

frontiers in Microbiology

ORIGINAL RESEARCH
published: 14 June 2016
doi: 10.3389/fmicb.2016.00928

Colonization of the Intestinal Tract of the Polyphagous Pest *Spodoptera littoralis* with the GFP-Tagged Indigenous Gut Bacterium *Enterococcus mundtii*

Beng-Soon Teh [1], Johanna Apel [2], Yongqi Shao [3] and Wilhelm Boland [1]*

[1] Department of Bioorganic Chemistry, Max Planck Institute for Chemical Ecology, Jena, Germany, [2] Clinic for Internal Medicine II, Department of Haematology and Medical Oncology, University Hospital Jena, Germany, [3] Laboratory of Invertebrate Pathology, College of Animal Sciences, Zhejiang University, Hangzhou, China

The alkaline gut of Lepidopterans plays a crucial role in shaping communities of bacteria. *Enterococcus mundtii* has emerged as one of the predominant gut microorganisms in the gastrointestinal tract of the major agricultural pest, *Spodoptera littoralis*. Therefore, it was selected as a model bacterium to study its adaptation to harsh alkaline gut conditions in its host insect throughout different stages of development (larvae, pupae, adults, and eggs). To date, the mechanism of bacterial survival in insects' intestinal tract has been unknown. Therefore, we have engineered a GFP-tagged species of bacteria, *E. mundtii,* to track how it colonizes the intestine of *S. littoralis*. Three promoters of different strengths were used to control the expression of GFP in *E. mundtii*. The promoter *ermB* was the most effective, exhibiting the highest GFP fluorescence intensity, and hence was chosen as our main construct. Our data show that the engineered fluorescent bacteria survived and proliferated in the intestinal tract of the insect at all life stages for up to the second generation following ingestion.

Keywords: ***Spodoptera littoralis*, green fluorescent protein, promoter, lactic acid bacteria, *Enterococcus mundtii*, intestinal tract**

OPEN ACCESS

Edited by:
Martin Grube,
Karl-Franzens-University Graz, Austria

Reviewed by:
Seungha Kang,
CSIRO, Australia
Morten Schiøtt,
University of Copenhagen, Denmark

***Correspondence:**
Wilhelm Boland
boland@ice.mpg.de

Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: *05 March 2016*
Accepted: *31 May 2016*
Published: *14 June 2016*

Citation:
Teh B-S, Apel J, Shao Y and Boland W (2016) Colonization of the Intestinal Tract of the Polyphagous Pest Spodoptera littoralis with the GFP-Tagged Indigenous Gut Bacterium Enterococcus mundtii. Front. Microbiol. 7:928. doi: 10.3389/fmicb.2016.00928

INTRODUCTION

Insects' guts harbor a wide range of microbial communities. Intestinal gut microbes contribute significantly to the development of their insect hosts by providing essential nutrients, aiding in food digestion, and protecting against other harmful pathogens. However, the functions of these microbes in the insect gut are still largely unknown due to the complexity and diversity of the microbes. In recent years, the agricultural pest, *Spodoptera littoralis* (Lepidoptera, Noctuidae) has been used as an experimental model insect to study gut microbiomes. The microbial composition in the gut of *S. littoralis* has been well characterized (Tang et al., 2012), yet the factors controlling its colonization are unknown.

Insect guts contain multiple compartments with different physicochemical conditions such as pH and oxygen availability which enrich for certain species of bacteria. The gut of certain lepidopteran, coleopteran, and dipteran is highly alkaline due to specific dietary preferences (Brune and Kühl, 1996; Harrison, 2001). The lepidopteran insects which feed on tannin-rich leaves have

alkaline midguts with pH as high as 11–12 (Appel and Martin, 1990; Harrison, 2001). A clear pH gradient occurs along the lepidopteran midgut from highly alkaline (pH ~ 10) anterior end to almost neutral posterior ends (Funke et al., 2008). The microbial community of *S. littoralis* (cotton leafworm) is dominated by *Pantoea* and *Citrobacter* from the phylum Proteobacteria in early-instar larvae (Shao et al., 2014). Bacteria in this phylum have the ability to degrade polysaccharide in insects (Anand et al., 2010; Adams et al., 2011; Engel et al., 2012). As insects aged toward late-instar, more than 97% of the total bacterial community shifted to Firmicutes, dominated mostly by *Enterococcus* and *Clostridium* sp. (Tang et al., 2012; Shao et al., 2014). The proliferation of *Clostridia* is linked to its role in cellulose digestion and fermentation of sugars (Watanabe and Tokuda, 2010). Interestingly, the alkaline midgut of gypsy moth larva also harbors *Enterococcus* (Broderick et al., 2004) while the Firmicutes dominates the midgut of the beetle *Pachnoda ephippiata* (Egert et al., 2003). Some insects harbor similar bacterial lineages in their alkaline guts.

To date, the genus *Enterococcus* is known to include more than 33 species (Kohler, 2007). The members of this genus are typically found in the intestinal tracts of humans and animals, in dairy products, and also in the environment: for example, in plant material, soil, and surface water (Giraffa, 2003; Ogier and Serror, 2008). *E. mundtii* is part of this genus. It is a non-motile, Gram-positive, facultative anaerobic organism that belongs to the group of lactic acid bacteria (LAB). It forms either cocci or rods, and is capable of producing lactic acid as a by-product of the fermentation of carbohydrates. The biological role of *E. mundtii* is still poorly understood, as most studies have focused on the model bacteria *Enterococcus faecalis* and *Enterococcus faecium,* which often cause human systemic infection (Arias and Murray, 2012).

Green fluorescent protein (GFP) originally isolated from *Aequorea victoria* has been extensively used as a reporter for gene expression in bacterial and mammalian cells (Yang et al., 1996; Valdivia and Falkow, 1997; Hazelrigg et al., 1998; Rolls et al., 1999). GFP is advantageous as it requires neither cofactors nor a substrate to be expressed in its host cells. Different variants of GFP, such as EGFP (enhanced green fluorescent protein) have been developed to improve fluorescent intensity (Cormack et al., 1996). The expression of GFP in several LAB has been successfully demonstrated (Scott et al., 2000; Hansen et al., 2001; Lun and Willson, 2004). In recent years GFP has been mostly used to investigate Gram-negative bacteria, and, less often, Gram-positive bacteria (Bubert et al., 1999; Freitag and Jacobs, 1999; Lewis and Marston, 1999; Fernandez de Palencia et al., 2000). Increasingly, GFP has been used to track how and where target bacterial species colonize the guts of several host insects (Thimm et al., 1998; Mumcuoglu et al., 2001; Husseneder and Grace, 2005; Kounatidis et al., 2009; McGaughey and Nayduch, 2009; Doud and Zurek, 2012).

In this work, we determine the fate of GFP-tagged *E. mundtii* within the digestive tract of *S. littoralis* when administered *in vivo*. In addition, we track the transmission route of the bacteria through all stages of the life cycle of *S. littoralis*. In fact, the incorporation of GFP-tagged *E. mundtii* provides a non-invasive monitoring of its survival in the insect gut, but still far from addressing the relationship between the insect and the bacterial symbiont. We are interested to further explore the underlying factors that drive this complex relationship by analyzing the bacterial and insect gut epithelial transcriptomes in future work. The transcriptome data will significantly expand our understanding of the functional roles of indigenous bacteria toward the development of the insect and other microbes. This can easily be done by identifying the insect- or microbe-derived compounds from the metabolic pathways resulted from the transcriptome data.

MATERIALS AND METHODS

Maintenance of Egg and Larvae

The eggs of *S. littoralis* were purchased from Syngenta Crop Protection Münchwilen AG (Münchwilen, Switzerland). Eggs were hatched at 14°C. Larvae were maintained at room temperature (24°C). Larvae were provided with sterile artificial diet made of white bean and essential nutrients without antibiotics and prepared based on Spiteller et al. (2000).

Bacterial Strains and Growth Conditions

Table 1 lists the bacterial strains and plasmids used in this study. *Escherichia coli* strain DH5α was used to maintain all GFP-containing plasmids. The plasmid pTRKH3-*erm*GFP (Addgene plasmid # 27169), pTRKH3-*slp*GFP (Addgene plasmid # 27168), and pTRKH3-*ldh*GFP (Addgene plasmid # 27167) were gifts from Michela Lizier. *E. mundtii* strain KD251 (isolated from the gut of *S. littoralis* at the Department of Bioorganic Chemistry, Max Planck Institute for Chemical Ecology) was used as the recipient of all plasmids (Shao unpublished). *E. coli* DH5α and *E. mundtii* were grown at 37°C with agitation (220 rpm) in Luria-Bertani (LB) and Todd-Hewitt Bouillon, THB (Roth, Karlsruhe, Germany) medium for both broth and agar, respectively. Antibiotics were used at the following concentrations: erythromycin, 50 μg ml^{-1} (for *E. coli*) or 5 μg ml^{-1} (for *E. mundtii*). All plasmids were extracted from *E. coli* using the GeneJet plasmid miniprep kit (Thermo Scientific, Vilnius, Lithuania). All strains were kept in glycerol stocks at −80°C for preservation and long-term storage.

Plasmids

All GFP expression vectors were derived from pTRKH3, a backbone shuttle vector for *E. coli* and various species of LAB, including *Streptococcus*, *Lactococcus*, *Enterococcus,* and *Lactobacillus* (O'Sullivan and Klaenhammer, 1993). The vector carries a gene for erythromycin resistance which is highly suitable for expression in *Enterococcus*. In addition, the vector possesses a modified GFP 5 (mGFP5) that is controlled by three constitutive promoters of different strengths. pTRKH3-*erm*GFP harbors EGFP that is controlled by a strong enterococcal erythromycin ribosomal methylase (ermB) promoter (Swinfield et al., 1990). The *Lactobacillus acidophilus* lactate dehydrogenase (*ldhL*) promoter (Kim et al., 1991) and surface layer protein (*slp*) promoter (Boot and Pouwels, 1996) constitutively control pTRKH3-*ldh*GFP and pTRKH3-*slp*GFP, respectively.

TABLE 1 | Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant properties	Reference or source
STRAINS		
E. mundtii KD251	Transformation host, isolated from the intestine of *S. littoralis*	Laboratory collection
E. coli DH5α	Transformed bacteria in stab cultures	Addgene
PLASMIDS		
pTRKH3	7.8 kB, *E. coli*-LAB shuttle vector, Em^r, Tet^r, pAMß1 origin, p15A origin	O'Sullivan and Klaenhammer, 1993
pTRKH3-*erm*GFP	Em^r, pAMß1 origin, p15A origin, pTRKH3 derivative containing *egfp* gene downstream of *ermB* promoter	Addgene plasmid 27169
pTRKH3-*ldh*GFP	Em^r, pAMß1 origin, p15A origin, pTRKH3 derivative containing *egfp* gene downstream of *ldhL* promoter	Addgene plasmid 27167
pTRKH3-*slp*GFP	Em^r, pAMß1 origin, p15A origin, pTRKH3 derivative containing *egfp* gene downstream of *slp* promoter	Addgene plasmid 27168

Em^r, erythromycin resistant; Tet^r, tetracycline resistant.

Electroporation of *Enterococcus mundtii*

Electroporation was carried out based on the modified protocol of *E. coli* (Dower et al., 1988). A single colony of *E. mundtii* was grown at 37°C in THB broth on a rotary shaker (Certomat BS-1 Sartorius, Goettingen, Germany) with agitation (220 rpm). An overnight culture was diluted 1:1000 in 100 ml of THB medium before being harvested by centrifugation at 4000 × g for 10 min (Sigma 3K18, Sigma, Germany) at 4°C when growth reached the exponential phase ($A_{600\ nm}$ approximately 2.2). The cells were washed with 100 ml of ice-cold distilled water, centrifuged as above and washed again with 50 ml of ice-cold water before being centrifuged again. The cells were then washed with 20 ml of 10% glycerol, centrifuged and finally suspended in 2 ml of 10% glycerol. The suspension was divided into 50 µl aliquots and stored at -80°C. Prior to electroporation, the frozen cells were thawed on ice and mixed with plasmid for 15 min before being transferred into a chilled 0.2 cm gap cuvette. Electroporation was performed by a single pulse at 1.8 kV (E = 9 kV/cm), 600 K and 10 µF, with a pulse length of 3.6 ms in an electroporator 2510 (Eppendorf, Hamburg, Germany). The concentration of purified plasmids used during electroporation was between 0.15 and 0.2 µg. The pulsed cells were immediately suspended with 950 µl of THB broth and further incubated for 2 h at 37°C with agitation (220 rpm) before 100 µl was plated on THB agar containing 5 µg ml^{-1} of erythromycin. The plates were incubated at 37°C for 48 h. Bacterial transformants containing target plasmids were verified by PCR screening.

Verification of Bacterial Identity by 16S rRNA Sequencing

All bacterial transformants were checked for identity by PCR to prevent contamination. Total DNA was extracted from GFP-tagged bacteria of three different constructs from overnight culture by using a MasterPure Complete DNA and RNA purification kit (Epicentre, Madison, WI, USA) according to the manufacturer's protocol. The bacterial 16S rRNA genes were amplified using universal primers, 27f (5'- AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'- GGTTACCTTGTTACGACTT-3'). PCR was performed in a final volume of 50 µl using 10 µM of each primer, 10 mM concentration of deoxynucleoside triphosphates, 50 mM $MgCl_2$, 1 U of *Taq* polymerase and buffer (Invitrogen, Carlsbad, CA, USA). Denaturation was performed at 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, annealing at 54°C for 30 s, and 72°C at 1 min 30 s. The final extension was at 72°C for 7 min. PCR products were purified using the PureLink Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen, Carlsbad, CA, USA). The purified PCR products were sent for Sanger sequencing. DNA sequences were assembled with DNA baser sequence assembly software (http://www.dnabaser.com). The assembled sequences were used for blast searches at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Feeding of *S. littoralis* Larvae with GFP Bacteria

A total of 50 first-instar larvae were fed artificial diet supplemented with antibiotics for 3 days in the following final concentration: ampicillin (5.75 µg ml^{-1}) and erythromycin (9.6 µg ml^{-1}). Each larva was fed small cubes (1 g) of artificial diet inoculated with *E. mundtii*-harboring pTRKH3-*erm*GFP for 1 day starting at day 6, followed by food without bacteria starting at day 7 until pupation. Control larvae were fed food without bacteria continuously. A single colony of bacteria was grown overnight in THB broth containing erythromycin (5 µg ml^{-1}) and diluted 1:10 in the same broth before being fed. A total of 100 µl from the 1:10 dilution broth ($A_{600\ nm}$ approximately 0.65) containing ~4.7×10^7 CFUs of GFP bacteria was applied to the food of the larvae. Every day, feces were removed to avoid re-inoculating the GFP bacteria.

Quantification of Bacteria from the Intestinal Tract

Larvae (n = 6 for each stage), adults or pupae (n = 3 for each stage), and a control (n = 1 for each stage) were killed by freezing at −20°C for 15 min. Each individual was surface sterilized in 70% ethanol and immediately rinsed in sterile distilled water. Guts were dissected in sterile 1 × PBS (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4.7H_20$, and 2 mM KH_2PO_4 [pH 7.4]), with sterile forceps under a stereomicroscope (Stemi 2000-C, Zeiss, Jena, Germany). Larval guts were excised into three sections: foregut, midgut, and hindgut. Gut tissues were aseptically homogenized in 100 µl of PBS. A serial dilution of 10-fold was performed by transferring 100 µl of the homogenized sample into 900 µl sterile PBS, vortexing vigorously, and spread-plating 100 µl of each dilution onto THB agar supplemented with

erythromycin (5 μg ml^{-1}). All plates were incubated at 37°C for 48 h. Total bacterial cells were counted as colony forming units (CFUs) for each intestinal tract region. Erythromycin resistance was used as selection marker for picking bacterial colonies. In addition, to verify the presence of plasmid-containing GFP, PCR screening was performed.

Tissue Cross-Sectioning

The fresh gut tissues were cut into sections (foregut, midgut, and hindgut) and frozen at −24°C in mounting medium for cryotomy (OCT compound, VWR, Leuven, Belgium) for 30 min. They were then cut with cryomicrotome (Microm Cryo-Star HM560 Cryostat, Walldorf, Germany) into 14–100 μm sections.

Fluorescence Microscopy

The cultures containing GFP-producing bacteria were harvested, and the pellets were suspended in 1 × PBS. Bacterial suspensions of 20 μl or slices of cross-sectioned tissue were mounted on microscope slides (Superfrost Plus, Thermo Scientific). Live cells were observed under an Axio Imager Z1 fluorescent microscope equipped with an AxioCam MRm camera (Zeiss, Jena, Germany). The GFP signal was detected using the filter set 10 (Cy2/GFP). All images were captured with a 63X magnification oil objective with an aperture of 1.4. The images were analyzed using the Axio Vision Rel 4.8 software (Zeiss, Jena, Germany). ImageJ, Fiji (Schindelin et al., 2012), an open-source software, was used to process all fluorescent images.

DNA Extraction and PCR Amplification of *gfp* Gene

Total DNA was extracted from larvae and pupae at all instars, and from adults, by using a DNA kit as mentioned above. The 735 bp of *gfp* gene was amplified using a set of primers consisting of GFP3fw (5'-TCGGAATTCATGAGTAAAGGAGAAGAA-3') and GFP3rev (5'- TCAGGATCCTTATTTGTATAGTTCATCC-3') (Lizier et al., 2010). An EcoRI and a BamHI site (underlined) were introduced for forward and reverse primers, respectively. The PCRs were performed in a final volume of 20 μl using 10 μM of each primer, 10 mM concentration of deoxynucleoside triphosphates, 50 mM $MgCl_2$, 1 U of *Taq* polymerase and buffer (Invitrogen, CA, USA). The following PCR conditions were used: 3 min at 94°C, followed by 35 cycles of 45 s at 94°C, 30 s at 60°C, and 2 min at 72°C, and final extension of 10 min at 72°C.

Western Blot

Bacterial cells were harvested from exponentially growing cultures. The cells were suspended in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) containing 20% sucrose, lysozyme (1 mg ml^{-1}), RNase (1 μg ml^{-1}), and DNase (1 μg ml^{-1}) and further disrupted by repeating a freeze-thaw cycle. The protein extracts were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 4–12% gel (Laemmli, 1970). The proteins were transferred onto a PVDF transfer membrane, pore size 0.45 μm (Thermo Scientific, Schwerte) with an electroblotter (Trans-blot Turbo Transfer System, BIO-RAD, Munich, Germany). The blots were blocked with 5% non-fat skimmed milk (NFDM) in TBS-T (Tris-buffer saline with Tween-20) for 1 h at room temperature. The membrane was then incubated for 16 h at 4°C with the mouse primary antibody anti-GFP (Roche Applied Science, Rotkreuz, Switzerland) diluted 1:2000 in blocking buffer. After three washes in blocking buffer, the membrane was incubated for 1 h at room temperature with Anti-mouse lgG, HRP-linked Antibody (Cell Signaling Technology, Cambridge, UK) diluted 1:5000 in blocking buffer. The membrane was washed three times in blocking buffer followed by incubation with the chemiluminescent reagent for 1 min. In the dark room, the membrane was transferred onto a foil, and X-ray film (CL-XPosure Film, Thermo Scientific, Schwerte, Germany) was placed on top of it. The film was developed after different exposure times (3 s–10 min).

Flow Cytometry

Bacteria from overnight cultures were re-suspended and diluted 1:10,000 in 1 × PBS. Fluorescence was determined in a CyFlow Space (Sysmex Partec, Görlitz, Germany). The data were analyzed using the CyFlow Space Operating Software FloMax. A blue laser (488 nm) was used for GFP fluorescence detection.

Statistical Analysis

Bacterial plate counts between 30 and 300 colonies were included in the calculation. Samples with colonies above 300 may not be distinguishable from one another on a plate count, whereas those below 30 may not be representative of the sample (Madigan et al., 2009). The total number of fluorescent *E. mundtii* recovered from each intestinal tract (foregut, midgut, and hindgut) across different larval stages was analyzed using JMP ^{R} 12.1.0[1]. Counts were analyzed using a one-way ANOVA test ($P < 0.05$). To further understand the different survival rates of GFP-*E. mundtii* at different larval stages, we compared the means of the combined three gut parts (foregut, midgut, and hindgut) as well as the means of individual gut regions of each larva using the Tukey–Kramer test ($P < 0.05$).

RESULTS

Comparison of Different GFP Constructs

Three different promoters, *ermB*, *ldhL,* and *slp* were used to control the expression of GFP, using pTRKH3 as a backbone shuttle vector. The strength of these GFP constructs was tested in *E. mundtii* by electroporation. This method was able to yield transformed colonies for all constructs. The recombinant bacterial colonies were picked and grown in THB at 37°C overnight before GFP fluorescence was visualized by epifluorescence microscopy. The highest fluorescence intensity was detected for *E. mundtii* transformed with pTRKH3-*erm*GFP (**Figure 1A**), followed by pTRKH3-*ldh*GFP (**Figure 1B**) and no signal for pTRKH3-slpGFP (**Figure 1C**) as well as wild-type *E. mundtii* (**Figure 1D**). The bacterial cultures were all grown simultaneously for 24 h, equivalent to stationary phase. The GFP content represents the same amount of cells which was measured as $OD_{600\ nm}$.

We analyzed the total expressed GFP in *E. coli* DH5α and *E. mundtii*. Western blot results showed that the GFP gene

[1]JMP ^{R} Version 12.1.0. SAS Institute Inc., Cary, NC, USA, 2015.

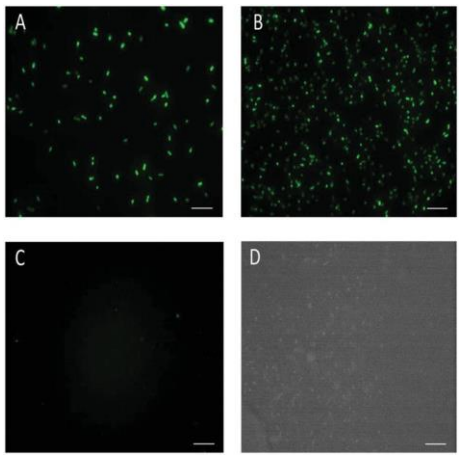


FIGURE 1 | Fluorescence micrographs of different constructs of GFP-expressing *E. mundtii* under the control of three constitutive promoters. (A) Strain of *E. mundtii*/pTRKH3-*erm*GFP, **(B)** Strain of *E. mundtii*/pTRKH3-*ldh*GFP, **(C)** Strain of *E. mundtii*/pTRKH3-*slp*GFP, **(D)** *E. mundtii* wild type (control) grown in THB for 24 h. All recombinant bacterial strains were grown in THB with erythromycin for 24 h. Scale bars: 10 μm. Magnification, 630X.

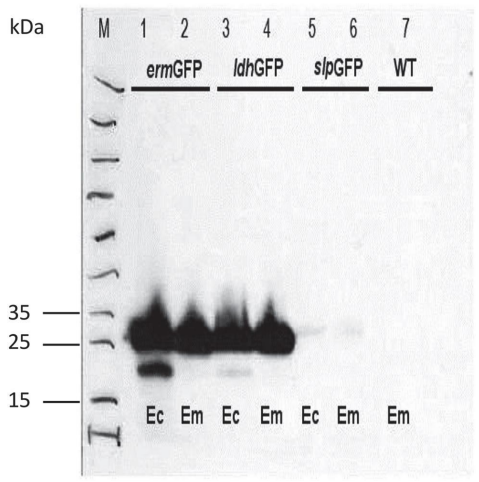


FIGURE 2 | Comparison of the level of recombinant GFP protein expression in *E. coli* DH5α and *E. mundtii* strains by western blot. Bacterial cell lysates from exponentially grown cultures were run through western blot. As immunoblotting gel shows, a significant amount of GFP protein is expressed by *E. coli* and *E. mundtii* strains harboring pTRKH3-*erm*GFP and pTRKH3-*ldh*GFP plasmids, whereas less protein expression can be detected for strains with pTRKH3-*slp*GFP, and no expression is shown for the wild-type strain. Ec, *E. coli*; Em, *E. mundtii*; WT, Wild type, and M, Page Ruler Prestained Protein Ladder. The molecular mass of GFP protein is approximately 27 kDa.

was expressed in large quantities in *E. mundtii* and *E. coli* DH5α cells when the cells were transformed with pTRKH3-*erm*GFP. A thick band associated with the production of large amounts of GFP protein was observed in the immuno-blotting gel for both bacterial cells transformed with pTRKH3-*ldh*GFP-expressing plasmid. Low quantities of protein were produced by the *slp* promoter controlling the GFP expression in both bacteria. The wild-type bacteria did not express GFP protein as expected (**Figure 2**).

Flow cytometry analysis confirmed the results obtained by epifluorescence microscopy and western blot. As expected, overnight cultures of *E. mundtti* cells with pTRKH3-*erm*GFP were highly fluorescent (38.5%), whereas cultures of pTRKH3-*ldh*GFP (21.7%) were slightly fluorescent and those of pTRKH3-*slp*GFP (0.65%) showed almost no fluorescence (**Figures 3A–C**). Due to the efficiency of pTRKH3-*erm*GFP, this construct was chosen to transform *E. mundtii* and used for feeding experiments.

Colonization of the Intestinal Tract of *S. littoralis* with Genetically Tagged Bacteria

GFP-tagged *E. mundtii* were fed to larvae of *S. littoralis* to visually monitor the persistence and fate of the bacteria within the digestive tract of different stages in the life cycle. We observed that fluorescent bacteria multiplied in the foregut, midgut, and hindgut regions after early ingestion of fluorescent bacteria. A high concentration of green fluorescent bacterial cells could be visualized in the foregut and midgut, but decreasing amounts toward the hindgut region of the third-instar larvae (data not shown). During this early stage of ingestion, the density of bacteria was high in most parts of the gut tissues. GFP bacteria were seen scattered in the foregut of the fourth-instar larvae, around the peritrophic membrane as well as entering the epithelium, adjacent to the hemocoel and fat body (**Figure 4A**).

In the early stages of ingestion the fluorescent bacteria could be seen accumulating in the midgut of fifth-instar larvae (**Figure 4B**) where they clumped together in the region of the peritrophic membrane. GFP-tagged bacteria were seen starting to double in larvae from fourth to fifth instars (data not shown). In sixth-instar larvae, strikingly, bacteria were trapped in the nodules of granular hemocytes, suggesting the occurrence of phagocytosis (**Figures 4C,D**). The number of bacteria was reduced significantly in sixth-instar larvae in most parts of the gut. A sharp reduction in the density of fluorescent enterococci occurred during pupation (**Figure 4E**). Bacteria went from clusters to free-standing groups by attaching to the fat body of pupae; at this stage there was no inner gut in the pupae to keep them from moving around.

Viable fluorescent cells of *E. mundtii* were detected in the tracheole of the adult insect (**Figure 4F**). This shows bacteria were successfully transmitted from pupal to new adult gut tissue, although there were few or no other bacteria found in the tracheole. We also tested the transmission route of recombinant

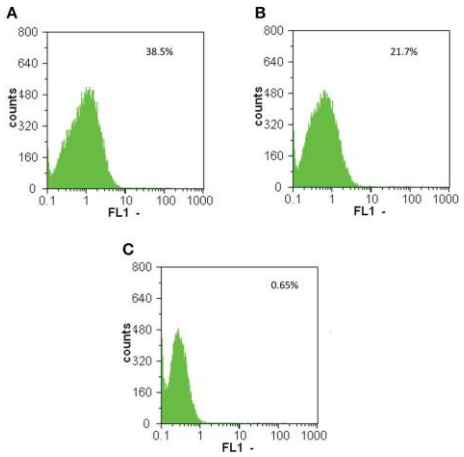


FIGURE 3 | Flow cytometry histograms of the GFP fluorescent intensities produced by overnight cultures of fluorescent *E. mundtii* cells harboring different promoter constructs with (A) pTRKH3/*erm*GFP, (B) pTRKH3/*Idh*GFP, and (C) pTRKH3/*slp*GFP. The intensities decrease from left to right, with the highest for contructs with *erm* promoter (38.5%), *Idh* (21.7%), and *slp* (0.65%) as the least efficient promoter. y-axis represents the number of bacterial counts and x-axis represents the fluorescence intensity.

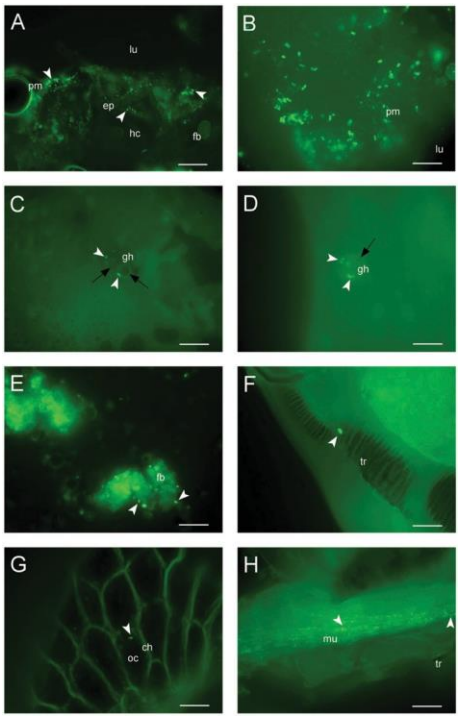


FIGURE 4 | Colonization of GFP-expressing *E. mundtii* in the *Spodoptera* intestinal tract. (A) Fluorescent image of the foregut region of fourth-instar larvae: bacteria are immobilized around the peritrophic membrane and gut epithelium (arrowheads) located adjacent to the hemocoel and fat body, scale bar = 20 μm. **(B)** In fifth-instar larvae, large clumps of bacteria are attached to the peritrophic membrane of the midgut tissue, scale bar = 10 μm. **(C,D)** Histological sections show fluorescent bacteria (arrowheads) are trapped within granular hemocytes containing nodules (black arrows) in the hindgut and midgut, leading to phagocytosis at the end of larval life, the sixth instar, scale bars = 10 μm. **(E)** A few bacteria are attached (arrowheads) to the fat body of pupae showing bacterial lysis occurs. **(F)** A single viable bacterial cell is immobilized in the tracheole of the adult, scale bar = 5 μm. **(G)** The fluorescent *E. mundtii* (arrowhead) is detectable in the oocyte of the eggs, scale bar = 10 μm. **(H)** Clusters of fluorescent bacteria (arrowheads) are scattered in the muscular tissue of the second generation first-instar offspring after hatching, scale bar = 10 μm. ch, chorion; ep, epithelium; fb, fat body; gh, granular hemocyte; hc, hemocoel; lu, gut lumen; mu, musculature; oc, oocyte; pm, peritrophic membrane; tr, tracheole. Magnification, **(A–G)**, 630X; **(H)**, 400X.

bacteria by allowing individual adults to mate. Remarkably, we observed that a few bacteria were detected in the oocyte (Knorr et al., 2015) and none in the chorion of the eggs (**Figure 4G**), which proves GFP-tagged bacteria can survive after almost 30 days at various stages of the entire life cycle of *S. littoralis*. We also showed that, after hatching, fluorescent bacteria were detected in the muscular tissue of the first-instar larvae of second generation offspring (**Figure 4H**).

Viable GFP Bacterial Cell Counts

Total fluorescent *E. mundtii* were recovered and counted from individual gut regions (foregut, midgut, and hindgut) on selective THB agar containing erythromycin. The mean of CFUs of bacteria recovered from each gut region showed significant difference between larval stages ($F = 15.38$; $df = 2$; $P < 0.0001$) by one-way ANOVA test. Further pairwise comparison revealed significant differences between the mean number of CFUs of combined gut parts between larvae in the fourth and fifth instars (likelihood ratio: 4.062; $P < 0.0001$) as well as the fifth and sixth instars (likelihood ratio: 3.048; $P = 0.0006$) but not between the fourth and sixth instars (likelihood ratio: 1.014; $P = 0.3853$; **Figure 5A**).

The number of *E. mundtii* in the foregut region was relatively low until to the fourth instar and transiently raised to $3.2 \pm 1.9 \times 10^6$ cells ($P < 0.0001$) during the fifth instar, followed by a decrease to $9.2 \pm 8.6 \times 10^4$ cells ($P < 0.0053$) during the sixth instar. A sharp decrease by 97.1% occurred toward the late-instar larval stage. The midgut CFU count rose during the fourth instar from a mean of $1.0 \pm 0.6 \times 10^5$ to $2.7 \pm 2.1 \times 10^7$ at the end of the fifth instar, representing a significant difference ($P = 0.0425$). In the sixth instar, bacterial counts fell to $4.0 \pm 3.7 \times 10^6$ and showed no significant difference to larvae in the fifth and sixth instars ($P = 0.1576$). Also in the hindgut region there was a transient increase of bacterial

counts from 2.2 ± 1.0 × 10^5 (fourth instar) to 1.5 ± 1.2 × 10^7 at the end of the fifth instar; this number fell by 94.5% to 2.2 ± 1.3 × 10^5 at the end of the sixth instar (P = 0.1087; **Figure 5B**). The ± values represent the standard error (SE). For some larvae, the hindgut region did not show any CFUs, possibly due to the high variation in the feeding behavior of individual larva. Overall, the number of bacteria in the intestinal tissues steadily increased from fourth- to fifth-instar larvae, but decreased tremendously during the sixth larval instar. The number of mean CFUs remained low during early pupation and slightly increased in the late pupation and adult stages (data not shown). No CFUs of fluorescent *E. mundtii* were detected from control larvae.

Tracking of Ingested GFP Bacteria by Colony PCR

The gut content of different stages of development of the insect was enumerated on selective agar plates. The bacterial colonies grown on agar were picked for colony-PCR experiments to verify the presence of GFP-containing plasmid. We were able to amplify the *gfp* gene of around ~735 bp from bacterial colonies at all stages of development (**Figure 6**). In addition, we could detect the GFP amplicon in fecal samples of all stages (data not shown), which confirmed that transgenic bacteria were present and could colonize the intestinal tract of *S. littoralis*.

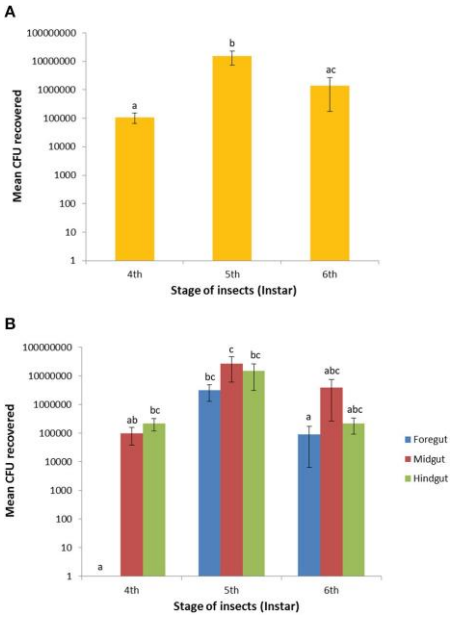


FIGURE 5 | Recovery of GFP-expressing *E. mundtii* in the foregut, midgut and hindgut across larval stages of *S. littoralis*. **(A)** Bacterial recoveries are based on the mean average of the combination of three gut regions of six independent larvae (n = 18 per larval stage). **(B)** Bacterial counts were determined in individual gut region within the same digestive tract of each stage (n = 6 per gut region). Different letters above error bars (SEM) denote significant differences between pairs (P < 0.05). CFUs = colony forming units.

DISCUSSION

The GFP-expressing plasmids used in this study were derived from a common backbone *E. coli*-enterococcal shuttle vector (pTRKH3), which was controlled by three constitutive promoters of different strengths. This vector contained moderate copy numbers (30–40) in *E. coli* and a high copy number (45–85) in *Streptococcus* and *Lactococcus* species (O'Sullivan and Klaenhammer, 1993; Papagianni et al., 2007). Moreover, it was stably maintained up to 25 generations without erythromycin and lost <4% after transformation into *Lactococcus lactis* (Papagianni et al., 2007). Our results showed that the strongest GFP expression signal was derived from the *ermB* promoter, which displayed the high fluorescence of recombinant bacteria upon detection by epifluorescence microscopy, western blotting and flow cytometry. This promoter is likely to be highly effective in many *Enterococcus* species, as it is derived from the broad-host range plasmid pAMß1 of *E. faecalis* (Swinfield et al., 1990). In *Staphylococcus aureus*, erythromycin resistance is caused by ribosome methylases encoded by *ermA*, *ermB,* and *ermC* genes which are involved in the methylation of 23S rRNA (Leclercq,

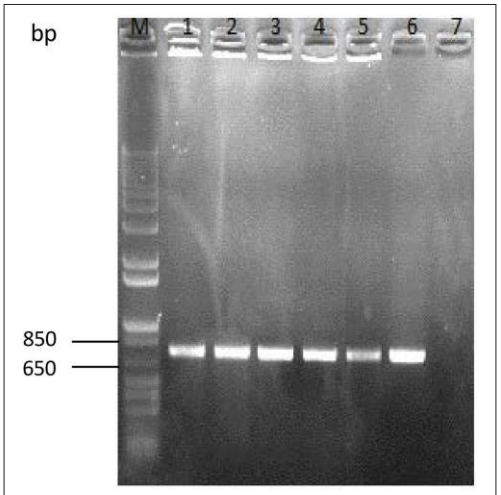


FIGURE 6 | Colony PCR-amplification of enumerated colonies of *E. mundtii* harboring GFP recovered from the intestinal tracts of larvae at different life stages. The *gfp* gene was amplified from fourth-, fifth,- and sixth-instar pupae and from adult insects. M, molecular weight marker (1-kb Plus DNA ladder, Invitrogen); Lane 1, fourth instar; Lane 2, fifth instar; Lane 3, sixth instar; Lane 4, pupa; Lane 5, adult; Lane 6, positive control (plasmid pTRKH3-*erm*GFP); Lane 7, negative control. The size of *gfp* gene is ~735 bp.

2002). The addition of erythromycin antibiotic as substrate may increase the expression of *ermB* gene in *E. mundtii*, thus activates the GFP expression. In addition, the pTRKH3 vector which originates from pAMß1 may be suitable for replication in Gram-positive bacteria. The strength of *gfp* gene expression controlled by these promoters was similar to that reported in *Lactobacillus reuteri* strains (Lizier et al., 2010).

The strength of promoter used to drive successful expression of heterologous proteins depends on strain and vary within LAB (McCracken and Timms, 1999). The constitutive *ldh* promoter is highly efficient in *Lactobacillus casei* (Pouwels et al., 2001) as well as in *E. mundtii*. It has been shown that the *ldh* gene is highly active in the logarithmic phase, but its expression decreases in the stationary phase in *Lactobacillus helveticus* (Savijoki and Palva, 1997). The low GFP expression signal from the *slp* promoter in our study may be due to different rate of transcription and translation of S-protein genes. In two *Lactobacillus* species, similar genes can be expressed with different regulatory mechanism (Pouwels et al., 1998). In some species of bacteria, the S-protein genes are controlled by multiple promoters (Vidgren et al., 1992), and some are preceded by a single promoter. The yield of mRNA controlled by multiple promoters might be higher than the yield directed by a single promoter. The regulation of S-protein gene expression is still not very well-known and may be growth-dependent. One of the five promoters upstream of S-protein gene in *Brevibacillus brevis* is active during all growth phases, while another promoter is only active during exponential growth (Adachi et al., 1989). It has been shown that the half-life of the S-protein mRNAs is different between bacterial species, *Aeromonas salmonicida* (22 min; Chu et al., 1993), *Caulobacter crescentus* (10–15 min; Fisher et al., 1988), and *L. acidophilus* (15 min; Boot et al., 1996). Another possible explanation of low GFP expression directed by a single *slp* promoter might be that *E. mundtii* do not synthesize S-layer protein which was also reported in *L. casei* as well (Masuda and Kawata, 1983).

Expression of *gfp* has effect on the physiology and fitness of the bacteria (Rang et al., 2003; Allison and Sattenstall, 2007). It was reported that the growth of *Salmonella* was suppressed due to constitutive expression of *gfp* (Oscar, 2003). In contrast, two case studies using *E. coli* and other pathogenic bacteria showed that the *gfp* expression did not affect bacterial survival (Leff and Leff, 1996; Valdivia et al., 1996). The use of erythromycin as an antibiotic selective marker has a number of drawbacks. It may cause toxic effects on the host insect and other gut microbes. The excessive use of antibiotics causes its spread in the environment and thus produces many antibiotic resistant pathogenic bacteria (Hamer and Gill, 2002; Livermore, 2007; Walsh and Fanning, 2008). Under laboratory conditions, it has been shown that the antibiotic resistance genes via a plasmid can be transferred into foodborne pathogenic bacteria by turning antibiotic sensitive strains into resistant ones (Van Meervenne et al., 2012).

Researchers have found that indigenous bacteria derived from the host insect could be reintroduced and could survive in the native gut environment (Chapco and Kelln, 1994; Dillon and Charnley, 1996; Martinez-Sanudo et al., 2011). In previous experiments, we introduced GFP-tagged *E. coli* into the gut of *S. littoralis* and were able to monitor the bacteria for up to 4 days after which they disappeared (Wallstein, 2014). Our observation was independently confirmed by others (Thimm et al., 1998), who found that genetically modified non-indigenous *E. coli* vanished within 1 day after introduction into the gut of collembola. In contrast, the indigenous *Alcaligenes faecalis* was able to colonize the intestinal tract of *Folsomia candida* (Collembola) for about 2 months (Thimm et al., 1998). In other studies, Husseneder and Grace failed to produce a persistent population of transgenic *E. coli* in the guts of termites (Husseneder and Grace, 2005). However, they successfully introduced the genetically modified indigenous *Alcaligenes faecalis* was able to colonize the intestinal tract of *Folsomia candida* (Collembola) for about 2 months indigenous *E. coli* to establish a stable population in the guts of termites may be due to resistance by the indigenous gut bacteria (Dillon and Dillon, 2004), and the fact that the non-indigenous bacteria might be outclassed by the natural microbial flora (Chao and Feng, 1990; Leff and Leff, 1996).

Since it is of interest to study the mode of transmission of GFP-labeled *E. mundtii* to the next generation, we also analyzed the occurrence of GFP-labeled cells in pupae, and oocytes of *S. littoralis*. The fluorescent bacteria were found, indeed, in the oocytes and were transmitted to the second-generation larvae. Recent examinations using fluorescent bacteria have found the bacteria to be transmitted from the gut into the eggs in *T. castaneum* (Knorr et al., 2015). In one hypothesis, the egg-smearing mode of vertical transmission, the surface of the eggs is contaminated with the environmental bacterial symbionts, which the freshly hatched larvae acquire by feeding on the eggshell (Douglas and Beard, 1997; de Vries et al., 2001). Bakula showed that the methylene blue dye used to stain the embryos of *Drosophila* was detected in the intestine of hatched first-instar larvae, suggesting that the larvae had ingested the embryos (Bakula, 1969). Transmission through parents also occurs, either from the mother to the offspring or from the father to mother and then to the offspring (Moran and Dunbar, 2006; Damiani et al., 2008). Damiani et al. also demonstrated that male-borne symbionts of the bacteria of the genus *Asaia* were transferred to females during mating of *Anopheles stephensi* mosquitoes (Damiani et al., 2008). These bacteria were then further transmitted from the mother to the offspring during sexual reproduction. In separate studies, Moran and Dunbar also showed that it is possible, though rare, for symbionts to be transferred from the father to the offspring (paternal transfer) in aphids (Moran and Dunbar, 2006).

Several factors—for instance, pH and oxygen availability can shape microbial colonization in different gut niches. It is known that the pH inside the gut of Lepidoptera such as *S. littoralis* is highly alkaline (pH ~ 8.5–10) in the foregut and midgut, and neutral (pH 7.0) in the hindgut (Funke et al., 2008). It has been reported that there is relatively low diversity of bacteria in the extremely alkaline guts of gypsy moth larvae, *Lymantria dispar* (Broderick et al., 2004) and high bacterial density in

the low-alkaline guts of larvae in beetles (Coleoptera), flies (Diptera), or bees (Apoidea) (Kadavy et al., 1999; Egert et al., 2003; Mohr and Tebbe, 2006). The survival of *E. mundtii* in alkaline environment shows that it has developed adaptation mechanisms.

It has been shown through FISH analyses that enterococci can form a biofilm-like structure by attaching themselves to the mucus layer of the gut epithelium (Koch and Schmid-Hempel, 2011; Engel et al., 2012; Shao et al., 2014). In our study, interestingly, most of the GFP-tagged bacteria did not spread throughout the whole gut content but were confined within the mucus layer of the peritrophic membrane. This membrane prevents the bacteria from gut lumen from entering the epithelium, as reported in the study of *Bactrocera oleae* (Mazzon et al., 2008). The peritrophic membrane was shown to have a defensive role against pathogens in *Drosophila melanogaster* (Kuraishi et al., 2011) and to act as a barrier against food particles and digestive enzymes (Lehane, 1997; Hegedus et al., 2009). In addition, the membrane was able to protect the bacteria from unfavorable gut conditions such as alkaline and acidic pH (Crotti et al., 2009). In our case, we observed that the fluorescent bacteria crossed the peritrophic membrane and invaded the gut epithelium of the fourth-instar larvae.

The composition and density of microorganisms changed as insects aged, for example in the case of the fruit fly, *D. melanogaster* (Ren et al., 2007; Buchon et al., 2009; Storelli et al., 2011; Wong et al., 2011). In our study, the number of fluorescent bacteria increased throughout the larval stage, from fourth- to fifth-instar larvae. This number was significantly higher in tissues from the midgut than from those in the foregut and hindgut, supporting the hypothesis that the midgut is a crucial region for digestion. Beneficial bacteria may be needed to aid in the metabolic activity of host insect. A strong decline of recombinant bacteria was observed in the sixth-instar larvae. This reduction prior to the pupal stage may be associated with the enhanced expression of antimicrobial peptide genes which has been shown in a few previous studies (Samakovlis et al., 1990; Tryselius et al., 1992; Tzou et al., 2000).

Humoral responses, such as the production of antimicrobial peptides, reactive oxygen species, and lysozymes, as well as activation of the prophenoloxidase system, are noticed when microorganisms invade (Jiang, 2008; Tsakas, 2010). Antimicrobial peptides can be repressed by transcription factors, including the homeobox gene *caudal,* in order to retain beneficial gut bacteria in the host insect (Ryu et al., 2008). Remarkably, we detected the encapsulation of fluorescent *E. mundtii* within the nodules of granule hemocytes which leads to bacterial lysis. The two most abundant hemocytes present in the larvae of Lepidoptera are granular cells (granulocytes) and plasmatocytes (Ratcliffe, 1993; Strand and Pech, 1995). The processes of phagocytosis, nodulation and encapsulation are hemocyte-mediated immune responses (Strand, 2008; Tsakas, 2010). Hemocytes encapsulate various cells ranging from bacteria to yeast and even synthetic beads and particles of India ink (Yokoo et al., 1995; Hernandez et al., 1999; Da Silva et al., 2000). We observed that the fluorescent enterococci survived pupation and became transmitted to the adults. Accordingly, the bacteria can be successfully transmitted during metamorphosis escaping the removal or reduction of midgut bacteria by colonizing sites farther away from the meconium. Another explanation may be that bacteria are resistant to the antimicrobial exuvial fluids that are consumed as part of the ecdysial process (Moll et al., 2001).

CONCLUSION

We have succeeded in tagging *E. mundtii* (strain KD251) with the *gfp* gene. The recombinant strain that harbors the pTRKH3-*erm*GFP plasmid was chosen to be reintroduced into *S. littoralis*. Interestingly, the fluorescent bacterial cells were able to colonize the intestinal tract of the host insect for nearly 30 days. These bacteria were efficiently transmitted from larval stages to the adult stage, where they survived up to the second generation. Increased knowledge of the distribution and transmission route of indigenous gut symbionts may lead us to better understand their biological role in the host insect.

AUTHOR CONTRIBUTIONS

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

ACKNOWLEDGMENTS

We thank Angelika Berg for laboratory assistance, Ewald Grosse-Wilde for the help in cryomicrotomy, Domenica Schnabelrauch for sequencing, and Grit Kunert for help with the statistics. We gratefully acknowledge editoral assistance by Emily Wheeler. This work was supported by the DFG (ChemBioSys, CRC 1127), the excellence graduate school Jena School for Microbial Communication (JSMC), and the Max Planck Society.

REFERENCES

Adachi, T., Yamagata, H., Tsukagoshi, N., and Udaka, S. (1989). Multiple and tandemly arranged promoters of the cell wall protein gene operon in *Bacillus brevis* 47. *J. Bacteriol.* 171, 1010–1016.

Adams, A. S., Jordan, M. S., Adams, S. M., Suen, G., Goodwin, L. A., Davenport, K. W., et al. (2011). Cellulose-degrading bacteria associated with the invasive woodwasp *Sirex noctilio*. *ISME J.* 5, 1323–1331. doi: 10.1038/ismej.2011.14

Allison, D. G., and Sattenstall, M. A. (2007). The influence of green fluorescent protein incorporation on bacterial physiology: a note of caution. *J. Appl. Microbiol.* 103, 318–324. doi: 10.1111/j.1365-2672.2006.03243.x

Anand, A. A., Vennison, S. J., Sankar, S. G., Prabhu, D. I., Vasan, P. T., 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, 107. doi: 10.1673/031.010.10701

Appel, H. M., and Martin, M. M. (1990). Gut redox conditions in herbivorous lepidopteran larvae. *J. Chem. Ecol.* 16, 3277–3290. doi: 10.1007/BF00982098

Arias, C. A., and Murray, B. E. (2012). The rise of the *Enterococcus*: beyond vancomycin resistance. *Nat. Rev. Microbiol.* 10, 266–278. doi: 10.1038/nrmicro2761

Bakula, M. (1969). The persistence of a microbial flora during postembryogenesis of *Drosophila melanogaster*. *J. Invertebr. Pathol.* 14, 365–374. doi: 10.1016/0022-2011(69)90163-3

Boot, H. J., Kolen, C. P., Andreadaki, F. J., Leer, R. J., and Pouwels, P. H. (1996). The *Lactobacillus acidophilus* S-layer protein gene expression site comprises two consensus promoter sequences, one of which directs transcription of stable mRNA. *J. Bacteriol.* 178, 5388–5394.

Boot, H. J., and Pouwels, P. H. (1996). Expression, secretion and antigenic variation of bacterial S-layer proteins. *Mol. Microbiol.* 21, 1117–1123. doi: 10.1046/j.1365-2958.1996.711442.x

Broderick, N. A., Raffa, K. F., Goodman, R. M., and 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. doi: 10.1128/AEM.70.1.293-300.2004

Brune, A., and Kühl, M. (1996). pH profiles of the extremely alkaline hindguts of soil-feeding termites (Isoptera: Termitidae) determined with microelectrodes. *J. Insect Physiol.* 42, 1121–1127. doi: 10.1016/S0022-1910(96)00036-4

Bubert, A., Sokolovic, Z., Chun, S. K., Papatheodorou, L., Simm, A., and Goebel, W. (1999). Differential expression of *Listeria monocytogenes* virulence genes in mammalian host cells. *Mol. Gen. Genet.* 261, 323–336. doi: 10.1007/pl00008633

Buchon, N., Broderick, N. A., Chakrabarti, S., and Lemaitre, B. (2009). Invasive and indigenous microbiota impact intestinal stem cell activity through multiple pathways in *Drosophila*. *Genes Dev.* 23, 2333–2344. doi: 10.1101/gad.1827009

Chao, W. L., and Feng, R. L. (1990). Survival of genetically engineered *Escherichia coli* in natural soil and river water. *J. Appl. Bacteriol.* 68, 319–325. doi: 10.1111/j.1365-2672.1990.tb02881.x

Chapco, W., and Kelln, R. A. (1994). Persistence of ingested bacteria in the grasshopper gut. *J. Invertebr. Pathol.* 64, 149–150. doi: 10.1006/jipa.1994.1086

Chu, S., Gustafson, C. E., Feutrier, J., Cavaignac, S., and Trust, T. J. (1993). Transcriptional analysis of the *Aeromonas salmonicida* S-layer protein gene *vapA*. *J. Bacteriol.* 175, 7968–7975.

Cormack, B. P., Valdivia, R. H., and Falkow, S. (1996). FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* 173, 33–38. doi: 10.1016/0378-1119(95)00685-0

Crotti, E., Damiani, C., Pajoro, M., Gonella, E., Rizzi, A., Ricci, I., et al. (2009). *Asaia*, a versatile acetic acid bacterial symbiont, capable of cross-colonizing insects of phylogenetically distant genera and orders. *Environ. Microbiol.* 11, 3252–3264. doi: 10.1111/j.1462-2920.2009.02048.x

Damiani, C., Ricci, I., Crotti, E., Rossi, P., Rizzi, A., Scuppa, P., et al. (2008). Paternal transmission of symbiotic bacteria in malaria vectors. *Curr. Biol.* 18, R1087–R1088. doi: 10.1016/j.cub.2008.10.040

Da Silva, J. B., De Albuquerque, C. M., De Araujo, E. C., Peixoto, C. A., and Hurd, H. (2000). Immune defense mechanisms of *Culex quinquefasciatus* (Diptera: Culicidae) against *Candida albicans* infection. *J. Invertebr. Pathol.* 76, 257–262. doi: 10.1006/jipa.2000.4980

de Vries, E. J., Jacobs, G., and Breeuwer, J. A. (2001). Growth and transmission of gut bacteria in the Western flower thrips, *Frankliniella occidentalis*. *J. Invertebr. Pathol.* 77, 129–137. doi: 10.1006/jipa.2001.5010

Dillon, R. J., and Charnley, A. K. (1996). Colonization of the guts of germ-free desert locusts, *Schistocerca Gregaria*, by the bacterium *Pantoea agglomerans*. *J. Invertebr. Pathol.* 67, 11–14. doi: 10.1006/jipa.1996.0002

Dillon, R. J., and Dillon, V. M. (2004). The gut bacteria of insects: nonpathogenic interactions. *Annu. Rev. Entomol.* 49, 71–92. doi: 10.1146/annurev.ento.49.061802.123416

Doud, C. W., and Zurek, L. (2012). *Enterococcus faecalis* OG1RF:pMV158 survives and proliferates in the house fly digestive tract. *J. Med. Entomol.* 49, 150–155. doi: 10.1603/ME11167

Douglas, A. E., and Beard, C. B. (1997). "Microbial symbiosis in the midgut of insects," in *Biology of the Insect Midgut*, ed M. Lehane (New York, NY: Academic Press), 315–333.

Dower, W. J., Miller, J. F., and Ragsdale, C. W. (1988). High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res.* 16, 6127–6145. doi: 10.1093/nar/16.13.6127

Egert, M., Wagner, B., Lemke, T., Brune, A., and Friedrich, M. W. (2003). Microbial community structure in midgut and hindgut of the humus-feeding larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). *Appl. Environ. Microbiol.* 69, 6659–6668. doi: 10.1128/AEM.69.11.6659-6668.2003

Engel, P., Martinson, V. G., and Moran, N. A. (2012). Functional diversity within the simple gut microbiota of the honey bee. *Proc. Natl. Acad. Sci. U.S.A.* 109, 11002–11007. doi: 10.1073/pnas.1202970109

Fernandez de Palencia, P., Nieto, C., Acebo, P., Espinosa, M., and Lopez, P. (2000). Expression of green fluorescent protein in *Lactococcus lactis*. *FEMS Microbiol. Lett.* 183, 229–234. doi: 10.1111/j.1574-6968.2000.tb08963.x

Fisher, J. A., Smit, J., and Agabian, N. (1988). Transcriptional analysis of the major surface array gene of *Caulobacter crescentus*. *J. Bacteriol.* 170, 4706–4713.

Freitag, N. E., and Jacobs, K. E. (1999). Examination of *Listeria monocytogenes* intracellular gene expression by using the green fluorescent protein of *Aequorea victoria*. *Infect. Immun.* 67, 1844–1852.

Funke, M., Buchler, R., Mahobia, V., Schneeberg, A., Ramm, M., and Boland, W. (2008). Rapid hydrolysis of quorum-sensing molecules in the gut of lepidopteran larvae. *Chembiochem* 9, 1953–1959. doi: 10.1002/cbic.200700781

Giraffa, G. (2003). Functionality of enterococci in dairy products. *Int. J. Food Microbiol.* 88, 215–222. doi: 10.1016/S0168-1605(03)00183-1

Hamer, D. H., and Gill, C. J. (2002). From the farm to the kitchen table: the negative impact of antimicrobial use in animals on humans. *Nutr. Rev.* 60, 261–264. doi: 10.1301/002966402320289395

Hansen, M. C., Palmer, R. J. Jr., Udsen, C., White, D. C., and Molin, S. (2001). Assessment of GFP fluorescence in cells of *Streptococcus gordonii* under conditions of low pH and low oxygen concentration. *Microbiology* 147, 1383–1391. doi: 10.1099/00221287-147-5-1383

Harrison, J. F. (2001). Insect acid-base physiology. *Annu. Rev. Entomol.* 46, 221–250. doi: 10.1146/annurev.ento.46.1.221

Hazelrigg, T., Liu, N., Hong, Y., and Wang, S. (1998). GFP expression in *Drosophila* tissues: time requirements for formation of a fluorescent product. *Dev. Biol.* 199, 245–249. doi: 10.1006/dbio.1998.8922

Hegedus, D., Erlandson, M., Gillott, C., and Toprak, U. (2009). New insights into peritrophic matrix synthesis, architecture, and function. *Annu. Rev. Entomol.* 54, 285–302. doi: 10.1146/annurev.ento.54.110807.090559

Hernandez, S., Lanz, H., Rodriguez, M. H., Torres, J. A., Martinez-Palomo, A., and Tsutsumi, V. (1999). Morphological and cytochemical characterization of female *Anopheles albimanus* (Diptera: Culicidae) hemocytes. *J. Med. Entomol.* 36, 426–434. doi: 10.1093/jmedent/36.4.426

Husseneder, C., and Grace, J. K. (2005). Genetically engineered termite gut bacteria (*Enterobacter cloacae*) deliver and spread foreign genes in termite colonies. *Appl. Microbiol. Biotechnol.* 68, 360–367. doi: 10.1007/s00253-005-1914-5

Jiang, H. (2008). The biochemical basis of antimicrobial responses in *Manduca sexta*. *Insect Sci.* 15, 53–66. doi: 10.1111/j.1744-7917.2008.00187.x

Kadavy, D. R., Plantz, B., Shaw, C. A., Myatt, J., Kokjohn, T. A., and Nickerson, K. W. (1999). Microbiology of the oil fly, *Helaeomyia petrolei*. *Appl. Environ. Microbiol.* 65, 1477–1482.

Kim, S. F., Baek, S. J., and Pack, M. Y. (1991). Cloning and nucleotide sequence of the *Lactobacillus casei* lactate dehydrogenase gene. *Appl. Environ. Microbiol.* 57, 2413–2417.

Knorr, E., Schmidtberg, H., Arslan, D., Bingsohn, L., and Vilcinskas, A. (2015). Translocation of bacteria from the gut to the eggs triggers maternal transgenerational immune priming in *Tribolium castaneum*. *Biol. Lett.* doi: 10.1098/rsbl.2015.0885. [Epub ahead of print].

Koch, H., and 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. doi: 10.1073/pnas.1110474108

Kohler, W. (2007). The present state of species within the genera *Streptococcus* and *Enterococcus*. *Int. J. Med. Microbiol.* 297, 133–150. doi: 10.1016/j.ijmm.2006.11.008

Kounatidis, I., Crotti, E., Sapountzis, P., Sacchi, L., Rizzi, A., Chouaia, B., et al. (2009). *Acetobacter tropicalis* is a major symbiont of the olive fruit fly (*Bactrocera oleae*). *Appl. Environ. Microbiol.* 75, 3281–3288. doi: 10.1128/AEM.02933-08

Kuraishi, T., Binggeli, O., Opota, O., Buchon, N., and Lemaitre, B. (2011). Genetic evidence for a protective role of the peritrophic matrix against intestinal bacterial infection in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* 108, 15966–15971. doi: 10.1073/pnas.1105994108

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685. doi: 10.1038/227680a0

Leclercq, R. (2002). Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. *Clin. Infect. Dis.* 34, 482–492. doi: 10.1086/324626

Leff, L. G., and Leff, A. A. (1996). Use of green fluorescent protein to monitor survival of genetically engineered bacteria in aquatic environments. *Appl. Environ. Microbiol.* 62, 3486–3488.

Lehane, M. J. (1997). Peritrophic matrix structure and function. *Annu. Rev. Entomol.* 42, 525–550. doi: 10.1146/annurev.ento.42.1.525

Lewis, P. J., and Marston, A. L. (1999). GFP vectors for controlled expression and dual labelling of protein fusions in *Bacillus subtilis*. *Gene* 227, 101–110. doi: 10.1016/S0378-1119(98)00580-0

Livermore, D. M. (2007). Introduction: the challenge of multiresistance. *Int. J. Antimicrob. Agents* 29(Suppl. 3), S1–S7. doi: 10.1016/S0924-8579(07)00158-6

Lizier, M., Sarra, P. G., Cauda, R., and Lucchini, F. (2010). Comparison of expression vectors in *Lactobacillus reuteri* strains. *FEMS Microbiol. Lett.* 308, 8–15. doi: 10.1111/j.1574-6968.2010.01978.x

Lun, S., and Willson, P. J. (2004). Expression of green fluorescent protein and its application in pathogenesis studies of serotype 2 *Streptococcus suis*. *J. Microbiol. Methods* 56, 401–412. doi: 10.1016/j.mimet.2003.11.012

Madigan, M., Martinko, J., Dunlap, P., and Clark, D. (2009). *Brock Biology of Microorganisms*. San Francisco, CA: Pearson Education, Inc.

Martinez-Sanudo, I., Savio, C., Mazzon, L., Girolami, V., Ciolfi, S., and Marri, L. (2011). Green fluorescent protein (GFP)-labeling of enterobacteria associated with fruit flies (Diptera: Tephritidae) and persistence in their natural host *Rhagoletis completa* Cresson. *Can. J. Microbiol.* 57, 969–973. doi: 10.1139/w11-057

Masuda, K., and Kawata, T. (1983). Distribution and chemical characterization of regular arrays in the cell walls of strains of the genus *Lactobacillus*. *FEMS Microbiol. Lett.* 20, 145–150. doi: 10.1111/j.1574-6968.1983.tb00105.x

Mazzon, L., Piscedda, A., Simonato, M., Martinez-Sanudo, I., Squartini, A., and Girolami, V. (2008). Presence of specific symbiotic bacteria in flies of the subfamily Tephritinae (Diptera Tephritidae) and their phylogenetic relationships: proposal of '*Candidatus Stammerula tephritidis*'. *Int. J. Syst. Evol. Microbiol.* 58, 1277–1287. doi: 10.1099/ijs.0.65287-0

McCracken, A., and Timms, P. (1999). Efficiency of transcription from promoter sequence variants in *Lactobacillus* is both strain and context dependent. *J. Bacteriol.* 181, 6569–6572.

McGaughey, J., and Nayduch, D. (2009). Temporal and spatial fate of GFP-expressing motile and nonmotile *Aeromonas hydrophila* in the house fly digestive tract. *J. Med. Entomol.* 46, 123–130. doi: 10.1603/033.046.0116

Mohr, K. I., and Tebbe, C. C. (2006). Diversity and phylotype consistency of bacteria in the guts of three bee species (*Apoidea*) at an oilseed rape field. *Environ. Microbiol.* 8, 258–272. doi: 10.1111/j.1462-2920.2005.00893.x

Moll, R. M., Romoser, W. S., Modrzakowski, M. C., Moncayo, A. C., and Lerdthusnee, K. (2001). Meconial peritrophic membranes and the fate of midgut bacteria during mosquito (Diptera: Culicidae) metamorphosis. *J. Med. Entomol.* 38, 29–32. doi: 10.1603/0022-2585-38.1.29

Moran, N. A., and Dunbar, H. E. (2006). Sexual acquisition of beneficial symbionts in aphids. *Proc. Natl. Acad. Sci. U.S.A.* 103, 12803–12806. doi: 10.1073/pnas.0605772103

Mumcuoglu, K. Y., Miller, J., Mumcuoglu, M., Friger, M., and Tarshis, M. (2001). Destruction of bacteria in the digestive tract of the maggot of *Lucilia sericata* (Diptera: Calliphoridae). *J. Med. Entomol.* 38, 161–166. doi: 10.1603/0022-2585-38.2.161

Ogier, J. C., and Serror, P. (2008). Safety assessment of dairy microorganisms: the *Enterococcus* genus. *Int. J. Food Microbiol.* 126, 291–301. doi: 10.1016/j.ijfoodmicro.2007.08.017

Oscar, T. P. (2003). Comparison of predictive models for growth of parent and green fluorescent protein-producing strains of *Salmonella*. *J. Food Prot.* 66, 200–207.

O'Sullivan, D. J., and Klaenhammer, T. R. (1993). High- and low-copy-number *Lactococcus* shuttle cloning vectors with features for clone screening. *Gene* 137, 227–231. doi: 10.1016/0378-1119(93)90011-Q

Papagianni, M., Avramidis, N., and Filioussis, G. (2007). High efficiency electrotransformation of *Lactococcus lactis* spp. lactis cells pretreated with lithium acetate and dithiothreitol. *BMC Biotechnol.* 7:15. doi: 10.1186/1472-6750-7-15

Pouwels, P. H., Leer, R. J., Shaw, M., Heijne den Bak-Glashouwer, M. J., Tielen, F. D., Smit, E., et al. (1998). Lactic acid bacteria as antigen delivery vehicles for oral immunization purposes. *Int. J. Food Microbiol.* 41, 155–167. doi: 10.1016/S0168-1605(98)00048-8

Pouwels, P. H., Vriesema, A., Martinez, B., Tielen, F. J., Seegers, J. F., Leer, R. J., et al. (2001). Lactobacilli as vehicles for targeting antigens to mucosal tissues by surface exposition of foreign antigens. *Methods Enzymol.* 336, 369–389. doi: 10.1016/S0076-6879(01)36602-8

Rang, C., Galen, J. E., Kaper, J. B., and Chao, L. (2003). Fitness cost of the green fluorescent protein in gastrointestinal bacteria. *Can. J. Microbiol.* 49, 531–537. doi: 10.1139/w03-072

Ratcliffe, N. A. (1993). "Cellular defense responses of insects: unresolved problems," in *Parasites and Pathogens of Insects*, *Vol. 1*, eds N. E. Beckage, S. N. Thompson and B. A. Feferici (San Diego, CA: Academic Press), 267–304.

Ren, C., Webster, P., Finkel, S. E., and Tower, J. (2007). Increased internal and external bacterial load during *Drosophila* aging without life-span trade-off. *Cell Metab.* 6, 144–152. doi: 10.1016/j.cmet.2007.06.006

Rolls, M. M., Stein, P. A., Taylor, S. S., Ha, E., McKeon, F., and Rapoport, T. A. (1999). A visual screen of a GFP-fusion library identifies a new type of nuclear envelope membrane protein. *J. Cell Biol.* 146, 29–44. doi: 10.1083/jcb.146.1.29

Ryu, J. H., Kim, S. H., Lee, H. Y., Bai, J. Y., Nam, Y. D., Bae, J. W., et al. (2008). Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in *Drosophila*. *Science* 319, 777–782. doi: 10.1126/science.1149357

Samakovlis, C., Kimbrell, D. A., Kylsten, P., Engstrom, A., and Hultmark, D. (1990). The immune response in *Drosophila*: pattern of cecropin expression and biological activity. *EMBO J.* 9, 2969–2976.

Savijoki, K., and Palva, A. (1997). Molecular genetic characterization of the L-lactate dehydrogenase gene (*ldhL*) of *Lactobacillus helveticus* and biochemical characterization of the enzyme. *Appl. Environ. Microbiol.* 63, 2850–2856.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 9, 676–682. doi: 10.1038/nmeth.2019

Scott, K. P.,Mercer, D. K.,Richardson, A. J.,Melville,C.M., Glover,L.A., andFlint, H. J. (2000). Chromosomal integration of the green fluorescent protein gene in lactic acid bacteria and the survival of marked strains in human gut simulations. *FEMS Microbiol. Lett.* 182, 23–27. doi: 10.1111/j.1574-6968.2000.tb08867.x

Shao, Y., Arias-Cordero, E., Guo, H., Bartram, S., and Boland, W. (2014). *In vivo* Pyro-SIP assessing active gut microbiota of the cotton leafworm, *Spodoptera littoralis*. *PLoS ONE* 9:e85948. doi: 10.1371/journal.pone.0085948

Spiteller, D., Dettner, K., and Bolan, 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. doi: 10.1515/BC.2000.096

Storelli, G., Defaye, A., Erkosar, B., Hols, P., Royet, J., and Leulier, F. (2011). *Lactobacillus plantarum* promotes *Drosophila* systemic growth by modulating hormonal signals through TOR-dependent nutrient sensing. *Cell Metab.* 14, 403–414. doi: 10.1016/j.cmet.2011.07.012

Strand, M. R. (2008). The insect cellular immune response. *Insect Sci.* 15, 1–14. doi: 10.1111/j.1744-7917.2008.00183.x

Strand, M. R., and Pech, L. L. (1995). Immunological basis for compatibility in parasitoid-host relationships. *Annu. Rev. Entomol.* 40, 31–56. doi: 10.1146/annurev.en.40.010195.000335

Swinfield, T. J., Oultram, J. D., Thompson, D. E., Brehm, J. K., and Minton, N. P. (1990). Physical characterisation of the replication region of the *Streptococcus faecalis* plasmid pAM beta 1. *Gene* 87, 79–90.

Tang, X., Freitak, D., Vogel, H., Ping, L., Shao, Y., Cordero, E. A., et al. (2012). Complexity and variability of gut commensal microbiota in polyphagous lepidopteran larvae. *PLoS ONE* 7:e36978. doi: 10.1371/journal.pone.0036978

Thimm, T., Hoffmann, A., Borkott, H., Munch, J. C., and Tebbe, C. C. (1998). The gut of the soil microarthropod *Folsomia candida* (Collembola) is a frequently changeable but selective habitat and a vector for microorganisms. *Appl. Environ. Microbiol.* 64, 2660–2669.

Tryselius, Y., Samakovlis, C., Kimbrell, D. A., and Hultmark, D. (1992). *CecC*, a cecropin gene expressed during metamorphosis in *Drosophila* pupae. *Eur. J. Biochem.* 204, 395–399. doi: 10.1111/j.1432-1033.1992.tb16648.x

Tsakas, S. (2010). Insect immunity and its signalling: an overview. *Invertebrate Surviv. J.* 7, 228–238.

Tzou, P., Ohresser, S., Ferrandon, D., Capovilla, M., Reichhart, J. M., Lemaitre, B., et al. (2000). Tissue-specific inducible expression of antimicrobial peptide genes in *Drosophila* surface epithelia. *Immunity* 13, 737–748. doi: 10.1016/S1074-7613(00)00072-8

Valdivia, R. H., and Falkow, S. (1997). Fluorescence-based isolation of bacterial genes expressed within host cells. *Science* 277, 2007–2011. doi: 10.1126/science.277.5334.2007

Valdivia, R. H., Hromockyj, A. E., Monack, D., Ramakrishnan, L., and Falkow, S. (1996). Applications for green fluorescent protein (GFP) in the study of host-pathogen interactions. *Gene* 173, 47–52. doi: 10.1016/0378-1119(95)00706-7

Van Meervenne, E., Van Coillie, E., Kerckhof, F. M., Devlieghere, F., Herman, L., De Gelder, L. S., et al. (2012). Strain-specific transfer of antibiotic resistance from an environmental plasmid to foodborne pathogens. *J. Biomed. Biotechnol.* 2012, 834598. doi: 10.1155/2012/834598

Vidgren, G., Palva, I., Pakkanen, R., Lounatmaa, K., and Palva, A. (1992). S-layer protein gene of *Lactobacillus brevis*: cloning by polymerase chain reaction and determination of the nucleotide sequence. *J. Bacteriol.* 174, 7419–7427.

Wallstein, M. (2014). *Transformation of Gut Bacteria for Visualization in the Intestine of Spodoptera littoralis*. Bachelor thesis, Jena: Friedrich-Schiller-Universität Jena, Biologisch Pharmazeutische Fakultät.

Walsh, C., and Fanning, S. (2008). Antimicrobial resistance in foodborne pathogens–a cause for concern? *Curr. Drug Targets* 9, 808–815. doi: 10.2174/138945008785747761

Watanabe, H., and Tokuda, G. (2010). Cellulolytic systems in insects. *Annu. Rev. Entomol.* 55, 609–632. doi: 10.1146/annurev-ento-112408-085319

Wong, C. N., Ng, P., and Douglas, A. E. (2011). Low-diversity bacterial community in the gut of the fruitfly *Drosophila melanogaster*. *Environ. Microbiol.* 13, 1889–1900. doi: 10.1111/j.1462-2920.2011.02511.x

Yang, T. T., Kain, S. R., Kitts, P., Kondepudi, A., Yang, M. M., and Youvan, D. C. (1996). Dual color microscopic imagery of cells expressing the green fluorescent protein and a red-shifted variant. *Gene* 173, 19–23. doi: 10.1016/0378-1119(95)00781-4

Yokoo, S., Goetz, P., and Tojo, S. (1995). Phagocytic activities of haemocytes separated by two simple methods from larvae of two lepidopteran species, *Agrotis segetum* and *Galleria mellonella*. *Appl. Entomol. Zool.* 30, 343–350.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4. Article II

www.nature.com/scientificreports

OPEN

Biodiversity and Activity of the Gut Microbiota across the Life History of the Insect Herbivore *Spodoptera littoralis*

Received: 27 January 2016
Accepted: 20 June 2016
Published: 08 July 2016

Bosheng Chen[1,*], Beng-Soon Teh[2], Chao Sun[3,*], Sirui Hu[1], Xingmeng Lu[1], Wilhelm Boland[2] & Yongqi Shao[1]

Microbes that live inside insects play critical roles in host nutrition, physiology, and behavior. Although Lepidoptera (butterflies and moths) are one of the most diverse insect taxa, their microbial symbionts are little-studied, particularly during metamorphosis. Here, using ribosomal tag pyrosequencing of DNA and RNA, we investigated biodiversity and activity of gut microbiotas across the holometabolous life cycle of *Spodoptera littoralis*, a notorious agricultural pest worldwide. Proteobacteria and Firmicutes dominate but undergo a structural "metamorphosis" in tandem with its host. *Enterococcus*, *Pantoea* and *Citrobacter* were abundant and active in early-instar, while *Clostridia* increased in late-instar. Interestingly, only enterococci persisted through metamorphosis. Female adults harbored high proportions of *Enterococcus*, *Klebsiella* and *Pantoea*, whereas males largely shifted to *Klebsiella*. Comparative functional analysis with PICRUSt indicated that early-instar larval microbiome was more enriched for genes involved in cell motility and carbohydrate metabolism, whereas in late-instar amino acid, cofactor and vitamin metabolism increased. Genes involved in energy and nucleotide metabolism were abundant in pupae. Female adult microbiome was enriched for genes relevant to energy metabolism, while an increase in the replication and repair pathway was observed in male. Understanding the metabolic activity of these herbivore-associated microbial symbionts may assist the development of novel pest-management strategies.

Insects are colonized by various microorganisms, and with the development of next-generation sequencing (NGS) technologies, a rapidly growing body of work, particularly on bees, ants and flies, has shown that these symbiotic associates have important effects on host nutrition, development and pathogen defense[1–5]. For example, the prevalence of bacterial gut symbionts, *Rhizobiales*, is tightly linked with the evolution of herbivory of ants, which supply additional nitrogen to the host[6]. Abundant lactic acid bacteria, maintained in biofilms in the foregut of Western honeybees (*Apis mellifera*), work in a synergistic manner to inhibit the proliferation of pathogens[7]. The phytophagous Lepidopterans, including butterflies and moths, are one of the most widespread and diverse taxa of insects on our planet, containing about 160,000 described species in 47 superfamilies[8], and are also major pests in agriculture; however, surprisingly, their associated microbial symbionts have not been studied much with modern molecular tools, making it difficult to identify the potential impacts that these microbes may have on host ecology and evolution.

Lepidopteran insects are holometabolous and develop through four life stages; each stage has its own morphology. The egg hatches into a larva, which feeds, molts and grows larger, pupates, then emerges as an adult insect that looks completely different from the larva. Currently most studies of the lepidopteran microbiota focus on microorganisms associated with the larval gut, providing only a single snapshot of the community. For example, conventional culture-dependent techniques have identified several proteolytic bacteria, including *Enterococcus spp.* and *Bacillus spp.*, from the gut of the velvetbean caterpillar *Anticarsia gemmatalis*[9]. Culture-independent techniques have revealed a core microbial community in the larval gut of the cotton leafworm, *Spodoptera littoralis*[10]. In contrast, almost nothing is known about the diversity and composition of microbial communities inhabiting

[1]College of Animal Sciences, Zhejiang University, 310058 Hangzhou, China. [2]Department of Bioorganic Chemistry, Max Planck Institute for Chemical Ecology, Beutenberg Campus, D-07745 Jena, Germany. [3]Institute of Biotechnology, Zhejiang University, 310058 Hangzhou, China. *These authors contributed equally to this work. Correspondence and requests for materials should be addressed to Y.S. (email: yshao@zju.edu.cn)

other stages, in particular, the adult microbiota; nor it is known how microbial populations may change over metamorphosis[11]. To address these gaps in our knowledge, in the present study we used a high-throughput sequencing-based approach to compare the structure of the bacterial community in replicate egg masses, larvae, pupae and adults of *S. littoralis*, a highly polyphagous lepidopteran pest found worldwide and also an important model system used in a variety of biological research[12]. To our knowledge, this is the first systematic survey of bacterial communities across the full life cycle of a moth species, providing a foundation for future studies of microbial symbiosis in this important insect group.

In addition to investigating how the *S. littoralis*-associated microbial community varies with life stages, we also aimed to determine the metabolically active populations within the community. Despite a proliferation of studies that document the 16S rRNA gene profiles of gut communities, few address whether taxa that are detected in the DNA pool are actually active cells (DNA extracted from samples can include DNA from dead or dominant bacteria, and extracellular DNA from lysed or degraded cells, which in fact do not have any metabolic activity.). While 16S rRNA from the RNA pool represents protein synthesis potential and can be used as an indicator of active microbes, which directly contribute to the current function of the microbiota. Thus the 16S rRNA/rRNA gene phylotype ratio (RNA/DNA) is, in principle, a measure of relative activity. Many studies have employed this methodology to characterize the active moiety of a microbial community from diverse environments[13–16]. For instance, Reid *et al.* used this method to evaluate the diversity of metabolically active and inactive bacteria in the wood-feeding beetle larval gut[15]. A comparison of the 16S rRNA gene versus 16S rRNA-derived data sets revealed that *Prochlorococcus* spp. play a more important role in the food web of oligotrophic sea than expected[16]. Taking advantage of the NGS technology, here we fully assessed the *S. littoralis* microbiota by examining both the DNA-based 16S rRNA gene and the RNA (cDNA)-based 16S rRNA with pyrosequencing, an ideal tool for exploring the vast majority and often uncultivable microbes in complex microbial communities. The recently developed software package, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt), was further used to delineate metabolic potentials of the organism represented by 16S rRNA sequence, based on the phylogenetic placement of that 16S rRNA sequence within a phylogeny of sequenced genomes, an approach demonstrated previously[17]. These comprehensive analyses of 16S rRNA sequences were expected to provide insights that have not been revealed by past studies into the total diversity and metabolic activity of Lepidoptera-associated microbial communities.

Results

Overview of *Spodoptera littoralis* microbiotas. Like all Lepidoptera, *S. littoralis* undergoes complete metamorphosis; larvae and adults are greatly differentiated in form and function (Fig. 1a). Larvae chew on plant leaves while adults often feed on nectar. Our study divided the relatively long larval stage into early-instar and late-instar stages; adults live for only a few days after eclosion. Neither culturing on nutrient agar plates nor PCR amplification using specific primers revealed any fungus or Archaea in any of our samples. While we found abundant bacteria persisting in all developmental stages of *S. littoralis*, the colony-forming unit (CFU) counts in the larva, pupa and adult were 6.3×10^7, 1.02×10^4, and 2.81×10^5 per sample, respectively. However, the diversity and composition of the *S. littoralis*-associated bacterial community (designated as microbiota) varies substantially across host developmental stages.

The microbial community in the egg masses was more diverse (43 OTUs per sample, analyzed at a 3% dissimilarity level) than the community identified in the early-instar larvae (34 OTUs per sample). Bacterial phylotype richness further decreased to 23 in late-instar larvae (Table 1). Pupae were associated with the lowest number of phylotypes, demonstrating an increasing reduction in the microbial diversity from egg to pupa. This trend was also true for Shannon diversity, phylotype evenness and phylogenetic diversity, all of which displayed similar patterns (Table 1 and Supplementary figure S1). In contrast, adults harbored high richness of bacteria with 73 and 46 phylotypes in females and males, respectively. The gradually flattening rarefaction curves confirmed that the vast majority of microbial diversity was captured in all samples (Fig. 1b).

A taxonomic analysis of sequences obtained by pyrosequencing revealed that the most prevalent phylum in the microbial community associated with *S. littoralis* egg masses was Proteobacteria (ca. 95% of the sequences), whereas the most prevalent phylum in the larvae was Firmicutes (ca. 59% of the sequences in the early-instar vs. 97% in the late-instar) (Fig. 1c). Notably, through metamorphosis, pupae also harbored a bacterial community rich in Firmicutes (ca. 99% of sequences). After eclosion, we further observed a remarkable change in the composition of the bacterial community. Mature adults, especially males, exhibited a large decrease in the relative abundances of Firmicutes and showed an increased abundance of Proteobacteria (Fig. 1c). The adult male gut microbiota was dominated by Proteobacteria (ca. 93% of the sequences), together with Actinobacteria (5%), whereas the adult female gut microbiota consisted of 56% Proteobacteria and 42% Firmicutes, respectively. Relative abundances of the most highly represented phyla including Proteobacteria, Firmicutes and Actinobacteria, changed significantly ($p < 0.001$) across life stages. The pattern of taxon distribution in each stage is described in detail in the following sections.

We compared community structures between samples using principal coordinate analysis (PCoA). Pairwise ecologic distances were calculated based on the β-diversity metrics of weighted and unweighted UniFrac, which takes into account both community membership and relatedness of community members[18]. After sequence jackknifing, these distances then were visualized by the Emperor PCoA plot, which displayed the similarity among communities (Fig. 2).

Variability of community composition within individuals. We first evaluated the variability of community composition among individuals by using denaturing gradient gel electrophoresis (DGGE) of amplified 16S rRNA genes, a commonly used molecular technique for rapid fingerprint analysis of the microbial community. A cultured gut bacterium, *Enterococcus mundtii*, representing the most prevalent taxon isolated from

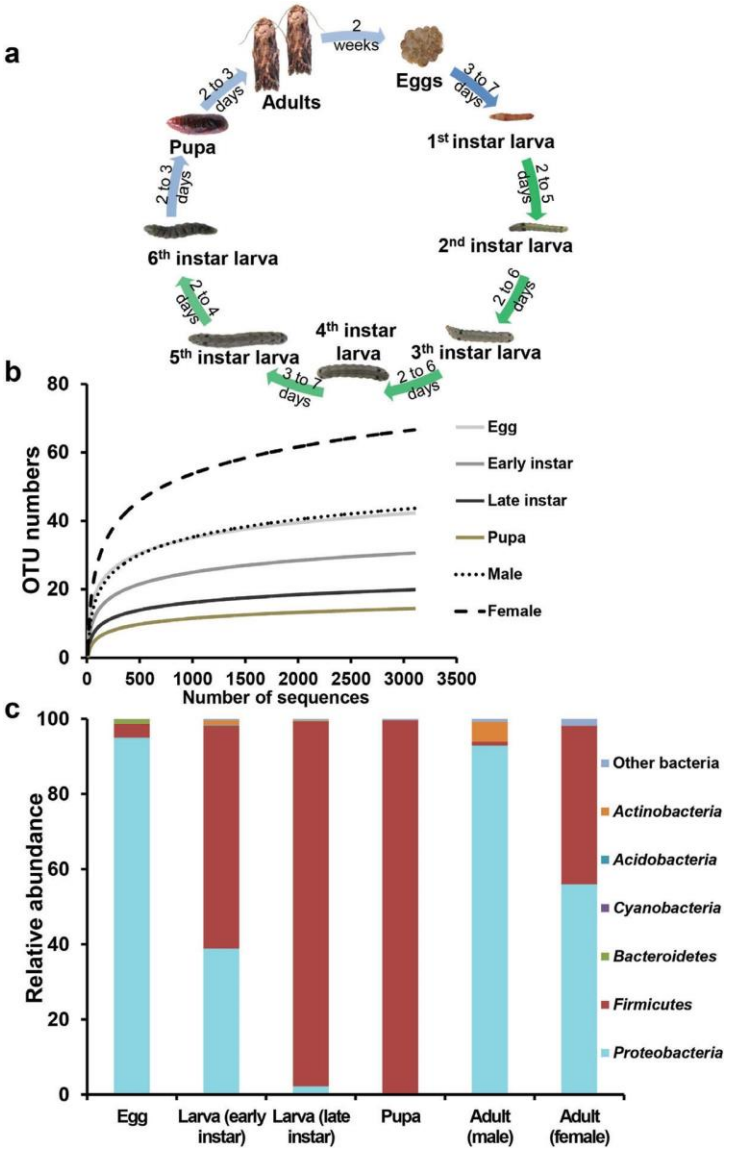


Figure 1. Changes in bacterial community diversity across life stages of *S. littoralis*. (a) Overview of development stages of the host. (b) Rarefaction curves depicted from randomly subsampled data sets with the same number of 16S sequences. The near saturated rarefaction curve indicates that the vastness of microbial diversity was retrieved from each sample. (c) Overview of the microbiota change during host development. Abundance of the 16S rRNA gene at each developmental stage at the phylum level. Relative abundances of the most dominant phyla including Proteobacteria, Firmicutes and Actinobacteria changed significantly ($p < 0.001$) across the life cycle.

Sample	Species richness indices		Species diversity indices	
	Observed	PD tree	Shannon	Simpson
Eggs	43	3	3.10	0.78
Early instar larva	34	3	1.85	0.60
Late instar larva	23	3	1.46	0.50
Pupa	15	1	0.26	0.06
Adult				
Male	46	2	1.71	0.42
Female	73	4	3.03	0.79

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

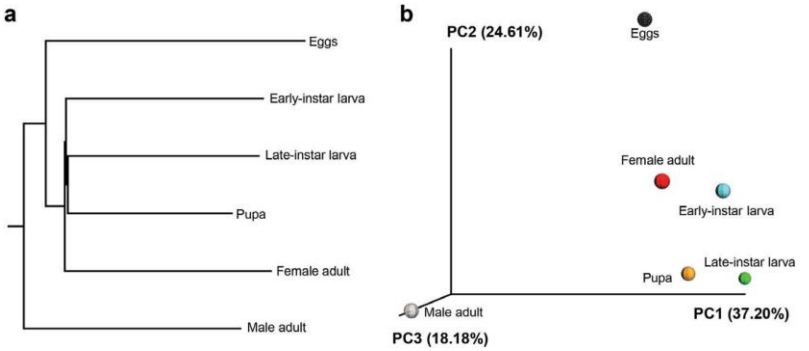


Figure 2. Similarity analysis of microbial communities. (**a**) UPGMA clustering of samples at different developmental stages according to community composition and structure. (**b**) Principal coordinates analysis (PCoA) plot visualizing the data based on β-diversity metrics of UniFrac.

S. littoralis in our previous study, was used as a DGGE standard. The DGGE profile showed that there was little variation among different individuals of a laboratory population reared under identical environmental conditions (Fig. 3a, larvae; b, adults). Similar DGGE band profiles indicated similar patterns of microbial community structure and diversity. The DGGE pattern of the larval gut microbiota was rather simple, consisting mostly of *E. mundtii* (the intense band, Fig. 3a). Likewise, similar DGGE band profiles were also observed among adults, both females and males. Although specific bacterial taxa existed in both populations, a more diverse microbial community was observed among females and a substantial difference was observed between female and male fingerprints (Fig. 3b). The three dominant bands in the female samples were weaker or absent in the male samples. The clustering analysis of DGGE profiles clearly differentiated female from male samples (Fig. 3c). The results from PCR-DGGE analysis showed that larvae subjected to the same conditions at the same life stage harbored communities that were highly similar in structure and membership, whereas among adults, the communities differed between males and females. Massive parallel pyrosequencing of the 16S was subsequently used to provide detailed taxonomic information (Figs 4–8).

Egg microbiota. *S. littoralis* lays a batch of eggs, not single eggs, on the leaves of plants, where the eggs are exposed to a wide range of environmental microbes. Eggs are attached to each other tightly and are covered with hair-like scales derived from the tip of the abdomen of the female moth (Fig. 1a and Supplementary video S1). A complex community of bacteria, dominated by the Proteobacteria, was associated with the egg masses. Large number of 16S sequences that were obtained by pyrosequencing was classified to the genus taxonomic level. The bacterial taxa were largely members of the genus *Pantoea* (53.5%), *Acinetobacter* (23.4%) and *Ralstonia* (9.2%), all belonging to the Proteobacteria (Fig. 4a). Other phylotypes within this phylum, classified as *Citrobacter*, *Klebsiella* and *Pseudomonas*, were also observed in the egg mass. Only a minor fraction of sequences were identified as *Enterococcus* (2.9%) and *Clostridium* (0.8%) belonging to the Firmicutes. Bacteria from other phyla, such as the genus *Sphingobacterium* in the Bacteroidetes phylum, were discovered in the egg samples too, but many taxa were present in low proportions (less than 0.1%).

The results from the RNA (cDNA)-based 16S rRNA data reflected the metabolically active bacterial populations, i.e., those with higher ribosomal content, suggesting candidates which may play key roles *in situ*. *Pantoea* occupied the highest relative abundance (71.1%) in the active microbial community (Fig. 4a).

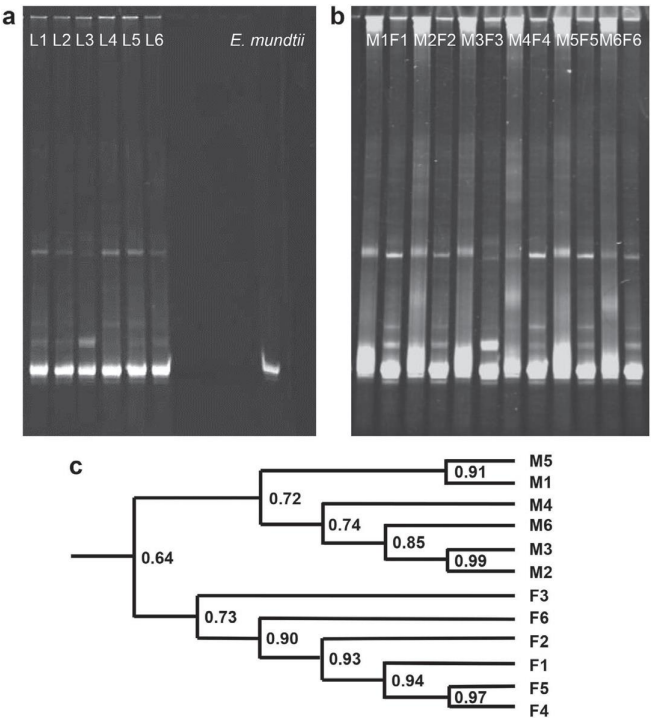


Figure 3. DGGE profiles of PCR-amplified 16S rRNA gene fragments of bacterial communities from *S. littoralis* larva and adult samples. (**a**) DGGE profile of the mature larval gut microbiota of different individuals (L = larva). (**b**) DGGE profile of the adult gut microbiota of different individuals (M = male adult and F = female adult). (**c**) Cluster analysis of the DGGE patterns of the male (M) and female (F) samples.

Citrobacter, *Klebsiella* and *Pseudomonas* were also more abundant in the RNA data set, implying their high activity. In contrast, *Ralstonia* dominated in the DNA data set (9.2%) but not in the RNA data set (0.4%), indicating low metabolic activity on the egg surface. *Acinetobacter* was also less abundant in the RNA data. Unsurprisingly, the strictly anaerobic *Clostridia* did not show activity since the egg was exposed to the air. These bacteria were present but not functioning *in situ*. The variation in bacterial composition between the DNA and RNA data sets was characterized using a Venn diagram (Fig. 4b). Overall, 35 OTUs were shared between groups. These shared OTUs represented the majority of sequences, indicating that the major phylotypes were metabolically active. There were more unique OTUs in the RNA data set (26) than in the DNA data set (9). However, most of these phylotypes were rare members of the community, representing <0.01% sequences in the total data set.

We observed that before the neonate larvae hatch out from the egg, they have already started feeding inside and have to bite enough eggshell material to make a hole before they can escape from the egg case (Supplementary video S1). Thus the microbiota associated with egg mass represents the maternal and environmental sources of gut bacteria.

Larval microbiota. Upon hatching, the neonate larva starts feeding and develops. Most bacteria associated with the eggmass were also observed in the early-instar larval gut microbiota, indicating gut symbionts are acquired by newborn hosts from the mother via egg. However, relative abundances of bacterial taxa differed largely between the microbiota of starting egg mass and that of larval gut. The taxonomic composition of the gut microbiota of early 2nd instar revealed a relatively low abundance of Proteobacteria sequences (approximately 38.9% of all sequences), in contrast to the higher level on the egg mass (95%). *Pantoea* decreased to just 23% in the larval gut (Fig. 5). Another dominant Proteobacteria that was closely related to *Citrobacter* comprised 15.6% of the community. In contrast, although only a minor fraction of *Enterococcus* was associated with the egg mass, it was particularly high in abundance (55.9%) in the larval gut. These major phylotypes were detected in both DNA and RNA data sets (Fig. 5). Although a low abundance of *Clostridium* was found in the DNA data set, it was one of dominant members of the RNA-derived fraction, indicating its high metabolic activity inside the gut.

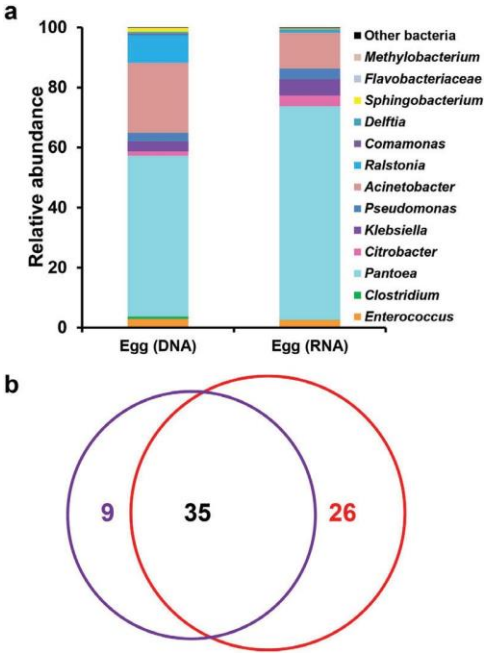


Figure 4. The microbiota associated with the egg mass of *S. littoralis*. (a) Relative abundance of major taxa (to genera level) in the DNA and RNA data sets. (b) Venn diagram showing overlaps of OTUs (at 97% similarity) between the DNA (purple circle) and RNA (red circle) data sets. Values are the numbers of OTUs calculated using the total data set.

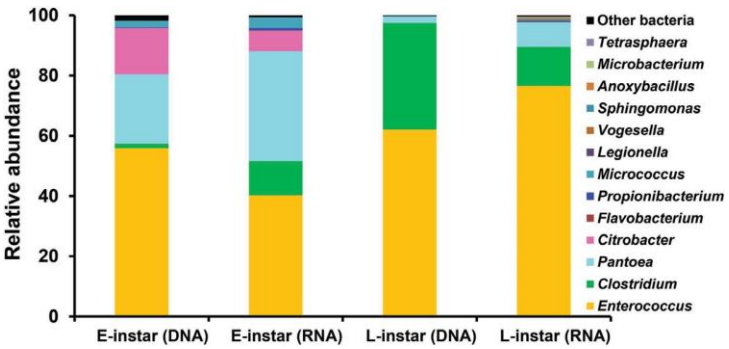


Figure 5. The larval gut microbiota of *S. littoralis*. Relative abundance of major taxa (to genera level) in the DNA and RNA data sets of early-instar larvae (E-instar) and late-instar larvae (L-instar).

The Firmicutes appeared to be replacing Proteobacteria as larvae developed. Taking advantage of PICRUSt, we predicted functional potentials of the microbial community associated with different developmental stages. The early-instar larval gut microbiome was more enriched for genes involved in cell motility, carbohydrate metabolism and transport pathways (Fig. 9). From a functional standpoint, the enrichment of these pathways could have a number of implications.

The gut microbiota consistently changed with host development. Late-instar larvae harbored a community with largely lower species diversity than that in early-instars. The Firmicutes flourished within the larval gut.

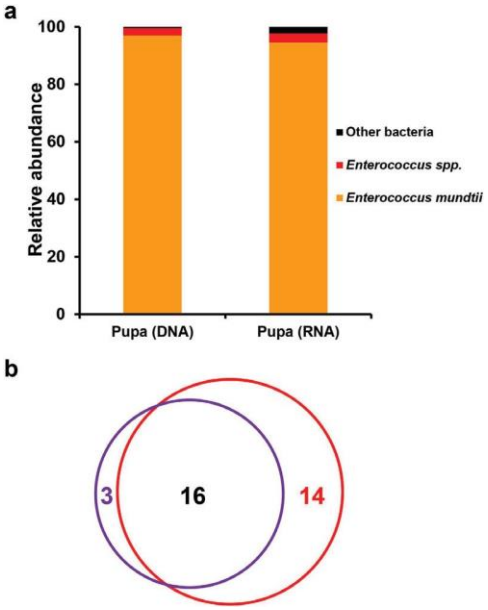


Figure 6. The microbiota associated with the pupae of *S. littoralis*. (a) Relative abundance of major taxa in the DNA and RNA data sets. (b) Venn diagram showing overlaps of OTUs (at 97% similarity) between the DNA (purple circle) and RNA (red circle) data sets.

Enterococcus was the most stable component in the microbiota, representing 62.1% of all sequences (Fig. 5). Similarly, a higher proportion (35.4%) of the gut bacterial community was found to belong to the *Clostridium* genus. On the contrary, members of the Proteobacteria phylum were significantly cleared from the larval gut microbiota; *Pantoea* was the exception, present in both stages. Each dominant genus also had a large fraction in the RNA-based data set. Functional differences were also observed in bacterial populations associated with different developmental stages. A relative increase in genes associated with amino acid, cofactor and vitamin metabolism pathways was observed in late-instar larvae (Fig. 9). Overall, despite the complex microbial diversity associated with the egg mass, the larval gut community became highly simplified through host development. The resulting gut communities were similar to each other within the population (Fig. 3a).

Metamorphosis. Each fully grown larva forms a cylindrical pupa (Fig. 1a). During metamorphosis, the overall body organization of the larva changes completely: most organs undergo deep remodeling or even completely degenerate, and differentiation processes are required to form the new body structure typical of the adult insect. The pupae show the presence of bacteria, and such bacterial populations seem to have no negative effect on traits related to the fitness of *S. littoralis*. However, the bacterial diversity in the pupal stage, indicated by the Shannon index, dropped to 0.26 from 1.46 in the late-larval stage, and 0.06 by the Simpson index (Table 1). Only enterococci dominated both RNA and DNA data sets, and they made up more than 97% of the sequences in both data sets (Fig. 6). Representative sequences of every *Enterococcus* OTU were separated from other sequences and compared with those available in the GenBank databases for more accurate identification. The representative of the most abundant OTU was identified as *E. mundtii* (Fig. 8). Several functional categories, such as those associated with carbohydrate metabolism, lipid metabolism, signal transduction, and membrane transport, diminished to 50% or more in the internal bacterial community of the pupa. Whereas genes involved in energy and nucleotide metabolism, transcription and translation pathways were abundant in the pupa, indicating a greater ability of the bacterial population to extract energy for surviving inside. After sampling, all the pupae successfully emerged and developed into adults.

Adult microbiota. After eclosion, adult moths were maintained under identical conditions for mating and fed on sugar solution because of their usual nectivorous lifestyle. The mature adults successfully mated and produced a normal amount of eggs. In light of the difference between male and female adults in their reproductive physiology and degree of nectar feeding, the internal bacterial communities associated with mature adults of each sex were studied separately. Overall, significant recolonization of the gut had been observed in adults and the richness of microbial species was restored (Table 1). However, a clear structural change in bacterial community

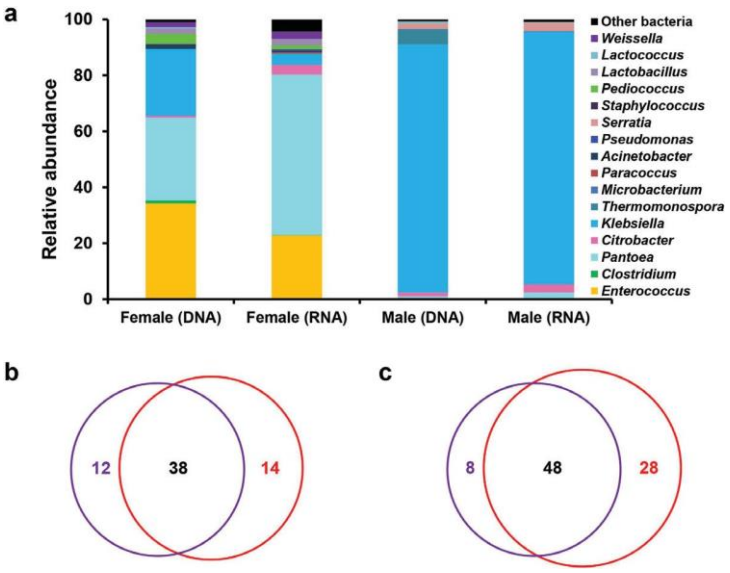


Figure 7. The adult gut microbiota of *S. littoralis*. (**a**) Relative abundance of major taxa (to genera level) in the DNA and RNA data sets of female and male adults. (**b**) Venn diagram showing overlaps of OTUs (at 97% similarity) between the DNA (purple circle) and RNA (red circle) data sets of male adult and female adult (**c**).

was identified between larva and adult, although enterococci prevailed into adulthood through metamorphosis. Phylotypes belonging to the bacterial family *Enterobacteriaceae* were overrepresented in adults relative to larvae. Furthermore, female and male adult gut microbiotas differed greatly in terms of the relative proportion of the most abundant bacteria each harbored (Fig. 7).

The bacterial community in female adults had a greater diversity relative to that in male adults. Besides *Enterococcus* occupied a considerable proportion (34.3%), other Firmicutes, including *Weissella*, *Pediococcus*, *Clostridium* and *Lactobacillus* spp., were also found in female adults (Fig. 7a). *Pantoea* were also well established in the gut flora, representing 29.7% of all sequences, while only a small proportion of *Citrobacter* was identified. The Gram-negative bacterium *Klebsiella* sp. was another prominent enterobacteria in the DNA data set (23.8%) but not in the RNA data set (4.0%). Main active bacterial taxa included *Pantoea*, *Enterococcus*, and *Citrobacter*. Interestingly, these bacteria were also found in the microbiota associated with the eggmass, indicating that certain bacteria might be transmitted to the filial generation. Venn diagrams showed that there were more OTUs in the RNA data set (Fig. 7b and c), but these phylotypes represented a low proportion of the total amount (less than 0.1%), indicating that rare phylotypes contributed to the metabolic processes in the gut.

Compared to female adults, male adults harbored a much higher proportion of microbes belonging to the familiar *Enterobacteriaceae*. Although *Pantoea* largely decreased in the male gut flora, *Klebsiella* sp. was particularly abundant in the male sample, representing >88% of all sequences, with the remaining percentage being made up of *Thermomonospora*, *Serratia* and *Citrobacter*. A similar profile was observed in the RNA data set, revealing that most taxa were active inside the gut. However, the Firmicutes, like *Enterococcus*, were maintained at a very low level in the males compared to in the females.

Using PICRUSt, we identified significant differences between the functional potentials of the bacterial community compositions (Fig. 9). These functional categories, including energy metabolism, membrane transport, transcription and biosynthesis of secondary metabolites, were enriched in the female adults, whereas in the male adults, the replication and repair pathway and associated relative gene copy numbers were increased by approximately 50%. The enrichment of several other pathways, including those for cell growth and death, and lipid metabolism, was observed in the male microbiome but not in the female.

Considering the robustness of enterococci across all stages of the host's development, we further screened the field-collected *S. littoralis* insects with the *Enterococcus*-specific primer. Field populations of *S. littoralis* were frequently associated with *E. mundtii* (Table 2), reflecting the significant role played by *E. mundtii* in host biology.

Discussion

Although accumulating studies have described the microbial diversity in the insect gut, to date there have been few reports comparing metabolic activities in the microbial populations associated with successive life stages. In the present work, we not only conducted microbial inventories of *S. littoralis* across the full host life cycle by

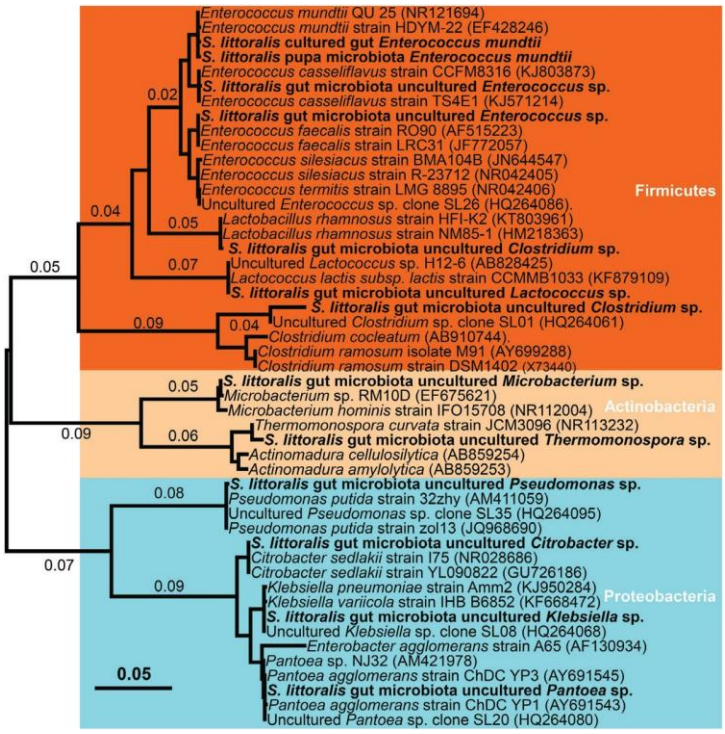


Figure 8. Phylogenetic analysis of dominant taxa identified from microbiotas associated with *S. littoralis*. Maximum-likelihood tree constructed on the basis of 16S rRNA gene sequences. Bootstrap values were obtained from a search with 500 replicates. Strain and accession numbers are given behind the species names.

sequencing the 16S rRNA gene, but we also systematically investigated metabolically active bacteria by evaluating 16S rRNA contents, which provide new insights into metabolic potentials of moth-associated microbial communities.

In general, a large proportion of OTUs (87%, representing 95% of sequences) were active within samples, indicating the host gut is a "hot spot" for diverse microbial activities. This result concurs with a previous study on the gut flora of wood-feeding huhu beetles (*Prionoplus reticularis*, Cerambycidae) that showed many bacterial phylotypes are active[15]. However, not all active bacteria could successfully colonize inside the host. Despite high diverse in starting egg mass, a significant reduction in bacterial diversity was observed during the development of *S. littoralis* from egg to pupa, highlighting the control the host has over its gut microbiota (Fig. 1). In addition, individuals subjected to the standard rearing conditions at the same developmental stages harbored communities that were highly conserved in structure and membership (Fig. 3). Overall, the microbiota of *S. littoralis* exhibits low phylum-level diversity compared to the microbiota of the wood-feeding termite or of the beetle *Odontotaenius disjunctus*[19,20]. Only a few bacterial species, mainly belonging to the phyla Firmicutes and Proteobacteria, were detected from *S. littoralis*, yet it is consistent with previous reports describing the low species richness of the microbiota in other lepidopterans. For example, tobacco hornworm, *Manduca sexta*, harbored a rather simple gut microflora consisting mostly of phylotypes belonging to *Enterococcus*[21]. A similar midgut bacterial community was revealed in the larvae of the gypsy moth (*Lymantria dispar* L.) fed on different diets, and distinct from its foliar diet[22]. The physiological and biochemical conditions within the host insect's alimentary tract appear to play an important role in structuring these communities. It is recognized that a straight alimentary canal contains fewer microorganisms. The extremely high pH (>10) in the lepidopteran larval gut could also act as a distinct selection pressure on microbial composition[23]. Considering that Lepidoptera are highly phytophagous insects, and the larval stage is most devastating, ingesting large amounts of plant materials and other potentially harmful microbes associated with their food, it makes sense for the host to efficiently control its gut microbiota and quickly clear invading microbes from its habitat. Therefore, the lepidopteran larval gut is a

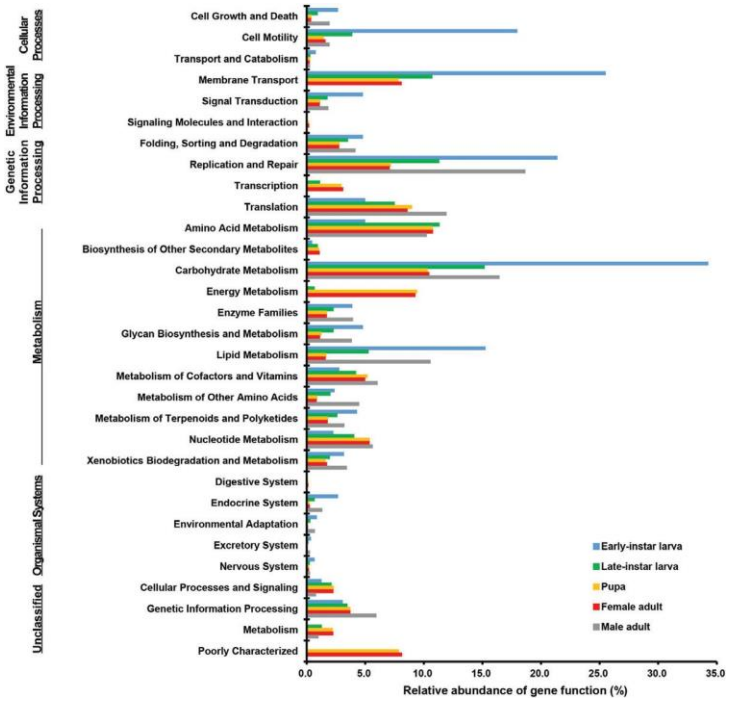


Figure 9. Inferred functions of bacterial communities associated with *S. littoralis*. All of the predicted KEGG metabolic pathways are shown at the second hierarchical level and grouped by major functional categories.

Stage of development	*n*	*Ent.* Positive	*Ent.* Positive (%)
Egg mass	5	5	100
Larva	10	8	80
Pupa	8	6	75
Adult			
Male	7	5	71
Female	9	8	89

Table 2. The frequency of association of *E. mundtii* in the field-collected *S. littoralis* samples as revealed by diagnostic PCR (*Ent.* = *E. mundtii*).

strongly selective eco-environment for its microbiome, and it may be common for larvae to maintain a relatively simple gut microbiota.

Furthermore, a developmental change in the most abundant species, from *Pantoea* and *Citrobacter* (Proteobacteria) in young larvae to *Enterococcus* and *Clostridium* (Firmicutes) in matured larvae was identified. All these dominant taxa are frequently detected in lepidopteran species. In particular, the *Enterococcus* genus successfully occupied the ecological niche and stably colonized the larval gut, despite its numerical inferiority in the egg microbiota. *Enterococci* have been found to be the most common gut bacteria in Lepidoptera, both wild and laboratory-reared populations[24–26]. For instance, *Enterococci* have been identified in the tobacco hornworm (Lepidoptera: Sphingidae), the gypsy moth (Lepidoptera: Erebidae), as well as the velvetbean caterpillar (Lepidoptera: Noctuidae), suggesting these bacteria perform some conserved functions in this highly phytophagous insect. Large amounts of *Citrobacter*, a genus within the *Enterobacteriaceae* family, occurred in the neonate larvae. Although this bacterium is known to form host associations with a variety of insects[21], its biological relevance remains unclear. *Pantoea*, another highly versatile and diverse enterobacteria, have been isolated from

many environments[27] and consist of taxa with known capacities for degrading and utilizing different types of plant materials. As such, *Pantoea*, are putatively helpful bacteria for herbivores. *Clostridium* emerged as dominant commensals only in the mature larval gut. In the late instar, the larval gut exhibits a prevailing anoxic atmosphere, which favors the development of anaerobic microorganisms, such as *Clostridia*, and facultative anaerobic enterococci[10]. This might be the dominant force influencing the shift of gut microflora composition from Proteobacteria to Firmicutes.

This difference in taxonomic membership may reflect divergent functional roles across particular life stages. Analyses of metabolic activity, based on the RNA, suggested these taxa actively function *in vivo* (Fig. 5). PICRUSt builds a predictive understanding of the functions of these symbionts within the host (Fig. 9). The gut microbiome was significantly enriched for genes involved in the carbohydrate metabolism pathway. The dominant *Gammaproteobacteria* in the family *Enterobacteriaceae* are well equipped to degrade major structural components of plant materials. *Pantoea* spp. can produce diverse enzymes, including β-galactosidases (GH2), α-xylosidases (GH31), α-mannosidases (GH47), and α-rhamnosidases (GH78), as well as pectinesterases (CE8) involved in the plant polymer degradation[28]. It has been discovered that *Citrobacter amalonaticus* is capable of breaking down chitin, reflecting the metabolic diversity of *Gammaproteobacteria*. These Proteobacteria symbionts could play similar functions in *S. littoralis* and might be important nutrient providers for host insects in their early life stages. Much research in insects and other animals has shown that increases in the Firmicutes are related to an increased ability to harvest energy from the diet. *Clostridia* species such as *C. thermocellum* and *C. ljungdahlii* are known to have a robust capacity to degrade cellulose and hemicellulose, and to metabolize amino acids[29]. The presence of a large proportion of *Clostridia* is likely to be important for efficient biomass utilization. Therefore, those bacterial symbionts likely also play important roles in nutrition. Data from PICRUSt is further supported by previous work, which employed comparative genomic analysis of the microbiome of the cutworm *Agrotis ipsilon* (Lepidoptera: Noctuidae)[30]. The predominance of *Enterococcus* and its high metabolic activity suggest that this bacterium has a functional significance with regard to its host. As members of the gypsy moth gut flora, enterococci have been shown to prevent colonization by pathogens[31]. In this study, we found that genes involved in the metabolism of terpenoids and polyketides are consistently expressed in the *S. littoralis* microbiome (Fig. 9). The isolated *E. mundtii* symbionts also have the ability to produce antimicrobials. Thus the dominant *E. mundtii* bacteria are most likely to be defensive mutualists. Altogether, the characteristic gut microbiota found in *S. littoralis* larvae may provide various benefits to the lepidopteran host ranging from nutrient supplementation to host defense.

Lepidopterans are holometabolous, and the transition from larvae to adult is a metabolically dynamic and complex process. The host gut microbiota also undergoes significant structural changes during metamorphosis and in the adult stage. Although the gut during the transition from larvae to adult is believed to undergo sterilization process and adults recruit new microbiota[32], it is interesting to observe here that *Enterococcus* species, mostly *E. mundtii*, are able to survive the metamorphosis and be transmitted to the emerged adults (Figs 6 and 7). Genes involved in energy and nucleotide metabolism, transcription and translation pathways were enriched in this enterococcal population. However, their exact roles inside the pupa are not understood and warrant further investigation.

The adult lepidopteran microbiota remains largely unexplored. There have been no previous culture-independent studies of microbial communities associated with adult moths. Because of their nectivorous lifestyle, the adult moth typically has a small and morphologically distinct gut in contrast to that of the larva. We found that *S. littoralis* adults host relatively complex bacterial communities, and the microbial community structure of the female adult differs from that of the male (Fig. 7). Firmicutes, mostly enterococci, formed a significant proportion of the female adult gut microbiota, while those bacteria remained at low levels in male adults. *Pantoea* and *Klebsiella* were another dominant taxa in female adults, whereas only *Klebsiella* was observed in male adults. Both *Pantoea* and *Klebsiella* belong to the Proteobacteria family *Enterobacteriaceae*, which occur widely in the guts of Lepidoptera and other herbivores and are potentially beneficial, nonpathogenic microbes[28,33]. Using level 2 KEGG predictions of ortholog function, differences between the functional potentials of the bacterial communities were also observed. The female adult microbiome was enriched for genes relevant to energy metabolism, while in the male adult, an increase in the replication and repair pathway was detected.

We know from studies on other holometabolous insect groups that adults may have similar microbiotas[34], or different microbiotas as larvae[35], or have sexually dimorphic microbiotas[36]. For example, the gut of adult cockchafer beetle *Melolontha hippocastani* housed the same microbial species that were present in the larval midgut, despite having metamorphosed from larva to beetle[34]. In contrast, a developmental change in the most abundant gut bacteria was identified in the fruitfly *Drosophila melanogaster*[35]. Notably, the bacterial composition of adult black flies *Simulium* spp. differed between males and females although they were collected from the same habitat[36]. Similarly, *Klebsiella* sp. was demonstrated to be relatively high in adult males of *Anopheles stephensi* but was not found in larvae and pupae[37]. Sexually dimorphic phenomena of the associated bacterial communities have also been reported in other animals. For example, female Antarctic seals harbor more Firmicutes in the gut, while males have more Fusobacteria[38]. Although the reasons for this shift are not well understood, several factors, including the radical change of internal physicochemical conditions in the digestive tract, host immune responses and disturbance might underlie this difference.

Interestingly, major taxa associated with the female adult, such as *Enterococcus*, were also detected in the eggmass, and these taxa further colonized the larval gut, suggesting that some gut symbionts are probably vertically transmitted. The maternal transmission of the core gut microbiota to the next generation might stabilize host-microbe interactions and facilitate co-evolution.

Recently, several comparative genomic and metagenomic studies of lepidopteran species have revealed an ancient and intimate relationship between bacteria and lepidopteran herbivores. It is reported that a gene encoding the enzyme that detoxifies plant-produced cyanide did not evolve in Lepidoptera but was horizontally

transferred from bacteria[39]. Clearly, the gut microbiota is an important source for diverse microbial activities and a "hot spot" for microbe-host interactions. A better understanding of the relationship of microbial symbionts to the lepidopteran host would lead to new concepts and approaches to control insect pests by manipulating their microbiota. Additionally, *S. littoralis* provides an attractive model for exploring complex microbial symbioses, as it has a simplified gut structure and microbial community, and is now genetically amenable[40]. The current study helps advance our understanding of ecological and evolutionary roles of gut symbionts in an important insect group.

Methods

Insect rearing, field collections and sample processing. *S. littoralis* larvae were hatched from eggs and reared on artificial diet as previously described[41]. Plastic cabinets with the diet and the larvae were kept at 23–25 °C under a regime of 16 h illumination and a dark period of 8 h. The emerged adults were supplied with a sucrose solution. The field population was collected from a vegetable gardening area in the vicinity of Hangzhou, China, in August 2015. The egg masses, larvae, pupae and adults were transported to the laboratory in Petri dishes and kept at −20 °C prior to dissection. The eggs' hatching process was recorded by a video camera.

For sample processing, all insects were first rinsed three times in sterile water, surface-sterilized in 70% ethanol for 30 s and rinsed again in sterile water. The whole gut tissue was dissected from each individual and homogenized for nucleic acid extraction, as previously described[41]. After dissection, the typical vitellogenic ovariole was observed in the mature female. The whole surface-sterilized pupa was used to investigate the internal bacteria. Egg masses were not surface-sterilized. The processed samples were first aseptically homogenized in 500 µL of sterile PBS. A serial dilution of 10-fold was performed by transferring 100 µL of the homogenized sample into 900 µL PBS, vortexing vigorously, and spread-plating 100 µL of each dilution onto Brain-heart infusion agar plates (8130, BD). All plates were incubated at 37 °C for 48 h. Total bacterial cells were counted as colony forming units (CFUs) for each sample.

Nucleic acid extraction and reverse transcription. The dissected insect tissues ($n = 6$ at each stage) were first ground under liquid N_2 with single-use, Eppendorf tube-adapted sterile pestles and then directly incubated with nucleic acid extraction solution, according to the manufacturer's protocols (MC85200, Epicentre) with minor modifications. An additional lysozyme incubation step (30 min at 37 °C) was included to break up Gram-positive bacterial cells. The quality of extracted total nucleic acid was checked on the agarose gel and quantified using a NanoDrop 1000 (Thermo Scientific). DNA and RNA were further purified from the extracted total nucleic acid following manufacturer's guidelines.

Extracted RNA was reverse-transcribed into cDNA using the QuantiTect Reverse Transcription kit (205311, Qiagen) according to the manufacturer's guidelines. RNA was first treated with genomic DNA Wipeout buffer at 42 °C for 2 min to eliminate any trace of co-extracted DNA. A volume of 7 µL of the DNase-treated RNA was used for reverse transcription to cDNA in a total reaction volume of 10 µL using random primers. Two negative controls were performed, including 7 µL of DNase-treated RNA with all RT reagents except for the reverse transcriptase and 7 µL of RT-PCR grade water instead of RNA.

Denaturing gradient gel electrophoresis (DGGE) of amplified 16S rRNA genes. PCR primers 968F/1401R were used to amplify the V6-V8 portion of 16S rRNA genes as previously described[26]. Archaea- and fungus-specific primers were used to amplify archaeal 16S and fungus ITS genes, respectively (Supplementary table S1)[42,43]. DGGE analysis was performed using the Bio-Rad DCode system. Electrophoresis was done using a 16 × 16 cm, 1 mm thick gel that contained 8% polyacrylamide with a 20 to 80% denaturant gradient (100% denaturant was 7 M urea and 40% (v/v) deionized formamide). The gels were run at 100 V for 16 h at 60 °C in TAE buffer (40 mM Tris–acetate, 1 mM EDTA; pH 7.4). After electrophoresis, the gels were stained for 30 min in TAE buffer with SYBR-Gold nucleic acid gel stain (S-11494, Invitrogen) for photographing. Gels were scanned using a GS-800 calibrated densitometer (Bio-Rad). Analysis of DGGE profiles (band match and clustering) was carried out using Quantity One software (version 4. 6.1; Bio-Rad), as described previously[44].

Pyrosequencing, data analysis and PCR screen. Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) was performed using a Roche 454 FLX instrument with Titanium reagents as described previously[41]. Basically, the hypervariable V1–V3 segment in the 16S rDNA was amplified using the fusion primer set Gray28F (5-GAGTTTGATCNTGGCTCAG-3) and Gray519r (5-GTNTTACNGCGGCKGCTG-3) extended with the respective primer Adaptor A/B and sample-specific multiplex identifiers (MID). The sequencing library was generated through one-step PCR with 30 cycles, using a Hot Start High Fidelity Taq Polymerase (Qiagen). Amplicons were sequenced based on the supplier protocol (Research and Testing Laboratory, Lubbock, TX, USA, http://www.researchandtesting.com). The reads extended from the forward direction (Gray28F), and all low-quality reads (quality cut-off = 25) and sequences <200 bp in length were removed following sequencing (Supplementary table S2).

The software package Quantitative Insight into Microbial Ecology (QIIME, 1.6.0 version) was used to process sequencing data and to calculate diversity[45]. Sequences first underwent quality control to remove potential artifacts and errors (the denoise_wrapper.py script in QIIME was used in our analysis) and trimmed of the part with low quality[46]. Chimera (detection method: ChimeraSlayer) and low abundance reads (<0.1%) were further removed from analysis[47]. Cdhit and uclust with 97% similarity cut-offs were used in multiple OTU picking to cluster the high-quality reads into operational taxonomic units (OTUs). For each OTU, the most abundant sequence was extracted as a representative sequence for each OTU picked and aligned to the Greengenes core set (http://greengenes.lbl.gov/) using PyNAST with the minimum sequence identity percent set to 75%[48]. The RDP classifier was employed to determine the highest resolution of taxonomy based on the Ribosomal Database

Project (http://rdp.cme.msu.edu/). 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. UniFrac was used for microbial community comparison according to Lozupone *et. al*[18]. Shared and unique OTUs are graphically represented in Venn diagrams described elsewhere[15]. The identification of OTUs that were significantly different in abundance was carried out in METASTATS using the nonparametric t-test against the taxonomic data extracted from QIIME[49]. The significance level to threshold (P value) was set at 0.05. Phylogenetic trees were calculated using the Maximum Likelihood method (Tamura-Nei model) with 500 bootstrap replicates in MEGA5[50].

To generate a synthetic metagenome, the observed 16S rDNA sequences were clustered into a collection of OTUs using the pick closed reference otus.py script in QIIME. The resultant biom-formatted OTU table was first normalized with respect to inferred 16S rRNA gene copy numbers and then used to predict metagenomic functional content based on the software package Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt)[17]. This computational approach exploits the relationship between phylogeny and function by combining 16s data with a database of reference genomes (Greengenes) to predict the presence of gene families. Functional predictions were exported as KEGG orthologs.

To show that *E. mundtii* is associated with the field population of *S. littoralis*, a primer specific for *E. mundtii* was utilized to screen for the symbiont using diagnostic PCR reactions (Table 2)[51]. PCR amplifications were conducted on a Mastercycler Gradient Thermocycler (Eppendorf, Germany) using 20 µL reactions, including 1 µL of DNA template, 1 × PCR buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl], 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.5 µM of each primer, and 0.1 µL of Taq DNA polymerase (18038, Invitrogen). The following cycle parameters were used: 3 min at 94 °C, followed by 35 cycles of 94 °C for 45 s, 60 °C for 30 s, and 72 °C for 1 min, and a final extension time of 10 min at 72 °C.

Deposition of nucleotide sequences. The sequences obtained in this study were deposited in the GenBank short-read archive (SRA), accession number SRR2886919 and 3260963.

References

1. Pinto-Tomas, A. A. *et al.* Symbiotic nitrogen fixation in the fungus gardens of leaf-cutter ants. *Science* **326,** 1120–1123 (2009).
2. Koch, H. & Schmid-Hempel, P. Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. *Proc Natl Acad Sci USA* **108,** 19288–19292 (2011).
3. Shin, S. C. *et al.* Drosophila microbiome modulates host developmental and metabolic homeostasis via insulin signaling. *Science* **334,** 670–674 (2011).
4. Zheng, L. *et al.* Bacteria mediate oviposition by the black soldier fly, Hermetia illucens (L.), (Diptera: Stratiomyidae). *Sci Rep* **3,** 2563 (2013).
5. Sun, Z. *et al.* Effects of BmCPV Infection on Silkworm Bombyx mori Intestinal Bacteria. *PLoS One* **11,** e0146313 (2016).
6. Russell, J. A. *et al.* Bacterial gut symbionts are tightly linked with the evolution of herbivory in ants. *Proc Natl Acad Sci USA* **106,** 21236–21241 (2009).
7. Vasquez, A. *et al.* Symbionts as major modulators of insect health: lactic Acid bacteria and honeybees. *PLoS One* **7,** e33188 (2012).
8. Krenn, H. W. Feeding mechanisms of adult Lepidoptera: structure, function, and evolution of the mouthparts. *Annu Rev Entomol* **55,** 307–327 (2010).
9. Visotto, L. E., Oliveira, M. G., Ribon, A. O., Mares-Guia, T. R. & Guedes, R. N. Characterization and identification of proteolytic bacteria from the gut of the velvetbean caterpillar (Lepidoptera: Noctuidae). *Environ Entomol* **38,** 1078–1085 (2009).
10. Tang, X. *et al.* Complexity and variability of gut commensal microbiota in polyphagous lepidopteran larvae. *PLoS One* **7,** e36978 (2012).
11. Hammer, T. J., McMillan, W. O. & Fierer, N. Metamorphosis of a butterfly-associated bacterial community. *PLoS One* **9,** e86995 (2014).
12. Legeai, F. *et al.* An Expressed Sequence Tag collection from the male antennae of the Noctuid moth Spodoptera littoralis: a resource for olfactory and pheromone detection research. *BMC genomics* **12,** 86 (2011).
13. Mattila, H. R., Rios, D., Walker-Sperling, V. E., Roeselers, G. & Newton, I. L. Characterization of the active microbiotas associated with honey bees reveals healthier and broader communities when colonies are genetically diverse. *PLoS One* **7,** e32962 (2012).
14. Gaidos, E., Rusch, A. & Ilardo, M. Ribosomal tag pyrosequencing of DNA and RNA from benthic coral reef microbiota: community spatial structure, rare members and nitrogen-cycling guilds. *Environ Microbiol* **13,** 1138–1152 (2011).
15. Reid, N. M., Addison, S. L., Macdonald, L. J. & Lloyd-Jones, G. Biodiversity of Active and Inactive Bacteria in the Gut Flora of Wood-Feeding Huhu Beetle Larvae (Prionoplus reticularis). *Appl Environ Microbiol* **77,** 7000–7006 (2011).
16. Rodriguez-Blanco, A., Ghiglione, J. F., Catala, P., Casamayor, E. O. & Lebaron, P. Spatial comparison of total vs. active bacterial populations by coupling genetic fingerprinting and clone library analyses in the NW Mediterranean Sea. *FEMS Microbiol Ecol* **67,** 30–42 (2009).
17. Langille, M. G. I. *et al.* Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat Biotechnol* **31,** 814–21 (2013).
18. Lozupone, C., Lladser, M. E., Knights, D., Stombaugh, J. & Knight, R. UniFrac: an effective distance metric for microbial community comparison. *ISME J* **5,** 169–172 (2011).
19. Warnecke, F. *et al.* Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. *Nature* **450,** 560–565 (2007).
20. Ceja-Navarro, J. A. *et al.* Compartmentalized microbial composition, oxygen gradients and nitrogen fixation in the gut of Odontotaenius disjunctus. *ISME J* **8,** 6–18 (2013).
21. Brinkmann, N., Martens, R. & Tebbe, C. C. Origin and diversity of metabolically active gut bacteria from laboratory-bred larvae of Manduca sexta (Sphingidae, Lepidoptera, Insecta). *Appl Environ Microbiol* **74,** 7189–7196 (2008).
22. Mason, C. J. & Raffa, K. F. Acquisition and structuring of midgut bacterial communities in gypsy moth (lepidoptera: erebidae) larvae. *Environ Entomol* **43,** 595–604 (2014).
23. Funke, M. *et al.* Rapid hydrolysis of quorum-sensing molecules in the gut of lepidopteran larvae. *Chembiochem* **9,** 1953–1959 (2008).
24. Broderick, N. A., Raffa, K. F., Goodman, R. M. & Handelsman, J. Census of the bacterial community of the gypsy moth larval midgut by using culturing and culture-independent methods. *Appl Environ Microbiol* **70,** 293–300 (2004).
25. Xiang, H. *et al.* Microbial communities in the larval midgut of laboratory and field populations of cotton bollworm (Helicoverpa armigera). *Can J Microbiol* **52,** 1085–1092 (2006).

26. Shao, Y., Arias-Cordero, E., Guo, H., Bartram, S. & Boland, W. *In vivo* Pyro-SIP assessing active gut microbiota of the cotton leafworm, Spodoptera littoralis. *PLoS One* **9,** e85948 (2014).
27. Walterson, A. M. & Stavrinides, J. Pantoea: insights into a highly versatile and diverse genus within the Enterobacteriaceae. *FEMS Microbiol Rev* **39,** 968–984 (2015).
28. Suen, G. *et al.* An insect herbivore microbiome with high plant biomass-degrading capacity. *PLoS genet* **6,** e1001129 (2010).
29. Fonknechten, N. *et al.* Clostridium sticklandii, a specialist in amino acid degradation: revisiting its metabolism through its genome sequence. *BMC Genomics* **11,** 555 (2010).
30. Shi, W. *et al.* Comparative genomic analysis of the microbiome [corrected] of herbivorous insects reveals eco-environmental adaptations: biotechnology applications. *PLoS genet* **9,** e1003131 (2013).
31. van Frankenhuyzen, K., Liu, Y. & Tonon, A. Interactions between Bacillus thuringiensis subsp. kurstaki HD-1 and midgut bacteria in larvae of gypsy moth and spruce budworm. *J Invertebr Pathol* **103,** 124–131 (2010).
32. Kohl, K. D., Cary, T. L., Karasov, W. H. & Dearing, M. D. Restructuring of the amphibian gut microbiota through metamorphosis. *Env Microbiol Rep* **5,** 899–903 (2013).
33. Chu, C. C., Spencer, J. L., Curzi, M. J., Zavala, J. A. & Seufferheld, M. J. Gut bacteria facilitate adaptation to crop rotation in the western corn rootworm. *Proc Natl Acad Sci USA* **110,** 11917–11922 (2013).
34. Arias-Cordero, E. *et al.* Comparative evaluation of the gut microbiota associated with the below- and above-ground life stages (larvae and beetles) of the forest cockchafer, Melolontha hippocastani. *PLoS One* **7,** e51557 (2012).
35. Wong, C. N., Ng, P. & Douglas, A. E. Low-diversity bacterial community in the gut of the fruitfly Drosophila melanogaster. *Environ Microbiol* **13,** 1889–1900 (2011).
36. Tang, X., Adler, P. H., Vogel, H. & Ping, L. Gender-specific bacterial composition of black flies (Diptera: Simuliidae). *FEMS Microbiol Ecol* **80,** 659–670 (2012).
37. Rani, A., Sharma, A., Rajagopal, R., Adak, T. & Bhatnagar, R. K. Bacterial diversity analysis of larvae and adult midgut microflora using culture-dependent and culture-independent methods in lab-reared and field-collected Anopheles stephensi-an Asian malarial vector. *BMC Microbiol* **9,** 96 (2009).
38. Nelson, T. M., Rogers, T. L., Carlini, A. R. & Brown, M. V. Diet and phylogeny shape the gut microbiota of Antarctic seals: a comparison of wild and captive animals. *Environ Microbiol* **15,** 1132–1145 (2013).
39. Wybouw, N. *et al.* A gene horizontally transferred from bacteria protects arthropods from host plant cyanide poisoning. *eLife* **3,** e02365 (2014).
40. Hakim, R. S., Baldwin, K. & Smagghe, G. Regulation of midgut growth, development, and metamorphosis. *Annu Rev Entomol* **55,** 593–608 (2010).
41. Shao, Y., Arias-Cordero, E. M. & Boland, W. Identification of metabolically active bacteria in the gut of the generalist Spodoptera littoralis via DNA stable isotope probing using 13C-glucose. *J Vis Exp* **81,** e50734 (2013).
42. Anderson, I. C. & Cairney, J. W. G. Diversity and ecology of soil fungal communities: increased understanding through the application of molecular techniques. *Environ Microbiol* **6,** 769–779 (2004).
43. Hershberger, K. L., Barns, S. M., Reysenbach, A. L., Dawson, S. C. & Pace, N. R. Wide diversity of Crenarchaeota. *Nature* **384,** 420 (1996).
44. Thompson, C. L., Wang, B. & Holmes, A. J. The immediate environment during postnatal development has long-term impact on gut community structure in pigs. *ISME J* **2,** 739–748 (2008).
45. Caporaso, J. G. *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7,** 335–336 (2010).
46. Reeder, J. & Knight, R. Rapidly denoising pyrosequencing amplicon reads by exploiting rank-abundance distributions. *Nat Methods* **7,** 668–669 (2010).
47. Haas, B. J. *et al.* Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Res* **21,** 494–504 (2011).
48. Caporaso, J. G. *et al.* PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* **26,** 266–267 (2010).
49. White, J. R., Nagarajan, N. & Pop, M. Statistical Methods for Detecting Differentially Abundant Features in Clinical Metagenomic Samples. *PLoS Comput Biol* **5,** e1000352 (2009).
50. Tamura, K. *et al.* MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28,** 2731–2739 (2011).
51. Kubota, H. *et al.* Detection of human intestinal catalase-negative, Gram-positive cocci by rRNA-targeted reverse transcription-PCR. *Appl Environ Microbiol* **76,** 5440–5451 (2010).

Acknowledgements

The assistance of Yitao He in the care of insects and collection of samples is gratefully acknowledged. We thank Jan Grueneberg for DGGE analysis; Angelika Berg for technical assistance; and Emily Wheeler for editorial assistance. This work was supported by the "Hundred Talents Program" (to Y. Shao) from Zhejiang University, China, the Jena School of Microbial Communication (JSMC), the Max Planck Society, the Modern Agricultural Technology System (No. CARS-22-ZJ0202) and the National Natural Science Foundation of China (Grant No. 31302033 to X. Lu).

Author Contributions

Y. S. and W. B. contributed to the initial design of the research. B. C. and B. T. conducted the experimental and C. S. performed bioinformatics analyses with guidance from Y. S. S. H. and X. L. collected field samples. Y. S. prepared the draft of this publication and all authors contributed to the subsequent stages of manuscript preparation.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Chen, B. *et al.* Biodiversity and Activity of the Gut Microbiota across the Life History of the Insect Herbivore *Spodoptera littoralis*. *Sci. Rep.* **6**, 29505; doi: 10.1038/srep29505 (2016).

5. Article III

frontiers in Microbiology

DATA REPORT
published: 25 October 2016
doi: 10.3389/fmicb.2016.01676

Draft Genome Sequence of *Enterococcus mundtii* SL 16, an Indigenous Gut Bacterium of the Polyphagous Pest *Spodoptera littoralis*

Bosheng Chen [1†], Chao Sun [2†], Xili Liang [1], Xingmeng Lu [1], Qikang Gao [2], Pol Alonso-Pernas [3], Beng-Soon Teh [3], Alexey L. Novoselov [3], Wilhelm Boland [3] and Yongqi Shao [1*]

[1] Laboratory of Invertebrate Pathology, College of Animal Sciences, Zhejiang University, Hangzhou, China, [2] Analysis Center of Agrobiology and Environmental Sciences, Zhejiang University, Hangzhou, China, [3] Department of Bioorganic Chemistry, Max Planck Institute for Chemical Ecology, Jena, Germany

Keywords: ***Enterococcus mundtii*, genome sequencing, symbiosis, *Spodoptera littoralis*, intestinal tract**

OPEN ACCESS

Edited by:
Malka Halpern,
University of Haifa, Israel

Reviewed by:
John Everett Parkinson,
Oregon State University, USA
Peng Bao,
Institute of Urban Environment (CAS), China

***Correspondence:**
Yongqi Shao
yshao@zju.edu.cn

[†] *These authors have contributed equally to this work.*

Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: *26 August 2016*
Accepted: *06 October 2016*
Published: *25 October 2016*

Citation:
Chen B, Sun C, Liang X, Lu X, Gao Q, Alonso-Pernas P, Teh B-S, Novoselov AL, Boland W and Shao Y (2016) Draft Genome Sequence of Enterococcus mundtii SL 16, an Indigenous Gut Bacterium of the Polyphagous Pest Spodoptera littoralis. Front. Microbiol. 7:1676. doi: 10.3389/fmicb.2016.01676

INTRODUCTION

Insects are the most abundant and diverse animal class on Earth, and they are associated with an amazing variety of symbiotic microorganisms, which participate in many relationships with the hosts (Douglas, 2015). For example, the fungal symbiont (*Leucoagaricus gongylophorus*) of leaf-cutting ants produces diverse enzymes for the degradation of plant material (Kooij et al., 2016). Similarly, *Bacillus pumilus* isolated from the gut of wood boring *Mesomorphus* sp. (Coleoptera: Tenebrionidae) exhibits significant cellulolytic and xylose isomerase activities (Balsingh et al., 2016).

The Lepidoptera, including moths and butterflies, is one of the most widespread and widely recognizable insect orders in the world. Although butterflies and moths play an important role in the natural ecosystem as pollinators and as food in the food chain, their leaf-chewing larvae are often problematic in agriculture, as their main source of food is live plants (Mithöfer and Boland, 2012). The leafworm *Spodoptera littoralis* (Lepidoptera: Noctuidae) is a highly polyphagous lepidopteran pest found worldwide and also an important model system used in a variety of biological research. Recent extensive surveys of its microbiome reveal that *Enterococcus mundtii* is one of the predominant gut microorganisms of *S. littoralis* and present at high frequency (Tang et al., 2012; Chen et al., 2016; Teh et al., 2016). Particularly, a stable isotope labeling-based approach suggested that this phylotype was also highly metabolically active inside the host across life history of *S. littoralis*, indicating the significant role played by *E. mundtii* in host biology (Shao et al., 2014). Therefore, the symbiotic *E. mundtii* probably constitutes a key factor for the success of this generalist herbivore in adapting to different environments and food sources. The aim of this study was to produce a genome sequence of the strain SL 16, which would assist in understanding of the

coevolution of the microbe and the insect host. The dataset has been submitted to NCBI Whole Genome Shotgun (WGS) projects and is reported here, providing an overview of the genome sequence and relevant features of gut symbiotic *E. mundtii*.

MATERIALS AND METHODS

Isolation of the Bacterial Strain

E. mundtii strain SL 16 was isolated from the mature 5th instar larva using standard microbiology methods. Briefly, the normal larvae were washed and sedated on ice for at least 1 h to anesthetize them. Then the whole gut sections were dissected from larvae using a fine Vannas scissor and forceps under a binocular microscope (Shao et al., 2013). The fresh gut tissues were put into phosphate buffered saline (PBS: 137 mM NaCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, and 2.7 mM KCl) and homogenized by hand with a sterile pestle. Bacterial isolates were made by plating the homogenized gut tissues on the *Enterococcus* Selective Agar (45183, Fluka). After incubation for 24 h at 30°C, the growing bacterial colonies were sub-cultured twice on the same agar medium. 2, 3, 5-Triphenyltetrazolium chloride (TTC) in the medium is reduced to insoluble formazan inside the bacterial cells, which gives pink or red coloration to enterococcal colonies. These purified enterococcal colonies were tested for key phenotypic traits including carbohydrate fermentation capability, motility, and pigment production as previously described (Manero and Blanch, 1999). Furthermore, the taxonomy was validated by colony PCR and sequencing of the amplified 16S rRNA gene. The representative *E. mundtii*

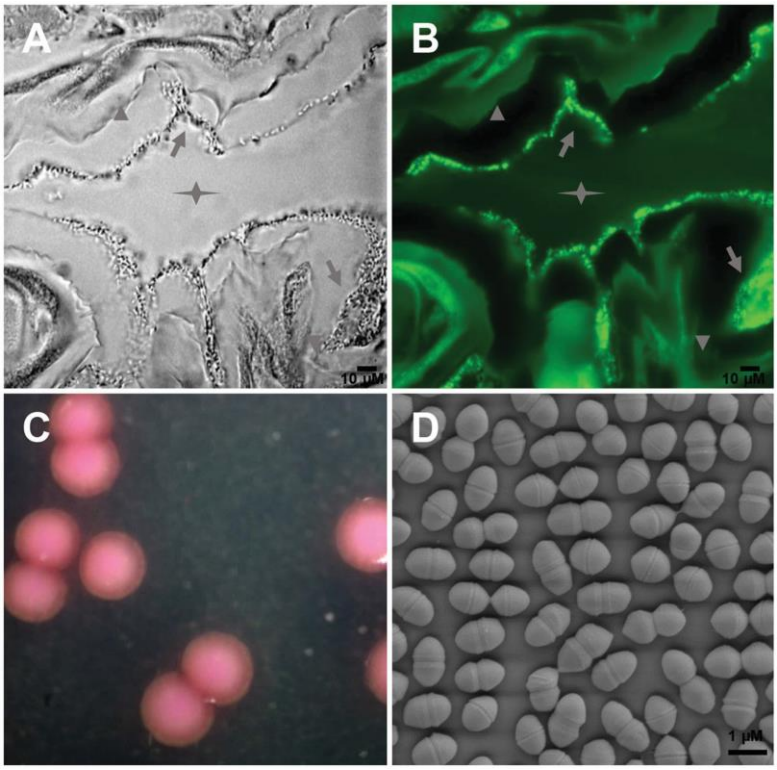


FIGURE 1 | Images of *E. mundtii* from *S. littoralis* reveal bacterial gut localization and phenotypic characteristics. (A) Phase-contrast micrograph and **(B)** FISH with a FITC-labeled Enterococcus-specific probe (green) show a high density of bacterial cells adhere on the mucus layer lining the gut epithelium. Star indicates the gut lumen, arrowhead indicates the gut epithelium tissue, and arrow indicates bacteria. **(C)** Photomicrograph of source organism on Enterococcus selective agar. 2, 3, 5-Triphenyltetrazolium chloride (TTC) in the agar is reduced to insoluble formazan inside the bacterial cells, which gives pink or red coloration to colonies. **(D)** Scanning electron micrograph of *E. mundtii* SL 16, showing cell division.

isolate, designated strain SL 16, was selected for this WGS project.

Fluorescence in situ Hybridization (FISH) was applied to localize the dominant enterococci as previously described (Shao et al., 2014). Shortly, 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). The sample was hybridized with 1.5 mM FITC-labeled *Enterococcus*-specific probe in hybridization buffer containing 900 mM NaCl, 20 mM Tris-HCl (pH 8.0), 20% formamide, 1% SDS. And images were taken with an Axio Imager Z1 microscope (Carl Zeiss, Jena, Germany). For scanning electron microscopy (SEM), cells were fixed in paraformaldehyde (1%), and glutaraldehyde (0.25%), dehydrated by ascending alcohol series and dried. After coating samples with gold, scanning electron micrographs were taken with a LEO 1525 instrument (Carl Zeiss, Jena, Germany).

Genomic DNA Isolation, Library Preparation and Sequencing

The genomic DNA was extracted from the cultured bacterium according to Pospiech and Neumann (1995). DNA quality was examined by 1% agarose gel electrophoresis and quantified using a NanoDrop™ spectrophotometer. The DNA library was constructed using the TruSeq™ DNA Sample Preparation Kit (Illumina Inc., San Diego, CA), and 5 μg of pure genomic DNA was prepared for a standard Illumina shotgun library construction. Briefly, genomic DNA was first sheared to a size ranging between 400 and 500 bp using the Covaris M220 per the manufacturer's recommendations. The fragmented DNA sample was end-repaired, dA-tailed, and ligated to multiplex adapters according to the manufacturer's instructions. The ligated products were purified and further enriched using PCR. The quality of the final amplified libraries were checked by running an aliquot (1 μL) on a high-sensitivity Bioanalyzer 2100 DNA Chip (Agilent Technologies). Paired-end sequencing was performed by using an Illumina MiSeq platform (Illumina Inc., San Diego, CA) at Majorbio Bio-pharm Technology Co., Ltd (Shanghai, China) according to the manufacturer's instructions (Zhang et al., 2016).

Preprocessing and Genome Assembly

The quality of sequence reads was evaluated using the FastQC tool as previously described (Balsingh et al., 2016). Reads with >10% Ns and/or 25–35 bases of low quality (≤Q20) were filtered out, and adapter and duplication contamination were removed as well as read ends were trimmed off. The filtered reads were assembled with Short Oligonucleotide Analysis Package (SOAP) *de novo* version 2.04 using a range of *k*-mer sizes (Li et al., 2009). Then GapCloser version 1.12 was used to close any internal gaps in the optimal scaffolded assembly. Repeats were predicted by RepeatMasker and Tandem Repeats Finder (TRF) tools (Rédou et al., 2016). Barrnap version 0.4.2 and tRNAscan-SE version 1.3.1 were employed to predict rRNAs and tRNAs respectively. The genome was annotated using Glimmer version 3.02 (Xu et al., 2014). The Clusters of Orthologous Groups of proteins (COG) categories were assigned to the SL 16 genome annotation using blastp (BLAST 2.2.28+) against the COG genes collection (Von Mering et al., 2005). The translations of the identified coding sequences (CDSs) were also used to search against the Protein family (Pfam) database with *E*-value cut-off of 1-e5. The metabolic pathway analysis was constructed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2014).

INTERPRETATION OF DATA SET

Whole Genome Sequencing of E. mundtii SL 16

Large amounts of *E. mundtii* closely adhere to the mucosal layer of *S. littoralis* gut epithelium, where they form a biofilm-like structure (**Figures 1A,B**). Strain SL 16 displays characteristic phenotypes of *E. mundtii*. It grows well on Slanetz and Bartley medium (Slanetz and Bartley, 1957), producing smooth, circular, glistening colonies (**Figure 1C**). The bacterial cells are 0.5–1.0 μm in diameter, and occur in the form of pairs (**Figure 1D**). Strain SL 16 could utilize various carbon sources, including xylose, cellobiose, and sucrose (**Table 1**).

Sequencing the genome of *E. mundtii* SL 16 produced a raw data set of 1,764,821,160 total bases. During the quality control, Illumina PCR adapter reads and low-quality reads were removed, and a total of 3,469,570 mate-pair reads (total bases 1,698,525,052 bp) were retained. The cleaned sequence reads were assembled with a *k*-mer setting of 125, which was determined by the optimal assembly result. The resulting genome sequence has an estimated size of 3,296,585 bp and a G+C content of 38.36%. 43,977 bp were repeats as predicted by RepeatMasker and TRF tools, which constituted 1.33% of the entire assembled genome.

TABLE 1 | *E. mundtii* SL 16 genome resources and characteristics.

	Name	Genome resources/characteristics
1	NCBI Bioproject ID	PRJNA337899
2	NCBI Biosample ID	SAMN05513637
3	NCBI Genome Accession Number	MCRG00000000
4	Sequence type	Illumina Miseq
5	Total number of Reads	3,515,580
6	Overall coverage	>100x
7	Estimated genome size (bp)	3,296,585
8	GC content (%)	38.36
9	Average of gene length (bp)	889
10	Protein coding genes	2939
11	tRNA coding genes	59
12	Motility	Non-motile
13	Cellobiose metabolism	Positive
14	Xylose metabolism	Positive
15	Arabinose metabolism	Positive
16	Sucrose	Positive

A total of 3125 genes with sequence length of 2,780,928 bp were predicted, which account for 84.4% of the genome, and 59 tRNA genes were identified by tRNAscan-SE. CDSs were searched against the NR, GO, string, Swiss-Prot, COG, and KEGG databases to analyze gene functions and metabolic pathways. In all, 1493 CDSs were assigned to COG families and 1411 CDSs were included in 154 pathways. Several physiological traits that may explain the successful adaptation of this bacterium to the environment of the gut have been found. In particular, a large amount of the coding capacity encountered in the genome of SL 16 (almost 12%) is dedicated to genes assigned to functions related to carbohydrate transport and metabolism, which matches well with the observed physiological characteristics of this strain (**Table 1**). This feature is shared with other colonic inhabitants, such as *Bacteroides fragilis* (Flint et al., 2008), and reflects the ecological niche of the organism presented inside a herbivore gut. The genome encodes several ABC-type sugar transporters, sugar-binding proteins, and a rich suite of glycosyl hydrolases, such as β-N-acetylhexosaminidase, α-galactosidase, β-glucosidase, β-galactosidase, and α-glucosidase. Moreover, the pyruvate dissipation pathways predicted for SL 16 include the capacity to produce L-lactate and several other fermentation metabolites, like short-chain fatty acids formate and acetate. This metabolic flexibility is expected to aid in efficient digestion and conversion of plant saccharides, thus promoting host development.

In conclusion, here we report a 3.30 Mbp draft genome sequence of *E. mundtii* strain SL 16, isolated from the generalist herbivore *S. littoralis*. The final *de novo* assembly is based on 1765 Mbp of Illumina data which provides an average coverage of 535 ×. Analysis of the genome shows high correlation between the genotypes and the phenotypes.

Direct Link to Deposited Data and Information to Users

The dataset submitted to NCBI include the assembled consensus sequence of E. mundtii SL 16 in Fasta format. The genome sequence can be accessed at DDBJ/EMBL/GenBank under the accession no. MCRG00000000. This paper describes the first version of the genome (https://www.ncbi.nlm.nih.gov/nuccore/MCRG00000000).

AUTHOR CONTRIBUTIONS

Work was planned by YS and WB, and executed jointly by BC and CS. XLi and BT were associated with isolation of the bacterium. AN and PA performed bioinformatics analyses. QG and XLu contributed to the DNA sequencing.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (Grant No. 31601906 to YS), the "Hundred Talents Program to YS" from Zhejiang University, the Modern Agricultural Technology System (No. CARS-22-ZJ0202) and the National Natural Science Foundation of China (Grant No. 31302033 to XLu).

REFERENCES

Balsingh, J., Radhakrishna, S., and Ulaganathan, K. (2016). Draft genome sequence of *Bacillus pumilus* ku-bf1 isolated from the gut contents of wood boring *Mesomorphus* sp. *Front. Microbiol.* 7:1037. doi: 10.3389/fmicb.2016.01037

Chen, B., Teh, B. S., Sun, C., Hu, S., Lu, X., Boland, W., et al. (2016). Biodiversity and activity of the gut microbiota across the life history of the insect herbivore *Spodoptera littoralis*. *Sci. Rep.* 6:29505. doi: 10.1038/srep29505

Douglas, A. E. (2015). Multiorganismal insects: diversity and function of resident microorganisms. *Annu. Rev. Entomol.* 60, 17–34. doi: 10.1146/annurev-ento-010814-020822

Flint, H. J., Bayer, E. A., Rincon, M. T., Lamed, R., and White, B. A. (2008). Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. *Nat. Rev. Microbiol.* 6, 121–131. doi: 10.1038/nrmicro1817

Kanehisa, M., Goto, S., Sato, Y., Kawashima, M., Furumichi, M., and Tanabe, M. (2014). Data, information, knowledge and principle: back to metabolism in KEGG. *Nucleic Acids Res.* 42, D199–D205. doi: 10.1093/nar/gkt1076

Kooij, P. W., Pullens, J. W., Boomsma, J. J., and Schiøtt, M. (2016). Ant mediated redistribution of a xyloglucanase enzyme in fungus gardens of *Acromyrmex echinatior*. *BMC Microbiol.* 16:81. doi: 10.1186/s12866-016-0697-4

Li, R., Yu, C., Li, Y., Lam, T. W., Yiu, S. M., Kristiansen, K., et al. (2009). SOAP2: an improved ultrafast tool for short read alignment. *Bioinformatics* 25, 1966–1967. doi: 10.1093/bioinformatics/btp336

Manero, A., and Blanch, A. R. (1999). Identification of *Enterococcus* spp. with a biochemical key. *Appl. Environ. Microbiol.* 65, 4425–4430.

Mithöfer, A., and Boland, W. (2012). Plant defense against herbivores: chemical aspects. *Annu. Rev. Plant Biol.* 63, 431–450. doi: 10.1146/annurev-arplant-042110-103854

Pospiech, A., and Neumann, B. (1995). A versatile quick-prep of genomic DNA from gram-positive bacteria. *Trends Genet.* 11, 217–218. doi: 10.1016/S0168-9525(00)89052-6

Rédou, V., Kumar, A., Hainaut, M., Henrissat, B., Record, E., Barbier, G., et al. (2016). Draft genome sequence of the deep-sea basidiomycetous yeast *Cryptococcus* sp. strain Mo29 reveals its biotechnological potential. *Genome Announc.* 4:e00461-16. doi: 10.1128/genomeA.00461-16

Shao, Y., Arias-Cordero, E., Guo, H., Bartram, S., and Boland, W. (2014). *In vivo* Pyro-SIP assessing active gut microbiota of the cotton leafworm, *Spodoptera littoralis*. *PLoS ONE* 9:e85948. doi: 10.1371/journal.pone.0085948

Shao, Y., Arias-Cordero, E. M., and Boland, W. (2013). Identification of metabolically active bacteria in the gut of the generalist *Spodoptera littoralis* via DNA stable isotope probing using 13C-glucose. *J. Vis. Exp.* e50734. doi: 10.3791/50734

Slanetz, L. W., and Bartley, C. H. (1957). Numbers of enterococci in water, sewage, and feces determined by the membrane filter technique with an improved medium. *J. Bacteriol.* 74, 591–595.

Tang, X., Freitak, D., Vogel, H., Ping, L., Shao, Y., Cordero, E. A., et al. (2012). Complexity and variability of gut commensal microbiota in polyphagous lepidopteran larvae. *PLoS ONE* 7:e36978. doi: 10.1371/journal.pone.0036978

Teh, B. S., Apel, J., Shao, Y., and Boland, W. (2016). Colonization of the intestinal tract of the polyphagous pest *Spodoptera littoralis* with the GFP-Tagged indigenous gut bacterium *Enterococcus mundtii*. *Front. Microbiol.* 7:928. doi: 10.3389/fmicb.2016.00928

Von Mering, C., Jensen, L. J., Snel, B., Hooper, S. D., Krupp, M., Foglierini, M., et al. (2005). STRING: known and predicted protein-protein associations, integrated and transferred across organisms. *Nucleic Acids Res.* 33, D433–D437. doi: 10.1093/nar/gki005

Xu, G., Hu, J., Fang, X., Zhang, X., Wang, J., Guo, Y., et al. (2014). Genome sequence of *Pseudomonas aeruginosa* strain LCT-PA220, which was selected after space flight by using biolog's powerful carbon source utilization technology. *Genome Announc.* 2:e00169-14. doi: 10.1128/genomeA.00169-14

Zhang, X., Li, G.-X., Chen, S.-C., Jia, X.-Y., Wu, K., Cao, C.-L., et al. (2016). Draft genome sequence of *Desulfitobacterium hafniense* strain DH, a sulfate-reducing bacterium isolated from paddy soils. *Genome Announc.* 4:e01693-15. doi: 10.1128/genomeA.01693-15

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6. Unpublished results Part I

Introduction

The lactic acid bacteria (LAB) are potential organisms for metabolic engineering due to small genomes and relatively simple metabolisms. Insect guts are a common place for plasmid transfer and transconjugation between bacterial strains (Watanabe and Sato, 1998). It has been reported that plasmid transfer occurs between strains of *B. thuringiensis* in *G. mellonella* and *S. littoralis* (Jarrett and Stephenson, 1990).

The allelic exchange is the usual method to generate chromosomal integration in LAB. The construction of flanking regions of homology is required for allelic exchange with the target gene on the chromosome. Several studies have shown successful integration of target genes in bacterial chromosomes through homologous recombination. A successful expression of *gfp* in bacteria requires the gene to be stably maintained. The expression of GFP using plasmids requires antibiotic as selection pressure which is quite cumbersome especially in *in vivo* applications. In addition to the construction of plasmid-borne GFP (Article I), I constructed a *gfp* gene cassette that was integrated into a target gene on the chromosome of *E. mundtii* by homologous recombination. The DNA-entry nuclease gene (*nuc*) was identified as potential target gene for integration without hampering the growth of *E. mundtii*.

Results

Construction of GFP expression vector by homologous recombination

In this thesis, the shuttle vector pTRKH2 (O'Sullivan and Klaenhammer, 1993) was used as the cloning vector as illustrated in Figure 6.1. This plasmid is an *E. coli*-gram positive bacteria shuttle vector. It replicates through theta-mode of replication with a size of 6.7 kb. In addition, it contains a p15A origin of replication for *E. coli* and a pAMβ1 origin of replication for gram positive bacteria. It encodes an erythromycin resistance gene (Em^r) as a selection marker.

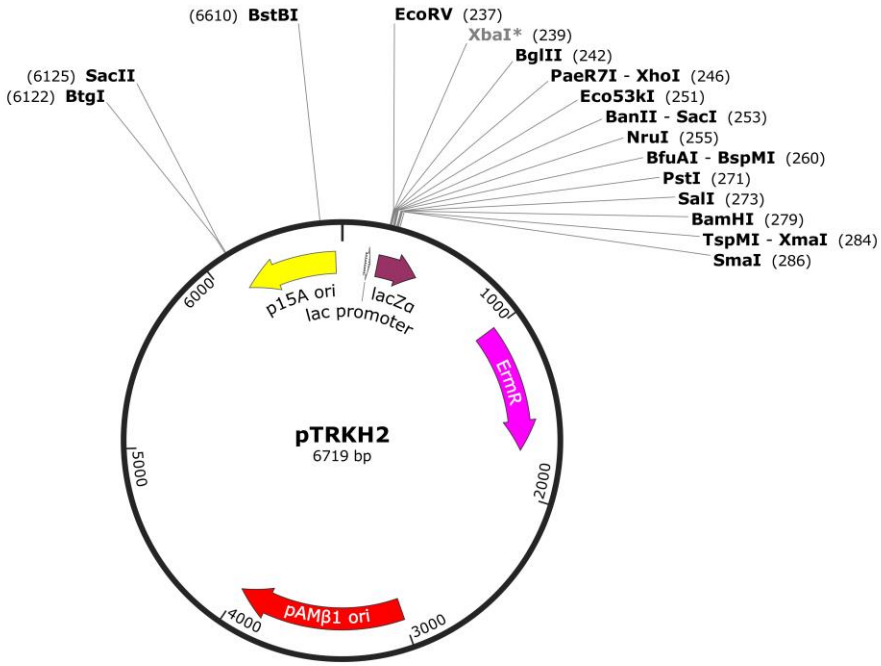


Figure 6.1. Map of the shuttle vector pTRKH2. The plasmid carries lacZα sequence containing a multiple cloning site (MCS) for target gene insertion.

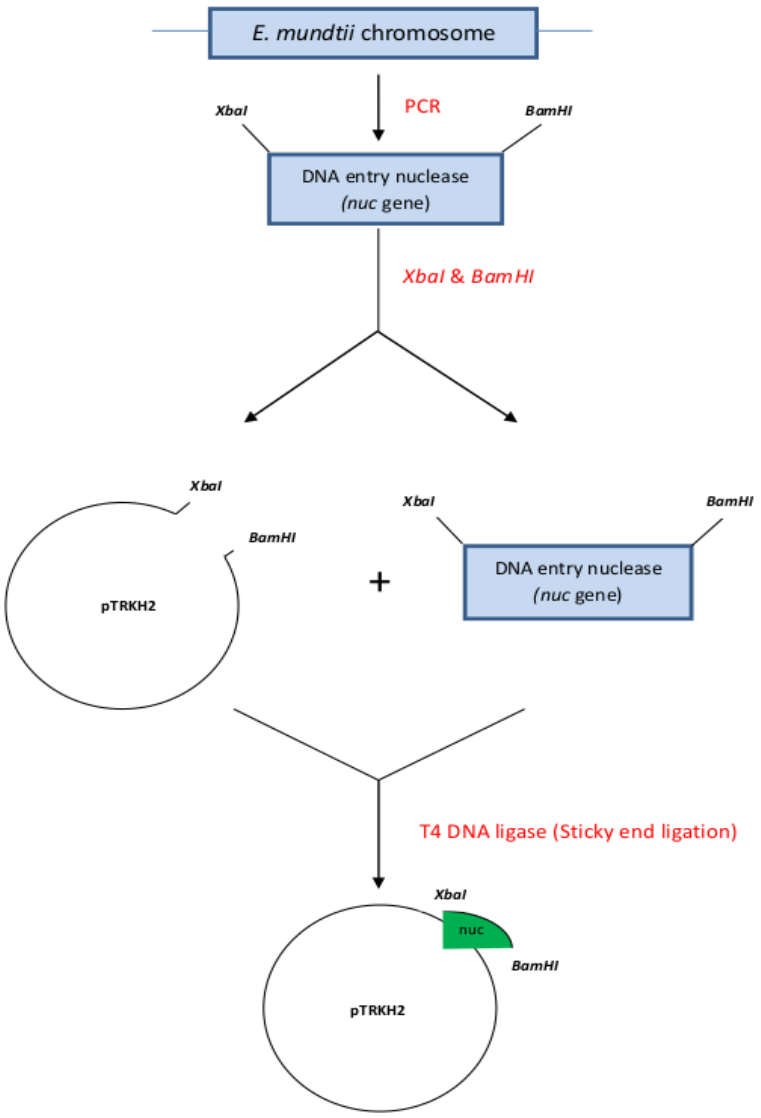


Figure 6.2. Workflow on the construction of recombinant cloning vector containing target gene of interest. The target gene DNA entry nuclease (*nuc*) was ligated to the plasmid vector pTRKH2.

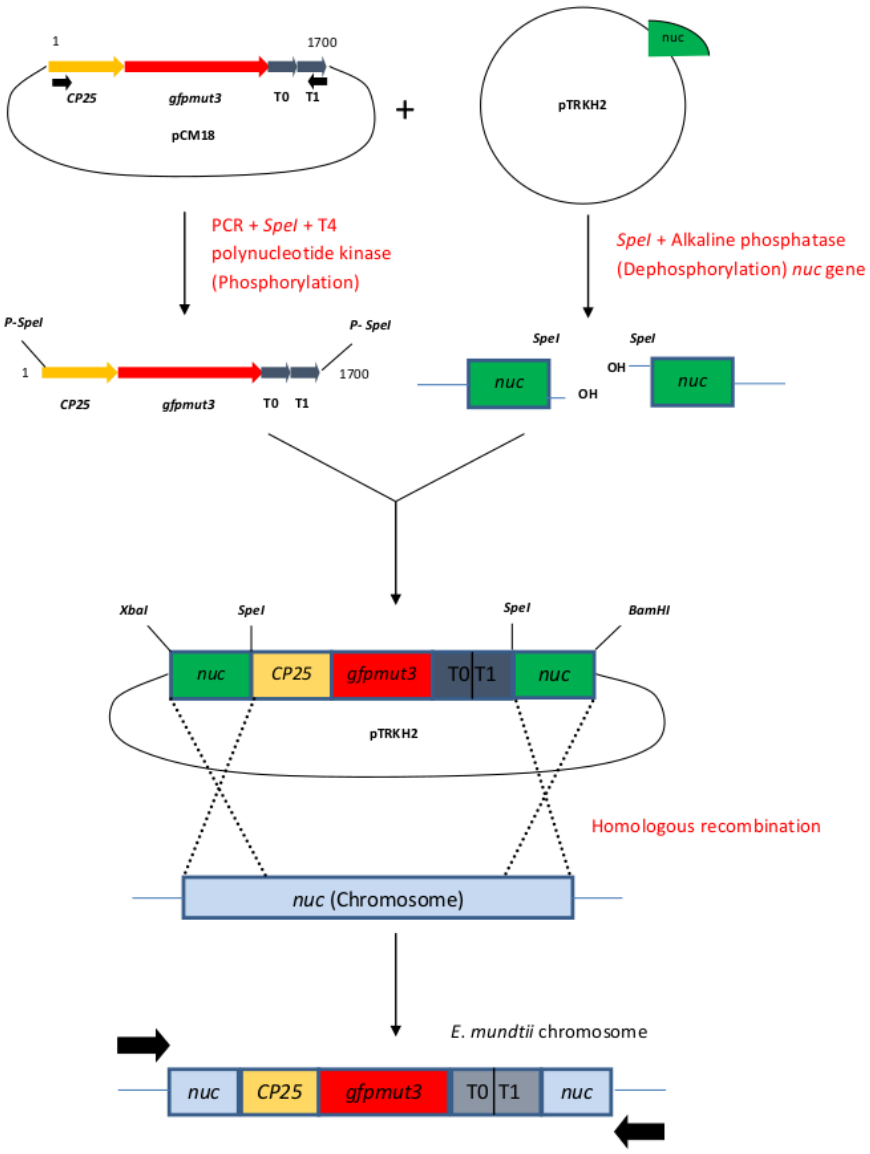


Figure 6.3. Integration of *gfp* gene flanked by upstream and downstream of *nuc* gene sequences on the chromosome of *E. mundtii* by homologous recombination.

All primers are listed in Table 6.2. The recombinant pTRKH2 containing *nuc* and *gfp* fragment was constructed as shown in Figure 6.2 and Figure 6.3. The entire P_{CP25}-RBSII-*gfp*mut3-T_0-T_1 terminator *gene* cassette (1.7 kb) was amplified from plasmid pCM18 (Hansen et al., 2001) by using primers ForCP25 and RevTT that contain the restriction sites *Spe*I,

respectively (Figure 6.4B). The target gene for homologous recombination was DNA-entry nuclease (*nuc*) as described (van Zyl et al., 2015). The *nuc* gene (0.72 kb) was amplified from genomic DNA of *E. mundtii* with primers ForEmuN and RevEmuN containing the restriction sites *Xba*I and *Bam*HI, respectively (Figure 6.4A). Initially, the *nuc* gene was ligated to the pTRKH2 shuttle vector (Figure 6.2). The recombinant plasmid pTRKH2-*nuc* was transformed into *Escherichia coli* DH5α and erythromycin resistance transformants were isolated. The presence of target gene in the recombinant plasmid was screened by PCR with primers ForEmuN and RevEmuN. The *nuc* gene was successfully amplified showing the success of ligation at *Xba*I and *Bam*HI sites of pTRKH2 plasmid.

Positive pTRKH2-nuc plasmid was digested at a single restriction site *Spe*I for *gfp*mut3 gene cassette insertion which was digested with the same enzyme prior to ligation with T4 DNA ligase. The ligation mixture was introduced into electrocompetent *E. coli* DH5α and *E. mundtii* through electroporation. Bacterial transformants with erythromycin resistant phenotype was selected. Primers ForEmuN and RevTT were used to screen for integration of *nuc*-*gfp*mut3-*nuc* gene cassette in the chromosome of *E. mundtii*. I could visualize high fluorescence from transformants of *E. coli* (Figure 6.5A), however, no fluorescent from *E. mundtii* (Figure 6.5B) by fluorescence microscopy. Although no fluorescence could be detected for *E. mundtii* transformants, further screening by PCR has shown that partial *nuc* and *gfp*mut3 gene fragments were amplified (Figure 6.4C), suggesting the likelihood of *gfp*mut3 being inserted in a specific site of *nuc* gene. Sequencing is necessary to verify those PCR amplicons. It is likely that *E. mundtii* transformants could either contain the autonomous plasmids or integrated but recombined *gfp*mut3 gene cassette. To further confirm whether the P_{CP25}-RBSII-*gfp*mut3-T_0-T_1

gene cassette was successfully integrated into *E. mundtii* chromosome, I screened for the presence of *nuc* gene. However, I could amplify a 720 bp fragment of *nuc* gene from genomic DNA. This shows that the *nuc* gene was not knocked out and no homologous recombination occurred.

Table 6.1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference or source
Strains		
Escherichia coli		
DH5α	Host strain for subcloning	Lab stock
DH5α pTRKH2*nuc*	Contains pTRKH2nuc plasmid	This study
Enterococcus mundtii		
KD251	Isolated from the gut of *S. littoralis*	Lab stock
KD251 pTRKH2*nucgfp*mut3	Contains pTRKH2nucgfpmut3 plasmid; Em^r	This study
Plasmids		
pCM18	*E. coli*-LAB shuttle vector; Em^r	Hansen et al., 2001
pTRKH2	6.7 kb; *E. coli*-LAB shuttle vector; Em^r; p15A and pAMβ1 origin of replication	O'Sullivan and Klaenhammer, 1993 (Addgene # 71312)
pTRKH2nuc	Plasmid carrying *E. mundtii* KD251 DNA-entry nuclease gene (*nuc*); Em^r	This study
pTRKH2*nucgfp*mut3	*E. coli*-LAB shuttle vector with P_{CP25}-RBSII-*gfp*mut3-T_0-T_1 terminator; Em^r	This study

Table 6.2. Primers used in this study

Target	Primer	Sequence 5' to 3'	Restriction sites	Product size (bp)
nuc	ForEmuN	TCA**TCTAGA**ACAAAAAAACAACCAAAACAATCATAG	*Xba*I	720
	RevEmuN	TCT**GGATCC**TTAGCTTGCCCCGTTTGATAG	*Bam*HI	
P_{CP25}-RBSII-*gfp*mut3-T_0-T_1	ForCP25	TCA**ACTAGT**CTTTGGCAGTTTATTCTTG	*Spe*I	1700
	RevTT	GA**ACTAGT**AGCGGCGGATTTGTCCT	*Spe*I	

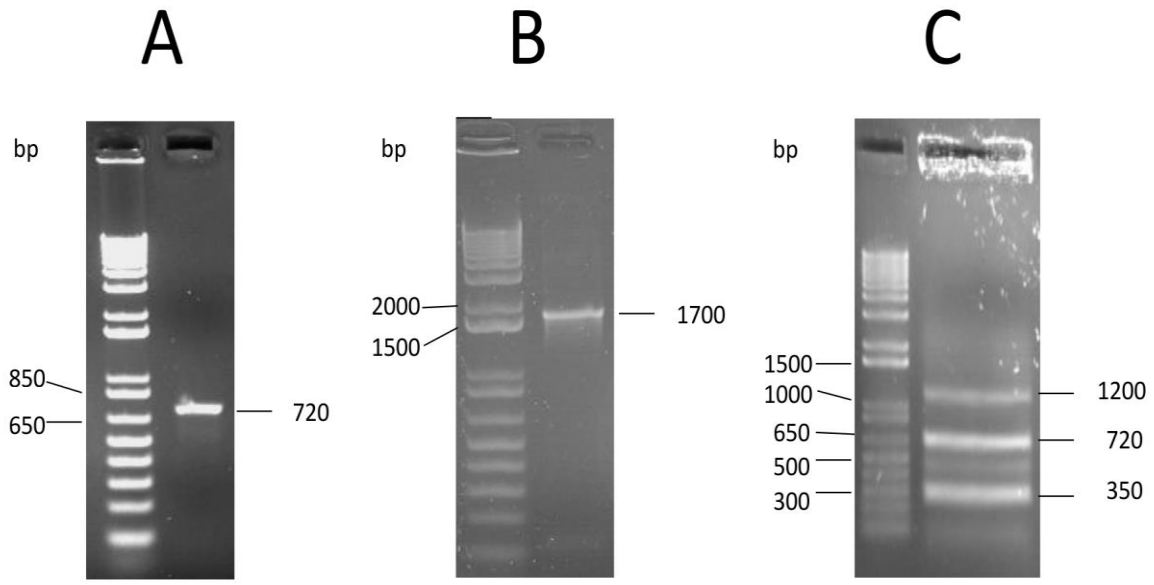


Figure 6.4. PCR amplification of several target genes. 1 kb Plus DNA ladder (Thermo Fisher Scientific) was used as marker. (A) *nuc* gene was amplified from genomic DNA of *E. mundtii* with primers ForEmuN and RevEmuN. (B) P_{CP25}-RBSII-*gfp*mut3-T_0-T_1 gene cassette was amplified from pCM18 plasmid with primers ForCP25 and Rev TT. (C) Three fragments of various sizes (1.2 kb, 0.72 kb and 0.35 kb) were amplified with primers ForEmuN and RevTT, implying the insertion of *gfp*mut3 gene cassette within *nuc* gene on the plasmid in *E. mundtii*.

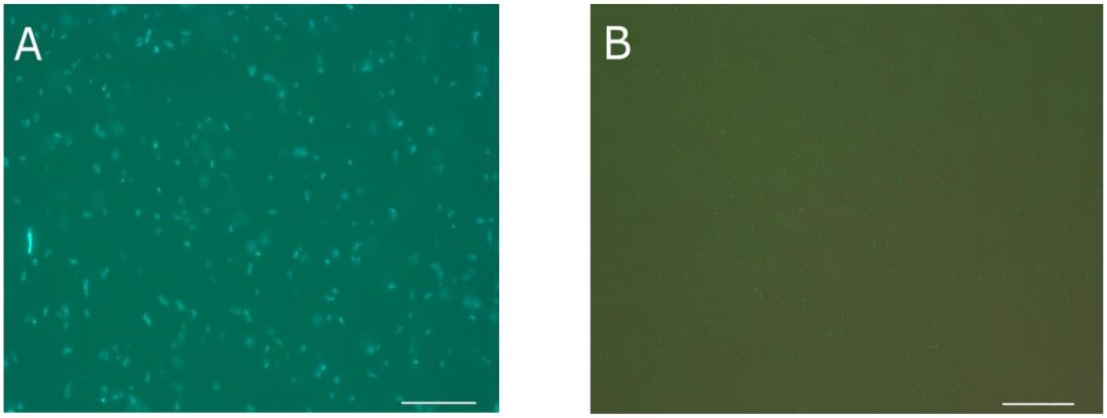


Figure 6.5. Fluorescence micrograph of bacteria harboring recombinant plasmid pTRKH2*nucgfp*mut3. (A) The recombinant *E. coli* DH5α was highly fluorescent, indicating the presence of *gfp*mut3 gene on the plasmid. (B) Although the same recombinant plasmid was electroporated into *E. mundtii*, however, no fluorescence was detected. Scale bars: 10 μm. Magnification, 400X.

7. Unpublished results Part II

Introduction

The gut bacteria of *S. littoralis* consists of a core microbial community predominated by *E. mundtii* across different life stages of the insect. However, the molecular mechanism of this successful colonization is unknown. The identification of bacterial diversity alone is not enough to decipher the ecological roles between gut symbionts and their host insect. Therefore, more powerful molecular approach notably the high throughput transcriptome sequencing, RNA-seq is necessary to gain better understanding of the bipartite symbiont-host interactions. Together with Tilottama Mazumdar, I analyzed the whole transcriptome of *E. mundtii* during interaction with the foregut and hindgut epithelial cells of the host insect. The GFP-tagged *E. mundtii* was integrated in the gut microbiome, and recovered using cell sorter (FACS). All results shown were based on two biological replicates for *in vivo* samples and three biological replicates for *in vitro* broth culture samples (control).

Results

Table 7.1. Number of Illumina reads aligned to the reference genome of *E. mundtii* QU25 using Tophat

Sample	Input (reads)	Aligned pairs	Aligned pairs (%)
Foregut 1 (F1)	13949493	2419614	17.60
Foregut 2 (F2)	10562548	6274715	59.40
Hindgut 1 (H1)	9491522	2609448	27.50
Hindgut 2 (H2)	9161970	4316215	47.10
Control 1 (C1)	9386723	3904877	41.60
Control 2 (C2)	9854743	3813786	38.70
Control 3 (C3)	9733545	4691569	48.20

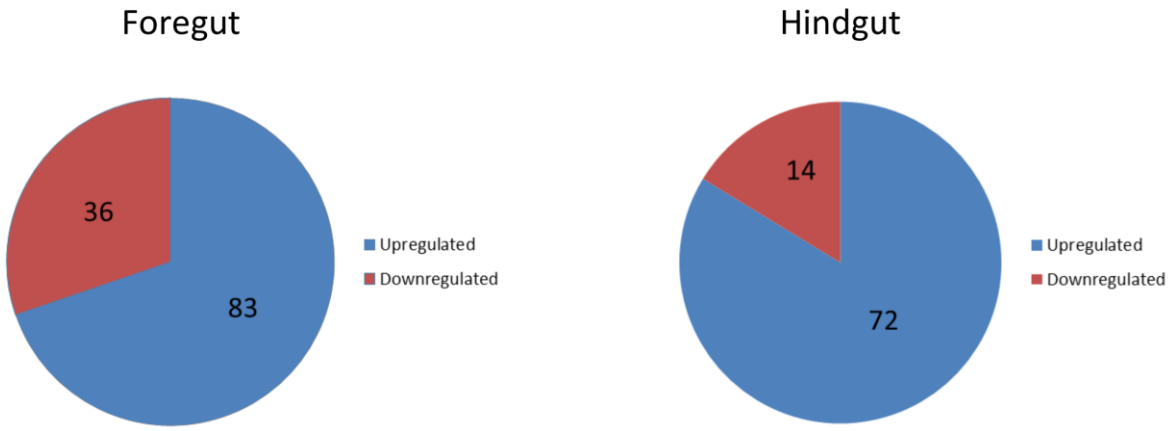


Figure 7.1. Differentially expressed genes of *E. mundtii* in the foregut and hindgut of *S. littoralis*. The fold-change (FC) values between control and *in vivo* samples were based on $\log_2$ FC ≥ 2, $p < 0.05$.

A total of 119 bacterial genes (83 up-regulated; 36 down-regulated) and 86 genes (72 up-regulated; 14 down-regulated) were differentially expressed (DEGs) in the foregut and hindgut of *S. littoralis*, respectively (Figure 7.1). These genes were further enriched for COG annotation. COG analysis showed the DEGs were grouped in 20 pathways. The largest COG groups were 'carbohydrate transport and metabolism' (283 genes), followed by 'transcription' (224 genes), 'replication, recombination and repair' (160 genes), 'translation, ribosomal structure and biogenesis' (158 genes), 'amino acid transport and metabolism' (150 genes), and others (Table 7.2). A striking up-regulation of genes was observed for several COG categories in the hindgut more than that of the foregut, including defense mechanisms, cell wall/membrane/envelope biogenesis, inorganic ion transport and metabolism, amino acid transport and metabolism, signal transduction mechanisms, and replication, recombination and repair. The induction of some of the bacterial genes in these pathways showed that the hindgut is a nutrient-limited environment.

KEGG database was searched for metabolic pathways used by *E. mundtii* for adaptation to gut environment. Pathway analysis is essential to explore the biological function of the genes at transcriptome level. Among up-regulated pathways in the foregut and hindgut were starch and sucrose metabolism, phosphotransferase system (PTS), biosynthesis of secondary metabolites, pyrimidine metabolism, purine metabolism, fructose and mannose metabolism, quorum sensing, and ABC transporters. In contrast, down-regulated pathways involved biosynthesis of antibiotics, carbon metabolism, biosynthesis of amino acids, and fatty acid metabolism and biosynthesis (Figure 7.2). More details about the genes involved in the metabolic pathways can be found in the general discussion (section 8.5).

Table 7.2. Number of genes that are differentially expressed based on Clusters of Orthologous Groups of protein (COG) classifications

COG category	Genes	Foregut		Hindgut	
		Upregulated	Downregulated	Upregulated	Downregulated
Lipid transport and metabolism	61	1	10	4	5
Nucleotide transport and metabolism	77	17	8	11	3
Carbohydrate transport and metabolism	283	39	46	48	14
Function unknown	253	32	24	28	31
Translation, ribosomal structure and biogenesis	158	11	10	9	20
Energy production and conversion	81	9	4	9	2
Transcription	224	16	39	40	19
Defense mechanisms	54	2	12	13	2
Cell wall/membrane/envelope biogenesis	134	5	21	25	2
Inorganic ion transport and metabolism	113	6	13	22	3
Intracellular trafficking, secretion and vesicular transport	40	4	9	8	1
Secondary metabolites biosynthesis, transport and catabolism	30	0	7	6	2
Cell motility	16	1	4	2	2
Amino acid transport and metabolism	150	9	22	22	9
Coenzyme transport and metabolism	63	3	9	11	1
General function prediction only	280	18	38	39	14
Signal transduction mechanisms	107	6	12	16	10
Posttranslational modification, protein turnover, chaperones	58	5	2	4	3
Replication, recombination and repair	160	6	20	14	9
Cell cycle control, cell division, chromosome partitioning	44	2	3	3	1

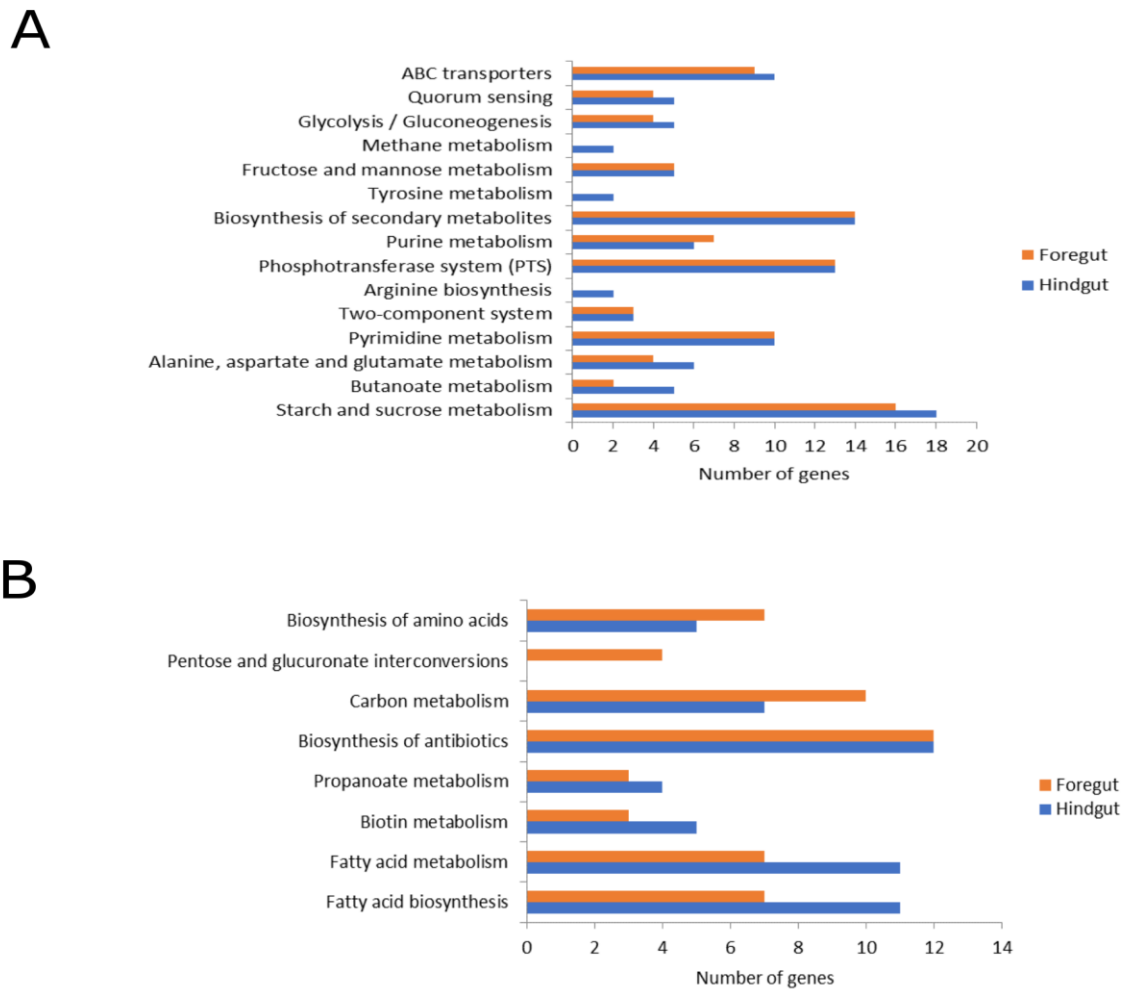


Figure 7.2. Comparison of KEGG pathways for the transcriptome sequences of *E. mundtii* in the foregut and hindgut of *S. littoralis*. (A) Up-regulated pathways. (B) Down-regulated pathways.

8. General discussion

8.1 Construction of GFP reporter bacteria

I constructed several strains of fluorescent *E. mundtii*, which were transformed with plasmid-containing *gfp* controlled by different promoters (Article I). I showed that the plasmid-based approach could be electro-transformed easily into *E. mundtii* with stable GFP fluorescent detection during its integration in the gut of the insect host, *S. littoralis*. The selection of an appropriate promoter to achieve a high level of GFP expression is crucial. Therefore, the use of constitutive or native-based promoters would be favorable, as these promoters could ensure the constant production of the target protein, especially in the gut environment. Several studies using homologous promoters have been reported to achieve efficient gene expression as the transcriptional signal induced by native promoters is recognized by the host bacteria (Chouayekh et al., 2009, Fang et al., 2008). Bacteria with the *gfp* gene cloned downstream of a native constitutive promoter express GFP efficiently in the broth culture (Article I, Figure 1A).

In addition, to reduce the use of antibiotic for plasmid maintenance in the bacterial cells, I have also constructed a *gfp* gene cassette integrated into a specific target gene on the chromosome of *E. mundtii* (Unpublished results part I). The construction of recombinant plasmid with a P_{CP25}-RBSII-*gfp*mut3-T_0-T_1 gene cassette flanked by upstream and downstream of target gene sequence (*nuc*) was successfully constructed in *E. coli* DH5α, resulted a highly fluorescent recombinant bacterium (Figure 6.5A). The same plasmid construct was introduced into *E. mundtii*, however, did not result in any fluorescence (Figure 6.5B), implying failure of homologous recombination to occur on the chromosome. Insertion of foreign gene (*gfp*) could pose a toxic effect; therefore, a rejection mechanism is induced to safeguard the bacterial metabolism. However, partial sequences of *gfp* flanked by *nuc* gene were detected by PCR,

showing ligation was successful probably on the plasmid (Figure 6.4C). Perhaps, the pTRKH2 shuttle vector or the promoter P_{CP25} or the *gfp* variant may work on other bacteria but not for *E. mundtii*.

Fluorescence imaging based on GFP protein is an emerging method to monitor microorganisms in live organism. The development of fluorescent microorganisms is particularly important to allow live monitoring of its survival and persistence in the host organism. *In vivo* imaging is a popular non-invasive method to track bacterial proliferation in animals. This technique has been widely used in bacterial infection studies involving *Salmonella typhimurium*, *Listeria monocytogenes*, *Pseudomonas aeruginosa,* and *Staphylococcus aureus* among others (Doyle et al., 2004).

Apart from fluorescent protein imaging, fluorescence *in situ* hybridization (FISH) has also been used in microbial ecology, for example to monitor localization of bacteria within the intestinal mucus of the mouse (Johansson et al., 2008). FISH is developed based on the design of specific primers or probes to target the unique regions of the 16S rRNA gene of a bacterial species. Our lab also uses FISH to track the colonization of *E. mundtii* in the intestinal tract of *S. littoralis* (Shao et al., 2014, Tang et al., 2012). However, this method has a limitation in which the bacterial cells labeled with probes become non-viable, and thus are non-recoverable using flow cytometer (FACS). Therefore, molecular method of labelling the bacterial with GFP is more suitable for the next aim of my PhD thesis that is to identify the global gene expression of *E. mundtii* living within the gut of *S. littoralis*.

Several methods have been used to introduce exogenous DNA into microbial cells; these include chemical treatment, electroporation, the use of a biolistic gun, ultrasound, polyethylene glycol, microwave and hydrogel (Singh et al., 2010). Of all the methods, electroporation is the

most efficient to transform many microorganisms. Electroporation is one of the transformation techniques for the rapid introduction of foreign DNA like plasmid into bacteria. The method uses an electric pulse that forms pores on the bacterial cell walls so that DNA can pass into the cell. In recent years, numerous lactic acid bacteria have been transformed using electroporation (Rixon and Warner, 2003). The success rate of electro-transformation depends on the permeability of the cell wall that allows sufficient DNA to enter the cell. In some cases, to improve electro-transformation efficiency, the cell wall is weakened by chemicals such as pretreatments with lysozyme (Rodriguez et al., 2007), threonine (Dornan and Collins, 1990), penicillin G (Wei et al., 1995), ethanol (Assad-Garcia et al., 2008), and glycine (Thompson and Collins, 1996). The cell wall weakening chemicals are effective only for certain bacteria species and not others. The electro-transformation efficiency of *Lactococcus lactis* was affected by several parameters such as the growth phase and cell density, the medium, the plasmid concentration, and the electric field strength (McIntyre and Harlander, 1989). The outcomes are different depends on the adjustment of these parameters. Hence, the protocol of electro-transformation needs to be optimized.

8.2 Niche-specific colonization of the gut microbiota

The variation of insect gut with its extreme physicochemical conditions (oxygen, pH and redox potential), defensive compounds secreted by the immune system and constant change in gut contents due to molting and metamorphosis all can affect the colonization of microorganisms.

It has been shown that the largest group of microorganisms is found in the hindgut in most insects. Microorganisms live in the hindgut benefit by the metabolites and ions transported

from the Malpighian tubules into the hindgut. The hindgut stores the nitrogenous waste and food waste possibly serve as nutrients for insect gut bacteria (Engel and Moran, 2013). The microbiota in the ileum of the hindgut of termites and scarab beetles metabolize plant polysaccharides into components that can be used by the insect (Brune and Kühl, 1996, Huang et al., 2010). In addition, some forms of structures like spines and plates of the hindgut are favorable for microbes to bind (Brune, 2006). In contrast to the hindgut, the midgut is more unfavorable for microorganisms to live in. Many antimicrobial peptides (Lemaitre and Miguel-Aliaga, 2013), dual oxidase enzyme (DUOX: NADPH oxidase) (Ha et al., 2005), and digestive enzymes (lysozymes) (Shanbhag and Tripathi, 2009) are secreted by the midgut epithelium cells of *D. melanogaster*.

In some insects, the pH of the gut is highly acidic with pH < 3 or highly alkaline (pH 8-12) for larval lepidopterans (Funke et al., 2008), which kills many microorganisms. The peritrophic matrix in the insect midgut is responsible to prevent the invasion of microorganisms. In some cases, microorganisms can penetrate the matrix with the help of chitinases (Dostalova and Volf, 2012). The presence of peritrophic matrix might explain why fluorescent *E. mundtii* cells are confined in that compartment, possibly the mechanism utilized by the insect to protect the gut epithelium from microorganisms (Article I, Figure 4). Some insects do not have a peritrophic matrix.

Spatial and temporal distribution of fluorescent *E. mundtii* was observed across all developmental stages, as well as in the foregut, midgut, and hindgut of *S. littoralis*, indicating its symbiotic relationship with the insect host (Article I, Figure 4). Data from the colony forming units (CFUs) showed that the density of fluorescent *E. mundtii* colonizing the gut of *S. littoralis* is relatively low in the foregut compared to the midgut and hindgut (Article I, Figure 5). This

phenomenon can be explained such that the microorganisms in the crop of the foregut can be present at high density compared to other gut regions (Kohler et al., 2012), but extremely unstable due to constant removal of food at this site. For examples, a bacterium, *Candidatus* Erwinia dacicola chooses to live in the cephalic bulb of the olive fly *Bactrocera oleae* and the pathogenic bacterium, *Xylella fastidiosa* inhabits the foregut of the leafhopper *Graphocephala atropunctata*.

The gut of *S. littoralis* is relatively simple with a straight tube in the absence of specialized structures called bacteriomes that harbor endosymbionts, such as in aphids, whiteflies, and other insects. The questions of how *S. littoralis* houses *E. mundtii* remain unknown as no compartment structures exist to protect the bacterium, for example the gut in the pupa is totally removed. The number of CFU counts of fluorescent *E. mundtii* remains extremely low in the pupa (data not shown). Similarly, this phenomenon has been shown in article II (Figure 1B), such that the diversity of bacterial phylotypes also decreases in the pupa. Several mosquito species that undergo metamorphosis show complete elimination of gut bacteria, especially in newly emerged adults (Moll et al., 2001). In article I, the symbiotic *Enterococcus* has been shown to be transmitted to the second-generation progeny, suggesting it co-evolves together with the insect host. The question of how a bacterial symbiont is transmitted from one generation to the next remains poorly understood. The symbiont that co-evolves with the host has a great chance to secure vertical transmission, for example the symbiosis between the aphid and endosymbiont *Buchnera*. One interesting point to highlight is that *E. mundtii* was transmitted in the eggs as its presence was detected during the tissue cross-sectioning (Article I, Figure 4G). It is not surprising that *Enterococcus* was also active in the gut and eggs of *Manduca sexta* (Brinkmann et al., 2008). Fluorescent bacteria were transmitted from the gut to the eggs in

Tribolium castaneum (Knorr et al., 2015). It has been shown that the bacterial symbiont, *Ishikawaella capsulata* is vertically transmitted through smearing of eggs by the female stinkbug. The newly hatched juveniles acquire the symbiont by ingesting the egg case (Fukatsu and Hosokawa, 2002).

8.3 The core gut microbiome

The host organism selects its own core microbes, which differ from one organism to another (Kostic et al., 2013). For example, the light organ of the Hawaiian bobtail squid, *Euprymna scolopes* is stably colonized only by a specific *V. fischeri* strain obtained from the environment and not from other organism such as fish (Kostic et al., 2013). The human-associated strains of *Lactobacillus reuteri* failed while the rat-associated strains could colonize the mouse gut (Frese et al., 2011). The bacterial symbionts of honey bees were unable to persist in the gut of bumble bees (Kwong et al., 2014).

Similarly, in our study, we failed to introduce the fluorescent *E. coli* strain in the gut of *S. littoralis* (Wallstein, 2014). This is most likely due to the presence of 1 mmol of iron chelator, 8-hydoxyquinoline-2-carboxylic acid (8-HQA) in the gut environment that inhibits *E. coli* growth. It has been shown that host genetics play important role in shaping the composition of core gut microbiota in zebrafish, apes, bees, termites, and *Drosophila* (Wong et al., 2013). The gut microbiome of insect is influenced by the host life stage, host phylogeny, and diet (Engel and Moran, 2013). Although diet causes major changes in gut microbial communities, however, a core microbiome remains stable. The indigenous gut bacteria of termites can adapt and live well in the gut of host termites (Husseneder and Grace, 2005). This is likely to be the reason that

causes the indigenous bacterium *E. mundtii* to persist well in the gastro-intestinal tract of *S. littoralis*.

It is common to find enterococci in the intestinal tract of lepidopteran larvae and other insects. The constant presence of enterococci signifies the unknown roles it plays towards the benefit of the host organisms. Apart from *Enterococcus* species, *Clostridium* sp. contributed to a large proportion of the gut microbiome of cotton leafworm (Tang et al., 2012). Thus far, the existence of *Clostridium* sp. in the late instar larvae has been unknown, but some of its function linked to cellulose degradation to help the insect to digest plant materials. It would be interesting to find out the interaction between *Enterococcus* and *Clostridium*, which remains obscure. Possibly, interaction in exchange of metabolites could occur between these two species of bacteria.

8.4 Mechanisms of gastro-intestinal colonization

Enterococci are well adapted to a wide range of environmental conditions, which make them one of the most successful gut commensals. The adverse conditions include variations in pH, temperatures, desiccation, and osmotic stress. All these variations are found in the gastro-intestinal tract of animals and insects. The normal gut microbiota competes with pathogenic bacteria for nutrients and attachment sites on the gut epithelium (Tasteyre et al., 2001) through a phenomenon known as colonization resistance. For example, commensal *E. coli* competes with pathogenic enterohaemorrhagic *E. coli* for nutrients in the form of amino acids and sugar (Momose et al., 2008, Fabich et al., 2008). It has been observed that *E. mundtii* forms biofilm on the mucus layer of the gut epithelium, suggesting a protection mechanism against pathogens (Teh et al., 2016, Shao et al., 2014).

Another possible defense mechanism of LAB strains is the production of antimicrobial peptides or bacteriocins against other bacteria (Shao et al., 2017, Caplice and Fitzgerald, 1999). For instance, the pupal midgut of *M. sexta* secretes a lot of antimicrobial compounds, which completely wipe out most microorganisms (Russell and Dunn, 1996). This could be one of the reasons that only few or no fluorescent bacterial cells were detected in the pupae of *S. littoralis* (Article I, Figure 4E). It remains unknown on how the insect host distinguishes between beneficial microorganisms and pathogens. The commensal symbionts might develop mechanism to suppress host immune system through the absence of peptidoglycan (PGN) structure on the bacterial surfaces. The insect host secretes the pattern recognition receptors (PRRs) that recognize the PGN structure of bacteria. For example, insects activate the Imd defense pathway, which produce antimicrobial peptides in response to PGN of Gram-negative bacteria. A good example to explain this selection is through the study of endosymbionts in tsetse flies, which involves the activation of antimicrobial responses against trypanosomes (Wang et al., 2009). The tsetse fly maintains homeostasis with its symbiotic bacterium *Wigglesworthia* through the peptidoglycan recognition protein LB (PGRP-LB) (Wang et al., 2009). The PGRP-LB could neutralize the antibacterial response towards *Wigglesworthia*. In contrast, PGRP-LB and other effectors control pathogenic microbes through activation of the IMD pathway (Wang et al., 2009).

It has been shown that commensal bacteria inhibit pathogen colonization by altering the host environmental conditions, such as pH. For example, bacteria of the genus Streptococci or Enterococci produce lactic acid to decrease the pH in the intestinal tract of *L. dispar* (Kodama and Nakasuji, 1971). Some commensal bacteria produce short chain fatty acids (SCFAs), which change the intestinal pH to inhibit pathogens (Shin et al., 2002).

8.5 Survival and adaptation strategies of *E. mundtii*

Microorganisms face environmental stresses, particularly those within the gastro-intestinal environment. The mechanisms these bacteria use to adapt to stress were explored by constructing the fluorescent reporter *E. mundtii*. The dominance and persistence of *E. mundtii* in the gut motivates us to look deeper into their gene expression system. Therefore, it is important to unravel the mechanisms used by microorganisms living within the gastro-intestinal environment. The fluorescence-activated cell sorting (FACS) enabled us to recover the GFP-tagged *E. mundtii* from a mixture of insect and other bacterial cells. The transcriptome data and differential gene expression of metabolic pathways were analyzed (Unpublished results part II).

Several *E. mundtii* genes encoding general stress protein (EMQU_1453, Ohr), universal stress proteins (EMQU_0268-EMQU_0269, EMQU_1232), and NADH peroxidase (EMQU_0459) were up-regulated under *in vivo* conditions. The *Ohr* has been shown to be involved in oxidative stress resistance in *E. faecalis* (Rince et al., 2001). Bacteria living within the host cells are exposed to reactive oxygen species (ROS) such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals. Organisms produce antioxidants and enzymes to reduce oxidative damage. A gene encodes for manganese catalase (EMQU_0568) was up-regulated. Catalases catalyze hydrogen peroxide into water and oxygen to reduce the formation of hydroxyl radical. Another enzyme fumarate reductase (EMQU_2110) was induced, implying its role in superoxide production to overcome oxidative stress. The *gls24* (EMQU_1475) corresponds to stress and starvation was also induced.

Several genes involved in iron transport were up-regulated, involving a ferric ABC transporter binding protein (EMQU_0143), ABC transporter permease (EMQU_0140-0141), and *fetC* (EMQU_0142), a ferric ABC transporter. The expression of *fetC* was higher in the hindgut

compared to that of the foregut. These genes (EMQU_0140-0143) were up-regulated when *E. faecalis* cells were grown in broth with limited iron conditions (Lopez et al., 2012). Moreover, a ferric uptake regulator family Fur was upregulated in the foregut (EMQU_1067).

Several genes of the phosphoenolpyruvate-dependent phosphotransferase (PTS) systems for cellobiose, mannose, and fructose were up-regulated. Starch and sucrose metabolism was induced as the larvae were fed with foods enriched with this carbohydrate (Figure 7.2A). The ABC sugar transporters were also up-regulated. Several carbohydrate-active enzymes (CAZy) were up-regulated more in the foregut than the hindgut. This suggests that the foregut is rich in different carbon sources, which require hydrolysis activity of CAZy. Most of the enzymes fall in the category of glycoside hydrolases (EMQU_0608, EMQU_0344, EMQU_0389, EMQU_1435). The genome of *E. mundtii* encodes numerous glycoside hydrolases to digest complex carbohydrates (Article III). Strikingly, two genes encoding a chitin-binding protein (EMQU_0940, EMQU_1285) were highly up-regulated more in the hindgut than the foregut. It has been shown that chitinases and chitin-binding proteins promote bacterial adherence to chitin-like molecules on the surface of mammalian cells. The chitinases secreted by *L. monocytogenes* suppress host innate immunity (Chaudhuri et al., 2013).

Surface proteins are important for bacteria to interact with host cells. These proteins help bacteria to colonize and adhere to the gut epithelium of the host. A sortase enzyme (EMQU_2188) was up-regulated in the gut. Sortase enzymes function as cysteine transpeptidases that attach proteins on the cell surface or join proteins to form hair-like fibres called pili to promote bacterial adhesion (Hendrickx et al., 2011). Sortases are potential drug target candidates as many surface proteins they interact are possible virulence factors (Suree et al., 2007).

The production of antimicrobial substances from insects or their resident symbionts is a survival strategy to keep pathogens at bay. The dominant gut bacterium *E. mundtii* has been shown to up-regulate the expression of an antimicrobial peptide called mundticin KS immunity protein (EMQU_2392), which is a stable class IIa bacteriocin. It establishes a chemical barrier, which prevents colonization by other bacterial competitors.

E. mundtii showed down-regulation in the biosynthesis of amino acids and fatty acid metabolism and biosynthesis (Figure 7.2B). However, genes encode for peptidase (EMQU_2717) and ABC transporters to transport amino acids or oligopeptides (EMQU_0123, EMQU_0127) were up-regulated to compensate the need for amino acids. The down-regulation of fatty acid biosynthesis suggests that the insect supplies fatty acid to *E. mundtii* for cell wall biosynthesis.

In future works, transposon mutagenesis could be an alternative to transcriptomics in search for essential genes required for gut establishment. Also, the midgut is another potential site for studying bacterial adaptation due to robust metabolic activities to occur.

9. Summary

Microbial symbionts are known to live in close associations with animals, plants, and insects. Insects harbor commensal microbes that provide the host with essential nutrients, aid in food digestion, and protection against pathogens. The cotton leafworm, *Spodoptera littoralis* (Lepidoptera: Noctuidae) is one of the most successful polyphagous insect pests that causes economic losses in agricultural industry. The gut microbial communities of *S. littoralis* are well characterized, and the insect is a popular model to study insect-microbe and microbe-microbe interactions. It is known that the gut of *S. littoralis* is predominated by *Enterococcus mundtii* and *Clostridium* sp. towards the late stage of larvae. However, the composition of gut microbiome of *S. littoralis* in other stages (eggs, pupae, and adults) was not defined in previous studies. The 16S rRNA amplicon sequencing based on DNA and cDNA levels has shown that *E. mundtii* was present in the eggs, pupae, and adults (Article II). Thus far, no study has been done to investigate the colonization of *E. mundtii* in the gut tissues across life stages of the host insect. To gain better understanding of its survival strategies, GFP-tagged *E. mundtii* was constructed to track its colonization in the intestine throughout different stages of development (larvae, pupae, adults, and eggs). Fluorescent bacteria survived and proliferated in the intestinal tract of the insect for all life stages, eventually entering second generation offspring following ingestion (Article I). This shows that symbiotic bacterium was vertically transmitted from the mother to progeny. However, the adaptation mechanisms of symbiotic *Enterococcus* in the gut environment are unknown. Hence, we sequenced the genome of *E. mundtii* to better decode genes that are important for gut colonization (Article III). To better explore real-time metabolic activities, transcriptome analysis of bacteria isolated from the foregut and hindgut of the insect by RNA-sequencing was performed (Unpublished results part II). Our results showed that *E. mundtii* expressed some

genes involved in carbohydrate transport and metabolism, oxidative stress, cell adhesion, defense, and iron transport for adaptation to the gut environments. In the future, the mechanisms involved in the cross-talk between host and microbe will be investigated by analyzing the insect's gut epithelium transcriptome.

10. Zusammenfassung

Mikrobielle Symbionten leben in enger Gemeinschaft mit Tieren, Pflanzen und Insekten. Insekten beherbergen kommensalische Mikroben die den Wirt mit essenziellen Nährstoffen versorgen, beim Verdau von Nahrung behilflich sind und Schutz gegen Pathogene bieten. Der afrikanische Baumwollwurm, *Spodoptera littoralis* (Lepidoptera: Noctuidae), verursacht in Form polyphagischer Insektenplagen wirtschaftliche Verluste in der Agrarindustrie. Die mikrobiellen Gemeinschaften im Darmtrakt von *S. littoralis* sind gut charakterisiert, und das Insekt gilt als beliebter Modellorganismus um die Interaktionen zwischen Insekt und Mikroben und Mikroben untereinander zu studieren. Es ist bekannt dass die mikrobielle Gemeinschaft im Darm von *S. littoralis* im späten Larvenstadium von *Enterococcus mundtii* und *Clostridium* sp. dominiert wird. Die Zusammensetzung der Darmgemeinschaft während der restlichen Stadien (Ei, Puppe und Erwachsenenstadium) wurde bisher jedoch nicht untersucht. Im Rahmen dieser Arbeit ergab die Sequenzierung von 16S rRNA, basierend auf DNA- und cDNA-Amplicons, dass *E. mundtii* während der Stadien des Eis, der Puppe und im Erwachsenenstadium nachweisbar ist (Artikel II). Um einen besseren Einblick in die bisher unbekannten Überlebensstrategien dieser Spezies zu erlangen, wurden GFP-markierte *E. mundtii*-Bakterien eingesetzt um die Darmkolonisierung während der verschiedenen Entwicklungsstadien (Larve, Puppe, Erwachsenenstadium, Ei) zu verfolgen. Die fluoreszierenden Bakterien überlebten und kolonisierten den Darmtrakt des Insektes zu allen Entwicklungsstadien, und waren später in Nachfahren der Folgegeneration nachweisbar (Artikel I). Dies beweist dass der Symbiont vertikal von den Weibchen an die Nachfahren weitergegeben wurde. Der letzte Teil der vorliegenden Arbeit setzte sich mit den Mechanismen der Anpassung symbiontischer *Enterococcus*-spezies im Darmtrakt auseinander. Dazu wurde das Genom von *E. mundtii* mit dem Ziel sequenziert, die Gene, die an der

Kolonisierung mitwirken, zu identifizieren (Artikel III). Um die metabolische Aktivität in Echtzeit zu untersuchen wurde das Transkriptom von Bakterien die aus dem Vorder- und Enddarm isoliert wurden über RNA-Sequenzierung analysiert (nicht publizierte Ergebnisse Teil II). Die Untersuchung ergab dass an der Anpassung an den Darmtrakt Gene beteiligt und exprimiert werden, die in zahlreichen Stoffwechselwegen involviert sind, wie z. B. dem Transport und Stoffwechsel von Kohlenhydraten, oxidativem Stress, Zelladhäsion, Abwehr und Eisentransport. Folgearbeiten werden sich mit dem Mechanismus des Cross-Talks zwischen Insekt und Mikroben auseinandersetzen. Dies wird über die Analyse des Transkriptoms des Darmepithels des Insekts erfolgen.

11. References

Alonso-Pernas, P., Bartram, S., Arias-Cordero, E. M., Novoselov, A. L., Halty-Deleon, L., Shao, Y. & Boland, W. 2017. *In vivo* isotopic labeling of symbiotic bacteria involved in cellulose degradation and nitrogen recycling within the gut of the forest cockchafer (*Melolontha hippocastani*). *Front Microbiol,* 8**,** 1970.

Assad-Garcia, J. S., Bonnin-Jusserand, M., Garmyn, D., Guzzo, J., Alexandre, H. & Grandvalet, C. 2008. An improved protocol for electroporation of *Oenococcus oeni* ATCC BAA-1163 using ethanol as immediate membrane fluidizing agent. *Lett Appl Microbiol,* 47**,** 333-8.

Barr, J. J., Auro, R., Sam-Soon, N., Kassegne, S., Peters, G., Bonilla, N., Hatay, M., Mourtada, S., Bailey, B., Youle, M., Felts, B., Baljon, A., Nulton, J., Salamon, P. & Rohwer, F. 2015. Subdiffusive motion of bacteriophage in mucosal surfaces increases the frequency of bacterial encounters. *Proc Natl Acad Sci U S A,* 112**,** 13675-80.

Baumann, P. 2005. Biology bacteriocyte-associated endosymbionts of plant sap-sucking insects. *Annu Rev Microbiol,* 59**,** 155-89.

Baumann, P., Baumann, L., Lai, C. Y., Rouhbakhsh, D., Moran, N. A. & Clark, M. A. 1995. Genetics, physiology, and evolutionary relationships of the genus *Buchnera*: intracellular symbionts of aphids. *Annu Rev Microbiol,* 49**,** 55-94.

Beard, C. B., Cordon-Rosales, C. & Durvasula, R. V. 2002. Bacterial symbionts of the triatominae and their potential use in control of Chagas disease transmission. *Annu Rev Entomol,* 47**,** 123-141.

Begley, M., Gahan, C. G. & Hill, C. 2005. The interaction between bacteria and bile. *FEMS Microbiol Rev,* 29**,** 625-51.

Borges, S., Silva, J. & Teixeira, P. 2014. The role of lactobacilli and probiotics in maintaining vaginal health. *Arch Gynecol Obstet,* 289**,** 479-89.

Bright, M. & Bulgheresi, S. 2010. A complex journey: transmission of microbial symbionts. *Nat Rev Microbiol,* 8**,** 218-30.

Brinkmann, N., Martens, R. & Tebbe, C. C. 2008. Origin and diversity of metabolically active gut bacteria from laboratory-bred larvae of *Manduca sexta* (Sphingidae, Lepidoptera, Insecta). *Appl Environ Microbiol,* 74**,** 7189-96.

Broderick, N. A., Raffa, K. F., Goodman, R. M. & 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.

Brooks, J. F., 2nd, Gyllborg, M. C., Cronin, D. C., Quillin, S. J., Mallama, C. A., Foxall, R., Whistler, C., Goodman, A. L. & Mandel, M. J. 2014. Global discovery of colonization determinants in the squid symbiont *Vibrio fischeri*. *Proc Natl Acad Sci U S A,* 111**,** 17284-9.

Brown, K. L. & Hancock, R. E. 2006. Cationic host defense (antimicrobial) peptides. *Curr Opin Immunol,* 18**,** 24-30.

Brucker, R. M. & Bordenstein, S. R. 2012. The roles of host evolutionary relationships (genus: *Nasonia*) and development in structuring microbial communities. *Evolution,* 66**,** 349-62.

Brune, A. 2006. Symbiotic associations between termites and prokaryotes. *In:* Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K. H. & Stackebrandt, E. (eds.) *The Prokaryotes.* New York: Springer.

Brune, A. & Kühl, M. 1996. pH profiles of the extremely alkaline hindguts of soil-feeding termites (Isoptera: Termitidae) determined with microelectrodes. *J Insect Physiol,* 42**,** 1121-27.

Buchner, P. 1965. Endosymbiosis of animals with plant microorganisms. Interscience, New York, NY.

Buchon, N., Broderick, N. A., Chakrabarti, S. & Lemaitre, B. 2009. Invasive and indigenous microbiota impact intestinal stem cell activity through multiple pathways in *Drosophila*. *Genes Dev,* 23**,** 2333-44.

Caplice, E. & Fitzgerald, G. F. 1999. Food fermentations: role of microorganisms in food production and preservation. *Int J Food Microbiol,* 50**,** 131-49.

Carvalho Mda, G., Steigerwalt, A. G., Morey, R. E., Shewmaker, P. L., Falsen, E., Facklam, R. R. & Teixeira, L. M. 2008. Designation of the provisional new *enterococcus* species CDC PNS-E2 as *Enterococcus sanguinicola* sp. nov., isolated from human blood, and identification of a strain previously named *Enterococcus* CDC PNS-E1 as *Enterococcus italicus* Fortina, Ricci, Mora, and Manachini 2004. *J Clin Microbiol,* 46**,** 3473-6.

Chandler, J. A., Lang, J. M., Bhatnagar, S., Eisen, J. A. & Kopp, A. 2011. Bacterial communities of diverse *Drosophila* species: ecological context of a host-microbe model system. *PLoS Genet,* 7**,** e1002272.

Chaudhuri, S., Gantner, B. N., Ye, R. D., Cianciotto, N. P. & Freitag, N. E. 2013. The *Listeria monocytogenes* ChiA chitinase enhances virulence through suppression of host innate immunity. *MBio,* 4**,** e00617-12.

Chouayekh, H., Serror, P., Boudebbouze, S. & Maguin, E. 2009. Highly efficient production of the staphylococcal nuclease reporter in *Lactobacillus bulgaricus* governed by the promoter of the *hlbA* gene. *FEMS Microbiol Lett,* 293**,** 232-9.

Clark, T. M. 1999. Evolution and adaptive significance of larval midgut alkalinization in the insect superorder. *J Chem Ecol,* 25**,** 1945-1960.

Coates, C. J., Jasinskiene, N., Miyashiro, L. & James, A. A. 1998. Mariner transposition and transformation of the yellow fever mosquito, *Aedes aegypti*. *Proc Natl Acad Sci U S A,* 95**,** 3748-51.

Colman, D. R., Toolson, E. C. & Takacs-Vesbach, C. D. 2012. Vesbach CD. 2012. Do diet and taxonomy influence insect gut bacterial communities? *Molecular Ecology,* 21**,** 5124-5137.

Cox, C. R. & Gilmore, M. S. 2007. Native microbial colonization of *Drosophila melanogaster* and its use as a model of *Enterococcus faecalis* pathogenesis. *Infect Immun,* 75**,** 1565-76.

Cullen, T. W., Schofield, W. B., Barry, N. A., Putnam, E. E., Rundell, E. A., Trent, M. S., Degnan, P. H., Booth, C. J., Yu, H. & Goodman, A. L. 2015. Gut microbiota. Antimicrobial peptide resistance mediates resilience of prominent gut commensals during inflammation. *Science,* 347**,** 170-5.

De Bary, A. 1879. Die Erscheinung der Symbiose. Strassburg: Verlag Trubner.

De Vuyst, L. & Vandamme, E. J. 1994. Antimicrobial potential of lactic acid bacteria. *In:* De Vuyst, L. & Vandamme, E. J. (eds.). London: Blackie Academic & Professional.

Delalibera, I., Vasanthakumar, A., Burwitz, B. J., Schloss, P. D., Klepzig, K. D., Handelsman, J. & Raffa, K. F. 2007. Composition of the bacterial community in the gut of the pine engraver, *Ips pini* (Say) (Coleoptera) colonizing red pine. *Symbiosis,* 43**,** 97-104.

Dillon, R. J. & Dillon, V. M. 2004. The gut bacteria of insects: nonpathogenic interactions. *Annu Rev Entomol,* 49**,** 71-92.

Dillon, R. J., Vennard, C. T. & Charnley, A. K. 2002. A note: gut bacteria produce components of a locust cohesion pheromone. *J Appl Microbiol,* 92**,** 759-63.

Dionne, M. S. & Schneider, D. S. 2008. Models of infectious diseases in the fruit fly *Drosophila melanogaster*. *Dis Model Mech,* 1**,** 43-9.

Dornan, S. & Collins, M. A. 1990. High efficiency electroporation of *Lactococcus lactis* subsp. *lactis* LM0230 with plasmid pGB301. *Lett Appl Microbiol,* 11**,** 62-4.

Dostalova, A. & Volf, P. 2012. *Leishmania* development in sand flies: parasite-vector interactions overview. *Parasit Vectors,* 5**,** 276.

Douglas, A. E. 2007. Symbiotic microorganisms: untapped resources for insect pest control. *Trends Biotechnol,* 25**,** 338-42.

Douglas, A. E. 2011. Lessons from studying insect symbioses. *Cell Host Microbe,* 10**,** 359-67.

Doyle, T. C., Burns, S. M. & Contag, C. H. 2004. *In vivo* bioluminescence imaging for integrated studies of infection. *Cell Microbiol,* 6**,** 303-17.

Dubilier, N., Bergin, C. & Lott, C. 2008. Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nat Rev Microbiol,* 6**,** 725-40.

El-Guindy, M. A., Abdel-Sattar, M. M. & El-Refai, A. R. M. 1983. The pattern of cross-resistance to insecticides and juvenile hormone analogues in a diflubenzuron-resistant strain of the cotton leaf worm *Spodoptera littoralis* Boisd. *Pestic Sci,* 14**,** 235-245.

Engel, P. & Moran, N. A. 2013. The gut microbiota of insects - diversity in structure and function. *FEMS Microbiol Rev,* 37**,** 699-735.

Fabich, A. J., Jones, S. A., Chowdhury, F. Z., Cernosek, A., Anderson, A., Smalley, D., Mchargue, J. W., Hightower, G. A., Smith, J. T., Autieri, S. M., Leatham, M. P., Lins, J. J., Allen, R. L., Laux, D. C., Cohen, P. S. & Conway, T. 2008. Comparison of carbon nutrition for pathogenic and commensal *Escherichia coli* strains in the mouse intestine. *Infect Immun,* 76**,** 1143-52.

Fang, F., Flynn, S., Li, Y., Claesson, M. J., Van Pijkeren, J. P., Collins, J. K., Van Sinderen, D. & O'toole, P. W. 2008. Characterization of endogenous plasmids from *Lactobacillus salivarius* UCC118. *Appl Environ Microbiol,* 74**,** 3216-28.

Fao/Who 2002. Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food. London, Ontario, Canada.

Fischetti, V. A., Novick, R. P., Ferretti, J. J., Portnoy, D. A. & Rood, J. I. 2006. *Gram-positive pathogens,* Washington, DC, American Society for Microbiology.

Franz, C. M., Holzapfel, W. H. & Stiles, M. E. 1999. Enterococci at the crossroads of food safety? *Int J Food Microbiol,* 47**,** 1-24.

Frese, S. A., Benson, A. K., Tannock, G. W., Loach, D. M., Kim, J., Zhang, M., Oh, P. L., Heng, N. C., Patil, P. B., Juge, N., Mackenzie, D. A., Pearson, B. M., Lapidus, A., Dalin, E., Tice, H., Goltsman, E., Land, M., Hauser, L., Ivanova, N., Kyrpides, N. C. & Walter, J. 2011. The evolution of host specialization in the vertebrate gut symbiont *Lactobacillus reuteri*. *PLoS Genet,* 7**,** e1001314.

Fukatsu, T. & Hosokawa, T. 2002. Capsule-transmitted gut symbiotic bacterium of the Japanese common plataspid stinkbug, *Megacopta punctatissima*. *Appl Environ Microbiol,* 68**,** 389-96.

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**,** 1953-9.

Funkhouser, L. J. & Bordenstein, S. R. 2013. Mom knows best: the universality of maternal microbial transmission. *PLoS Biol,* 11**,** e1001631.

Gage, D. J. 2004. Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiol Mol Biol Rev,* 68**,** 280-300.

Gilmore, M. S., Lebreton, F. & Van Schaik, W. 2013. Genomic transition of enterococci from gut commensals to leading causes of multidrug-resistant hospital infection in the antibiotic era. *Curr Opin Microbiol,* 16**,** 10-6.

Grossman, G. L., Rafferty, C. S., Clayton, J. R., Stevens, T. K., Mukabayire, O. & Benedict, M. Q. 2001. Germline transformation of the malaria vector, *Anopheles gambiae*, with the *piggyBac* transposable element. *Insect Mol Biol,* 10**,** 597-604.

Ha, E. M., Oh, C. T., Bae, Y. S. & Lee, W. J. 2005. A direct role for dual oxidase in *Drosophila* gut immunity. *Science,* 310**,** 847-50.

Hamdi, C., Balloi, A., Essanaa, J., Crotti, E., Gonella, E., Raddadi, N., Ricci, I., Boudabous, A., Borin, S., Manino, A., Bandi, C., Alma, A., Daffonchio, D. & Cherif, A. 2011. Gut microbiome dysbiosis and honeybee health. *J Appl Entomol,* 135**,** 524-533.

Hammer, T. J., Mcmillan, W. O. & Fierer, N. 2014. Metamorphosis of a butterfly-associated bacterial community. *PLoS One,* 9**,** e86995.

Hansen, M. C., Palmer, R. J., Jr., Udsen, C., White, D. C. & Molin, S. 2001. Assessment of GFP fluorescence in cells of *Streptococcus gordonii* under conditions of low pH and low oxygen concentration. *Microbiology,* 147**,** 1383-91.

Hartenstein, V. 1993. *Atlas of Drosophila development,* Cold Spring Harbor, Cold Spring Harbor Laboratory Press.

Hendrickx, A. P., Budzik, J. M., Oh, S. Y. & Schneewind, O. 2011. Architects at the bacterial surface - sortases and the assembly of pili with isopeptide bonds. *Nat Rev Microbiol,* 9**,** 166-76.

Hongoh, Y., Sharma, V. K., Prakash, T., Noda, S., Toh, H., Taylor, T. D., Kudo, T., Sakaki, Y., Toyoda, A., Hattori, M. & Ohkuma, M. 2008. Genome of an endosymbiont coupling N2 fixation to cellulolysis within protist cells in termite gut. *Science,* 322**,** 1108-9.

Horn, M., Collingro, A., Schmitz-Esser, S., Beier, C. L., Purkhold, U., Fartmann, B., Brandt, P., Nyakatura, G. J., Droege, M., Frishman, D., Rattei, T., Mewes, H. W. & Wagner, M. 2004. Illuminating the evolutionary history of chlamydiae. *Science,* 304**,** 728-30.

Huang, J. Y., Sweeney, E. G., Sigal, M., Zhang, H. C., Remington, S. J., Cantrell, M. A., Kuo, C. J., Guillemin, K. & Amieva, M. R. 2015. Chemodetection and destruction of host urea allows *Helicobacter pylori* to locate the epithelium. *Cell Host Microbe,* 18**,** 147-56.

Huang, S. W., Zhang, H. Y., Marshall, S. & Jackson, T. A. 2010. The scarab gut: A potential bioreactor for bio-fuel production. *Insect Sci,* 17**,** 175-183.

Husseneder, C. & Grace, J. K. 2005. Genetically engineered termite gut bacteria (*Enterobacter cloacae*) deliver and spread foreign genes in termite colonies. *Appl Microbiol Biotechnol,* 68**,** 360-7.

Jarrett, P. & Stephenson, M. 1990. Plasmid transfer between strains of *Bacillus thuringiensis* infecting *Galleria mellonella* and *Spodoptera littoralis*. *Appl Environ Microbiol,* 56**,** 1608-14.

Jing, X., Wong, A. C., Chaston, J. M., Colvin, J., Mckenzie, C. L. & Douglas, A. E. 2014. The bacterial communities in plant phloem-sap-feeding insects. *Mol Ecol,* 23**,** 1433-44.

Johansson, M. E., Phillipson, M., Petersson, J., Velcich, A., Holm, L. & Hansson, G. C. 2008. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc Natl Acad Sci U S A,* 105**,** 15064-9.

Jones, R. T., Sanchez, L. G. & Fierer, N. 2013. A cross-taxon analysis of insect-associated bacterial diversity. *PLoS One,* 8**,** e61218.

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**,** 8618-22.

Klein, G. 2003. Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract. *Int J Food Microbiol,* 88**,** 123-31.

Knorr, E., Schmidtberg, H., Arslan, D., Bingsohn, L. & Vilcinskas, A. 2015. Translocation of bacteria from the gut to the eggs triggers maternal transgenerational immune priming in *Tribolium castaneum*. *Biol Lett,* 11.

Kodama, R. & Nakasuji, Y. 1971. Further studies on the pathogenic mechanism of bacterial diseases in gnotobiotic silkworm larvae. *IFO Res Comm,* 5**,** 1-9.

Kohler, T., Dietrich, C., Scheffrahn, R. H. & Brune, A. 2012. High-resolution analysis of gut environment and bacterial microbiota reveals functional compartmentation of the gut in wood-feeding higher termites (*Nasutitermes* spp.). *Appl Environ Microbiol,* 78**,** 4691-701.

Kojima, A. & Hirose, E. 2012. Transmission of cyanobacterial symbionts during embryogenesis in the coral reef ascidians *Trididemnum nubilum* and *T. clinides* (Didemnidae, Ascidiacea, Chordata). *Biol Bull,* 222**,** 63-73.

Kommineni, S., Bretl, D. J., Lam, V., Chakraborty, R., Hayward, M., Simpson, P., Cao, Y., Bousounis, P., Kristich, C. J. & Salzman, N. H. 2015. Bacteriocin production augments niche competition by enterococci in the mammalian gastrointestinal tract. *Nature,* 526**,** 719-22.

Kostic, A. D., Howitt, M. R. & Garrett, W. S. 2013. Exploring host-microbiota interactions in animal models and humans. *Genes Dev,* 27**,** 701-18.

Kwong, W. K., Engel, P., Koch, H. & Moran, N. A. 2014. Genomics and host specialization of honey bee and bumble bee gut symbionts. *Proc Natl Acad Sci U S A,* 111**,** 11509-14.

Lazzaro, B. P. 2008. Natural selection on the *Drosophila* antimicrobial immune system. *Curr Opin Microbiol,* 11**,** 284-9.

Lemaitre, B. & Hoffmann, J. 2007. The host defense of *Drosophila melanogaster*. *Annu Rev Immunol,* 25**,** 697-743.

Lemaitre, B. & Miguel-Aliaga, I. 2013. The digestive tract of *Drosophila melanogaster*. *Annu Rev Genet,* 47**,** 377-404.

Leroy, P. D., Sabri, A., Heuskin, S., Thonart, P., Lognay, G., Verheggen, F. J., Francis, F., Brostaux, Y., Felton, G. W. & Haubruge, E. 2011. Microorganisms from aphid honeydew attract and enhance the efficacy of natural enemies. *Nat Commun,* 2**,** 348.

Ley, R. E., Peterson, D. A. & Gordon, J. I. 2006. Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell,* 124**,** 837-48.

Liu, L., Tong, H. & Dong, X. 2012. Function of the pyruvate oxidase-lactate oxidase cascade in interspecies competition between *Streptococcus oligofermentans* and *Streptococcus mutans*. *Appl Environ Microbiol,* 78**,** 2120-7.

Liu, W., Zhang, K., Li, Y., Su, W., Hu, K. & Jin, S. 2017. Enterococci mediate the oviposition preference of *Drosophila melanogaster* through sucrose catabolism. *Sci Rep,* 7**,** 13420.

Lopez, G., Latorre, M., Reyes-Jara, A., Cambiazo, V. & Gonzalez, M. 2012. Transcriptomic response of *Enterococcus faecalis* to iron excess. *Biometals,* 25**,** 737-47.

Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K. & Knight, R. 2012. Diversity, stability and resilience of the human gut microbiota. *Nature,* 489**,** 220-30.

Ludwig, W., Schleifer, K. H., Whitman, W. B. & Al, E. 2009. Family IV. *Enterococcaceae* fam. nov. *In:* De Vos, P., Garrity, G. M. & Jones, D. (eds.) *Bergey's Manual of Systematic Bacteriology.* 2nd ed. New York: Springer.

Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., Pavlov, A., Pavlova, N., Karamychev, V., Polouchine, N., Shakhova, V., Grigoriev, I., Lou, Y., Rohksar, D., Lucas, S., Huang, K., Goodstein, D. M., Hawkins, T., Plengvidhya, V., Welker, D., Hughes, J., Goh, Y., Benson, A., Baldwin, K., Lee, J. H., Diaz-Muniz, I., Dosti, B., Smeianov, V., Wechter, W., Barabote, R., Lorca, G., Altermann, E., Barrangou, R., Ganesan, B., Xie, Y., Rawsthorne, H., Tamir, D., Parker, C., Breidt, F., Broadbent, J., Hutkins, R., O'sullivan, D., Steele, J., Unlu, G., Saier, M., Klaenhammer, T., Richardson, P., Kozyavkin, S., Weimer, B. & Mills, D. 2006. Comparative genomics of the lactic acid bacteria. *Proc Natl Acad Sci U S A,* 103**,** 15611-6.

Martin, J. D. & Mundt, J. O. 1972. Enterococci in insects. *Appl Microbiol,* 24**,** 575-80.

Mcintyre, D. A. & Harlander, S. K. 1989. Genetic transformation of intact *Lactococcus lactis* subsp. *lactis* by high-voltage electroporation. *Appl Environ Microbiol,* 55**,** 604-10.

Moll, R. M., Romoser, W. S., Modrzakowski, M. C., Moncayo, A. C. & Lerdthusnee, K. 2001. Meconial peritrophic membranes and the fate of midgut bacteria during mosquito (Diptera: Culicidae) metamorphosis. *J Med Entomol,* 38**,** 29-32.

Momose, Y., Hirayama, K. & Itoh, K. 2008. Competition for proline between indigenous *Escherichia coli* and *E. coli* O157:H7 in gnotobiotic mice associated with infant intestinal microbiota and its contribution to the colonization resistance against *E. coli* O157:H7. *Antonie Van Leeuwenhoek,* 94**,** 165-71.

Morales-Jimenez, J., Zuniga, G., Villa-Tanaca, L. & Hernandez-Rodriguez, C. 2009. Bacterial community and nitrogen fixation in the red turpentine beetle, *Dendroctonus valens* LeConte (Coleoptera: Curculionidae: Scolytinae). *Microb Ecol,* 58**,** 879-91.

Moran, N. A., Mccutcheon, J. P. & Nakabachi, A. 2008. Genomics and evolution of heritable bacterial symbionts. *Annu Rev Genet,* 42**,** 165-90.

Mosallanejad, H. & Smagghe, G. 2009. Biochemical mechanisms of methoxyfenozide resistance in the cotton leafworm *Spodoptera littoralis*. *Pest Manag Sci,* 65**,** 732-6.

Moya, A., Pereto, J., Gil, R. & Latorre, A. 2008. Learning how to live together: genomic insights into prokaryote-animal symbioses. *Nat Rev Genet,* 9**,** 218-29.

Mullard, A. 2009. Microbiology: Tinker, bacteria, eukaryote, spy. *Nature,* 459**,** 159-61.

Muller, S. & Nebe-Von-Caron, G. 2010. Functional single-cell analyses: flow cytometry and cell sorting of microbial populations and communities. *FEMS Microbiol Rev,* 34**,** 554-87.

Mundy, L. M., Sahm, D. F. & Gilmore, M. S. 2000. Relationship between enterococcal virulence and antimicrobial resistance. *Clin Microbiol Rev,* 13**,** 513-522.

Nallapareddy, S. R., Wenxiang, H., Weinstock, G. M. & Murray, B. E. 2005. Molecular characterization of a widespread, pathogenic, and antibiotic resistance-receptive *Enterococcus faecalis* lineage and dissemination of its putative pathogenicity island. *J Bacteriol,* 187**,** 5709-18.

Nemergut, D. R., Costello, E. K., Hamady, M., Lozupone, C., Jiang, L., Schmidt, S. K., Fierer, N., Townsend, A. R., Cleveland, C. C., Stanish, L. & Knight, R. 2011. Global patterns in the biogeography of bacterial taxa. *Environ Microbiol,* 13**,** 135-44.

Nyholm, S. V. & Mcfall-Ngai, M. J. 2003. Dominance of *Vibrio fischeri* in secreted mucus outside the light organ of *Euprymna scolopes*: the first site of symbiont specificity. *Appl Environ Microbiol,* 69**,** 3932-7.

Nyholm, S. V. & Mcfall-Ngai, M. J. 2004. The winnowing: establishing the squid-*vibrio* symbiosis. *Nat Rev Microbiol,* 2**,** 632-42.

O'callaghan, D. & Vergunst, A. 2010. Non-mammalian animal models to study infectious disease: worms or fly fishing? *Curr Opin Microbiol,* 13**,** 79-85.

O'sullivan, D. J. & Klaenhammer, T. R. 1993. High- and low-copy-number *Lactococcus* shuttle cloning vectors with features for clone screening. *Gene,* 137**,** 227-31.

Papadimitriou, K., Zoumpopoulou, G., Foligne, B., Alexandraki, V., Kazou, M., Pot, B. & Tsakalidou, E. 2015. Discovering probiotic microorganisms: *in vitro*, *in vivo*, genetic and omics approaches. *Front Microbiol,* 6**,** 58.

Parekh, S., Ziegenhain, C., Vieth, B., Enard, W. & Hellmann, I. 2016. The impact of amplification on differential expression analyses by RNA-seq. *Sci Rep,* 6**,** 25533.

Pickard, J. M. & Chervonsky, A. V. 2015. Intestinal fucose as a mediator of host-microbe symbiosis. *J Immunol,* 194**,** 5588-93.

Priya, N. G., Ojha, A., Kajla, M. K., Raj, A. & Rajagopal, R. 2012. Host plant induced variation in gut bacteria of *Helicoverpa armigera*. *PLoS One,* 7**,** e30768.

Rince, A., Giard, J. C., Pichereau, V., Flahaut, S. & Auffray, Y. 2001. Identification and characterization of *gsp65*, an organic hydroperoxide resistance (*ohr*) gene encoding a general stress protein in *Enterococcus faecalis*. *J Bacteriol,* 183**,** 1482-8.

Rixon, J. & Warner, P. J. 2003. Background, relevant genetic techniques and terms. *In*: Wood B. J. B, Warner P. J. (Eds). *Genetics of lactic acid bacteria*. Kluwer Academic/Plenum Publishers, New York, NY. 1-24.

Rodriguez, M. C., Alegre, M. T. & Mesas, J. M. 2007. Optimization of technical conditions for the transformation of *Pediococcus acidilactici* P60 by electroporation. *Plasmid,* 58**,** 44-50.

Russell, J. A., Moreau, C. S., Goldman-Huertas, B., Fujiwara, M., Lohman, D. J. & Pierce, N. E. 2009. Bacterial gut symbionts are tightly linked with the evolution of herbivory in ants. *Proc Natl Acad Sci U S A,* 106**,** 21236-41.

Russell, J. A., Weldon, S., Smith, A. H., Kim, K. L., Hu, Y., Lukasik, P., Doll, S., Anastopoulos, I., Novin, M. & Oliver, K. M. 2013. Uncovering symbiont-driven genetic diversity across North American pea aphids. *Mol Ecol,* 22**,** 2045-59.

Russell, J. B. & Rychlik, J. L. 2001. Factors that alter rumen microbial ecology. *Science,* 292**,** 1119-22.

Russell, V. & Dunn, P. E. 1996. Antibacterial proteins in the midgut of *Manduca sexta* during metamorphosis. *J Insect Physiol,* 42**,** 65-71.

Sabree, Z. L., Kambhampati, S. & Moran, N. A. 2009. Nitrogen recycling and nutritional provisioning by *Blattabacterium*, the cockroach endosymbiont. *Proc Natl Acad Sci U S A,* 106**,** 19521-6.

Schleifer, K. H. & Kilpper-Balz, R. 1984. Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the genus *Enterococcus* nom. rev. as *Enterococcus faecalis* comb. nov. and

Enterococcus faecium comb. nov. *International Journal of Systematic and Evolutionary Microbiology,* 34**,** 31-34.

Schoenian, I., Spiteller, M., Ghaste, M., Wirth, R., Herz, H. & Spiteller, D. 2011. Chemical basis of the synergism and antagonism in microbial communities in the nests of leaf-cutting ants. *Proc Natl Acad Sci U S A,* 108**,** 1955-60.

Schwartzman, J. A. & Ruby, E. G. 2016. Stress as a normal cue in the symbiotic environment. *Trends Microbiol,* 24**,** 414-24.

Shanbhag, S. & Tripathi, S. 2009. Epithelial ultrastructure and cellular mechanisms of acid and base transport in the *Drosophila* midgut. *J Exp Biol,* 212**,** 1731-44.

Shao, Y., Arias-Cordero, E., Guo, H., Bartram, S. & Boland, W. 2014. *In vivo* Pyro-SIP assessing active gut microbiota of the cotton leafworm, *Spodoptera littoralis*. *PLoS One,* 9**,** e85948.

Shao, Y., Chen, B., Sun, C., Ishida, K., Hertweck, C. & Boland, W. 2017. Symbiont-derived antimicrobials contribute to the control of the lepidopteran gut microbiota. *Cell Chem Biol,* 24**,** 66-75.

Sharon, G., Segal, D., Ringo, J. M., Hefetz, A., Zilber-Rosenberg, I. & Rosenberg, E. 2010. Commensal bacteria play a role in mating preference of *Drosophila melanogaster*. *Proc Natl Acad Sci U S A,* 107**,** 20051-6.

Sherman, J. M. 1937. The Streptococci. *Bacteriol Rev,* 1**,** 3-97.

Shibata, S., Yip, E. S., Quirke, K. P., Ondrey, J. M. & Visick, K. L. 2012. Roles of the structural symbiosis polysaccharide (*syp*) genes in host colonization, biofilm formation, and polysaccharide biosynthesis in *Vibrio fischeri*. *J Bacteriol,* 194**,** 6736-47.

Shin, R., Suzuki, M. & Morishita, Y. 2002. Influence of intestinal anaerobes and organic acids on the growth of enterohaemorrhagic *Escherichia coli* O157:H7. *J Med Microbiol,* 51**,** 201-6.

Singh, M., Yadav, A., Ma, X. & Amoah, E. 2010. Plasmid DNA transformation in *Escherichia coli*. Effect of heat shock temperature, duration, and cold incubation of CaCl2 treated cells. *Int J Biotechnol Biochem,* 6**,** 561-568.

Storelli, G., Defaye, A., Erkosar, B., Hols, P., Royet, J. & Leulier, F. 2011. *Lactobacillus plantarum* promotes *Drosophila* systemic growth by modulating hormonal signals through TOR-dependent nutrient sensing. *Cell Metab,* 14**,** 403-14.

Suree, N., Jung, M. E. & Clubb, R. T. 2007. Recent advances towards new anti-infective agents that inhibit cell surface protein anchoring in *Staphylococcus aureus* and other gram-positive pathogens. *Mini Rev Med Chem,* 7**,** 991-1000.

Tan, C. K., Lai, C. C., Wang, J. Y., Lin, S. H., Liao, C. H., Huang, Y. T., Wang, C. Y., Lin, H. I. & Hsueh, P. R. 2010. Bacteremia caused by non-*faecalis* and non-*faecium enterococcus* species at a Medical center in Taiwan, 2000 to 2008. *J Infect,* 61**,** 34-43.

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

Tasteyre, A., Barc, M. C., Collignon, A., Boureau, H. & Karjalainen, T. 2001. Role of FliC and FliD flagellar proteins of *Clostridium difficile* in adherence and gut colonization. *Infect Immun,* 69**,** 7937-40.

Teh, B. S., Apel, J., Shao, Y. & Boland, W. 2016. Colonization of the intestinal tract of the polyphagous pest *Spodoptera littoralis* with the GFP-tagged indigenous gut bacterium *Enterococcus mundtii*. *Front Microbiol,* 7**,** 928.

Thompson, K. & Collins, M. A. 1996. Improvement in electroporation efficiency for *Lactobacillus plantarum* by the inclusion of high concentrations of glycine in the growth medium. *J Microbiol Methods,* 26**,** 73-79.

Truman, J. W. & Riddiford, L. M. 1999. The origins of insect metamorphosis. *Nature,* 401**,** 447-52.

Tyrrell, G. J., Turnbull, L., Teixeira, L. M., Lefebvre, J., Carvalho Mda, G., Facklam, R. R. & Lovgren, M. 2002. *Enterococcus gilvus* sp. nov. and *Enterococcus pallens* sp. nov. isolated from human clinical specimens. *J Clin Microbiol,* 40**,** 1140-5.

Van Zyl, W. F., Deane, S. M. & Dicks, L. M. 2015. Use of the mCherry fluorescent protein to study intestinal colonization by *Enterococcus mundtii* ST4SA and *Lactobacillus plantarum* 423 in mice. *Appl Environ Microbiol,* 81**,** 5993-6002.

Venn, A. A., Loram, J. E. & Douglas, A. E. 2008. Photosynthetic symbioses in animals. *J Exp Bot,* 59**,** 1069-80.

Wallstein, M. 2014. Transformation of gut bacteria for visualization in the intestine of *Spodoptera littoralis*. Bachelor Thesis. Jena, Germany: Friedrich-Schiller-Universität Jena, Biologisch Pharmazeutische Fakultät.

Wang, J., Wu, Y., Yang, G. & Aksoy, S. 2009. Interactions between mutualist *Wigglesworthia* and tsetse peptidoglycan recognition protein (PGRP-LB) influence trypanosome transmission. *Proc Natl Acad Sci U S A,* 106**,** 12133-12138.

Warnecke, F., Luginbuhl, P., Ivanova, N., Ghassemian, M., Richardson, T. H., Stege, J. T., Cayouette, M., Mchardy, A. C., Djordjevic, G., Aboushadi, N., Sorek, R., Tringe, S. G., Podar, M., Martin, H. G., Kunin, V., Dalevi, D., Madejska, J., Kirton, E., Platt, D., Szeto, E., Salamov, A., Barry, K., Mikhailova, N., Kyrpides, N. C., Matson, E. G., Ottesen, E. A., Zhang, X., Hernandez, M., Murillo, C., Acosta, L. G., Rigoutsos, I., Tamayo, G., Green, B. D., Chang, C., Rubin, E. M., Mathur, E. J., Robertson, D. E., Hugenholtz, P. & Leadbetter, J. R. 2007. Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. *Nature,* 450**,** 560-5.

Watanabe, K. & Sato, M. 1998. Plasmid-mediated gene transfer between insect-resident bacteria, *Enterobacter cloacae*, and plant-epiphytic bacteria, *Erwinia herbicola*, in guts of silkworm larvae. *Curr Microbiol,* 37**,** 352-5.

Webster, N. S., Taylor, M. W., Behnam, F., Lucker, S., Rattei, T., Whalan, S., Horn, M. & Wagner, M. 2010. Deep sequencing reveals exceptional diversity and modes of transmission for bacterial sponge symbionts. *Environ Microbiol,* 12**,** 2070-82.

Wei, M. Q., Rush, C. M., Norman, J. M., Hafner, L. M., Epping, R. J. & Timms, P. 1995. An improved method for the transformation of *Lactobacillus* strains using electroporation. *J Microbiol Methods,* 21**,** 97-109.

Wieczorek, H., Beyenbach, K. W., Huss, M. & Vitavska, O. 2009. Vacuolar-type proton pumps in insect epithelia. *J Exp Biol,* 212**,** 1611-9.

Wollenberg, M. S., Claesen, J., Escapa, I. F., Aldridge, K. L., Fischbach, M. A. & Lemon, K. P. 2014. Propionibacterium-produced coproporphyrin III induces *Staphylococcus aureus* aggregation and biofilm formation. *MBio,* 5**,** e01286-14.

Wong, A. C., Chaston, J. M. & Douglas, A. E. 2013. The inconstant gut microbiota of *Drosophila* species revealed by 16S rRNA gene analysis. *ISME J,* 7**,** 1922-32.

Wong, C. N., Ng, P. & Douglas, A. E. 2011. Low-diversity bacterial community in the gut of the fruitfly *Drosophila melanogaster*. *Environ Microbiol,* 13**,** 1889-900.

Xiang, H., Wei, G. F., Jia, S., Huang, J., Miao, X. X., Zhou, Z., Zhao, L. P. & Huang, Y. P. 2006. Microbial communities in the larval midgut of laboratory and field populations of cotton bollworm (*Helicoverpa armigera*). *Can J Microbiol,* 52**,** 1085-92.

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.

_____

Beng-Soon Teh

Jena, den 03.12.2017

13. Acknowledgments

It is truly an exciting PhD journey for me over the past 5 years. I would like to take this opportunity to express my gratitude to Professor Boland for his supervision, discussion, and guidance towards the completion of my PhD works. The facilities and equipment available at the department are fantastic, which allow me to perform experiments without problems. I would like to acknowledge the financial assistance from JSMC and MPI that support my living expenses in Germany. Of course, I would like to thank my parents for their unconditional love and support throughout my PhD journey. Their patience and words of encouragement have motivated me to undertake the doctoral study.

Colleagues and technicians are simply awesome. It has been invaluable experiences to work with Johanna Apel, Michael Wallstein, Yongqi Shao, Erika Arias-Cordero, Pol Alonso, Alexey Novosolev, and Tilottama Mazumdar. I received good advices from Yongqi and Erika on the experimental setup and discussion. I would like to attribute the contribution, particularly to Tilottama who helped me a lot in the lab works, transcriptome data analysis, and sharing of ideas. A big thank to Angelika Berg who helps to prepare insect larvae for experiments. It is great to acquire some input from Anne-Kathrin regarding the plasmid construction and bacterial physiology. There are many people to thank at the Department of Bioorganic Chemistry. Also, special thanks to our collaborators, Yvonne Schlenker (Universitätsklinikum Jena) for flow cytometry assistance and Wolfgang Schmidt-Heck (Hans-Knöll-Institute Jena) for bioinformatics analyses. Outside the lab, social life will not be fun without friends like Felix, Federica, Sara, Reyna, and Matthias. In addition, special thanks to friends and relatives in Malaysia for their continuous support.

14. Curriculum Vitae

Personal data

Name : Beng-Soon Teh

Date of birth : February 12, 1985

Place of birth : Penang, Malaysia

Home address : Ernst-Zielinski-Str. 19, 07745 Jena

Education and Qualifications

2013-2017 Jena School for Microbial Communication (JSMC) fellow
PhD student at the Max Planck Institute for Chemical Ecology, Department of Bioorganic Chemistry, Jena, Germany

2009-2011 Master of Science (MSc) – Biotechnology
Centre for Chemical Biology (CCB@USM), University Science of Malaysia, Penang, Malaysia

2005-2008 Bachelor of Applied Science (Honors) – Biotechnology
School of Biological Sciences, University Science of Malaysia, Penang, Malaysia

Award

Jena School for Microbial Communication (JSMC) PhD fellowship

Member of society

2017 American Society for Microbiology (ASM) member

Workshop attended

2016 EMBL course: Next Generation Sequencing: RNA Sequencing Library Preparation that took place from 17-19 February 2016 at EMBL Heidelberg, Germany

Presentations

Oral presentations

Teh B.S. (2017). Mechanisms of survival of *Enterococcus mundtii* in the intestinal tract of *Spodoptera littoralis*. Talk presented at the American Society for Microbiology (ASM) Microbe 2017, New Orleans, LA, USA

Teh B.S. (2016). The secret lives of microbial gut communities across the life cycle of the insect pest *Spodoptera littoralis*. Talk presented at 51st Doktorandenworkshop: Naturstoffe: Chemie, Biologie, Ökologie, Universität Bayreuth, Bayreuth, Germany

Teh B.S. (2015). *Enterococcus mundtii* - functionally important gut bacterium in the *Spodoptera littoralis* intestinal tract. Talk presented at 5th MiCom International Student Conference, Jena, Germany

Teh B.S. (2014). Unraveling the secret lives of core bacteria within the gut of *Spodoptera littoralis*. Talk presented at JSMC Symposium, Jena, Germany

Poster presentations

Teh B.S., Mazumdar T., Boland W. (2017). Microbes-associated survival strategies in the gut of *Spodoptera littoralis*. Poster presented at 6th MiCom International Student Conference, Jena, Germany

Teh B.S., Mazumdar T., Boland W. (2016). Microbes-associated survival strategies in the gut of *Spodoptera littoralis*. Poster presented at SAB Meeting 2016, MPI for Chemical Ecology, Jena, Germany

Teh B.S., Mazumdar T., Boland W. (2016). Microbes-associated survival strategies in the gut of *Spodoptera littoralis*. Poster presented at ICE Symposium, MPI for Chemical Ecology, Jena, Germany

Teh B.S. (2016). Colonization of the GFP-tagged indigenous gut bacterium *Enterococcus mundtii* in the intestinal tract of *Spodoptera littoralis*. Poster presented at INRA-ROWETT Gut Microbiology Symposium, Clermont-Ferrand, France

Teh B.S., Boland W. (2016). Colonization of indigenous gut bacterium *Enterococcus mundtii* in the intestinal tract of *Spodoptera littoralis*. Poster presented at Annual Conference 2016 of the Association for General and Applied Microbiology (VAAM), Vereinigung für Allgemeine und Angewandte Mikrobiologie (VAAM), Jena, Germany

Teh B.S., Alonso P., Boland W. (2015). Microbes associated with the gut of generalist and specialist insects. Poster presented at ICE Symposium, MPI for Chemical Ecology, Jena, Germany

Arias Cordero E., Alonso P., Shao Y., **Teh B.S.**, Apel J., Boland W. (2014). Microbes associated with the gut of generalist and specialist insects. Poster presented at ICE Symposium, MPI for Chemical Ecology, Jena, Germany

Arias Cordero E., Alonso P., Shao Y., **Teh B.S.**, Boland W. (2014). Microbes associated with the gut of generalist and specialist insects. Poster presented at SAB Meeting 2014, MPI for Chemical Ecology, Jena, Germany

Teh B.S. (2013). Unraveling the secret lives of *Enterococcus* within the gut of *Spodoptera littoralis*. Poster presented at JSMC Symposium, Jena, Germany

List of publications

Chen, B., Sun, C., Liang, X., Lu, X., Gao, Q., Alonso, P., **Teh, B. S.**, Novoselov, A., Boland, W. & Shao, Y. (2016). Draft genome sequence of *Enterococcus mundtii* SL 16, an indigenous gut bacterium of the polyphagous pest *Spodoptera littoralis*. *Frontiers in Microbiology*. 7: 1676

Chen, B., **Teh, B. S.**, Hu, S., Lu, X., Boland, W. & Shao, Y. (2016). Biodiversity and activity of the gut microbiota across the life history of the insect herbivore *Spodoptera littoralis*. *Scientific Reports*. 6: 29505

Teh, B. S., Apel, J., Shao, Y. & Boland, W. (2016). Colonization of the intestinal tract of the polyphagous pest *Spodoptera littoralis* with the GFP-tagged indigenous gut bacterium *Enterococcus mundtii*. *Frontiers in Microbiology*. 7: 928

Teh, B. S., Lau, N. S., Ng, F. L., Abdul Rahman, A. Y., Wan, X., Saito, J., Hou, S., Teh, A. H., Najimudin, N. & Alam, M. **(**2015). Complete genome sequence of the thermophilic *Thermus* sp. strain CCB_US3_UF1 from a hot spring in Malaysia. *Standards in Genomic Sciences*. 10:76

Abdul Rahman, A. Y., Usharraj, A. O., Misra, B. B, Thottathil, G. P., Jayasekaran, K., Feng, Yun., Hou, S., Ong, S. Y., Ng, F. L., Lee, L. S., Tan, H. S., Muhd Sakaff. M. K. L., **Teh, B. S.**, Khoo, B. F., Badai, S. S., Ab Aziz, N., Yuryev, A., Knudsen, B., Dionne-Laporte, A., Mchunu, N. P., Yu, Q., Langston, B.J., Freitas, T. A. K., Young, A. G., Chen, R., Najimudin, N., Saito, J. A. & Alam, M. (2013). Draft genome sequence of the rubber tree *Hevea Brasiliensis*. *BMC Genomics*. 14(75):1-15

Teh, B. S., Abdul Rahman, A. Y., Saito, J. A., Hou, S. & Alam, M. (2012). Complete genome sequence of the thermophilic bacterium *Thermus* sp. strain CCB_US3_UF1. *Journal of Bacteriology*. 194(5):1240

Book Chapter:

Mazumdar T., **Teh, B. S.** & Boland, W. (2017). The microbiome of *Spodoptera littoralis*: development, control and adaptation to the insect host. In "*Metagenomics for Gut Microbes"*. ISBN 978-953-51-5653-6. Edited by Ranjith Kumavath. In Press

Teaching experience

May 2017	Supervised a high school student: Philipp Neusens for 2 weeks
March 2017	Conducted one-week microbiology course for a group of Master's degree students from Friedrich Schiller University of Jena.
October 2013 - September 2014	Supervised a Master of Science (Msc) student: Johanna Apel for a year.
May 2014 – August 2014	Supervised a Bachelor of Science (BSc) student: Michael Wallstein for 3 month