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Control of gut microbiome by Lepidopteran pest *Spodoptera littoralis*

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List of abbreviations

µF	Micro farad
8-HQA	8-hydroxy-2-quinoline carboxylic acid
AIP	Auto inducing proteins
aRNA	Amplified Ribonucleic acid
asp	Alkaline shock protein
ATP	Adenosine triphosphate
bgl	β-Glucosidase gene
cDNA	Complimentary Deoxyribonucleic acid
celA	Cellulose biosynthesis gene
CFU/ml	Colony forming units per milliliter
COG	Clusters of orthologous group
CTAB	Cetyl trimethylammonium bromide
ddH_2O	Double distilled water
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
ermB	Erythromycin ribosomal methylase promoter
FACS	Fluorescent associated cell sorting
FAD	Flavin adenine dinucleotide
fetC	Ferric ATP binding cassette transporter gene
FP, RP	Forward primer, reverse primer

FPKM	Fragments Per Kilobase of transcript per Million mapped reads
fruK	1-phosphofructokinase gene
Fts	Cell division protein
FUR	Ferric uptake regulation protein
GFP	Green Fluorescent Protein
glcK	Glucokinase
gls	General stress proteins
GO	Gene Ontology
HCl	Hydrochloric acid
HiSeq	High throughput sequencing
HPK	Histidine protein kinases
kDa	Kilo Dalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
KMO	Kynurenine-3-monoxygenase
LAB	Lactic acid producing bacteria
ldh	Lactate dehydrogenase gene
LPxTG	Sortase (Leu-Pro-any-Thr-Gly)
Lux	Quorum sensing protein family
MutS	DNA mismatch repair protein
NADPH	Nicotinamide adenine dinucleotide phosphate
NGS	Next Generation Sequencing
NH4OAc	Ammonium acetate

nha	Sodium/hydrogen exchanger family protein
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
pfk	Phosphofructokinase gene
PTS	Phosphotransferase system
PVP	Polyvinylpyrrolidone
Rec	Recombinant protein
RNAi	RNA interference
RNase	Ribonuclease
rRNA	Ribosomal Ribonucleic acid
S	Svedberg constant
SecE	Protein translocase complex
SfsA	Sugar fermentation stimulation protein
slp	Surface (S)-layer protein promoter
THB	Todd Hewitt Broth medium
usp	Universal shock proteins
VirD4	Type IV secretion system-coupling protein
WT	wild-type
WxL	Cell wall binding domain
YafQ	mRNA interferase toxin protein

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1 Introduction

1.1 Basics of gut microbiota

The term microbiota refers to the complete microbial population localized in a particular system. Microbial population involves bacteria, fungi, archaea, viruses and protozoans. Humans and other higher eukaryotes are colonized by diverse microbial consortia (1). The relationship of a host gut with the indigenous microbial consortium is a result of co-evolution over the past millions of years (2). These microbes are obtained during and after birth. Even though the host encounters a continued contact with a vast variety of microbes during its growth and development, it is still able to maintain a state of homeostasis (3).

The host and its microflora have two types of interactions; pathogenic and symbiotic. Pathogenic is when the microbial consortia is fatal to the host, whereas symbiotic could be 'mutualism' or 'commensalism'. Pathogenic microbes like *Wolbachia pipientis* in Arthropods could lead to sperm-egg cytoplasmic incompatibility and male killing (4). Gut microbes were found to be responsible for obesity in human and mice (5). It has been estimated that the portion of pathogenic microbes is much smaller amongst the biota colonizing animal hosts (6). Mutualistic relationship is like the gut flora of herbivores, which induces the reliance of herbivores for the cellulose digestion. This kind is a benefit to both the host organism and the symbionts. However, a majority of gut microbes are neither pathogenic nor symbiotic, which makes them 'commensal', which is neither harmful nor advantageous to both the host and the associated microbes (7).

The structure of the gut occurs in a way that it separates the symbionts from the host to prevent any pathogenic infection by the harmful microflora. The modulation of this microbial landscape is the result of dynamic interactions throughout life including diet, environment, antibiotic use, host immunity and disease. These microbes have been studied to be substantially beneficial to the host, for example, their contribution to the ability to digest the indigestible plant polysaccharides (8). The human gut microbiota influences host physiology, metabolism, nutrition and immunity. Changes in the gut microbiome have been linked with obesity, malnutrition, and other gastrointestinal conditions. Experiments on mice have proven that a normal gut microflora is necessary to keep pathogenic infections by *Salmonella typhimurium* at bay (9).

Lower vertebrates and invertebrates also have specific interactions with their respective gut microbes. Having said this, the duration of bacterial retention depends on the size of the gut, gut conditions and the host life cycle (10).

1.2 Insect gut microbiome

Insects are the predominantly known animal species residing in wide range of terrestrial habitats. Microbes have coevolved with insects forming a symbiosis that aids in supplying the host with essential nutrients, maintenance of host fitness, aids in digestion, pheromone production, host defence, metabolism and so on (10). Herbivorous insects prove to be a large niche for microbial inhabitants due to their high consumption of plant material. For example, termites depend on their intestinal microbes for plant cell wall digestion. House crickets depend on their symbiont *Acheta domesticus* for metabolism. *Burkholderia* species in Langriina beetles have antifungal properties that help the beetles against infections by *P. lilacinum* (11). The gut bacterial symbiont, *Rhizobiales* is hugely associated with providing additional nitrogen to ants. Lactic acid bacteria (LAB) species present in the gut of Western honeybees, *Apis mellifera* help to inhibit pathogen proliferation (12). Symbiont elimination in leaf beetle, *Cassida rubiginosa* results in drastic reduction of host survival which indicates the impact of symbionts on host fitness (13).

Gut microbes can be vertically transmitted, where the bacterial transfer occurs via the egg shells (also called egg smearing) and hence passes on to the succeeding generations of the insects. In vertical transmission, insects excrete symbiotic bacteria from anus to smear and contaminate the egg surfaces. The symbiont transfer can also be horizontal, which occurs through the insect's development based on the diet, social behaviour and environment (14). This could lead to a competition between the native indigenous microbial population and the non-natives in order to survive in the insect gut (9).

Symbionts are obligates and/or facultative in nature. Obligate symbionts have a major role in host fitness, whereas facultative ones might have the ability to negatively affect the insect host (15). The obligate symbionts are mostly maternally (or vertically) transmitted hence they have a co-evolutionary impact on the insect host. Obligate symbionts might have reduced genome sizes due to coevolution with the host (13). Facultative ones are either maternally or horizontally transmitted, they are either beneficial or harmful to the hosts. Because of such distant association with host, the facultative symbionts do not have reduced genomes and therefore have a free-living ability (16).

The endocellular associations are more evolutionarily related to the insect host than the extracellular symbionts. Therefore the genome evolution of the insect-symbionts is hugely affected by the endocellular or extracellular nature of the symbionts (15). Extensive genetic connection is found between extracellular symbiont interactions and host fitness that likely plays a key role in gut colonization (17). Extracellular associations are also vulnerable to replacement by non-indigenous or horizontally acquired microbes (18).

1.3 Factors determining the gut community

Microbial colonization is based on physicochemical conditions in the lumen of the insect gut, and possess extreme variation in both pH and oxygen availability. The diverse microbial community in insect gut include protists, fungi, archaea, bacteria and viruses. The bacterial phyla in the gut mostly include *Gammaproteobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, *Bacteroidetes*, *Clostridia*, *Actinomycetes*, *Spirochetes*, *Actinobacteria*, Firmicutes including *Lactobacillus* and *Bacillus* species and many others (10). Significant differences were found in the relative abundances of microbes in insects and were classified according to the criteria of host environmental habitat, diet, developmental stage, and phylogeny (19). Insect gut community diversity was also observed to depend on the complexity of gut structure. For example, Lepidoptera have a simple gut structure compared to Hemiptera. This suggests the presence of more diverse and complex gut microbe in the latter than former (10).

Apart from lack of oxygen and gut pH, the gut composition of insect are regulated by several other factors, including, presence of digestive enzymes, insect's immune system and antimicrobial compounds produced by certain gut communities (9, 20). For example, the immune system of *Drosophila melanogaster* not only prevents the insect from pathogens but also regulates its bacterial community. This involves the intestinal *Caudal* gene which aids in regulating the resident gut population. RNAi silencing of this gene proved to induce a reduction in the microbial population in the gut due to the overexpression of antimicrobial production. Also, the bumblebee gut population helps in the host defence against the common intestinal parasite *Crithidia bombi* (8). Also, produced by *Lactobacillus lactis* is a lantibiotic bacteriocin which is more effective than the conventional vancomycin antibiotic against *Streptococcus pneumoniae* infection in a mouse model (9).

1.4 Lepidopteran insect hosts

The phytophagous Lepidoptera is a widely diverse insect taxon that includes butterflies and moths. Their association with symbiotic microbes have not been intensively studied.

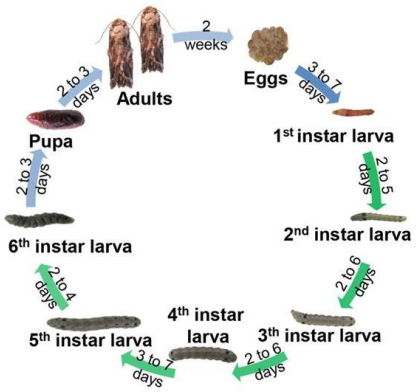


Figure 1. Life cycle of Lepidopteran insects (12). The cycle involves hatching of eggs and emergence of 1st instar larvae followed by 2nd instar through 6th instar larvae followed by pupation and hence emergence of adults and so forth.

Lepidopteran insects possess four life stages including, the egg that hatches into a larva that feeds and grows into succeeding larval instars, pupates and hence emerges as adults as shown in Figure 1 (12). Holometabolous insects undergo a dynamic microbial community turnover during a complex process called metamorphosis. This results in the increment and decrement of the microbial diversity pattern across the life stages (8).

S. littoralis is a polyphagous agricultural pest that has been reported to have evolved to resist insecticide treatments (21). It is known that controlling the insects from being pests could be possible by manipulating their gut microbial communities on a molecular level. This could include antibiotic ingestion by the pest, hereby diminishing endosymbionts which could in theory, reduce the pest activity. But this procedure is only possible *in-vitro* as antibiotics could drastically affect the insect fitness. But in some cases like in Mosquitoes, *Wolbachia* has been used to incorporate transposable elements via germline transmission to regulate parasite infection (22). Hence, the understanding of the core intestinal microbiome of *S.littoralis* might give a complete insight and ability to manipulate insect's detrimental effects on agricultural crops.

1.5 Model organism: *Spodoptera littoralis*

Spodoptera littoralis, also commonly known as the cotton leafworm is a well known agricultural pest that feed on a wide variety of plant species (as shown in Figure 2A). They

are widely used as experimental models in ecological and physiological studies (23). They possess a very simple, tube-like longitudinal gut structure which is divided into fore-, mid- and hindgut as shown in Figure 2B. The gut lacks compartmentalization (8).

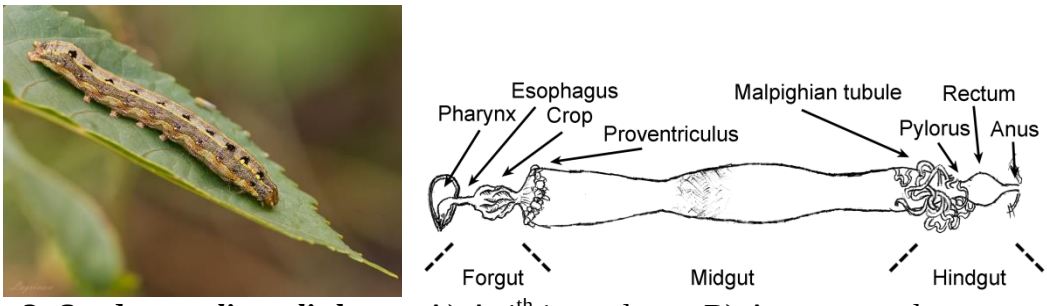


Figure 2. *Spodoptera littoralis* larva. A) A 4th instar larva. B) Anatomy and structure of the *S. littoralis* larvae (23).

The generalist herbivore Lepidopteran *S. littoralis* larvae have a foregut size of about 8 mm, midgut of about 14 mm followed by hindgut of about 8 mm. A large bacterial population of more than 10^7 CFU/mL (colony forming units per millilitre) is prevalent in the gut irrespective of the simple gut structure. The pH conditions of the three gut sections of the larvae were determined using miniaturised glass electrodes.

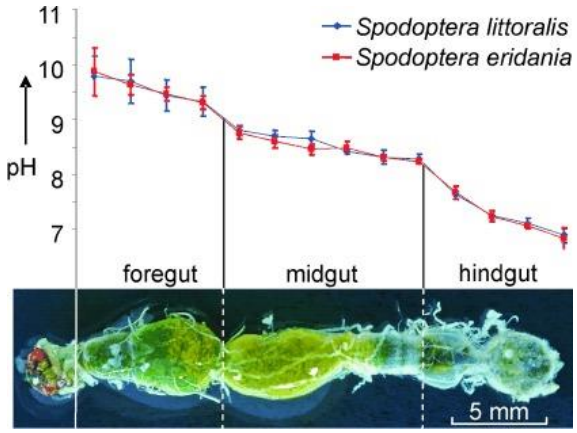


Figure 3. pH profile of *S. littoralis* larval gut. The pH of the foregut, midgut and hindgut of the larvae were measured to be 10, 8.25-8.75 and 7-7.50 respectively (24).

The foregut (regurgitate) pH was observed to be highly alkaline about pH 10±0.5. Along the gut structure, a nearly constant reduction in the pH could be observed from the foregut to the hindgut as shown in Figure 3. The pH of midgut was a moderately reduced range from 8.75 to 8.25. While in the posterior gut sections, almost neutral pH values ranging from 7.55 to 6.58 were observed (24). Because of vast pH variance, *S. littoralis* is an interesting Lepidopteran model to study complex microbial symbioses due to the simple gut structure

with an attractive gut population. The *S. littoralis* larvae maintained at 24˚C in an alternate 16 h light and 8 h dark period, that are reared in lab are fed with an artificial diet consisting of white beans, paraben and formalin (25).

High-throughput techniques have revealed the diverse gut microbial community of *S. littoralis*. The egg mass being highly diverse in bacterial community, the diversity faces a huge reduction when the insect develops from egg to pupa suggesting the fact that the host controls the microflora as it grows. The major phyla observed amongst the gut community of the larvae are Firmicutes and *Clostridia* species (23). The development of an anoxic environment in the growing larval gut clearly suggests the increasing presence of such anaerobic microbes. Firmicutes have an increasing ability to harvest energy from the diet and *Clostridia* species like *C. thermocellum* and *C. ljungdahlii* have the capacity to digest cellulose and hemicellulose and also amino acid metabolism (12). Clostridia are also eminent gut bacteria in termites (23).

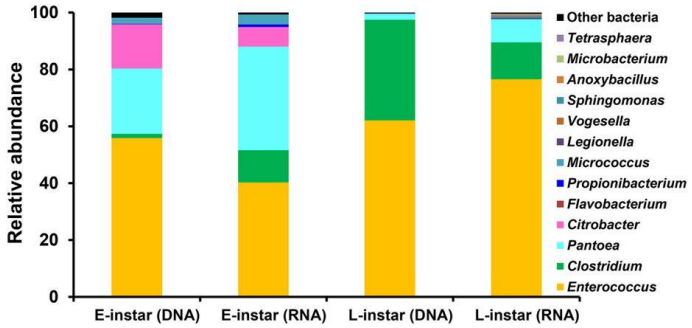


Figure 4. Larval gut microbial distribution of *S. littoralis*. The relative abundances of bacterial taxa of DNA and RNA data sets of early instar (E-instar) and late instar (L-instar) *S. littoralis* larvae (12).

Amongst the Firmicutes, *Enterococci* have been noticed to be dominant and present throughout the insects developmental stages as shown in Figure 4. *Enterococci* are the predominant gut bacteria that colonize a variety of hosts, including humans, animals and insects like Drosophila, ground beetles and desert locusts. For example, tobacco hornworm, *Manduca sexta* possess a very simple and less diverse gut microbiome with a major occurrence of *Enterococcus* species. As *Enterococci* are LAB species, they have essential probiotic properties that are advantageous to the host gastrointestinal tract (8).

Enterococcus, being the major taxon associated with the female adults, egg mass, larval gut, and hence the succeeding generations, may suggest a probably vertical transmission of the symbionts. The maternal associated symbiont transfer or vertical transmission makes the symbiosis stable and also facilitates co-evolution (12).

1.6 Predominant gut symbiont of *S.littoralis*: *Enterococcus mundtii*

The core gut bacteria of the generalist herbivore *S. littoralis* are *Enterococcus mundtii*, which prevail throughout the insect's life cycle regardless of diet as shown in Figure 5. For example, *E. mundtii* was also found to be the dominant gut in the Lepidopteran *Galleria mellonella* (26).

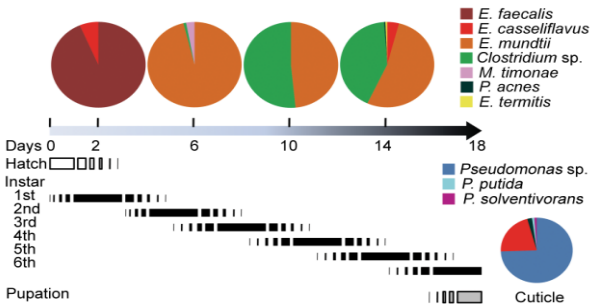


Figure 5. Temporal variation in bacterial population along the gut of *Spodoptera littoralis.* The composition of bacterial community of *S. littoralis* by cloning and sequencing from insects at various life stages is shown. *Enterococcus* species are the dominating bacteria in the insect gut (23).

E. mundtii is a gram positive, yellow pigmented, non-motile, LAB species. These species are suitable to dairy and phyto-environment. These enterococci species could be isolated from hands of milkers, soil, cow dungs and plants. It is not much well known regarding the pathogenicity, but they have been isolated during chronic thigh abscess, sinus mucosa and endophthalmitis infection in humans (27). *E. mundtii* is culturable *in vitro* (8).

The gut communities evolve strategies in order to compete and survive in the host gut. One of such effective strategies is the production of antimicrobial compounds. This *E. mundtii* SL strain produces a stable class IIa bacteriocin, Mundticin KS that helps it to compete with the coexisting pathobionts residing in the gut. Bacteriocins are evolutionarily conserved antimicrobial compounds that a great alternatives to conventional antibiotics (8).

The bacteria *Enterococcus faecalis* and *Enterococcus casseliflavus* are potential pathogens that are found during the early larval stages of *S. littoralis*. they reduce in number with the insect development (Figure 5) because mundticin KS produced by *E. mundtii* which inhibits the growth of the native coexisting Enterococci pathogens and hereby protect the Lepidopteran host. A few strains of *E. faecalis* have also been observed to cause lethal infections in Lepidopteran hosts (8). For example, in the larval development of the housefly, *E. faecalis* was studied to have deleterious effects. Specifically *E. faecalis* SL strain carries a highly virulent factor called enterococcus gelatinase that has the ability to decompose host's

extracellular matrix. These potential pathogens that could either by orally acquired via diet by *S. littoralis* or from the surrounding environment prove to be a challenge to the host survival. Which is why, *E. mundtii* helps in host defence and confers benefit to the insect (27).

Mundticin KS is selective against some pathogenic bacteria including *E. faecalis*, *Streptococcus*, and *Lactobacillus* but not any other indigenous gut residents, leading to the normal gut development of the insect host. This ability of a targeted approach towards pathogen clearance directly complements host defence. However, the production of bacteriocin was only observed in case of strain SL, which suggests that not every *E. mundtii* strain has the ability to exhibit antimicrobial activity (9).

1.7 Study of colonization and localization of *E. mundtii* by GFP based reporter method

Green Fluorescent Protein or GFP isolated from Jellyfish *Aequorea victoria* is most commonly used for fluorescent reporter based gene expression studies, localization and structural analyses of living cells. The GFP when exposed to light in blue to UV range excites at a wavelength of 395 nm and emits green fluorescence at 508 nm. It has a molecular weight of 27 kDa containing 238 amino acids. Only oxygen is required by GFP as a cofactor to be able to form chromophore and it is also stable at temperatures up to 65˚C and pH of 6-11 range. It is also non-toxic to cells and does not affect cellular growth. The first ever *gfp* gene was cloned in 1992 (8).

Lactic acid producing bacteria (LAB) include *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus* and others are widely used as probiotics that have been studied to benefit human and animal health. Due to the immense role of LAB bacteria in pathogen elimination, it is necessary to study their survival and colonizing strategies *in vivo* by development of fluorescent based reporter constructs. Plasmids are present in most of the LAB species with varying sizes (0.87 kb to >250 kb) and copy numbers (1 or more per cell). Enterococci possess plasmids that are resistant to various antibiotics like erythromycin, vancomycin, tetracyclin and gentamicin. Some of these plasmids encode for toxins, virulence factors, sex pheromones and bacteriocins. The choice of expression vector depends on mode of replication, copy number and stability (8).

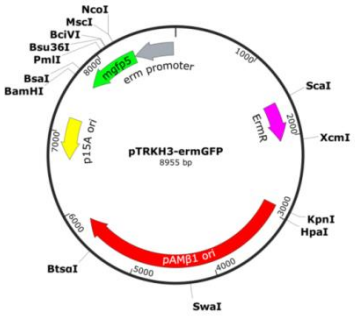


Figure 6. Plasmid map of pTRKH3 vector. The *E. coli* shuttle vector pTRKH3 consists of *mgfp5* gene regulated by the *ermB* promoter. The plasmid has p15A and pAMβ1 as the origins of replication (8).

pTRKH3 is a shuttle vector for *Escherichia coli* (*E. coli)* and some LAB species, having a copy number of ~45-85 in *Lactococcus* and *Streptococcus* species also carrying a gene for erythromycin resistance. Three different promoters, *ermB, ldhL*, and *slp* were used to check the GFP expression, using pTRKH3 as a backbone shuttle vector. The recombinant bacterial colonies that were picked and grown in THB at 37°C overnight were then inspected for the highest fluorescence intensity by epifluorescence microscopy. The highest fluorescence intensity was detected in *E. mundtii* transformed with pTRKH3-*erm*GFP, hence it was chosen as the promoter (28). Expression of mutated *gfp* gene (*mgfp5*) on a pTRKH3 plasmid controlled by a strong promoter, erythromycin ribosomal methylase promoter (*ermB*) in *Enterococcus mundtii* was carried out as in Figure 6 (8).

1.8 Transformation of *E. mundtii* KD251

Introduction of exogenous DNA into microbial cells could be accomplished by various methods including, chemical treatment, electroporation, biolistic gun method, ultraviolet rays (UV), polyethylene glycol (PEG), hydrogel and microwave radiations. Out of all, electroporation most efficiently transforms a broad array of microbes by introducing foreign plasmid into the host bacteria. This method involves electric pulse which results in transient pores on the bacterial cell walls allowing the DNA to pass through (8).

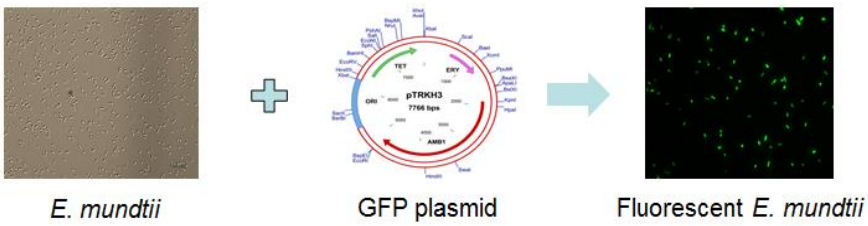


Figure 7. Workflow of transgenic E. mundtii KD251 reporter strain preparation (28)

Conventional use of the electroporation method was used to transform *E. mundtii* KD251 strain that was retrived from the *S. littoralis* larval gut (Figure 7). The bacterial cells were grown till exponential phase, diluted and pelleted down then washed with ice cold distilled water. This step was conducted twice, followed by addition of 10% Glycerol for preservation. 0.15-0.2 µg plasmid concentration was considered optimum for the electroporation. The competent cells were mixed with pTRKH3 plasmid DNA and then transferred to 02 cm plastic cuvette for transformation at an electric pulse of 1.8 kV, 600 Ω paralle resistance and 10 µF capacitance. The pulsed cells were obtained in fresh THB broth medium and the cell suspension was incubated at 37˚C for 2 h before plating them on THB agar plates containing 5 µg/ml erythromycin antibiotic. After 48 h of incubation, the transformed colonies were screened for the plasmid containg the *gfp* gene. The complete transformation protocol of *E. mundtii* is meantioned in (28).

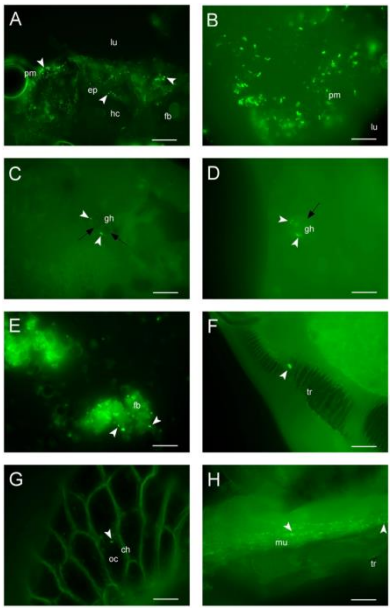


Figure 8. Colonization pattern of *E. mundtii* reporter in the intestinal tract of *S. littoralis*. A) Fluorescent bacterial cells of reporter strain in 4th instar larvae arrowheads show gut epithelium. B) GFP labelled bacterial cells in the midgut tissue of 5th instar larvae. C,D) White and black arrowheads showing fluorescent bacteria in midgut and hindgut tissues of 6th instar larvae. E) Arrowheads depicting very few labelled *E. mundtii* cells in pupae. F) A single viable cell observed in adult insect gut tissue. G) *E. mundtii* cells (arrowheads) in oocyte of *S. littoralis* eggs and H) Fluorescent bacteria in the 1st instar larvae of second generation (28)

The gut microbiome of *S.littoralis* was monitored under an Epifluorescence Microscope by incorporating a florescent tagged symbiont, *E. mundtii* as a reporter organism (Figure 8). This GFP-labelled strain could readily integrate to the intestinal tract, form a bio-film like structure and hence, colonize to sustain throughout the insects developmental stages. As this

reporter also is visualized in the successive generations, a possible vertical transmission of this bacteria in *S. littoralis* was hypothesised. This reporter organism could be recovered for further transcriptome-based analyses. Fluorescent *E. mundtii* was also observed in fecal samples of the larvae indicating their successful travel along the intestinal tract of the insect (8).

1.9 Fluorescent Activated Cell Sorting (FACS)

Flow cytometry is the technology which makes it possible to recover the reporter bacteria which are integrated to the gut of *S. littoralis* larvae. Fluorescence-activated cell sorting method (FACS) makes it possible to sort the GFP-tagged *E. mundtii* reporter from a mixture of microbial communities residing in the host gut. Flow cytometry separates cells based on their size, complexity, granularity and in particular fluorescence. The sample which has to be sorted is passed through a flow cell. The sheath fluid brings down the cells in the sample, through a channel where they encounter a laser beam. Detectors measure the scattering of light measuring the cell size and granularity. Therefore, the flow cytometer qualitatively and quantitatively analyses the samples (8).

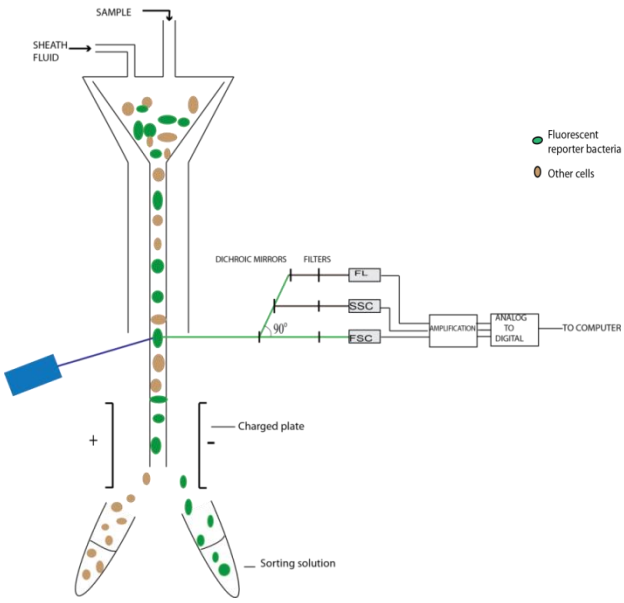


Figure 9. Illustration of Fluorescent activated cell sorting work flow. Fluorescently labelled single bacterial cells sorted through flow cytometer (8).

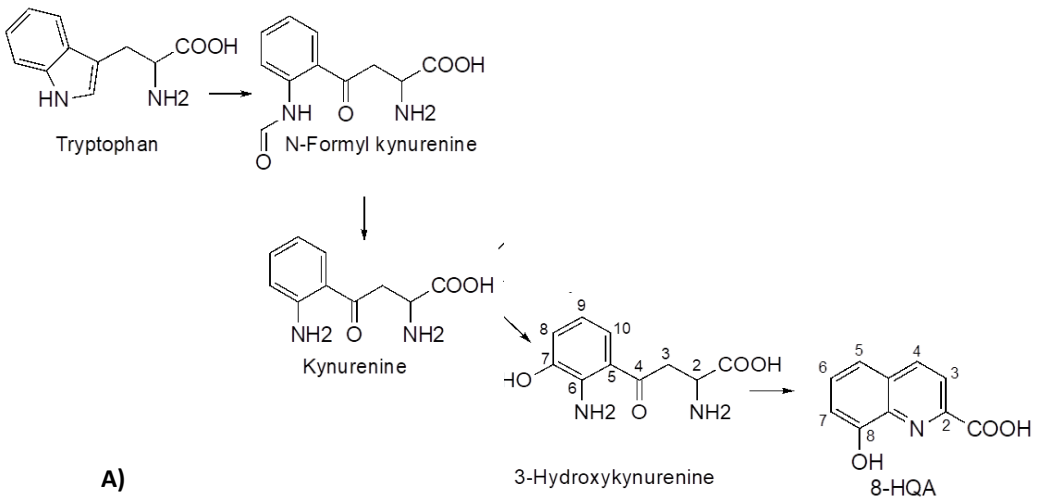
The cells of interest that are fluorescently tagged by GFP are separated from the mixture of gut homogenate when passed through the flow channel. The pressure from an adjustable compressor forces the sample through laser beam where scattering of the beam takes place as shown in Figure 9. Scattering occurs depending on the chosen excitation wavelength of the

fluorophore. The measurement of forward scatter (FSC) refers to light refracted by the cell based on the cell size, whereas the side scatter (SSC) measurement refers to light refracted based on fluorescence and granularity. More scattered light indicated more granularity of the cell. Each cell is enclosed in a droplet which corresponds to the charge depending on the cell's deflection after passing through an electric field. Uncharged droplets are discarded in the waste. Detectors are adjusted to be able to view florescence emitted by GFP. The single cells that are sorted and collected from the Flow cytometer could then be further studied (8).

1.10 8-hydroxyquinoline-2-carboxylic acid production by insects

Quinolinic carboxylic acid derivatives are widely found in plants, insects and bacteria. Foregut homogenate (regurgitate) analysis of *S. littoralis* revealed the presence of 0.5–5 mM amounts of 8-hydroxyquinoline-2-carboxylic acid. 8-HQA is a siderophore, which is not produced by the gut bacteria, but the insect host to possible control its microbial community. Even though the biological importance of this compound has not been known but the biosynthetic pathway of the compound has been successfully studied (29).

The insect *S. littoralis* produces large amounts of 8-HQA from the tryptophan via the kynurenine pathway. The schematic diagram of the biosynthesis of the compound from tryptophan is shown in Figure 10A. Kynurenine-3-monoxygenase (KMO) is an FAD-dependent enzyme that catalyses the 3-hydroxylation of kynurenine in the presence of NADPH and molecular oxygen (30). It was predicted that 3-hydroxykynurenine is the precursor of 8-HQA as shown in Figure 10A. Two possible pathways as shown in Figure 10B were hypothesized for 8-HQA synthesis from 3-hydroxykynurenine. When KMO is absent, the step involving kynurenine to 3-hydroxykynurenine, is inhibited, there is a huge reduction (about 85%) in 8-HQA synthesis (29).



A)

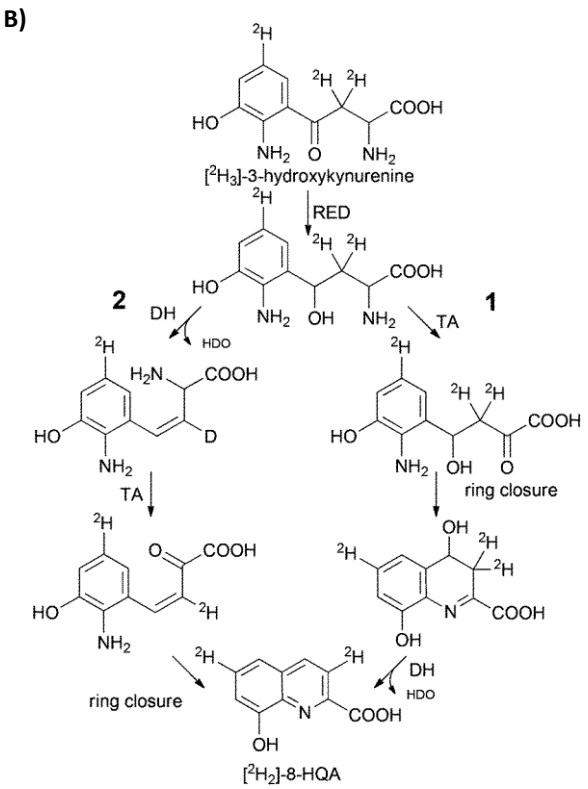


Figure 10. Biosynthesis of 8-hydroxy-2carboxylic acid from Tryptophan. A) Predicted 8-HQA production from tryptophan metabolism and the precursor that forms 8-HQA being 3-hydroxykynurenine. B) Two alternative pathways suggesting 8-HQA production from 3-hydroxykynurenine via enzymatic ring closure step; RED: reductase, DH: dehydratase, TA: transaminase (29)

Apart from a diverse pH gradient and lack of oxygen content in the *S. littoralis* gut, the larvae produce high amounts of 8-HQA (8-hydroxyquinoline-2-carboxylic acid). Tryptophan is the source of this 8-HQA production. This compound is an iron chelator that presumably controls the iron concentration in the insect gut.

Iron is one of the key elements for bacterial metabolism and hence, survival. Iron is an essential element in oxygen quenching, oxidative metabolism in Citric Acid cycle, electron transport chain, assimilation of nitrogen and many others (31). This suggests that there must be a reduction in the Fe-dependent gut bacterial growth. However, it does not seem to be the case as the insect gut population, predominantly *E. mundtii* is readily able to survive this iron limitation condition in the gut (29). Hence, to study the gut community in the absence of a Fe quencher (8-HQA), metagenomic information of KMO knockout strains would provide a primary idea regarding the bacterial diversity and survival.

1.11 16S rRNA gene amplification

16S rRNA gene is the most conserved and the least variable gene present in all the cells.

This can also be called 16S rDNA gene because it is transcribed and translated to form ribosomal subunit. The ribosome in prokaryotes is 70S which consists of 30S small subunit and 50S large subunit. The 30S consists of 16S rRNA and 50S consists of 5S and 23S rDNA. Whereas the ribosome in eukaryotes is 80S, comprising of 40S small subunit containing 18S rRNA and 60S large subunit containing 5S, 5.8S and 28S rDNA. Here, S is the Svedberg constant which is the measure of sedimentation rate upon the application of centrifugal force (32).

The 16S rRNA gene contains V1-V9 variable regions which could be amplified by Polymerase Chain Reaction (PCR) method. The study of these variable regions can help in determining homology amongst different organisms. V4 region amplification is used in this study, which is followed from the already optimised protocol of the Earth Microbiome project (33). In this protocol, primers are designed complimentary to the conserved regions that flank the target variable region, hereby giving access to easy amplification of the hypervariable region. The analysis includes species identification, assessment of taxonomy, phylogeny and other important characteristics.

1.12 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction or PCR amplification is a very important molecular biology tool founded by Kary B. Mullis in 1983 (34). It involves amplification of a target site with the help of specific primers that are extended by oligonucleotide addition by a DNA polymerase enzyme. Primers are essential to initiate the strand synthesis and DNA polymerase adds nucleotides to the free 3'-hydroxyl end of the template strand. GC content of the primers, annealing temperature and the reaction buffer have major roles in a PCR reaction.

The three basic steps that make this PCR reaction are denaturation, annealing and elongation. Denaturation is when the two strands of the template DNA separate when subjected to high temperature (97°C) for 15-60 seconds. In annealing step, the primers anneal or integrate to the DNA template strand at 65°C for 15-60 seconds. And finally, in elongation step, the DNA polymerase extends and synthesizes new complimentary strands at 72°C for 2-5 minutes. In principle, PCR product yields about thousands to millions of amplicons after 30-40 cycles (35). There are several other types of PCR, including Real time PCR, Quantitative PCR, Reverse Transcription PCR that also follow the same principle of the conventional PCR but with minute variations.

1.13 *In vitro* transcription amplification (Reverse transcription PCR)

RNA amplification is a necessary prerequisite for effective transcriptome analysis of RNA samples with low concentrations. The steps involved in this *in vitro* based amplification protocol are as follows: (a) Bacterial RNA does not possess poly A tail hence, *E. coli* poly (A) polymerase adds the tail at the ends of RNA. (b) Primers complimentary to poly A tail are used to synthesize the 1st strand of cDNA by the process called reverse transcription. (c) RNase H enzyme is added to degrade RNA from RNA-cDNA pair and hence DNA polymerase is used to synthesize the second strand of cDNA, resulting in double stranded cDNA. (d) cDNA is purified by removing fragmented DNA, salts and enzymes. (e) Transcription of cDNA to antisense RNA occurs in the presence of DNA dependent RNA polymerase. This step is optimum at 37˚C and the reaction time depends on the amount of amplified RNA required. (f) Finally, the purification of amplified RNA is done by removing residual enzymes, salts and unutilized dNTPs. After the RNA amplification, the samples are subjected to precipitation with 5 M NH_4OAc and ethanol. This step is carried out to increase the concentration of purified aRNA (8).

1.14 Transcriptomic and Genomic data analysis

DNA Sequencing began with the Sanger approach in 1977. But in recent years, due to advancements in technology, the sequencing methodology has taken a huge leap forward. Next Generation Sequencing (NGS) is regarded as High throughput sequencing.

To study the metabolically active gut bacterial populations, 16S rRNA gene profiling is necessary. This method is a clear indicator of active microbes which directly contribute to the current function. Next generation Illumina sequencing technology (NGS) is a rapidly growing methodology to study the symbiotic associates in greater depth (12). This was carried out with great sensitivity and with deep sequencing (Hiseq), which increases the ability to detect less abundant transcripts. Illumina helps in dealing with small picogram amounts of RNA with great resolution ability. The amplified RNA from GFP-tagged reporter *E.mundtii* that goes through this Hiseq sequencing helps to identify differentially regulated genes and hence to understand the story behind its adaptation in the harsh gut environment of *S. littoralis* (8).

The metagenomics analysis along with NGS technology provides in-depth knowledge about symbiotic microbial diversity analysis and reveals groups of unculturable microbes (18). Gene targeted techniques include primers to specifically amplify targeted gene. In our case,

the target gene is the conserved 16S rRNA gene from the metagenomics DNA of KMO knocked out and wild-type *S. littoralis* guts.

1.15 Aims of the thesis

There are two aims of this thesis; 1) Survival strategies of *Enterococcus mundtii* in the gut of *Spodoptera littoralis* larvae and 2) Investigating if 8-HQA is responsible for dictating gut microbial community of *S. littoralis*.

No comprehensive study of *E. mundtii* has been conducted to investigate how it resists the adverse stresses in the gut. Therefore, the first aim of this thesis work is to study the differential gene expression analysis of *E. mundtii* subjected to the gut environment of *S. littoralis* compared to the *in vitro* grown bacteria in Todd Hewitt Broth (THB) media, by a transcriptomic approach. Furthermore, investigating the variation between the gut flora of 8-HQA lacking *S. littoralis* insects and the wild-type insects is necessary to know more about the effects of iron limitation on the insect gut flora. This is carried out by analysing the comparative metagenomic data of the whole gut community of the KMO knockout line and the wild-type *S. littoralis* insects is the second aim of the thesis. This analysis is based on the sequencing of 16S rRNA amplicons derived from the extracted and purified metagenomics DNA of whole guts of two larval instars, pupae and adults of one complete generation.

2 Materials and Methods

2.1 Materials

The eggs of *S. littoralis* insect for the first part of thesis were obtained from Syngenta Crop Protection Munchwielen AG , Switzerland and for the second part were obtained from Department of Entomology, MPI-CE, Jena, Germany. An entire list of all the chemicals, devices and kits along with the manufacturing industry are mentioned below in Tables 1, 2 and 3.

2.1.1 Kits

Table 1. Information of the kits used during the study

Item	**Manufacturing Company**
DNA & RNA Purification Kit	Master Pure™ Complete, Epicentre, Madison, USA
MessageAmp ™ II aRNA Amplification Kit	Invitrogen, Life Technologies, USA
QIAquick® Gel Extraction Kit	Qiagen, Germany
Zymo Research RNA Clean & Concentrator™-5	The Epigenetics Company, USA
RNAeasy® Mini Kit	Qiagen, Germany

2.1.2 Devices and equipment

Table 2. Details of the devices and equipment used in this study

Item	**Manufacturing Company**
37˚C Shaker Incubator CERTOMAT® BS-1	B. Braun Biotech International, Germany
-80˚C Sanyo Freezer	Innovationstechnik GmbH, Germany
Biophotometer	Eppendorf, Germany
Centrifuge 5415D	Eppendorf AG, Germany
Centrifuge 5415R	Eppendorf AG, Germany
Corning™ Sterile Cell Strainers (40 microns)	Fischer Scientific, Thermo Fischer Scientific, UK
Electrophoresis power supply	Amarsham Pharmacia Biotech, UK

Fluorescence Microscope	Zeiss, Jena, Germany
Freezer (-20°C)	Liebherr, Germany
Fume Hood	Erlab, France
Gel Doc XR+ System	Bio-RAD Laboratories, Inc., Germany
Gel Documentation viewer TFT Display	Sony, Japan
Gel electrophoresis (Bio-RAD Wide Mini-Sub® Cell GT)	Bio-RAD Laboratories, Inc., Germany
GeneAmp® PCR System 2700	Applied Biosystems, USA
Glass slides	Superfrost Plus, Thermo Scientific, Lithuania
Heraeus Laminar chamber	Caverion Deutschland GmbH, Germany
Microcentrifuge Kisker Spraut	Biozym & Carl Roth, Germany
Microwave (MW 800)	Continent, Germany
Nanodrop One	Thermo Scientific, USA
Sample plex Genogrinder 2010	Metuchen, NJ
Sigma 3-18K centrifuge	Sigma Laboratory Centrifuges GmbH, Germany
Thermomixer	Epperndorf Thermostate plus, Germany
Thermostatic water bath	Tried Electric, Israel
Vapo.protect PCR machine	Eppendorf, Germany
Vortex genie 2	Scientific Industries, Inc., USA
Weighing Balance	Sortorius lab Instruments GmbH, Germany

2.1.3 Chemicals, solutions and reagents

Table 3. Chemicals, solutions and reagents used during the course of this study

Item	**Manufacturing Company**
dNTP mix	Invitrogen, USA
2-propanol	Carl Roth GmbH + Co. KG, Germany
5X HF Buffer	Thermo Fischer Scientific Baltics UAB, Lithuania
Agarose	Bio&SELL GmbH, Germany
5 M Ammonium acetate	Merck KGaA, Germany

Betaine	Sigma Life Sciences, Germany
Chloroform	Carl Roth GmbH + Co. KG, Germany
CTAB Buffer	Carl Roth GmbH + Co. KG, Germany
DNA Gel Loading Dye (6X)	Life Technologies, USA
Ethanol absolute	VWR Chemicals, France
Ethylene diamine tetraacetic acid (EDTA)	Carl Roth GmbH + Co. KG, Germany
F515 primer (5'-TATGGTAATTGTGTGYCAGCMGCCGCGGTAA -3')	Eurofins Genomics, Germany
Gene Ruler 1kb Plus DNA Ladder	New England Biolabs® GmbH, Germany
Isoamylalcohol	Carl Roth GmbH + Co. KG, Germany
Midori Green Advance DNA Stain	Nippon Genetics Europe GmbH, Germany
Polyvinylpyrrolidone (PVP)	Sigma Chemical Company, USA
R806 primer (5'-AGTCAGCCAGCCGGACTACNVGGGTWTCTAAT -3')	Eurofins Genomics, Germany
RNA Later™	Invitrogen by Thermo Fischer Scientific Baltics UAB, Lithuania
RNase – DNase free water Marker	5 PRIME, Inc., USA
RNase ZAP™	Sigma Life Sciences, Lithuania
Rotiphorese® TAE Buffer	Carl Roth GmbH + Co. KG, Germany
Sodium chloride (NaCl)	Carl Roth GmbH + Co. KG, Germany
Thermo Scientific Phusion-High Fidelity DNA Polymerase	Thermo Fischer Scientific Baltics UAB, Lithuania
Todd-Hewitt Bouillon (THB) Media	Carl Roth GmbH + Co. KG, Germany
Tris HCl	Promega Quality Chemicals, USA
β-mercaptoethanol	Carl Roth GmbH + Co. KG, Germany

2.2 Methods

The methods belonging to first and second aims of the thesis work have been addressed as 1 and 2 respectively, in the following section.

2.2.1 Maintenance of eggs and larvae

1. Transcriptome analysis of *Enterococcus mundtii*

Spodoptera littoralis eggs were obtained from Switzerland (see page 28). Hatching of the eggs occurred at 14˚C and larvae were maintained at 23-25˚C under a regime of 16 h light and 8 h dark period. The reared larvae were fed on small cubes (1g) of agar-based artificial diet containing white Lima beans, paraben, formalin (36). 100 µl of antibiotic with concentrations of 9.6 µg/ml erythromycin and 5.75 µg/ml ampicillin was spiked into the artificial diet. This antibiotic based diet was fed to the larvae twice on alternative days to clean the indigenous gut microbial population already present in the larval guts.

2. Metagenomics of wild-type and KMO knockout *S.littoralis*

Hatched *S. littoralis* eggs of the KMO knockout line and wild-type (WT) were obtained from the Department of Entomology, MPI-CE, Jena, Germany, where the knockout was carried out by CRISPR/Cas9 method. KMO knockout and WT larvae were grown in separate petri dishes containing layer of White Lima bean based artificial diet (36). The larvae were separately reared and maintained based on family numbers to prevent them from inter-family mating. 3 families (or biological replicates) per each KMO knockout and WT lines were maintained at room temperature.

2.2.2 GFP reporter *E. mundtii* strain

1. Transcriptome analysis of *Enterococcus mundtii*

Enterococcus mundtii KD251 which was isolated from the gut of *S. littoralis* was transformed with Green Fluorescent Protein (GFP) containing expression vector, pTRKH3-ermGFP. This GFP tagged reporter *E. mundtii* strain was grown on Todd-Hewitt Bouillon medium (THB medium), both broth and 1.5 % Agar in the presence of 5 µg/ml Erythromycin antibiotic. The glycerol stock of the strain was preserved at -80˚C (28).

2.2.3 Feeding of larvae with the GFP reporter strain

1. Transcriptome analysis of *Enterococcus mundtii*

The reporter *E. mundtii* inoculated on THB broth with 5 µg/ml erythromycin was allowed to reach the stationary phase by overnight incubation in 37˚C shaker incubator. The stationary phase culture was then re-inoculated in THB broth with 5 µg/ml erythromycin and incubated

at 37˚C till the culture reached mid-log phase with the optical density (OD) of 0.5-0.6. The culture was then centrifuged at 5000 RCF for 10 minutes and the pellet was resuspended in distilled water. As the *S. littoralis* larvae reached 2nd instar, they were fed with ~10^{10} cells of the resuspended GFP reporter solution by pipetting 100 µl into small cubes of the artificial diet (28). Removal of feces was done regularly in order to avoid re-inoculation of the GFP bacteria.

2.2.4 Tissue sectioning and Fluorescent Microscopy

1. Transcriptome analysis of *Enterococcus mundtii*

Sections of foregut and hindgut were cut from fresh gut tissues of 5th instar *S. littoralis* larvae and were frozen at -24˚C. The control culture containing GFP-producing *E. mundtii* was harvested on THB broth and pellets were suspended in 1X PBS. 20 µl bacterial suspension and slices from cross-sections fresh larval gut tissues were mounted on glass slides. Live cells were observed under Axio Imager Z1 fluorescent microscope with AxioCam MRm camera, the GFP signals were using the Cy2/GFP filter set option. All the images were analysed and captured with 63X magnification oil objective with an aperture of 1.4 (28).

2.2.5 Sample preparation for Flow Cytometry

1. Transcriptome analysis of *Enterococcus mundtii*

About thirty of 5th instar larvae were collected and frozen at -20˚C for 20 min. The dissection was carried out under the laminar hood which was thoroughly cleaned with RNase ZAP™ to avoid contamination by potential RNases present. The larvae were washed by dipping them in ethanol followed by ddH_2O using sterile tweezers. Larvae were dissected using sterile scissors and tweezers and the dissected guts were collected in three separate falcon tubes, each for fore-, mid- and hindguts. These dissected guts were resuspended in 1:1 ratio of RNAlater and 6 % Betaine solution and were homogenized in separate and sterile pestle and mortar. Using 40 µm Corning™ Sterile Cell Strainers, the homogenized foregut, midgut and hindgut samples were filtered and collected in three separate falcons respectively. The foregut and hindgut samples were used for cell sorting and further analyses as the transcriptome of *E. mundtii* at extreme gut pH conditions could be analysed. A control sample was also prepared for sorting, where the control contained GFP containing *E. mundtii* strain grown *in vitro* on THB media at 37˚C incubator shaker.

2.2.6 Fluorescence associated cell sorting (FACS)

1. Transcriptome analysis of *Enterococcus mundtii*

The foregut and hindgut homogenates along with control were sorted using BD FACSAria™. This uses Ion laser emission at 480 nm wavelength and a 502 long pass filter. The volts for forward and side scatters were 451 V and 390 V respectively. The GFP emission occurred at 530 nm wavelength. The flow rate of the cell sorting was within the range of 10 µl/min – 80 µl/min. Single cell mode sorting was conducted and the sorted single cells containing the GFP were collected in 5 ml sterile Greiner tubes. The cells were collected in 1 ml RNAlater for 3 hours which corresponded to 6000-7000 events/sec. PBS buffer at 7.4 pH was used as the sheath fluid for sorting. 250,000 cells were sorted per sample and collected in an RNA-protective reagent.

2.2.7 Nucleic acid extraction

1. Transcriptome analysis of *Enterococcus mundtii*

The single cells sorted from foregut, hindgut and control samples were then subjected to RNA extraction using RNAeasy® Mini Kit . The entire extraction procedure was carried out in RNase free area and RNase ZAP™ was used to clean all equipment and also the work space before starting the extraction. Before elution, RNA was subjected to density gradient centrifugation using phenol, chloroform, isoamyl alcohol and finally, ethanol precipitation. RNA was eluted from two samples of foregut and hindgut homogenates each, in 15 µl of RNase free water (37). After extraction, the sample concentrations were measured by Nanodrop One.1 µl of sample was subjected to quantification in duplicates. As the quantified measurements were quite low, it was necessary to increase the concentration.

2. Metagenomics of wild-type and KMO knockout *S.littoralis*

20 larvae from 3rd instar, 6 larvae 5th instar, 6 pupae and 6 adult stages of each KMO mutants and WT lines were reared and collected for DNA extraction. This was done for three different families (or replicates) per stage. The insects were frozen for 20 min before the dissection. The whole guts of 3rd instar, 5th instar larvae and adults of KMO and WT lines were dissected using sterile scalpel and tweezers and collected separately in respectively labelled falcon tubes. For pupal samples, whole pupae were collected. Six sample tubes for every stage was prepared including three for KMO and three for WT lines. Liquid N_2 was added to the

falcons containing the samples along with different sizes of sterile stainless steel grinding beads and stored at -80˚C. CTAB based DNA extraction protocol was used in the next steps.

A Geno/Grinder® 2010 was used for the cell lysis step for the samples due to high amounts of tissue/cuticle content. Based on the sample size, 2-5 ml CTAB lysis buffer was used, followed by grinding in Geno/Grinder® for 2-5 min at 1000-1150 rpm. The CTAB buffer was freshly prepared using 1 M Tris HCL pH 8.0, 5 M NaCl, 0.5 M EDTA and CTAB salt dissolved in distilled water. Required amounts of Polyvinylpyrrolidone (PVP) and β-mercaptoethanol were dissolved in to the CTAB buffer. The grinding process was done till the samples looked homogenous and without any clumps from the gut or pupal cuticles.

The samples after being ground and homogenized, were treated with 24:1 (v/v) ratio of chloroform:isoamyl alcohol solution (cleaning solution) and centrifuged at 16,000 g for required time. The aqueous phase were then collected in fresh tubes and treated with 9:2 (v/v) ratio of isopropanol:5 M ammonium acetate (precipitant solution) and incubated for a while, followed by centrifugation. The supernatants were discarded in a phenol waste jar and the pellets were treated with 70% ethanol and then centrifuged. The pellets were again washed with 95% ethanol and then centrifuged. The pellets were then air dried and finally re-suspended in ultra-pure water. The volumes required to add the cleaning, precipitation solutions, ethanol and water were decided based on the sample volumes. After the extraction, the DNA concentrations of all the samples were measured using Nanodrop One and stored at -20˚C in 500 µl aliquots for 16S rRNA PCR.

2.2.8 RNA concentration

1. Transcriptome analysis of *Enterococcus mundtii*

Foregut and the hindgut RNA samples were increased in concentration using the Zymo Research RNA Clean & Concentrator™-5 kit. The procedure was followed as per the protocol of the kit. Sample volumes were increased up to 50 µl by adding required amounts of RNase free water. After the samples were concentrated, the quantification was carried out using Nanodrop One. About four fold increments in the concentrations were observed.

2.2.9 PCR amplification

1. Transcriptome analysis of *Enterococcus mundtii*

RNA amplification was a necessary prerequisite for effective transcriptome analysis for RNA samples with low concentrations. The *in vitro* based transcription method was conducted using the MessageAmp ™ II aRNA Amplification Kit. The amplification protocol was followed according to the MessageAmp ™ II aRNA Amplification Kit.

After the RNA amplification, the samples were subjected to NH_4OAc and ethanol precipitation. This step was carried out to increase the concentration of purified aRNA (amplified RNA). This step is also mentioned in the MessageAmp ™ II aRNA Amplification Kit protocol. At the end of this step, the RNA concentration for the foregut and hindgut samples ranged within 80-85 ng/µl concentration, which was then sent for sequencing and library preparation.

2. Metagenomics of wild-type and KMO knockout *S.littoralis*

16S rRNA PCR amplification for the samples was tried out using numerous combinations of primers and polymerases. The different polymerases that were tried included TaKaRa Taq, Pfx polymerase, Hotstar HighFidelity polymerase, Platinum Superfi polymerase and finally, Phusion polymerase. The finally optimized PCR was conducted using forward F515 primer (5'-TATGGTAATTGTGTGYCAGCMGCCGCGGTAA-3') and reverse R806 primer (5'-AGTCAGCCAGCCGGACTACNVGGGTWTCTAAT-3') along with Phusion polymerase. The samples used were diluted to 1:10 ratio and *E.coli* DNA was used as a positive control. The negative control was conducted with the same composition as the sample, except the DNA template was substituted with distilled water. The PCR amplification of each KMO and WT lines per stage was conducted in triplicate. About 15 ng DNA templates were used for amplification. The master mix was prepared for 20 µl reaction according to the Table 4.

Table 4. Reagents required for PCR reaction mix preparation

PCR reagents	**Final concentration**
5X Buffer	1X
10 mM dNTPs	200 µM
10 mM FP	0.5 mM
10 mM RP	0.5 mM
DNA template	~ 15 ng
Polymerase	0.4 U

The V4 region amplification was carried out in an Eppendorf Mastercycler nexus PCR cycler and the reactions were performed with an initial denaturation step at 98°C for 3 sec, followed 45 cycles of 98°C for 10 sec, 64.3°C for 30 sec, and a final elongation step at 72°C for 12 sec. Finally, an extended elongation step at 72°C for 10 min. The DNA marker that was used to determine the band size was Gene Ruler 1 kb Plus DNA Ladder. 3 µl samples were mixed with 2 µl DNA gel loading dye to run on 2% Agarose gel containing ~3-5 µl Midori green Advance DNA stain for visualization under UV illumination. The gel electrophoresis Bio-RAD Wide Mini-Sub® Cell GT chamber was used to run the samples at voltage of 150 mV and 120 A current for 35-40 min. The gel was visualized using Gel Doc™ XR+ System and documented.

2.2.10 Gel extraction and quantification

2. Metagenomics of wild-type and KMO knockout *S.littoralis*

The bands obtained at ~390 kb size had to be extracted from the gel. The protocol followed was according to QIAquick® Gel Extraction Kit manual. Before the extraction procedure, the sterile eppendorfs were weighed then the bands were carefully cut while observing under UV illumination machine and collected in respective eppendorfs. The weight was again noted, in order to calculate the weight of the gels retrieved. According to the gel weights, the reagents were added following the DNA extraction protocol. Finally, the sample concentrations were measured using Nanodrop One and sent for sequencing and further analyses.

2.2.11 Sequencing and analysis

1. Transcriptome analysis of *Enterococcus mundtii*

The amplified RNA samples from foregut, hindgut and control were sent to Max Planck Genome Centre, Cologne, for sequencing and library preparation. 300-1000 ng of amplified RNA was used for library preparation using Illumina ultralow RNA library preparation method and the sequencing of the library was done on Illumina Hiseq2500 platform. A total of 10 million paired end reads of length 250 bp each were sequenced.

During the bioinformatic analysis, the following tools were used for respective studies. FastQC was used for initial analysis of the reads and LINUX command line was used for the complete analysis work starting from trimming off the adapters to gene expression profile studies. Trimmomatic 0.36 was used to trim off the adapters. The trimmed reads were then

assembled using Tophat 2.1.0 tool and mapped to the already available genome of *Enterococcus mundtii* QU25 using Cufflinks 2.2.0. Normalization of the read counts was done based on fragments of Kilobase of transcripts per Million mapped reads (FPKM) value. The assemblies were then merged using Cuffmerge and Cuffdiff was used for computation of differentially expressed genes comparing the *E. mundtii* from gut samples and the *in vitro* grown. Clusters of Orthologous group (COG) was used to group the proteins. The genes were also mapped against Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to predict metabolic pathways and also for annotation. Gene Ontology (GO) was also used for annotation as it provided details about functional characteristics. Clusterprofiler package of R version 3.3.3 was used to visualize enriched pathway data.

R package (CummeRbund 2.0) was used to visualize and plot the data. Dendrograms, PCA plots, histogram, heat maps and box plots were generated using the R package. A fold change of 2 was optimized as the threshold to study the differentially expressed gene profiles of *E. mundtii*.

2. Metagenomics of wild-type and KMO knockout *S.littoralis*

Among the three replicates of 3^{rd} instar, 5^{th} instar larvae, pupae and adult samples that were sent for sequencing, only a part of the results were achieved. Except for the 3^{rd} instar larvae data, others were obtained on time from Max Planck Genome Centre, Cologne. Sequencing data of all the three replicates of 5^{th} instar larvae, pupae and adult samples of WT and KMO lines were obtained and analysed.

The QIIME 2.0 platform was used to analyse the sequencing data of the gut microbial metagenome of wild-type and KMO knockout *S. littoralis* insects. Similar to the transcriptome analysis, R package was used to plot graphs and deduce conclusions from the metagenomics data. The bar plots of the bacterial abundances in the 5^{th} instar larvae, pupae and adult samples of WT and KMO lines were plotted using R studio software. To analyse the amount of diversity within the alpha diversity of the samples, a box plot was plotted using the QIIME2 pipeline. For the comparison of individual samples with the others, Pairwise Kruskal-wallis test was carried out and tabulated.

3. Results

3.1 Transcriptome analysis of *Enterococcus mundtii*

3.1.1 Epifluorescence Microscopy

The gut tissues of the 5th instar larvae along with the control containing smear of *E. mundtii* culture grown *in vitro* were observed under Fluorescence Microscope. Fluorescent bacterial cells of cocci shaped *E. mundtii* could be observed. They occurred in short chains and also as single cells as shown in Figure 11. Apart from the bacterial cells, a lot of auto-fluorescence was observed during the microscopy. Hence, to verify if the fluorescence emitting cells were the *E. mundtii* reporter strains, positive control was observed as in Figure 11C. When compared to the control, the observed bacterial cells were confirmed to be *E. mundtii* reporter, confirming successful integration and colonization of the GFP tagged reporter fed through artificial diet.

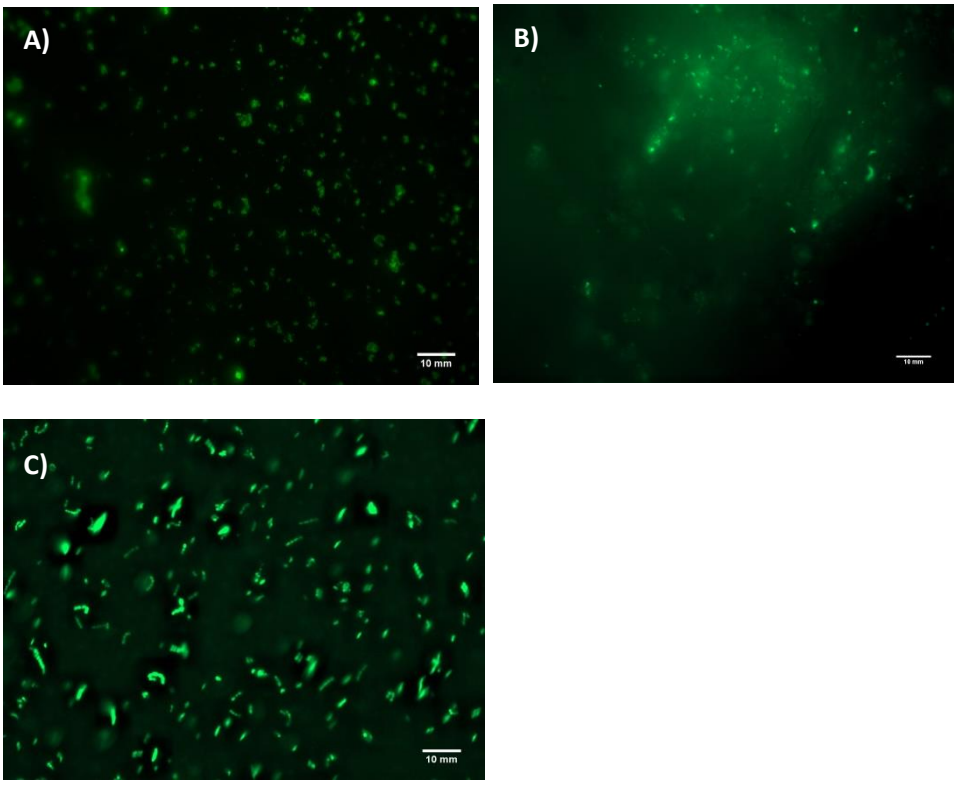


Figure 11. Localization of GFP labelled *E. mundtii* in the intestinal tract of 5th instar *S. littoralis* larvae. A) and B) Reporter bacterial cells occurring as single cells and short chains in the foregut and hindgut of 5th instar larval gut tissues respectively. C) Positive control showing *in vitro* grown GFP tagged *E. mundtii* cells. (Resolution: 63X and scale bar: 10 mm)

3.1.2 FACS analysis

It was necessary to understand the differential gene expression profiles of *E. mundtii* reporter from the foregut and hindgut of *S. littoralis* and to compare them to the control or the *in vitro* grown *E. mundtii* culture. The reason for choosing two ends of the insect gut was to study the profile at highly alkaline pH of about 10 in the foregut and neutral in hindgut. For this to happen, it was necessary to selectively target the GFP tagged bacterial cells amongst the insect and other indigenous microbial cells. The whole experiment was done using triplicates. This thesis contributed to the results of one complete replicate out of the three biological replicates of transcriptome profiles of foregut, hindgut and control samples.

250,000 single cells of GFP tagged *E. mundtii* reporter were sorted per sample. These individual cells contributed to 2-4% of the total gut homogenate. The Fluorescence based sorting profiles of the control culture along with foregut and hindgut homogenates can be seen in Figure 12.

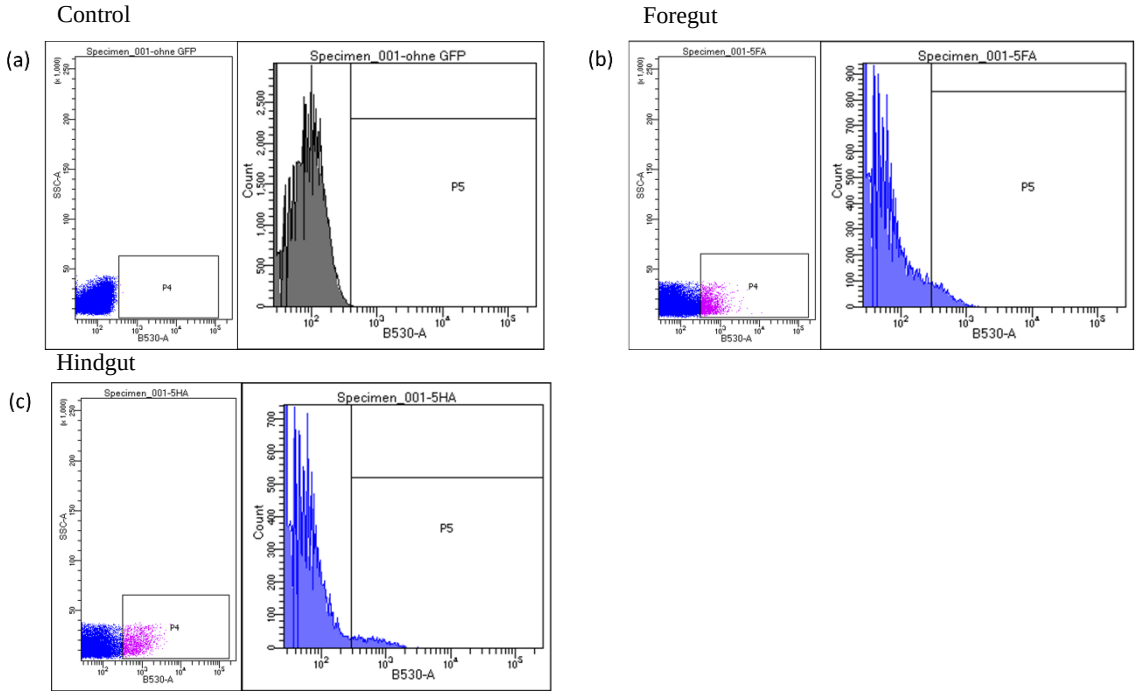


Figure 12. Fluorescent sorting profiles of *E. mundtii*-pTRKH3 from *in vitro* and *in vivo* samples. A) Sorting profile of control sample of *E. mundtii* reporter from broth culture. B) and C) Sorting profiles of reporter strain obtained from foregut and hindgut homogenates respectively. P4 and P5 regions correspond to density of fluorescent bacterial cells detected and sorted by the cytometer, respectively.

The scattered points lying in the P4 area and the peaks lying in P5 area represent the density of fluorescent cells that are sorted from the samples (Figure 12B, 12C). Therefore, in control no peak or points are observed in the P5 region or P4 region (Figure 12A). The clusters of

points and peaks lying outside the P4 and P5 regions are the result of auto fluorescence or fluorescence emitted from non GFP-tagged *E. mundtii* cells.

3.1.3 RNA Concentrations

After RNA extraction from the sorted samples of foregut, hindgut and control samples, the concentrations were observed as shown in Table 5. The concentrations were measured in duplicates. Absorbance values of 260/280 ratio indicates presence of protein impurity and 260/230 ratio indicates organic solvent contamination.

Table 5. RNA concentrations of foregut and hindgut sorted samples in duplicates along with control

Sample	**Concentration (ng/µl)**	**A260/280**	**A260/230**
Foregut	4.80	2.00	0.00
Foregut	4.30	2.10	0.00
Hindgut	13.20	1.50	0.30
Hindgut	13.00	1.80	0.20
Control	126.70	2.10	0.40

The RNA concentrations measured after increasing the RNA concentration by following the Research RNA Clean & Concentrator™-5 kit protocol (Table 6).

Table 6. RNA concentration of samples after conducting kit based purification and clean up

Sample	**Concentration (ng/µl)**	**A260/280**	**A260/230**
Foregut	4.40	1.80	0.10
Foregut	4.50	2.00	0.10
Hindgut	28.60	2.50	0.10
Hindgut	28.40	2.60	0.10
Control	188.70	2.10	1.00

The RNA concentrations measured after the *in vitro* transcription based PCR amplification method, followed by purifications of the amplified RNA by 5 M NH_4OAc and ethanol are as in Table 7.

Table 7. Concentrations of RNA measured after amplification and treatment with NH_4OAc and ethanol

Sample	**Concentration (ng/µl)**	**A260/280**	**A260/230**
Foregut	25.60	2.30	2.20
Foregut	25.40	2.30	2.30
Hindgut	84.60	2.40	2.30
Hindgut	85.30	2.40	2.30
Control	3478.00	2.00	2.10

As the RNA concentration of the control sample was really high, it was diluted to 1:10 before sending for sequencing.

3.1.4 Transcriptome assembly and data analysis

After the sequence reads were mapped against the fully available genome sequence of *Enterococcus mundtii* QU25 (38) the sequence read alignment was carried out (Table 8). The number of reads generated per sample was 10 million and paired ended.

Table 8. Alignment percentages of foregut, hindgut and control samples after mapping

Sample	**Number of reads from Trimmomatic**	**Percentage alignment by Tophat pipeline (%)**
Foregut	10623050.00	73.40
Hindgut	10187317.00	55.50
Control	973355.00	48.30

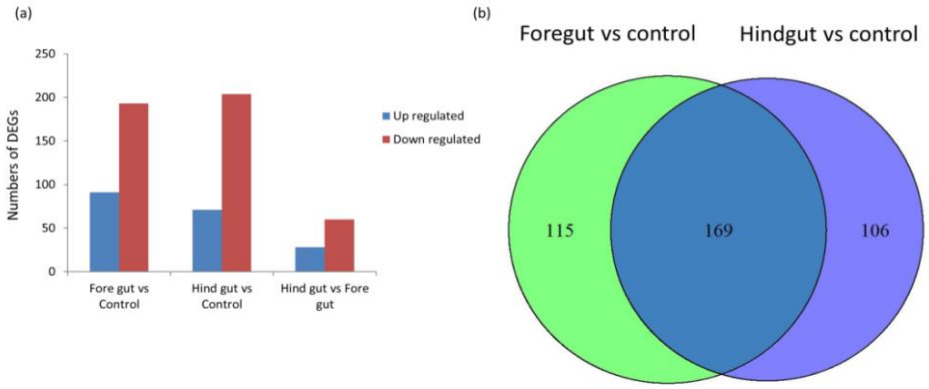


Figure 13. Comparison of differential gene expression profile data of *E. mundtii* obtained from *in vivo* and *in vitro* conditions. A) Bar plot showing up and downregulated genes ($p \leq 0.05$) comparing foregut vs control, hindgut vs control and foregut vs hindgut. B) Venn diagram depicting overlapping number of genes in *E. mundtii* obtained from foregut and hindgut compared to control samples.

Differential gene expression analyses were carried out and numerous genes were found differentially regulated on comparing *E. mundtii* obtained from the foregut and hindgut of the *S. littoralis* larvae with respect to the *in vitro* grown culture. Three different types of comparison were made to check the numbers of differentially regulated genes common between; 1) foregut and hindgut, 2) foregut and control and 3) hindgut and control, as in Figure 13. 284 and 275 genes are significantly differentially regulated in *E. mundtii* from *in vivo*. 61% of genes were observed to be common amongst foregut and hindgut profiles. As shown in Fig, about 169 differentially expressed genes are common between the foregut and hindgut samples compared to the control.

To check the reproducibility of the GFP reporter based system adopted in this work, a PCA plot and dendrogram were plotted between the individual replicates of foregut, hindgut and control samples. In the PCA plot, the clustering of the three replicates of foregut and hindgut samples were observed to occur much farther and differently from the control sample as shown in Figure 14A. Obeying the previous result of PCA plot, the dendrogram also showed a similar trend. In the dendrogram, the control samples were clustered away from the foregut and hindgut samples as in Figure 14B. The gene expression profiles from the foreguts and hindguts almost overlapped yet clustered away from the control samples.

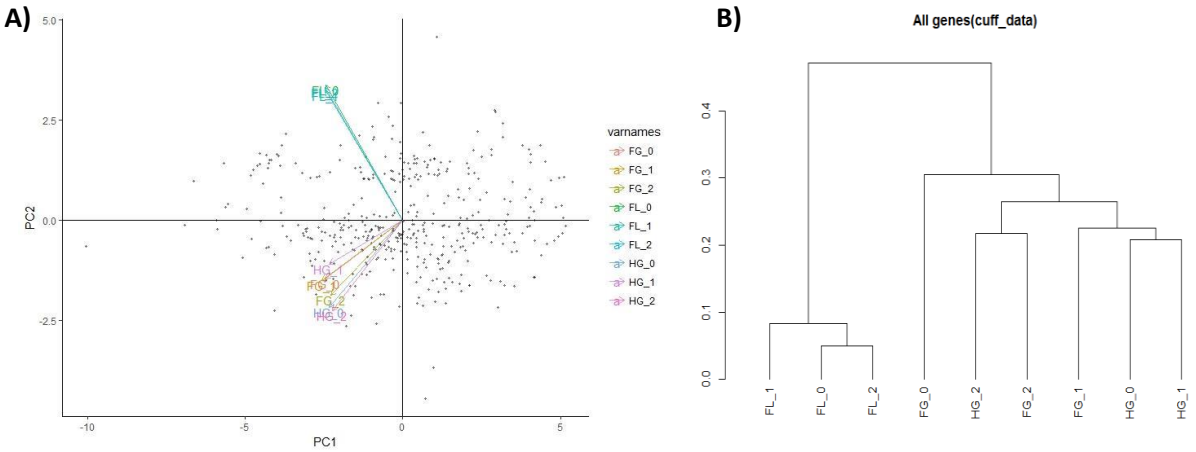


Figure 14. Clustering of gene expression profile data of *E. mundtii* transcriptome obtained from foregut, hindgut and control conditions. A) and B) indicate PCA plot and dedrogram of clustering of three replicates of transcriptome data from foregut and hindgut of *S. littoralis* larvae and *in vitro* grown control, respectively.

3.1.5 Survival strategies of *E. mundtii* in the insect gut

The differentially expressed genes by *E. mundtii* present in the foregut and hindguts of *S. littoralis* when compared to *in vitro* grown *E. mundtii* directly indicate the adaptive strategies followed by the bacteria in order to survive. Data addressing colonization, stress responses to various stresses and metabolism adopted by *E. mundtii in vivo* versus *in vitro* are discussed in the following.

i) Colonization ability

The first step to successful colonization of bacterial cells include adhesion to the gut epithelium of the insect and hence preventing the bacteria from getting flushed from the host system. Various well characterized adhesins, conserved motifs and domains have been studied that contribute to adhesion.

Genes encoding an LPxTG motif and sortase enzymes were also observed to be upregulated along with the WxL domains in the *E. mundtii* retrieved from the insect gut on comparison with the one grown on broth culture as shown in Figure 15. Also, the cell wall associated biofilm protein showed upregulation.

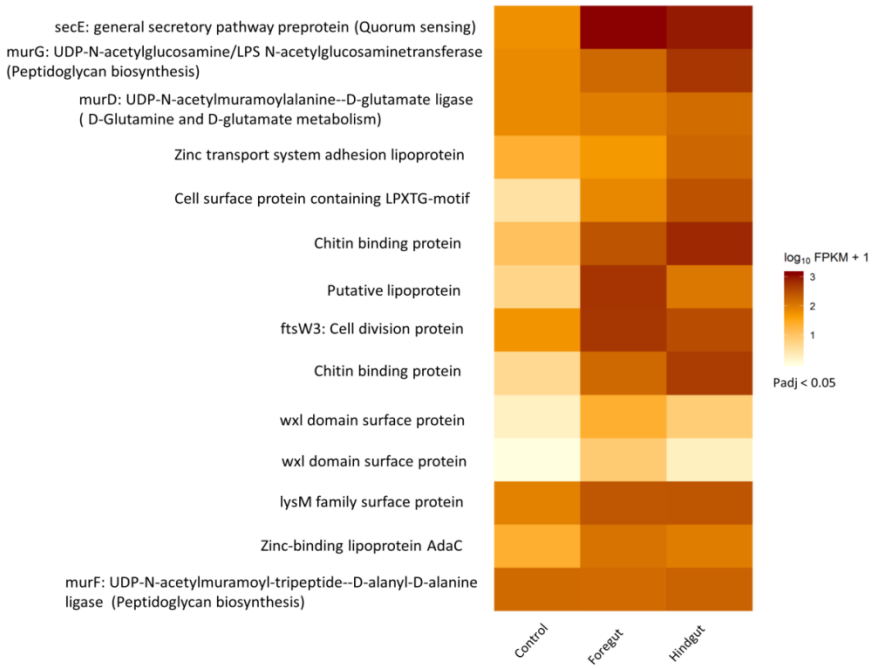


Figure 15. Heat map showing differential regulation of certain adhesion associated genes of *E. mundtii* in the insect gut. When compared to the *in vitro* grown *E. mundtii*, genes that aid in attachment of bacterial cells to the gut epithelium of the insect show upregulation *in vivo* conditions.

Cell surface anchor family of proteins and also Chitin binding proteins were observed to be upregulated in *E. mundtii* from foregut and hindgut samples compared to control. Also, the Fts family needed for proper cell division and also for cell wall connection are upregulated *in vivo* compared to *in vitro* samples.

ii) Various stress responses

Differentially expressed stress related genes indicate the strategies followed by *E. mundtii* in order to survive adverse stress in the *S. littoralis* gut as shown in Figure 16. The genes for a Two component system and quorum sensing (*agr* family) were upregulated in *E. mundtii* from foregut and hindgut when compared to the control. The Two-component system is a signal transduction system also responsible for quorum sensing mechanism. LuxS and LuxR for quorum sensing were upregulated as well.

Reactive oxygen species including superoxide and hydroxyl radicals, hydrogen peroxide were observed to be overly produced by *E. mundtii* in the gut as compared to *in vitro* culture. DNA starvation proteins are upregulated. Stress proteins including the general stress proteins and universal stress proteins were over expressed in the *E. mundtii* obtained from foregut and

hindgut of *S. littoralis* when compared to *E. mundtii* grown *in vitro*. YafQ and DNA damage inducible protein J were upregulated too.

SecE and VirD4 necessary for intracellular secretion and transport were upregulated in foregut and hindgut. Repair proteins like MutS, RecU and RecG responsible for DNA replication, recombination and repair were observed to be upregulated.

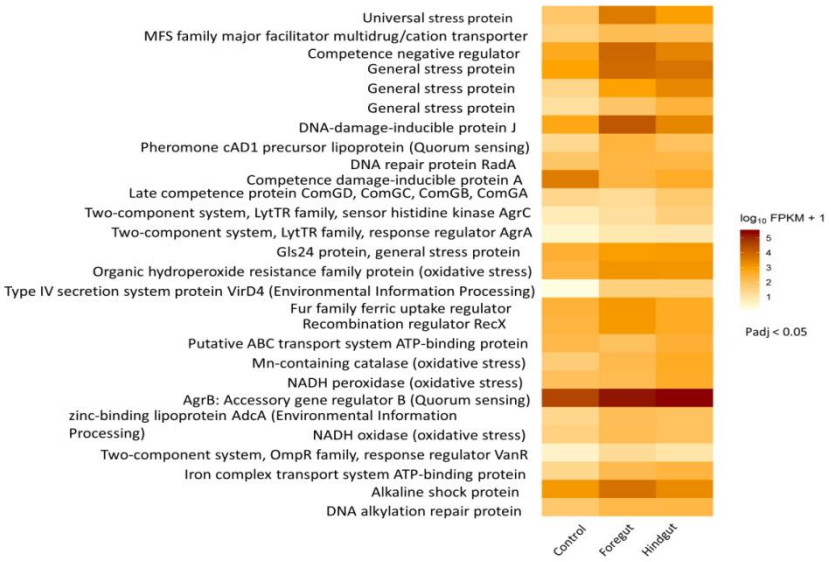


Figure 16. Heat map showing differential gene regulation profiles of stress tolerance associated genes in *E. mundtii* obtained from foregut and hindgut when compared to control. As the bacteria experience various stresses in the gut, over expression of stress related genes occur compared to *in vitro* grown bacteria.

Alkaline stress proteins were more expressed in foregut compared to hindgut. Cation transporters were observed to be downregulated along with the ATP binding protein. Whereas, upregulation was observed in adenosine and cytidine deaminases, purine & pyrimidine metabolism and penicillin binding proteins.

iii) Metabolic pathways

Glycolytic genes like glucokinase (*glcK*), 1-phosphofructokinase (*fruK*), 6-phospho-beta-glucosidase (*celA*, *bglP*, *bglB*, *bglG*) were downregulated. But the expression of 6-phosphofructokinase (*pfkA*) and glucose-6-phophate isomerase were more expressed as shown in Figure 17. Lactase dehydrogenase (*ldhA*) and sugar fermentation stimulation protein (SfsA) were upregulated. Alcohol dehydrogenases from fermentation were also upregulated.

Also, the genes encoding phosphotransferase (PTS) systems were found upregulated along with other sugar metabolism pathways in *E. mundtii* grown *in vivo*. Sucrose specific PTS transporter, sucrose-6-phosphate dehydrogenase and alpha-amylase enzyme neopullanase were found to be upregulated too.

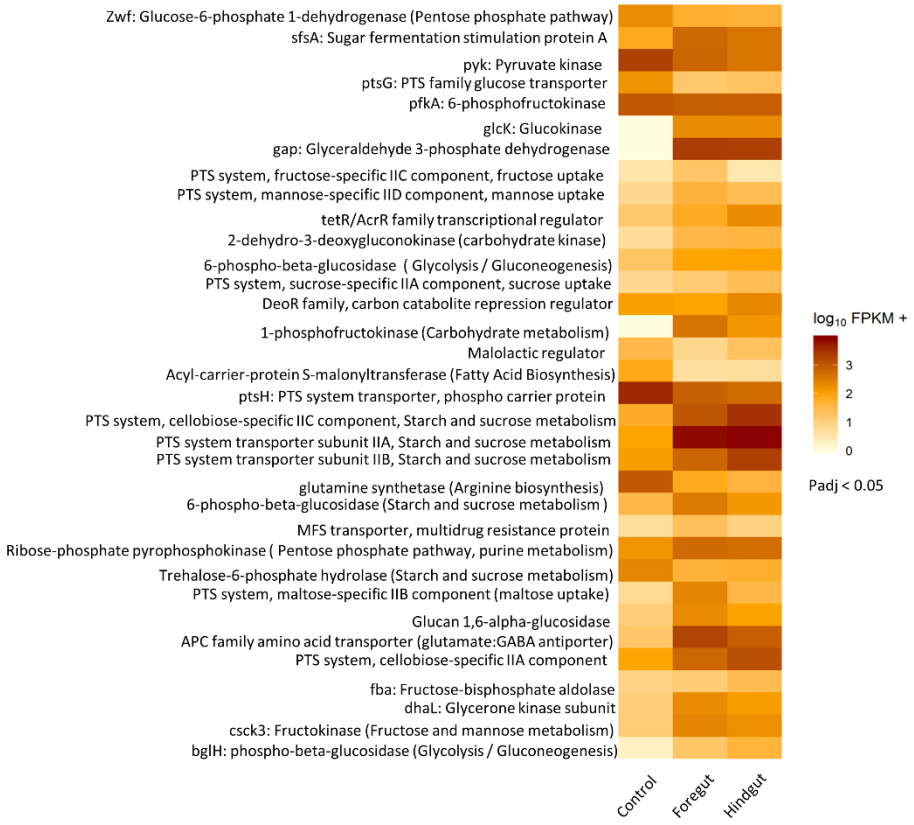


Figure 17. Heat map indicating regulation of metabolism associated genes in *E. mundtii* present in the gut compared to control. Certain genes responsible for metabolism were observed to be upregulated while some downregulated in foregut and hindgut samples compared to control.

Upregulation of *fetC* permease and FUR family transcriptional regulator were observed in the foregut and hindgut samples. Now, to compare the gene expression profile based on pH, Figure 18 shows differential gene regulation of certain genes between foregut and hindgut samples. Apart from the *asp* gene which was highly upregulated in the foregut (alkaline conditions), all the other genes were downregulated when compared to hindgut. The other slightly downregulated genes in foregut compared to hindgut, include *atp* genes, stress proteins genes, and the H+ antiporter *nha* gene.

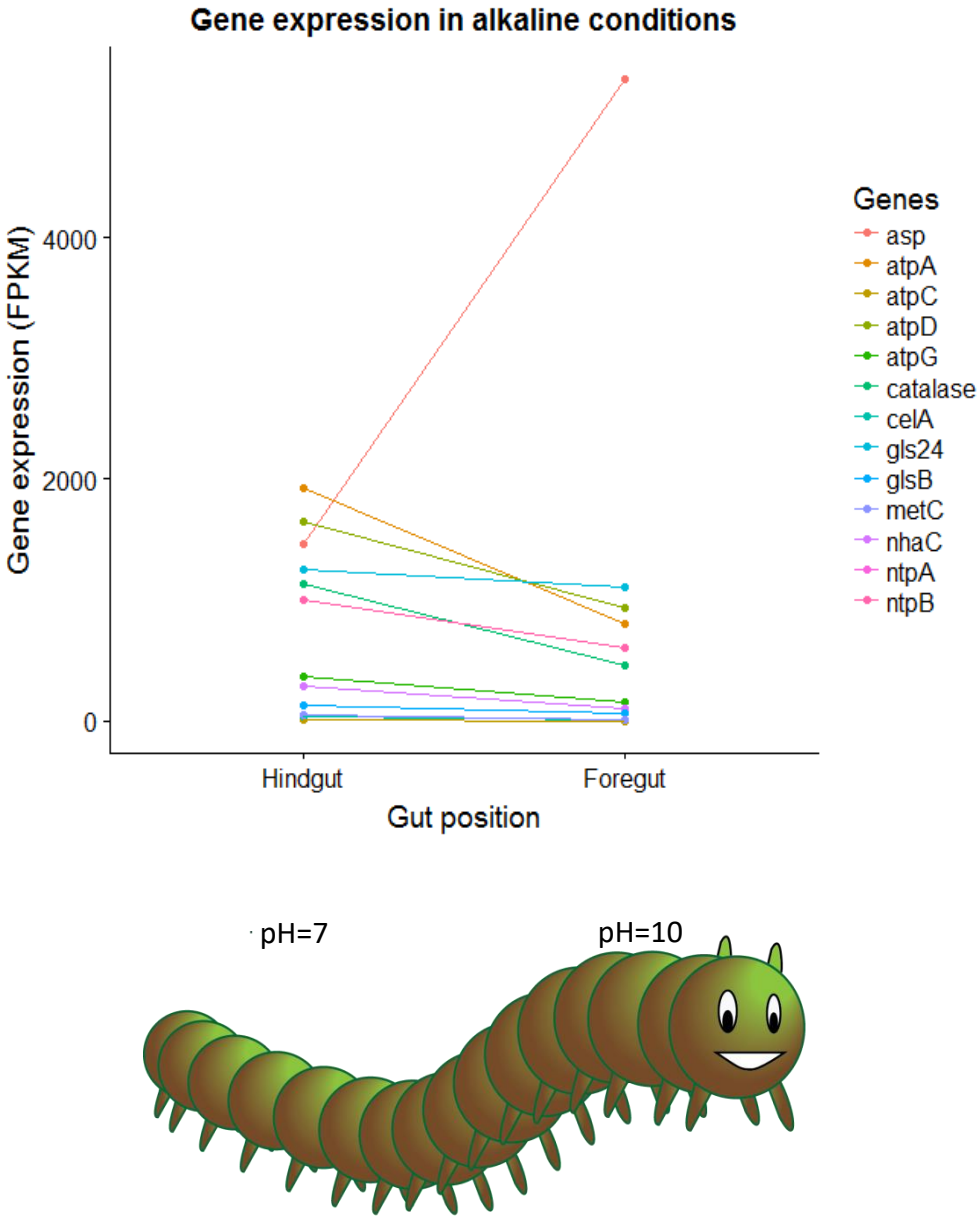


Figure 18. Graph showing regulations of certain genes in *E. mundtii* obtained from highly alkaline foregut (pH = 10) compared to neutral hindgut (pH = 7). When *E. mundtii* profiles at extreme gut pH conditions of *S. littoralis* larvae were compared, certain genes were observed to be differentially regulated.

3.1.6 Gene Ontology (GO) and KEGG annotations

The genes annotated by Gene Ontology (GO) were based on molecular functions, biological process and cellular components (Figures 19, 20). Peptidoglycan biosynthesis and N-acetylmuramoyl-L-alanine are upregulated in foregut and hindgut. Several enzymes for purine and pyrimidine biosynthesis are upregulated. Response to oxidative stress as shown in

Figure 19 is more expressed in hindgut compared to foregut and control. Starch and sucrose metabolism are upregulated. Amino acids biosynthesis (like phenylalanine, glutamate, tyrosine and tryptophan) and fatty acid production seem to be downregulated. Overall, metabolic genes seem to be downregulated in the symbiont.

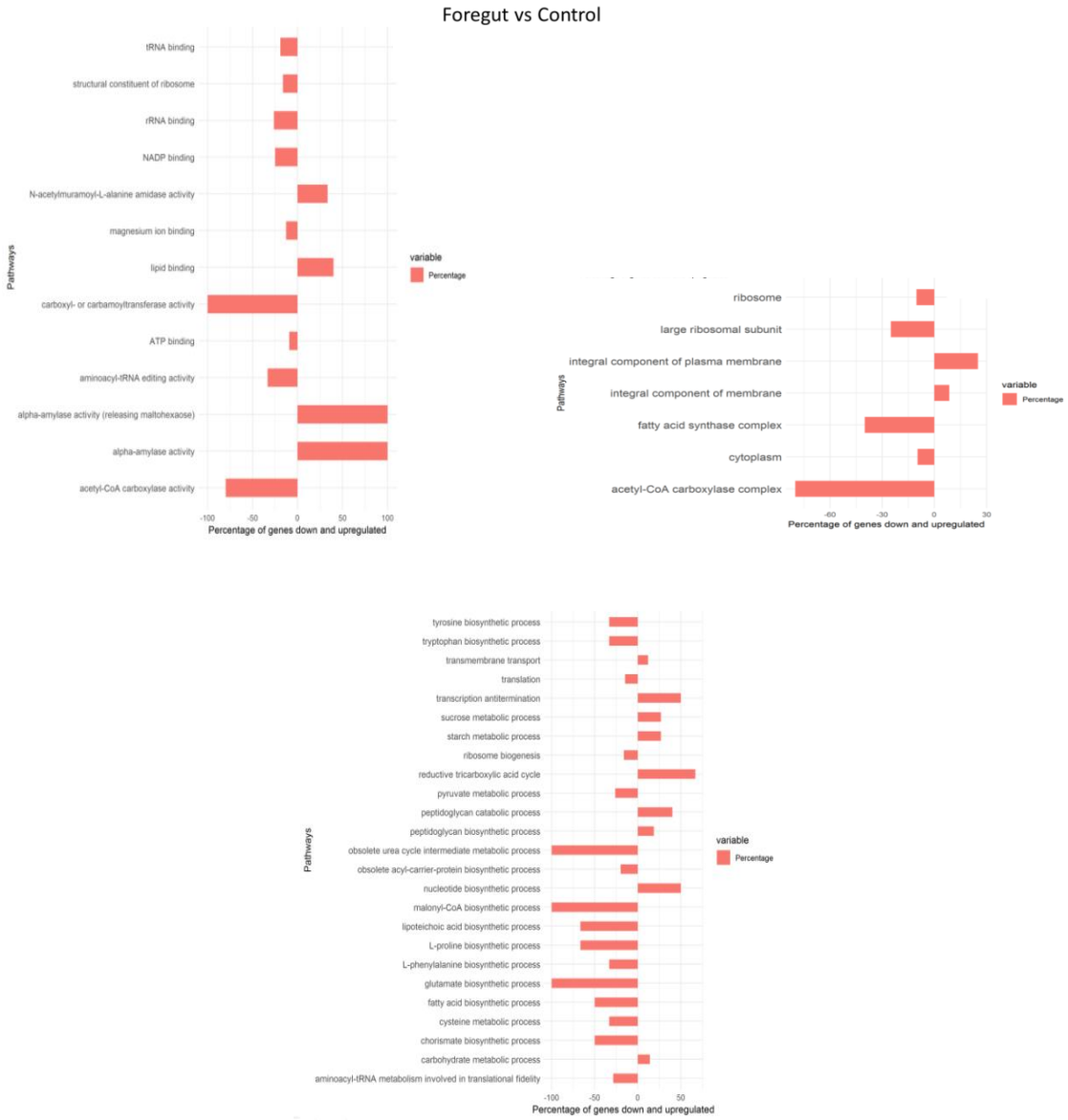


Figure 19. Graphs showing differential gene expression of *E. mundtii* obtained from *in vivo* and *in vitro* conditions based on Gene Ontology classification.

938 differentially expressed genes in *E. mundtii* obtained from the insect gut were mapped to KEGG pathways to understand the biological significance. 44 and 29 pathways were upregulated in foregut and hindgut whereas, 52 and 46 pathways were downregulated in foregut and hindgut respectively as shown in Figure 21.

Several amino acids and fatty acid biosynthesis are downregulated along with biosynthesis of antibiotics, propionate metabolism and secondary metabolite synthesis. Surprisingly, *E. mundtii* from hindgut seemed to biosynthesize lysine via the diaminopimelate pathway.

Figure 20. Graphs showing up and downregulation of assembled *E. mundtii* genes obtained from foregut & hindgut compared to control

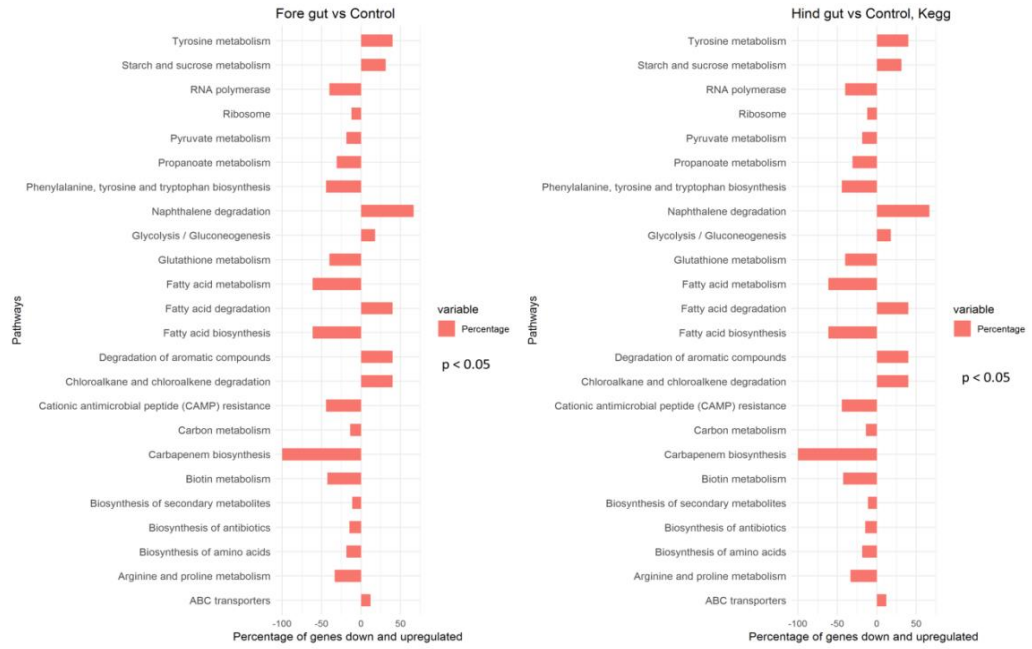


Figure 21. KEGG Orthology classification of assembled unigenes annotated from transcriptome profiles of *E. mundtii*. The graphs show both up and downregulation of certain assembles genes when compared to control conditions.

3.2 Metagenomics of wild-type and KMO knockout *S.littoralis*

After the survival mechanism of *E. mundtii* in the presence of oxidative stress, alkaline pH, Fe limitation and other gut stresses was observed, it was necessary to further examine the *S. littoralis* gut microbial composition under individual stress conditions. First stress of the *S. littoralis* gut that was targeted was the lack of Fe, which was a result of the compound 8-HQA produced by the insect. A comparison of gut microbial communities of *S. littoralis* that could produce the Fe chelating compound 8-HQA and the insects that were knocked down of 8-HQA, was carried out. This could give us an overall insight regarding the survival of the bacteria in the presence of Fe limiting conditions in the insect gut.

3.2.1 DNA concentrations

Concentrations of the DNA were measured in triplicates after being extracted from the KMO and WT lines of 3rd, 5th instar larvae, pupae and adults. The measured concentrations are as follows. 3, 5, P and A denote the four stages of the insect's life cycle followed by the family number (indicated by a 3-digit number)

Table 9. Measurements of DNA concentrations from samples after extraction based on the CTAB/PVP method

Samples	**Concentrations (ng/µl)**	**A 260/280**	**A 260/230**
3 KMO 442	7843.90	1.90	1.90
3 KMO 432	2065.00	2.00	1.90
3 KMO 432 (2)	2065.00	2.00	1.90
3 WT 426	2296.00	2.10	2.30
3 WT 445	670.00	2.00	2.30
3 WT 445 (2)	670.00	2.00	2.30
5 KMO 442	1484.00	1.30	0.50
5 KMO 434	299.70	1.60	0.60
5 KMO 431	425.00	1.50	0.50
5 WT 435	937.00	1.40	0.40
5 WT 445	698.00	1.40	0.50
5 WT 428	661.70	1.80	1.10
P KMO 434	822.00	1.60	0.90
P KMO 431	939.90	1.50	0.90
P KMO 442	321.00	1.80	1.40
P WT 428	447.80	1.60	0.90
P WT 435	989.00	1.60	1.00
P WT 445	1838.00	0.60	0.50
A KMO 434	700.30	1.90	1.40
A KMO 431	1419.90	1.10	0.90
A KMO 442	1079.90	0.80	0.60
A WT 428	1357.30	0.90	0.80
A WT 445	1312.90	1.00	0.80
A WT 435	887.10	1.10	0.90

(Here, (2) indicates the use of different gut homogenate sample from the same family)

After the 16S rRNA PCR amplification was conducted followed gel extraction, the DNA concentrations of the samples were again measured and noted.

Table 10. DNA concentrations measured after 16S rRNA gene amplification and gel extraction

Samples	**Concentrations (ng/µl)**
3 KMO 442	7.90
3 KMO 432	8.30
3 KMO 432	30.00
3 WT 426	5.10
3 WT 445	19.50
3 WT 445 (2)	33.50
5 KMO 442	33.00
5 KMO 434	3.40
5 KMO 431	41.00
5 WT 435	16.60
5 WT 445	56.70
5 WT 428	6.70
P KMO 434	30.00
P KMO 431	69.50
P KMO 442	9.00
P WT 428	19.00
P WT 435	10.00
P WT 445	10.00
A KMO 434	26.00
A KMO 431	7.00
A KMO 442	6.80
A WT 428	59.00
A WT 445	3.80
A WT 435	18.70

3.2.2 16S rRNA PCR amplification

After the V4 region of the 16S rRNA genes were amplified using the universal primers F515 and R806, bands were observed with the size of ~390 bp. Except for the negative control,

which did not have bands. Figure 22 shows the amplified product run on the gel for some of the samples. These bands were then carefully used to extract DNA from the gel and hence send them for sequencing.

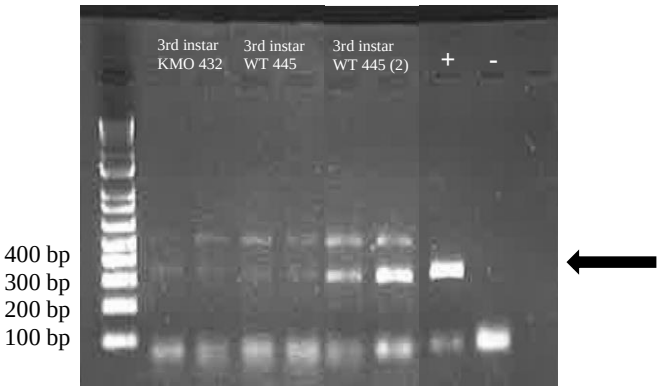


Figure 22. Gel electrophoresis image of 16SrRNA amplification of samples. The bands observed at about 390 bp size, falling between 400bp and 300bp are the amplified 16S rRNA gene amplification bands of 3rd instar larvae samples in duplicates, followed by positive and negative controls.

3.2.3 Genomic data sequencing and analysis

The sequence reads obtained were 2 million with paired ends. This genomic data analysis of the sequences of the KMO and WT samples was conducted by QIIME 2.0. The bacterial community diversity was observed by plotting graphs based on relative abundance of the 16S rRNA gene in the three replicates of each KMO and WT lines from 5th instar larvae, pupae and adult stages. The averages of the three replicates per stage of KMO and WT lines were considered for calculation of relative abundance to plot the bar graphs.

Alpha diversity metric, Faith Phylogenetic Diversity analysis suggests suggests that KMO 434 adult has the highest within-sample diversity (Figure 23). Pairwise Kruskal-wallis test has been performed to analyse how significantly different in bacterial diversity each pair is ($p <= 0.05$) (Table 11). The significantly different pairs have been emboldened.

Table 11. Pairwise Kruskal-wallis test results

Group 1	Group 2	H	p-value	q-value
KMO 432 3rd	WT 435 adult	1.00	0.30	0.40
	WT 435 5th	2.00	0.20	0.20
	KMO 431 adult	1.00	0.30	0.40
	WT 428 adult	2.00	0.20	0.20

	KMO 434 adult	2.00	0.20	0.20
	WT 428 pupa	1.80	0.20	0.20
	KMO 434 pupa	2.00	0.20	0.20
	KMO 431 5th	1.80	0.20	0.20
	WT 435 pupa	0.00	1.00	1.00
	KMO 442 5th	1.80	0.20	0.20
	WT 445 5th	1.50	0.20	0.30
	KMO 431 pupa	1.80	0.20	0.20
	WT 445 pupa	1.50	0.20	0.30
WT 435 adult	WT 435 5th	2.00	0.20	0.25
	KMO 431 adult	1.00	0.30	0.40
	WT 428 adult	0.50	0.50	0.50
	KMO 434 adult	2.00	0.20	0.20
	WT 428 pupa	1.80	0.20	0.20
	KMO 434 pupa	2.00	0.20	0.20
	KMO 431 5th	1.80	0.20	0.20
	WT 435 pupa	1.50	0.20	0.30
	KMO 442 5th	1.80	0.20	0.20
	WT 445 5th	1.50	0.20	0.30
	KMO 431 pupa	0.20	0.60	0.70
	WT 445 pupa	1.50	0.20	0.30
WT 435 5th	KMO 431 adult	0.00	1.00	1.00
	WT 428 adult	3.00	0.10	0.20
	KMO 434 adult	5.30	**0.02**	0.20
	WT 428 pupa	4.50	**0.02**	0.20
	KMO 434 pupa	5.30	**0.02**	0.20
	KMO 431 5th	0.50	0.50	0.50
	WT 435 pupa	3.40	0.10	0.20
	KMO 442 5th	0.50	0.50	0.50
	WT 445 5th	3.40	0.10	0.20
	KMO 431 pupa	0.50	0.50	0.50
	WT 445 pupa	3.40	0.10	0.20

KMO 431 adult	WT 428 adult	2.00	0.20	0.20
	KMO 434 adult	2.00	0.20	0.20
	WT 428 pupa	1.80	0.20	0.20
	KMO 434 pupa	2.00	0.20	0.20
	KMO 431 5th	1.80	0.20	0.20
	WT 435 pupa	1.50	0.20	0.30
	KMO 442 5th	0.20	0.60	0.70
	WT 445 5th	1.50	0.20	0.30
	KMO 431 pupa	0.20	0.60	0.70
	WT 445 pupa	1.50	0.20	0.30
WT 428 adult	**KMO 434 adult**	5.30	**0.02**	0.20
	WT 428 pupa	4.50	**0.02**	0.20
	KMO 434 pupa	5.30	**0.02**	0.20
	KMO 431 5th	2.00	0.20	0.20
	WT 435 pupa	3.40	0.10	0.20
	KMO 442 5th	4.50	**0.03**	0.20
	WT 445 5th	3.40	0.10	0.20
	KMO 431 pupa	3.10	0.10	0.20
	WT 445 pupa	3.40	0.10	0.20
KMO 434 adult	**WT 428 pupa**	4.50	**0.03**	0.20
	KMO 434 pupa	3.00	0.10	0.20
	KMO 431 5th	4.50	**0.03**	0.20
	WT 435 pupa	3.40	0.10	0.20
	KMO 442 5th	4.50	**0.03**	0.20
	WT 445 5th	3.40	0.10	0.20
	KMO 431 pupa	4.50	**0.03**	0.20
	WT 445 pupa	3.40	0.10	0.20
WT 428 pupa	KMO 434 pupa	2.00	0.20	0.20
	KMO 431 5th	3.90	**0.04**	0.20
	WT 435 pupa	3.00	0.10	0.20
	KMO 442 5th	3.90	**0.05**	0.20
	WT 445 5th	3.00	0.10	0.20

	KMO 431 pupa	3.90	**0.05**	0.20
	WT 445 pupa	3.00	0.10	0.20
KMO 434 pupa	**KMO 431 5th**	4.50	**0.03**	0.20
	WT 435 pupa	3.40	0.10	0.20
	KMO 442 5th	4.50	**0.03**	0.20
	WT 445 5th	3.40	0.10	0.20
	KMO 431 pupa	4.50	**0.03**	0.20
	WT 445 pupa	3.40	0.10	0.20
KMO 431 5th	WT 435 pupa	3.00	0.10	0.20
	KMO 442 5th	3.90	**0.05**	0.20
	WT 445 5th	3.00	0.10	0.20
	KMO 431 pupa	0.40	0.50	0.60
	WT 445 pupa	3.00	0.10	0.20
WT 435 pupa	KMO 442 5th	3.00	0.10	0.20
	WT 445 5th	0.00	1.00	1.00
	KMO 431 pupa	3.00	0.10	0.20
	WT 445 pupa	0.00	1.00	1.00
KMO 442 5th	WT 445 5th	3.00	0.10	0.20
	KMO 431 pupa	0.40	0.50	0.60
	WT 445 pupa	3.00	0.10	0.20
WT 445 5th	KMO 431 pupa	3.00	0.10	0.20
	WT 445 pupa	0.00	1.00	1.00
KMO 431 pupa	WT 445 pupa	3.00	0.10	0.20

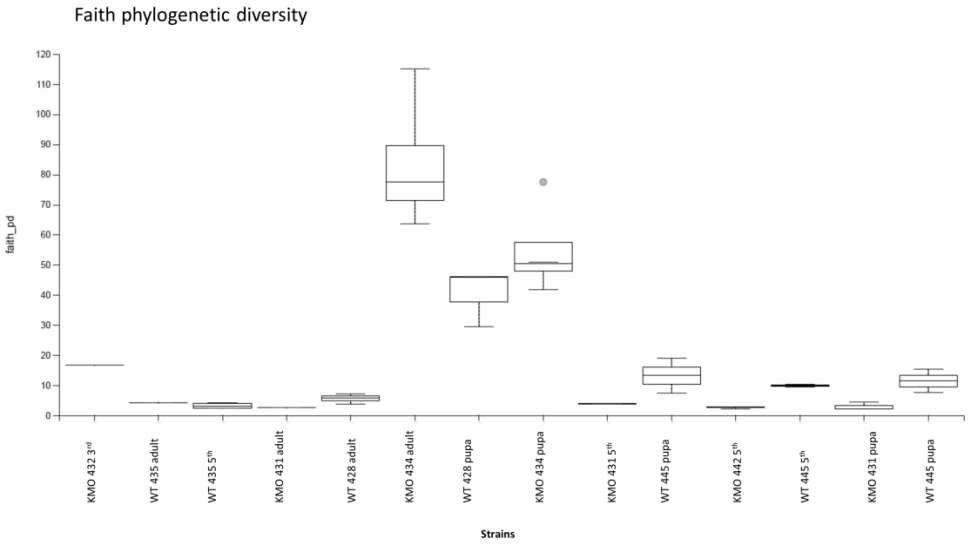


Figure 23. Alpha diversity metric, Faith Phylogenetic Diversity analysis. This box plot suggests that KMO 434 adult has the highest within-sample diversity.

Figure 24 shows the bacterial abundance profile in the KMO and WT lines of 5th instar larvae. As presented, most of the bacteria remain unclassified. A small fraction of Proteobacteria could be seen in the KMO larvae. In WT larvae, a bit more that 25% of Proteobacteria could be seen along with a tiny fraction of Firmicutes.

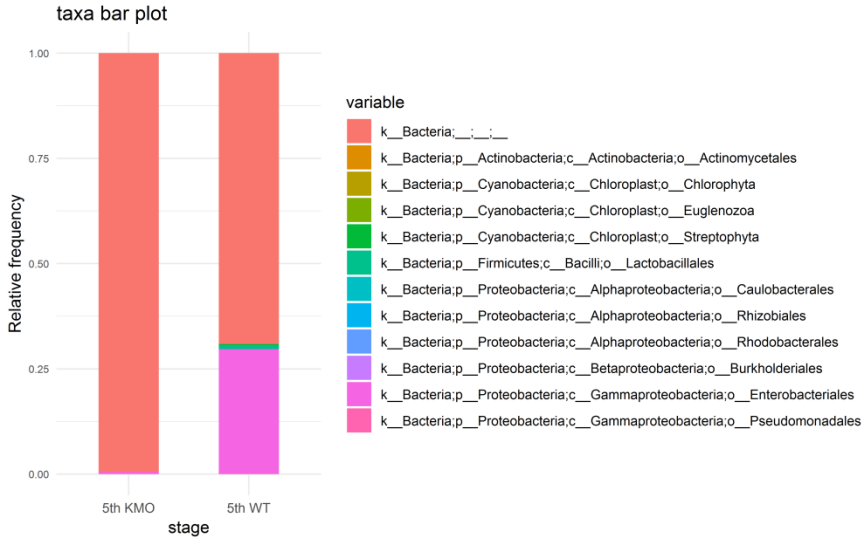


Figure 24. Relative bacterial abundance in 5th instar larval gut of wild-type and KMO knocked down *S. littoralis*

The Figure 25 shows the abundances of bacterial diversity in KMO and WT lines of the pupal stage. Just like the 5th instar larvae, here also a huge percentage of bacteria remain

unclassified in both KMO and WT pupae. A very tiny fraction of Firmicutes could be observed in KMO pupae whereas about 50% of bacteria in WT lines fall in the Firmicutes category. About 12-13% of KMO pupae comprise of Proteobacteria, which is nowhere to be seen in the WT pupae.

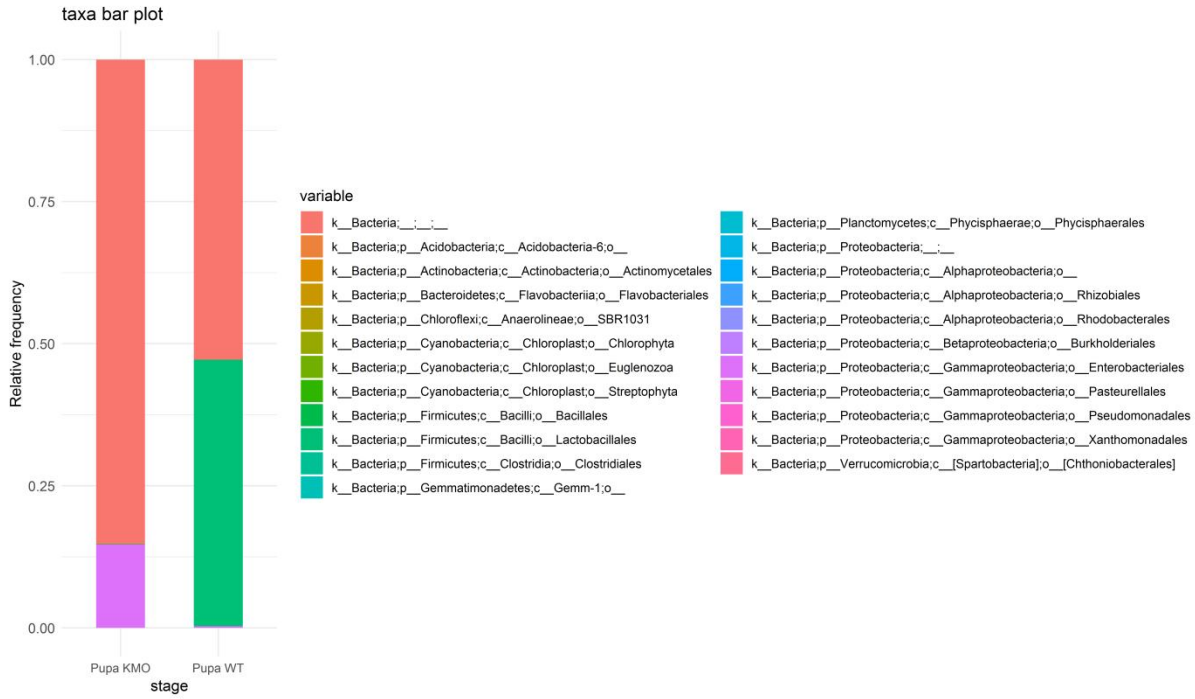


Figure 25. Relative bacterial abundance in the wild-type and KMO knockout lines of *S. littoralis* pupae samples.

The following figure shows the relative bacterial abundance in KMO and WT lines of adult *S. littoralis* insects. According to the graphs plotted, a little more than about 50% of the bacteria seem unclassified in both KMO and WT adults. A very little fraction in the KMO line fall under Firmicutes category and the remaining bacteria are Proteobacteria. Also in WT lines, Proteobacteria and Firmicutes could be observed at abundances of about 10% and 25% respectively.

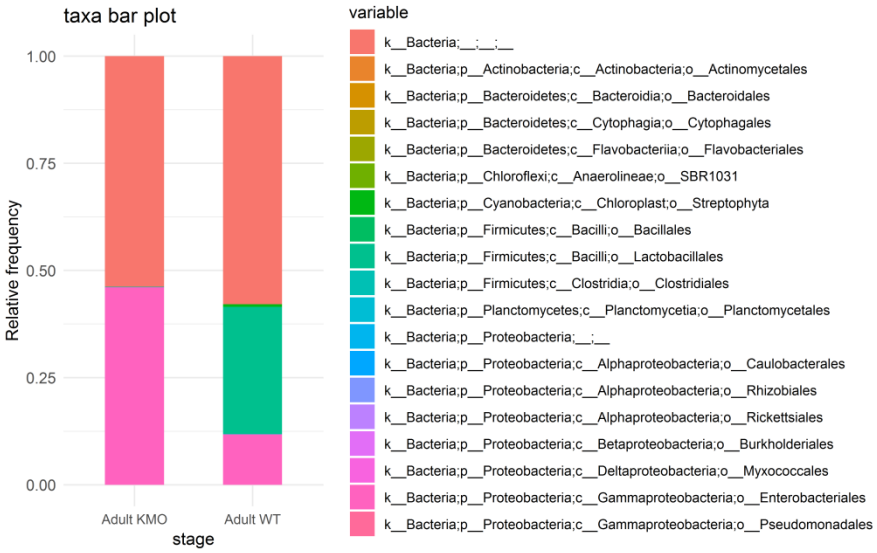


Figure 26. Relative abundance of bacterial community present in wild-type and KMO knockout lines of *S. littoralis* adults

4 Discussions

4.1 Transcriptome analysis of *E. mundtii*

The first aim of this thesis involved delivery of specifically probed *E. mundtii* symbiont with a GFP label. This was done to find answers to the question about how this endosymbiont could dominate and survive the stressful conditions of the larvae of *Spodoptera littoralis* (Figure 26). A workflow was optimized to specifically target one specific gut bacteria out of several others by help of sorting out the fluorescently labelled *Enterococcus mundtii* KD251 strain. The reporter strain after being fed to the larvae at early stage of 2nd instar, it was expected that the GFP tagged bacteria would colonize and modulate its gene expression profile according to the insect's gut conditions. *E. mundtii* expression profiles at two different gut pH conditions were checked in this work as the foregut was measured to be highly alkaline and the hindgut being neutral. The results obtained could confirm the hypothesis.

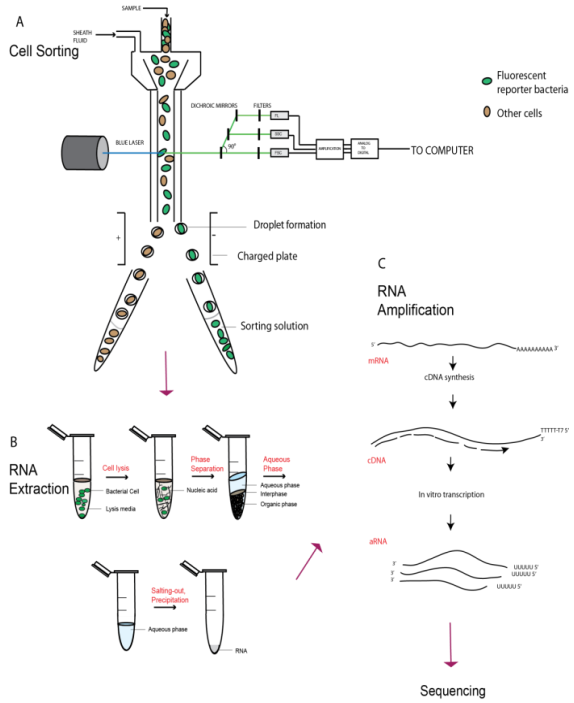


Figure 27. Workflow for transcriptome analysis of fluorescent tagged *E. mundtii*. A) Sorting of single GFP tagged cells using Flow Cytometry. B) Total bacterial RNA extraction from the sorted samples. C) In vitro reverse transcription amplification of total bacterial RNA (8)

Regarding the reproducibility of the GFP tagged bacterial strain methodology, PCA plot and dendrogram proved that the clustering of *E. mundtii* obtained from the insect guts was farther away from the *in vitro* grown culture. This verified the different in the gene expression profiles of *E. mundtii in vivo* and *in vitro*. A variety of gene expression profile data was obtained from the transcriptome sequencing study conducted to know more about the survival strategies of *E. mundtii* in the gut of the insect.

E. mundtii in order to colonize the gut of the host, adhesion of the bacteria to the gut epithelial surface is necessary. Certain motifs and domains are responsible to carry out this function of adhesion in the endosymbiont communities. These proteins are mostly cell wall associated surface proteins which act as signal peptides for the adhesion process. LPxTG is one of such motifs. Sortase is an endopeptidase which is responsible to cleave and link the peptides to the peptidoglycan of the bacterial cell wall resulting in adhesion. Hence, the up-regulation of genes encoding LPxTG motif and also sortase enzymes prove the attachment ability of *E. mundtii* to the insect gut wall surface and potential biofilm formation. WxL domains, which are also observed to be upregulated in *E. mundtii* obtained from the gut, is also crucial for the bacterial adhesion to the gut epithelium and also for adaptation to varied environmental stress conditions (8). C- terminal of conserved LPxTG motif (EMQU_1297, fms22, EMQU_1456) and WxL domains (EMQU_0485, EMQU_0541) are two well characterized contributors for adhesion. Class of proteins belonging to cell surface anchoring family (EMQU_0540, EMQU_2160), Ftf family of genes (ftsL, ftsA, ftsZ, ftsW3) and chitin binding proteins also contribute to cell surface attachment and cell wall connection (39, 40). Biofilm protein (EMQU_2682) that helps in matrix formation and stress management showed upregulation. This goes hand in hand with the ABCD two component systems (41).

The Two-component systems play a key role I n living organisms in order to survive varied environmental conditions. These are the signal transduction mechanisms that are induced in bacteria during stress conditions in the environment. The key players of this system are auto-inducing proteins (AIPs), histidine protein kinases (HPKs) and response regulators. On encountering stress, the AIPs tend to interact with the HPKs and hence sending the signal to response regulators that ultimately produce factors/ proteins that helps the bacteria survive irrespective of the stress (8).

The two-component systems are also responsible for quorum sensing as AIPs play a major role. There is an increase the AIPs when the bacterial cell density increases which is followed by a signalling cascade leading to cooperative gene expression by the bacterial cells. Accessory gene regulator (*agr*) recognizes AIPs and eventually leading to signal production for quorum sensing. Quorum sensing is a mechanism where the bacterial cells communicate to form aggregates. This is also a stress survival strategy where the bacteria divide the labor and conserve their energy to survive (8). The *agr* family that is responsible for quorum sensing was up regulated. LuxS (EMQU_04) and LuxR (EMQU_0921) that are also responsible for quorum sensing were upregulated (42). Bacterial competence and quorum are

known to be interlinked (43) VanS/VanR (EMQU_0353, EMQU_0354) system that provide defence in the presence of glycopeptide type antibiotics like vancomycin, were upregulated. They function in first line defence providing resistance to antibiotics (44).

E. mundtii, in order to survive under oxidative stress caused by the host metabolism upregulates certain enzymes. These include Superoxide dismutase (EMQU_0929), catalase (EMQU_0568), NADH oxidase-peroxidase cycle (EMQU_0335, EMQU_0459, EMQU_1279, and EMQU_1851), organic hydroperoxide resistance family (ohr) (EMQU_1453), thioredoxin family protein, peptide-methionine (R)-S-oxide reductase (EMQU_0165), and H_2O_2 resistance protein (EMQU_1453)

Stress proteins like the general stress proteins (glsB, glsB1, gls33) and universal stress proteins (uspA2) also influence the survival of bacteria in adverse conditions. Both the proteins were observed upregulated in the *E. mundtii* obtained from the insect gut. The general stress proteins help the bacteria to survive through oxidative stress, heat stress, salt stress whereas, the universal stress proteins aid bacterial survival under temperature fluctuations, heat and hypoxia (8). The usp family also depends on bacterial density caused by quorum sensing (45).

Genes for other functions like intracellular trafficking and transport include secE and virD4 for type IV secretion system were observed to be upregulated in *E. mundtii in vitro*. Other repair proteins like MutS (EMQU_2803), recA operon (EMQU_2752) for DNA repair from oxidative stress were observed to be upregulated. These play a role in recombination, repair and maintenance of DNA (46). Also, recU (EMQU_1307) and recG (EMQU_0120) help in DNA replication and repair were upregulated (47, 48). DNA starvation protein dps (EMQU_2828) prevents bacteria from multiple stresses, yafQ (EMQU_3002) and DNA damage inducible protein J (EMQU_0492, EMQU_3001) play role in biofilm formation were all upregulated.

E. mundtii from foregut was expected to highly express genes that prevent the bacteria from alkaline pH. The over expression of alkaline stress protein by *E. mundtii* could be observed in foregut (pH 10) when compared to hindgut (neutral pH). There was a downregulation of Na + H+ antiporter NhaC family (EMQU_2152) that aid in methionine transport and synthesis. This was mainly observed in alkaline conditions.

Downregulation of ATP binding protein was expected as the proton motive force is decreased as H+-ATPase activity lowers and the proton potential is zero at pH 10. Under these conditions the cation antiporters do not function therefore causing impairment in the cytoplasmic alkalization activity in order for the bacterial cells to survive in alkaline stress (49). When the foregut gene expression profile was compared to the hindgut, atpACDG were observed to be downregulated confirming the theory.

Facultative anaerobes like *E. mundtii* have the ability to switch between respiration and fermentation for energy production based on the amounts of oxygen present. Hence it can be understood why glucokinase (glcK), 1-phosphofructokinase (fruK), 6-phospho-beta-glucosidase (celA, bglP, bglB, bglG) were downregulated (49). But the expression of 6-phosphofructokinase (pfkA) and glucose-6-phophate isomerase suggest that glycolysis could still occur.

Bacteria express certain sugar transportation mechanisms depending on the carbohydrate sources available. Phosphotransferase (PTS) systems are responsible for sugar transportation allowing respective transporters to act on the respective sugar sources that are present. These systems are specific to the sugar source in order to help the bacteria sustain in the presence of complex carbohydrate conditions. The transport system uses energy from phosphoenopyruvate (PEP) via oxidative phosphorylation (8). As the *S. littoralis* was fed with the artificial Lima bean paste diet, which is a source of high sugar content, upregulation of the PTS genes in *E. mundtii* was expected. Several of the PTS systems (EMQU_2136) were observed to be over expressed in the *E. mundtii* grown *in vivo* compared to *in vito* culture hereby obeying the hypothesis. Upregulation of sucrose-6-phosphate dehydrogenase (scrB) and alpha-amylase enzyme neopullanase indication the ability of sucrose and starch metabolism (8).

Downregulation of amino acid and fatty acid metabolism may be because the bacteria do not waste energy as it is readily available from the host. Exception of amino acid Lysine was observed to be upregulated in the hindgut. Also many of the metabolic pathways that were observed to be downregulated in the *E. mundtii* also maybe because the insect does the work, hereby, helping the bacteria.

When *E. mundtii* gene expression profiles in foregut and hindgut samples were compared, except for *asp* gene, all the other genes were observed downregulated in foregut with respect to hindgut. This confirms the over expression of alkaline shock protein (*asp* gene) related

genes in foregut due to highly alkaline pH of 10 when compared to neutral in hindgut. Figure 16

In a nutshell, the gut of *S. littoralis* was observed to be predominantly colonized by *E. mundtii* and *Clostridia* species. The bacteria adhere to the gut epithelium in order to colonize and multiply as shown in Figure 27. *Enterococcus mundtii* uses survival strategies in order to compete and survive in the insect gut. Primarily, it produces anti bacteriocin to destroy potential pathobionts like *Enterococcus faecalis* and *Enterococcus Casseliflavus* which were initially successfully colonizing the early instar larval guts.

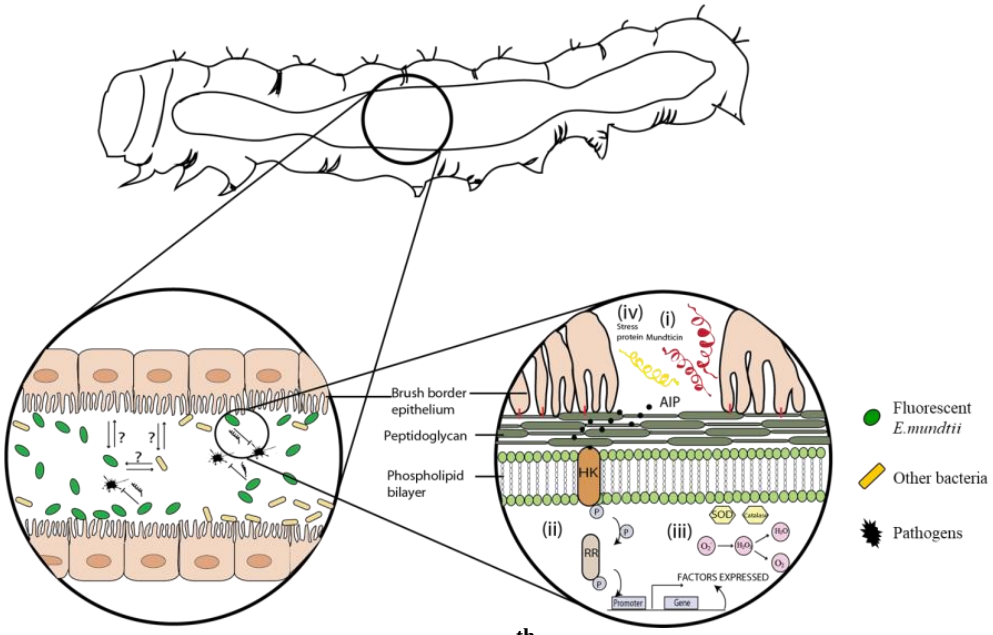


Figure 28. Overview of gut structure of 5th instar *S. littoralis* larvae (8). Reporter *E. mundtii* adhere to the gut epithelium of the insect host and successfully colonizes. The bacteria over expresses genes such as *agr* system for adaptation, superoxide dismutase and catalase to resist oxidative stress, general and universal stress proteins to survive varied stresses along with Fe limitation and alkaline stress resistance genes. These comprise of the stress survival strategies of *E. mundtii* in order to sustain and dominate the host gut.

It also increases the expression of certain genes in order to survive adverse stress conditions in the insects gut. A two component system involving *agr* system that helps in phosphorylation of histidine kinases leads to activation of certain transcription factors required to adapt adverse gut environment. The over expression of genes encoding superoxide dismutase and catalases by *E. mundtii* in the insect gut help in the survival of bacteria by converting superoxide radicals to water and oxygen. Additionally, *E. mundtii* overexpressed general stress proteins and universal stress proteins to adapt to different stress like oxygen limitation, heat and alkaline pH conditions of the insect gut. Also, over expression of genes for survival of bacteria in iron limiting conditions were found in the

foregut and hindgut samples when compared to the *in-vitro* expression profiles. Hence, the differential gene expression profiles observed in *E. mundtii* obtained from *in vivo* samples and *in vitro* samples provide a useful insight towards the survival mechanism of the bacteria in the insect gut. Over expression of certain stress related genes *in vivo* suggest the ability of *E. mundtii* to sustain even in oxidative stress, lack of iron, alkaline pH conditions of the *S. littoralis* gut.

4.2 Metagenomics of wild-type and KMO knockout *S.littoralis*

The 16S rRNA gene amplification method was used followed by Hiseq sequencing technology in order to analyse the variation in the gut microbial community of KMO knockout lines and WT lines of *S. littoralis*. This methodology was necessary to determine whether 8-HQA plays a major role in the gut community regulation of the Lepidopteran insect host.

Species richness and abundance were studied by analysis of alpha and beta diversity. Alpha diversity signifies the richness of the species that indicate how similar or different the bacterial species are, while the beta diversity signifies the differences in microbial abundances between two samples.

Based on the bacterial abundances observed in the KMO and WT lines of 5^{th} instar larvae, pupae and adults, predominant bacteria remain unclassified. The remaining bacterial populations present in all the stages belong to either Firmicutes or Proteobacteria. Not much regarding the bacterial diversity amongst the KMO and WT lines could be said as most of them were not classified. This could be also improved by using genus specific pipelines in order to retrieve more data regarding the bacteria present in the guts of the insects of different stages. The depth of the sequencing also plays a role in the preciseness of the sequencing data results. As in this our case, only 2 million paired end reads were obtained which could also lead to the non-identification of most of the bacterial species.

According to the Pairwise Kruskal-wallis test for alpha diversity analysis, significant differences among samples with p value ≤ 0.05 were observed. As further analysis is required to deduce from the data regarding the role of 8-HQA in the gut microbiome regulation, nothing much can be immediately concluded.

4.3 Conclusions and future prospects

Lactic acid bacteria (LAB) play a major role in production of dairy products and fermented food. They are also potential probiotics which benefit the health of human beings and they could also be the source of live vaccination. *Enterococcus* species are LAB species which are studied in this experiment in the gut of the organism *Spodoptera littoralis*. In this thesis, to study the potential host-microbial symbiotic interactions, the predominant endosymbiont *Enterococcus mundtii* was tagged with a GFP label to analyse its survival strategies in the insect gut. Interestingly, as the reporter *E. mundtii* that was fed to the insect could be visualized in the succeeding generations, it was hypothesized that the transmission occurring was vertical. Vertical transmission is when the mother smears the egg shell surface with the gut symbiont community, a tiny part of which is then ingested by the larvae while emerging from a hatching egg (8). Gut microbial community is moulded by several factors including pH, diet, simple gut structure of *S. littoralis* and host secretions. Availability of oxygen, insect's immunity. Also, metamorphosis leads to either an overall turnover of the gut population or selection of the competent ones over others. Above all of these adversities, microbes evolve with strategies in order for their survival and sustenance in the host gut (8).

E. mundtii seemed to dominate, exercising a colonization resistance towards some other species of the gut. Hence, it was necessary to study the survival mechanism of *E. mundtii* species in the insect gut and to study if the *S. littoralis-E. mundtii* interaction is a mutualistic one, benefiting the insect and also the bacteria. Transcriptome analysis of the reporter based *E. mundtii* construct obtained from the gut of the insect helped in understanding the mechanism in a better way. This reporter based system enables understanding of other species specific interaction studies in the future.

The current work involving the sequencing of 16S rRNA gene of the microbial flora from different life stages of WT and KMO knockout insect lines is yet to be completed. More analysis needs to be done before concluding regarding the microbial diversity present in the 8-HQA producing and non-producing *S. littoralis* gut. Effect of iron on the gut microbial community could provide more insight regarding the dependability of gut microbiota on 8-HQA. As 8-HQA is an iron chelator, it would be better to know about the iron content in the guts of the WT larvae. *Enteroccocus mundtii* could be specifically targeted and analysed in KMO and WT conditions in order to deduce more details about *S. littoralis-E. mundtii* interactions.

5 Summary

Spodoptera littoralis is a well-known Lepidopteran pest that feeds on a wide variety of agricultural crops. The microbial community of the larval stages of *S. littoralis* was elucidated. On analysing the temporal variation of the bacterial community present in the intestinal tract of the insect, *Enterococcus* and *Clostridia* species were observed to be more dominant. *Enterococcus faecalis* and *Enterococcus casseliflavus* were observed to be the dominant ones in the early larval stages of the insect that were eventually outnumbered by *Enteroccus mundtii* which became the dominant bacteria in the later larval instars and hence through the adults. The ability of *E. mundtii* to produce an antimicrobial peptide called Mundticin helped it to destroy the earlier dominant *E. faecalis* and *E. casseliflavus* that could prove lethal to the insect. To know more about the *E. mundtii* bacteria and its survival strategies in the host gut, we constructed a GFP tagged reporter strain using *E. mundtii* retrieved from the *S. littoralis* gut. We then fed the fluorescent reporter at early larval instars, retrieved it back after letting the reporter integrate into the gut and modify its expression profiles based on the gut environment.

When we compared the transcriptome data of *E. mundtii* reporter retrieved from the insect gut to the *in vitro* grown reporter strain, several genes were found to be differentially expressed. This included overexpression of stress related genes in the expression profiles of the *in vivo* grown bacteria suggesting potential survival mechanisms of the bacteria in the adverse conditions of *S. littoralis* gut. The gut stresses include high pH, presence of oxidative stress, limitation of Fe and many more. Furthermore, a comparative study of genomic data of the gut microbiome of Fe chelator producing *S. littoralis* and Fe chelator non-producing insects was carried out. This was conducted to investigate if 8-HQA (a Fe chelator compound produced by *S. littoralis*) is a factor in regulation of *S. littoralis* gut microbiome. The 16S rRNA gene amplification results of the gut microbial community of wild type and mutant lines helped us to further analyse alpha and beta diversity of the samples. As a lot of work for in-depth analysis of the results is still required to be done, it would be too soon to deduce and confirm anything from the currently presented data.

6 Zusammenfassung

Spodoptera littoralis ist ein bekannter Pflanzenschädling, der sich von einer Vielzahl landwirtschaftlicher Kulturen ernährt. Die mikrobielle Gemeinschaft in allen Larvenstadien von *S. littoralis* wurde aufgeklärt. Bei der Analyse der zeitlichen Veränderung der im Insektendarm vorhandenen Bakteriengemeinschaft fand man, dass *Enterococcus*- und *Clostridia*-Arten vorherrschten. Es wurde beobachtet, dass *Enterococcus faecalis* und *Enterococcus casseliflavus* die frühen Larvenstadien des Insekts dominierten, die schließlich in den späteren Larvenstadien und dadurch auch im Erwachsenenstadium von *Entercoccus mundtii* abgelöst wurden. Durch die Fähigkeit von *E. mundtii* ein antimikrobielles Peptid namens Mundticin herzustellen, half die früher dominierenden *E. faecalis* und *E. casseliflavus* zu zerstören, die sich für das Insekt als tödlich erweisen könnten. Um mehr über die *E. mundtii*-Bakterien und ihre Überlebensstrategien im Darm des Wirts zu erfahren, konstruierten wir einen GFP-markierten Reporter-Stamm unter Verwendung von *E. mundtii*, der aus dem *S. littoralis*-Darm gewonnen wurde. Dann fütterten wir den fluoreszierenden Reporter in frühen Larvenstadien, holten ihn zurück nachdem der Reporter in den Darm integriert und sein Expressionsprofil basierend auf der Darmumgebung modifiziert wurde.

Als wir die Transkriptomdaten des aus dem Insektendarm gewonnenen *E. mundtii*-Reporters mit dem in vitro gezüchteten Reporterstamm verglichen, wurden verschiedene Gene mit unterschiedlichen expressionsniveau sichtbar. Dies beinhaltete die Überexpression von stressbedingten Genen, die in den Expressionsprofilen der in vivo gezüchteten Bakterien beobachtet wurden und auf potentielle Überlebensmechanismen der Bakterien unter den nachteiligen Bedingungen von *S. littoralis* schließen lassen. Zu den widrigen Darmbedingungen zählen ein hoher pH-Wert, das Vorhandensein von oxidativem Stress, die Limitation von Fe und viele andere mehr. Darüber hinaus wurde eine vergleichende Studie der genomischen Daten des Darm-Mikrobioms von Fe-Chelator produzierenden *S. littoralis*, und den Insekten die keine Fe-Chelator produzieren, durchgeführt. Dies wurde durchgeführt, um zu untersuchen, ob 8-HQA (eine von *S. littoralis* hergestellte Fe-Chelator-Verbindung) ein Faktor bei der Regulation des *S. littoralis*-Darmmikrobioms ist. Die Ergebnisse der 16S-rRNA-Genamplifikation der Darmmikrobengemeinschaft von Wildtyp- und Mutantenlinien halfen uns bei der weiteren Analyse der Alpha- und Beta-Diversität der Proben.

References

1. Hooper LV, Midtvedt T, Gordon JI. How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annu Rev Nutr. 2002;22:283-307.

2. Hooper LV. Bacterial contributions to mammalian gut development. Trends Microbiol. 2004;12(3):129-34.

3. El Aidy S, Hooiveld G, Tremaroli V, Backhed F, Kleerebezem M. The gut microbiota and mucosal homeostasis: colonized at birth or at adulthood, does it matter? Gut microbes. 2013;4(2):118-24.

4. Stouthamer R, Breeuwer JAJ, Hurst GDD. Wolbachia pipientis: Microbial manipulator of arthropod reproduction. Annu Rev Microbiol. 1999;53:71-102.

5. Tilg H, Kaser A. Gut microbiome, obesity, and metabolic dysfunction. J Clin Invest. 2011;121(6):2126-32.

6. Ochman H, Moran NA. Genes lost and genes found: Evolution of bacterial pathogenesis and symbiosis. Science. 2001;292(5519):1096-8.

7. Heintz C, Mair W. You Are What You Host: Microbiome Modulation of the Aging Process. Cell. 2014;156(3):408-11.

8. Mazumdar T, Teh BS, Boland W. The microbiome of Spodoptera littoralis: development, control and adaptation to the insect host. Metagenomics for Gut Microbes: IntechOpen; 2018. p. 77-103.

9. Shao YQ, Chen BS, Sun C, Ishida K, Hertweck C, Boland W. Symbiont-Derived Antimicrobials Contribute to the Control of the Lepidopteran Gut Microbiota. Cell Chem Biol. 2017;24(1):66-75.

10. Engel P, Moran NA. The gut microbiota of insects - diversity in structure and function. Fems Microbiol Rev. 2013;37(5):699-735.

11. Florez LV, Scherlach K, Gaube P, Ross C, Sitte E, Hermes C, et al. Antibiotic-producing symbionts dynamically transition between plant pathogenicity and insect-defensive mutualism. Nat Commun. 2017;8.

12. Chen BS, Teh BS, Sun C, Hu SR, Lu XM, Boland W, et al. Biodiversity and Activity of the Gut Microbiota across the Life History of the Insect Herbivore Spodoptera littoralis. Sci Rep-Uk. 2016;6.

13. Salem H, Bauer E, Kirsch R, Berasategui A, Cripps M, Weiss B, et al. Drastic Genome Reduction in an Herbivore's Pectinolytic Symbiont. Cell. 2017;171(7):1520-+.

14. Kikuchi Y, Hosokawa T, Nikoh N, Meng XY, Kamagata Y, Fukatsu T. Host-symbiont co-speciation and reductive genome evolution in gut symbiotic bacteria of acanthosomatid stinkbugs. Bmc Biol. 2009;7.

15. Kikuchi Y, Hosokawa T, Nikoh N, Meng XY, Kamagata Y, Fukatsu T. Host-symbiont co-speciation and reductive genome evolution in gut symbiotic bacteria of acanthosomatid stinkbugs. BMC biology. 2009;7:2.

16. Kim JK, Lee BL. Insect Symbiosis and Immunity: The Bean Bug-Burkholderia Interaction as a Case Study. Adv Insect Physiol. 2017;52:179-97.

17. Powell JE, Leonard SP, Kwong WK, Engel P, Moran NA. Genome-wide screen identifies host colonization determinants in a bacterial gut symbiont. P Natl Acad Sci USA. 2016;113(48):13887-92.

18. Shi WB, Syrenne R, Sun JZ, Yuan JS. Molecular approaches to study the insect gut symbiotic microbiota at the 'omics' age. Insect Sci. 2010;17(3):199-219.

19. Yun JH, Roh SW, Whon TW, Jung MJ, Kim MS, Park DS, et al. Insect Gut Bacterial Diversity Determined by Environmental Habitat, Diet, Developmental Stage, and Phylogeny of Host. Appl Environ Microb. 2014;80(17):5254-64.

20. Dillon RJ, Dillon VM. The gut bacteria of insects: Nonpathogenic interactions. Annu Rev Entomol. 2004;49:71-92.

21. Mosallanejad H, Smagghe G. Biochemical mechanisms of methoxyfenozide resistance in the cotton leafworm Spodoptera littoralis. Pest Manag Sci. 2009;65(7):732-6.

22. Grossman GL, Rafferty CS, Clayton JR, Stevens TK, Mukabayire O, Benedict MQ. Germline transformation of the malaria vector, Anopheles gambiae, with the piggyBac transposable element. Insect Mol Biol. 2001;10(6):597-604.

23. Tang XS, Freitak D, Vogel H, Ping LY, Shao YQ, Cordero EA, et al. Complexity and Variability of Gut Commensal Microbiota in Polyphagous Lepidopteran Larvae. Plos One. 2012;7(7).

24. Funke M, Büchler R, Mahobia V, Schneeberg A, Ramm M, Boland W. Rapid hydrolysis of quorum-sensing molecules in the gut of lepidopteran larvae. Chembiochem. 2008;9(12):1953-9.

25. Mithöfer A, Wanner G, Boland W. Effects of feeding Spodoptera littoralis on lima bean leaves. II. Continuous mechanical wounding resembling insect feeding is sufficient to elicit herbivory-related volatile emission. Plant Physiol. 2005;137(3):1160-8.

26. Johnston PR, Rolff J. Host and Symbiont Jointly Control Gut Microbiota during Complete Metamorphosis. Plos Pathog. 2015;11(11).

27. Higashide T, Takahashi M, Kobayashi A, Ohkubo S, Sakurai M, Shirao Y, et al. Endophthalmitis caused by Enterococcus mundtii. J Clin Microbiol. 2005;43(3):1475-6.

28. Teh BS, Apel J, Shao YQ, Boland W. Colonization of the Intestinal Tract of the Polyphagous Pest Spodoptera littoralis with the GFP-Tagged Indigenous Gut Bacterium Enterococcus mundtii. Front Microbiol. 2016;7.

29. Pesek J, Svoboda J, Sattler M, Bartram S, Boland W. Biosynthesis of 8-hydroxyquinoline-2-carboxylic acid, an iron chelator from the gut of the lepidopteran Spodoptera littoralis. Org Biomol Chem. 2015;13(1):178-84.

30. Giorgini F, Huang SY, Sathyasaikumar KV, Notarangelo FM, Thomas MA, Tararina M, et al. Targeted deletion of kynurenine 3-monooxygenase in mice: a new tool for studying kynurenine pathway metabolism in periphery and brain. The Journal of biological chemistry. 2013;288(51):36554-66.

31. Skaar EP. The Battle for Iron between Bacterial Pathogens and Their Vertebrate Hosts. Plos Pathog. 2010;6(8).

32. Woo PCY, Lau SKP, Teng JLL, Tse H, Yuen KY. Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. Clin Microbiol Infec. 2008;14(10):908-34.

33. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. Isme J. 2012;6(8):1621-4.

34. Mullis KB, Faloona FA. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods in enzymology. 1987;155:335-50.

35. Cullen DW, Lees AK, Toth IK, Duncan JM. Conventional PCR and real-time quantitative PCR detection of Helminthosporium solani in soil and on potato tubers. Eur J Plant Pathol. 2001;107(4):387-98.

36. Maffei M, Bossi S, Spiteller D, Mithöfer A, Boland W. Effects of feeding Spodoptera littoralis on lima bean leaves. I. Membrane potentials, intracellular calcium variations, oral secretions, and regurgitate components. Plant Physiol. 2004;134(4):1752-62.

37. Uhlenhaut C, Kracht M. Viral infectivity is maintained by an RNA protection buffer. J Virol Methods. 2005;128(1-2):189-91.

38. Shiwa Y, Yanase H, Hirose Y, Satomi S, Araya-Kojima T, Watanabe S, et al. Complete Genome Sequence of Enterococcus mundtii QU 25, an Efficient L-(1)-Lactic Acid-Producing Bacterium. DNA Res. 2014;21(4):369-77.

39. Sanchez B, Gonzalez-Tejedo C, Ruas-Madiedo P, Urdaci MC, Margolles A. Lactobacillus plantarum Extracellular Chitin-Binding Protein and Its Role in the Interaction between Chitin, Caco-2 Cells, and Mucin. Appl Environ Microb. 2011;77(3):1123-6.

40. Erickson HP, Anderson DE, Osawa M. FtsZ in Bacterial Cytokinesis: Cytoskeleton and Force Generator All in One. Microbiol Mol Biol R. 2010;74(4):504-28.

41. Li YH, Tian XL. Quorum Sensing and Bacterial Social Interactions in Biofilms. Sensors-Basel. 2012;12(3):2519-38.

42. Koch B, Lijefors T, Persson T, Nielsen J, Kjelleberg S, Givskov M. The LuxR receptor: the sites of interaction with quorum-sensing signals and inhibitors. Microbiol-Sgm. 2005;151:3589-602.

43. Lee MS, Morrison DA. Identification of a new regulator in Streptococcus pneumoniae linking quorum sensing to competence for genetic transformation. J Bacteriol. 1999;181(16):5004-16.

44. Jordan S, Hutchings MI, Mascher T. Cell envelope stress response in Gram-positive bacteria. Fems Microbiol Rev. 2008;32(1):107-46.

45. Kim H, Goo E, Kang Y, Kim J, Hwang I. Regulation of Universal Stress Protein Genes by Quorum Sensing and RpoS in Burkholderia glumae. J Bacteriol. 2012;194(5):982-92.

46. Ishag HZA, Xiong QY, Liu MJ, Feng ZX, Shao GQ. E-coli recA gene improves gene targeted homologous recombination in Mycoplasma hyorhinis. J Microbiol Meth. 2017;136:49-56.

47. Ayora S, Carrasco B, Doncel E, Lurz R, Alonso JC. Bacillus subtilis RecU protein cleaves Holliday junctions and anneals single-stranded DNA. P Natl Acad Sci USA. 2004;101(2):452-7.

48. Ishioka K, Iwasaki H, Shinagawa H. Roles of the recG gene product of Escherichia coli in recombination repair: Effects of the Delta recG mutation on cell division and chromosome partition. Genes Genet Syst. 1997;72(2):91-9.

49. Ran SJ, Liu B, Jiang W, Sun Z, Liang JP. Transcriptome analysis of Enterococcus faecalis in response to alkaline stress. Front Microbiol. 2015;6.

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Eigenständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig angefertigt, nicht anderweitig zu Prüfungszwecken vorgelegt und keine anderen als die angegebenen Hilfsmittel verwendet habe. Sämtliche wissentlich verwendeten Textausschnitte, Zitate oder Inhalte anderer Verfasser wurden ausdrücklich als solche gekennzeichnet.

Jena, 15th Januar 2019 _____

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