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Transcriptomics Reveal the Survival Strategies of *Enterococcus mundtii* in the Gut of *Spodoptera littoralis*

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Abstract

The complex interaction between a higher organism and its resident gut flora is a subject of immense interest in the field of symbiosis. Many insects harbor a complex community of microorganisms in their gut. Larvae of *Spodoptera littoralis,* a lepidopteran pest, house a bacterial community that varies both spatially (along the length of the gut) and temporally (during the insect's life cycle). To monitor the rapid adaptation of microbes to conditions in the gut, a GFP-tagged reporter strain of *E. mundtii*, a major player in the gut community, was constructed. After early-instar *S. littoralis* larvae were fed with the tagged microbes, these were recovered from the larval fore- and hindgut by flow cytometry. The fluorescent reporter confirmed the persistence of *E. mundtii* in the gut. RNA-sequencing of the sorted bacteria highlighted various strategies of the symbiont's survival, including upregulated pathways for tolerating alkaline stress, forming biofilms and two-component signaling systems for quorum sensing, and resisting oxidative stress. Although these symbionts depend on the host for amino acid and fatty acids, differential regulation among various metabolic pathways points to an enriched lysine synthesis pathway of *E. mundtii* in the hindgut of the larvae.

Keywords *Enterococcus mundtii* · *Spodoptera littoralis* · Flow cytometry · Transcriptomics · Foregut · Hindgut

Introduction

Insects comprise the largest phylum of arthropods on earth, according to the IUCN red list. Microorganisms are known to form symbiotic relationships with insects by supplying them with essential nutrients, protection against pathogens, and aid in digesting organic matter. They contribute significantly to insects' ability to act as potential pathogens to animals, pests or pollinators of food crops, and as cyclers of carbon and nitrogen during the decomposition of plant biomass (Engel and Moran 2013).

Insects with a straight, tube-like gut usually possess a less diverse microbial population than species with invaginations and deep pouches (Engel and Moran 2013). Other factors that shape the gut population include the following: oxygen level, gut pH, the presence of digestive enzymes, antimicrobial compounds and insect diet (Dillon and Dillon 2004; Paniagua Voirol et al. 2018; Shao et al. 2017). Although most bacteria have an affinity for neutral pH, several acidophiles and alkalophiles have adapted to extreme pH conditions. In case of Lepidopterans, their guts have been repeatedly found to be alkaline in nature (Mason et al. 2020; Paniagua Voirol et al. 2018).

Vertical transmission of symbionts allows bacterial transfer (from the ovaries to the egg shells) to the next generation (Lee et al. 2015), whereas horizontal transmission occurs over the course of the life cycle, through diet and social behavior (Mason and Raffa 2014). Regardless of how bacteria are transmitted, microbial populations may be unstable during early developmental stages. For example, the gut of holometabolous lepidopterans undergoes complete metamorphosis from pupa to adult, resulting in microbial turnover and

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variable microbial counts (Tang et al. 2012). Influence of diet and a dynamic trend of gut microbiota because of holometaboly have also been studied in lepidopterans like *Spodoptera exigua* (Martínez-Solís et al. 2020), *Lymantria dispar* (Mason and Raffa 2014), *Spodoptera frugiperda* and *Helicoverpa zea* (Mason et al. 2020) and *Brithys crini* (González-Serrano et al. 2019). In *Brithys crini*, the developmental stage played the main role in determining the bacterial population in their guts (González-Serrano et al. 2019). *Grapholita molesta*'s dynamic trend in microbiome is apparent when the larvae grow to third- fifth instars (Wang et al. 2020).

The cotton leafworm, *Spodopera littoralis,* a holometabolous lepidopteran (Paniagua Voirol et al. 2018) that feeds on a broad range of plants, is a prevalent pest in the tropical and subtropical regions of the world. Despite the seemingly simple structure of the gut, it has a pH gradient: the anterior part and midgut of lepidopteran larvae, are highly alkaline, with a pH range of 11–12 (Wieczorek et al. 2009), but the posterior part is neutral (Funke et al. 2008). Such a gradient might restrict the survival of many microbial species. Despite their alkaline pH, bacteria of the phylum Firmicutes, notably Enterococci and *Clostridium* sp., are found to be the core microbiome in the larval gut of *S. littoralis* (Tang et al. 2012). The abundance of Enterococci was also observed in numerous other Lepidopterans (Paniagua Voirol et al. 2018), (Ugwu et al. 2020;Rozadilla et al. 2020), or butterflies such as *Galleria melonella* (Johnston and Rolff 2015), *Bombyx mori* (Sun et al. 2017), and *Grapholita molesta* (Wang et al. 2020). The examples are in agreement with our model insect, *Spodoptera littoralis*, where *Enterococcus mundtii* has been shown to dominate the gut (Tang et al. 2012;Teh et al. 2016;Shao et al. 2017). *Enterococcus mundtii* is a gram-positive, non-motile lactic acid bacterium, well adapted to dairy and plant environments (Magni et al. 2012). It is found on the human navel, cow teats and the hands of milkers; in soil and in the gastrointestinal tracts of humans, animals and several species of Lepidopterans, *e.g. Ephestia kuehniella* and *Plutella xylostella* (Johnston and Rolff 2015;Grau et al. 2017;Ishag et al. 2017) They can exert probiotic, positive effects which have been shown in humans (Hanchi et al. 2018), but also in insects. *E. mundtii* produce an antimicrobial peptide, mundticin KS, that keeps potential pathobionts like *Enterococcus fecalis* and *Enterococcus casseliflavus* at bay. These pathobionts are apparent in first-instar larvae, but their early colonization success is brief, owing to mundticin (Shao et al. 2017; Tang et al. 2012). In addition, larvae of several lepidopteran species produce high concentrations of 8-hydroxyquinoline-2-carboxylic acid, an iron chelator that is derived from tryptophan and found in the larval gut and regurgitate (Pesek et al. 2015). Since iron is one of the main elements in several metabolic pathways, such as those responsible for the quenching of reactive oxygen species, oxygen metabolism in TCA cycle, electron transport and nitrogen assimilation among others (Anzaldi and Skaar 2010), this chelator, along with mundticin may contribute to control the microbiome in larval guts.

In this paper we used a GFP-tagged *E. mundtii* (Teh et al. 2016) to visualize how the test organism adapts to the gut environment of the host insect. In the digestive tract the reporter organism is permanently exposed to the high- and low-molecular-weight compounds from the plant food, from the whole ensemble of the other gut microbes, to compounds produced and released by the insect, as well as to digestion products which altogether contribute to the gene expression of the test organism. Following FACS-sorting and sequencing of the RNA of the recovered bacteria this approach allowed us for the first time in a lepidopteran an in depth analysis of the adaptive strategies of the symbiont. In particular, responses to alkaline stress, biofilm formation and the induction of signaling systems for quorum sensing and oxidative stress are relevant elements of the mutual host/guest interaction.

Materials and Methods

Maintenance of Eggs and Larvae

The eggs of *S. littoralis* were obtained from Syngenta Crop Protection Munchwielen AG (Munchwielen, Switzerland). Eggs were hatched at 14° C and the larvae were maintained at 24° C in an alternate 16 h light period and 8 h dark period. Larvae were reared on an agar-based artificial diet containing white beans, as described by Maffei et al. (2004).

Bacterial Strain

A fluorescent strain of *E. mundtii* KD251 (isolated from the gut of *S. littoralis* in the Department of Bioorganic Chemistry) was constructed by transforming a GFP-containing expression vector pTRKH3-ermGFP, as described (Teh et al. 2016). This strain was grown in Todd-Hewitt Broth (THB) (Roth, Karlsruhe, Germany) medium for both broth and 1.5% agar (Roth, Karlsruhe, Germany), and in the presence of 5 μg ml^{-1} of erythromycin (Acros Organics, NJ, USA). The strain was preserved as a glycerol stock at −80° C.

Introduction of the Reporter Bacteria into the Insect Microbiome

A stationary phase culture of fluorescent reporter *E. mundtii* in THB broth containing 5 μg ml^{-1} of erythromycin was grown till mid-log phase with $OD_{600} \sim 0.5$–0.6 at 37 °C with shaking at 220 rpm. The culture was pelleted at 5000 x g for 10 min at 4 °C and resuspended in distilled water. First-instar *S. littoralis* larvae ($n = 120$) were fed small cubes of artificial

diet supplemented with two antibiotics, ampicillin (5.75 μgml^{-1}) (EMD Millipore corp., Billerica, MA, USA) and erythromycin (9.6 μgml^{-1}) for 3 days, to reduce the already existing bacterial load, before (at the second instar) being fed with100 μl from the 1:10 dilution broth (~10^{10} cells) containing fluorescent *E. mundtii* as described (Teh et al. 2016). These larvae were allowed to grow until the fifth instar, when samples were prepared for FACS.

Sample Preparation for FACS

A total of 30 fifth-instar larvae for each gut region – foregut and hindgut – were dissected with sterile forceps and scissors in a sterile clean bench. Following dissection, the gut tissues were immediately submerged in 10 ml of RNAlater solution (Invitrogen, Vilnius, Lithuania). Tissues submerged in RNAlater solution were mixed with 2 ml of 6% (*w*/*v*) betaine (Sigma Aldrich, St. Louis, MO, USA) and placed on ice prior to being crushed with mortar and pestle until gut homogenates were formed. Thereafter, fluorescent *E. mundtii* were separated from the intestinal debris by filtration through 40 μm pore-size cell strainers (Falcon, NY, USA). The filtrates were then separated into aliquots of 600 μl each and kept at -80 °C for the sorting experiment.

As controls, *E. mundtii* broth cultures (10 ml, $n = 3$) were grown to exponential growth ($OD_{600} \sim 0.5$–0.6) and centrifuged at 5000 x g for 15 min at 4 °C to pellet the bacterial cells. Bacterial cells were washed once with sterile phosphate-buffered saline (PBS) and resuspended with RNAlater to a concentration of approximately 10^{10} CFU ml^{-1}.

Cell Sorting by FACS

The gut homogenates were analyzed using BD FACSAria™ Fusion Cell Sorter (Becton Dickinson, Heidelberg, Germany). The machine relies on an ion laser emitting a 488 nm wavelength, and a 502 long pass filter, followed by a 530/30 band pass filter. The green fluorescent protein emits light with a peak wavelength of 530 nm. Prior to loading each sample in the FACS machine, the homogenate was thawed, and 1:5 dilution of the homogenate was made in sterile PBS, followed by vortexing for 10 s for proper mixing and to dislodge the bacteria from tissue. The cells were sorted at a flow rate ranging from10 μl/min to 80 μl/min. The sorting was done in a single-cell mode, and the sorted cells were collected in 5 ml sterile polypropylene round-bottom tubes (Falcon, Mexico). The cells were collected for a period of 3 h, which corresponded to an acquisition of 6000–7000 events/s. The flow cytometry grade of PBS buffer (Thermo Fischer, Wilmington, DE, USA) at pH of 7.4 was used as the sheath fluid. A total of ~250, 000 cells were sorted from each sample of control, fore and hindgut homogenates into 1 ml of RNA Protect solution (Qiagen, Hilden, Germany).

RNA Extraction and Sequencing

The FACS-sorted fluorescent bacterial cells (~ 250,000) from each control, foregut and hindgut ($n = 3$) were pelleted by centrifugation at 5000 x g for 10 min at 4 °C, leaving the sorting solution and RNA protect in the supernatant. The supernatent was removed from the sorted cells prior to RNA isolation, and total RNA was isolated from the pelleted cells using the RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, with some modifications. Pelleted bacterial cells were lysed enzymatically for 15 min at 37 °C (enzymatic mix: 1X TE buffer, pH 8 (Applichem GmbH, Darmstadt, Germany), pH 8.0, 5 μg ml^{-1} lysozyme (Sigma Aldrich, St. Louis, MO,USA) and 50 Uml^{-1}mutanolysin (Sigma Aldrich, St. Louis, MO,USA)). All samples were DNase-treated with on-column DNase digestion per the manufacturer's protocol prior to RNA isolation. The concentration of total RNA in controls was diluted to match the bacterial concentration at the level of a single cell. RNA was further cleaned and concentrated using a concentrator kit (Zymo Research, USA) and yielding about 12 μl in final volume (~10 ng). The purified RNA was linearly amplified using MessageAmp II bacterial RNA amplification kit (Invitrogen, Vilnius, Lithuania) and 10 ng of total RNA following the manufacturer's instructions. The amplified RNA (aRNA) was concentrated by precipitation with 5 M ammonium acetate. The quality and quantity of the total RNA was measured with a NanoDrop One Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). RNA samples were sent to the Max Planck Genome Centre in Cologne for RNA sequencing. A total of 0.3 μg - 1 μg of amplified RNA was used for cDNA library preparation using the Ultra-Low Input RNA kit following the Illumina protocol at the Max Planck Genome Centre, Cologne. Sequencing was carried out on the HiSeq 2500 sequencer at Cologne, and a total of approximately 10 million paired-end reads (2 × 150 bp) were generated for each sample.

RNA-seq Data Analysis

FastQC was done for an initial quality analysis of the reads. Analysis of the reads, including trimming of adapters and differential gene expression analysis, was done on LINUX-based Command line interface, following the Tuxedo protocol (Trapnell et al. 2012). The adapters were trimmed using Trimmomatic 0.36; trimmed reads were assembled using Tophat 2.1.0 and mapped to the genome of *E. mundtii* QU25 (Shiwa et al. 2014) using Cufflinks 2.2.0. The read counts were normalized with FPKM (fragments of kilobase of transcripts per million mapped reads) (supplementary S7), and assemblies were merged using Cuffmerge. Cuffdiff was used to compute the differentially expressed genes between

E. mundtii from the larval gut and *E. mundtii* grown *in vitro*. Based on homology to protein families, the proteins that were predicted for *E. mundtii* were categorized under gene ontology terms (http://geneontology.org). The genes were also mapped to the KEGG database to predict the pathways (supplementary). Gene annotation information of *E. mundtii* was obtained from the KEGG-FTP server. The results of differentially expressed genes were visualized using R-package CummeRbund 2.0, on R version 3.3.3 (2017-03-06). This R-package generated all the plots: dendrograms, PCA plot and heatmaps. A fold-change of ≥2 was used as a threshold to analyze the differentially expressed genes. Pathway analysis was performed using the R-package, clusterProfiler (Yu et al. 2012). The enricher () and enrichKEGG () functions performed enrichment tests with gene ontology categories and KEGG databases, respectively, and grouped enriched pathways based on the number of significantly expressed (p value cut-off = 0.05) genes in the *in vivo* conditions as compared to control (Boyle et al. 2004;Yu et al. 2012).

Data Availability

The raw transcriptome data has been deposited to NCBI Short Read Archive (SRA). The BioProject ID is: PRJNA622409.

Results

The bacterial strain *Enterococcus mundtii*, a dominant symbiont of *S. littoralis*, was employed as a reporter organism in order to follow its colonization of the insect gut. The approach provides direct information on the mode and pathways required for the bacteria to adapt to the adverse conditions encountered. GFP-tagged bacteria (Teh et al. 2016) were fed to second-instar larvae. At fifth instar, flow cytometry was used to sort the reporters to compare their gene expression with those of *E. mundtii* grown *in vitro* (supplementary S1).

Sorting of GFP-Tagged *E. mundtii* Cells from the Gut of Experimentally Colonized *S. littoralis* Larvae by Flow Cytometry

After *E. mundtii* exposed to the gut conditions of *S. littoralis* larvae were sorted and isolated using flow cytometry and their transcriptomes were compared to those of bacteria grown in Todd Hewitt Broth (THB). We chose THB-cultured *E. mundtii* grown in a shaker incubator at 37 degree Celsius and 220 RPM as a control, because these are ideal, stress-free conditions (Restrepo et al. 2005). The bacteria grown in the media has been termed as "Control" all throughout the study. The bacteria were harvested at OD 0.5–0.6 when the bacterial load was 10^{10} CFU/ml. In THB, a complete medium, bacteria grow reliably, using dextrose as the source of energy. Since the *S. littoralis* foregut is alkaline and hindgut, neutral, we focused on *E. mundtii* growing at these two regions.

From the gut homogenates containing the fluorescent reporter *E. mundtii*, 250,000 fluorescent cells were sorted by a flow cytometer. The collected cells constituted 2 to 4% of the total homogenate. In addition, for comparison, 250,000 fluorescent *E. mundtii* cells grown *in vitro* were sorted and for differential gene expression was analyzed (Fig. S1).

RNAseq Analysis Revealed Many Differentially Expressed Genes between *E. mundtii* Growing *In Vitro* vs. *In Vivo*

To understand the mechanisms underlying the process by which *E. mundtii* adapts to the fore and hind gut of *S. littoralis* larvae, we analyzed gene expression between bacteria growing *in vitro* and *in vivo*. The RNA extracted from the FACS (Fluorescence Activated Cell Sorting)-sorted *E. mundtii* cells was sequenced using the Illumina Ultra-Low Input RNA kit, and the resulting 10 million short reads per treatment and replicates were processed and aligned against the fully sequenced genome of *E. mundtii* QU25 (Shiwa et al. 2014). Supplementary Table S2 shows the alignment percentages of these reads against the genome.

The numbers of significantly up- and downregulated genes between *E. mundtii* cells exposed to different *S. littoralis* gut sections is shown in Table 1. Out of 2696 assembled genes, 284 and 275 genes are significantly differentially regulated (fold change = 2, $p \leq 0.05$) in *E. mundtii* in the fore- and hindgut, respectively. The density plot in Fig. S3(a) shows the distribution of differentially expressed genes in foregut, hindgut and control.

There are 168 genes in common between the *E. mundtii* exposed to the fore- and hindguts that are differentially regulated when compared to the control. Most of these common

Table 1 Number of differentially expressed genes – up- and downregulated ($p \leq 0.05$) – in *Enterococcus mundtii* compared according to the following conditions: *E. mundtii* living in foregut *vs*. control, hindgut *vs*. control and foregut *vs*. hindgut

	E. mundtii in foregut *vs*. control ($p<0.05$)	*E. mundtii* in hindgut *vs*.control ($p<0.05$)	*E. mundtii* in foregut *vs*. hindgut ($p<0.05$)
No. of genes upregulated	91	71	28
No. of genes downregulated	193	204	60
Total no. of genes ($p<0.05$)	284	275	88

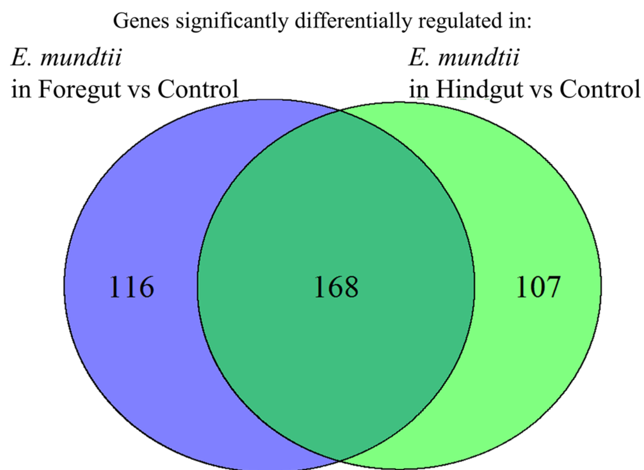


Fig. 1 Venn diagram showing overlap of differentially expressed genes in the following two conditions: *E. mundtii* living in foregut *vs*. control, and *E. mundtii* living in hindgut *vs*. control

genes belong to adaptive processes required by *E. mundtii* to colonize by adhering to the gut wall, avoid stresses, and to acquire iron and complex carbohydrates. Out of these 168 genes, ones with functional annotation (by the KEGG database) are shown in supplementary S8 ((Fig.1), (supplementary S8)).

To test for biological and technical variability, individual replicates were analyzed, and a PCA plot (Fig. 2) and dendrogram (Fig. S3 (b)) were generated. The gene expression profiles of *E. mundtii* from the insect gut and the control form separate clusters and nodes.

Gene Enrichment Analysis Revealed Several Pathways Differentially Expressed between *E. mundtii* Growing *In Vitro vs. In Vivo*

The differentially expressed genes were subjected to pathway analysis to determine the up- and downregulated pathways in *E. mundtii* when they are adapting to the gut conditions. Hence, the genes with functional annotation were classified according to three categories of gene ontology: molecular function, biological process and cellular component. We discuss only the category "biological processes" because it highlights the major pathways of *E. mundtii* living in the gut of the host.

To classify assembled genes with functions into different pathways, we used clusterprofiler R package. Gene annotation information of *E. mundtii* was obtained from the KEGG-FTP server and used to categorize the differentially expressed genes from our results into pathways, followed by an enrichment test by the clusterprofiler function Enricher (), (p value cut-off = 0.05). Out of 2696 assembled genes of *E. mundtii*, 1590 were functionally annotated and classified according to pathway. Of the 284 and 275 (Table 1) differentially regulated genes (p value cut-off = 0.05) in fore- and hind guts,

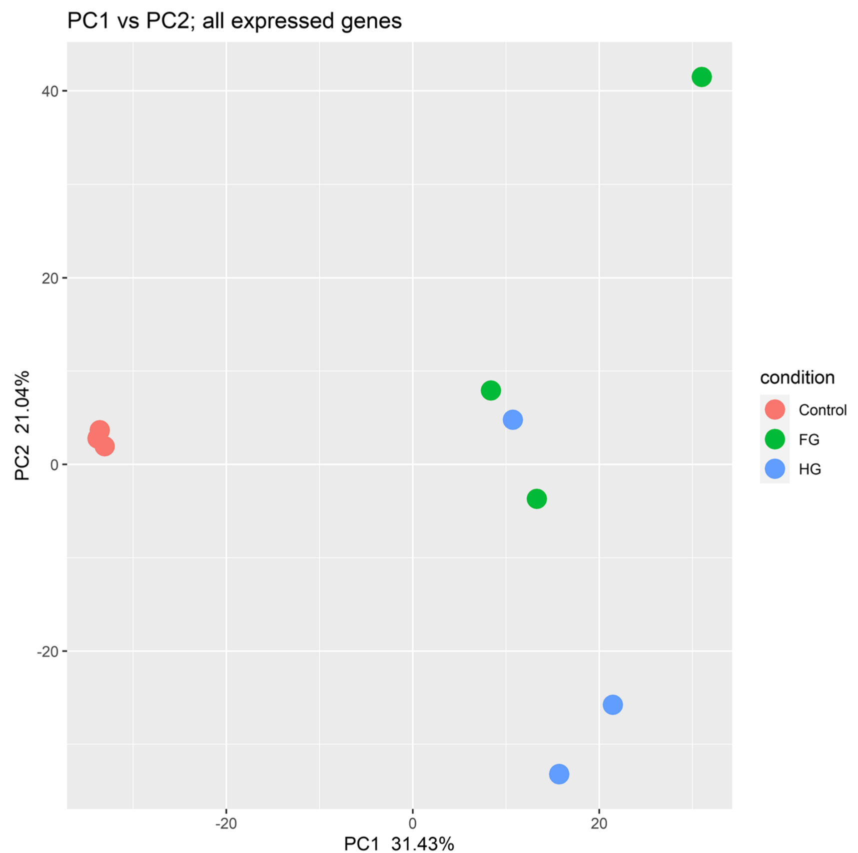


Fig. 2 PCA plot showing clustering of the transcriptomic profiles among the three replicates of *E. mundtii* obtained from the foregut (FG), hindgut (HG) and control

respectively, 199 and 190 were functionally annotated in the category of biological processes. The pathways that are significantly enriched (*p* value cut-off = 0.05) are shown in Fig. 3; percentages were calculated as such: number of genes up- or downregulated in a pathway divided by the total number of genes of that pathway that were annotated in the category. This fraction of up/down regulated genes in each significantly enriched pathway (p value cut-off = 0.05) is shown in y-axis in Fig. 3.

The upregulated genes in both fore- and hindguts represent several pathways, including the reductive TCA cycle, nucleotide biosynthetic processes, carbohydrate metabolic processes, peptidoglycan turnover, starch and sucrose metabolism and transmembrane transport (Fig. 3, Supplementary sheet S9, S10.)

There are several notable enriched pathways in the hindgut: lysine biosynthesis via the diaminopimelate pathway might indicate the bacteria are producing the amino acid (Fig. 3, S4); cell adhesion, which could indicate that the bacteria are adhering to the host epithelium to keep from being flushed out of the host gut; and oxidative stress response.

Not only the synthesis of amino acids, such as phenylalanine, glutamate, tyrosine and tryptophan (though not lysine), but also of fatty acids (shown by the downregulation of acetyl CoA carboxylase activity, malonyl CoA biosynthetic activity) and metabolism in general seem to be downregulated in the symbiont. Moreover, when *E. mundtii* lives in the gut, a down regulation of fatty acid biosynthesis is accompanied with enhanced fatty acid degradation (Fig.3, S4). We hypothesize that, by obtaining these by-products from the host, symbionts avoid the energy costs associated with these processes of fatty and amino acid biosynthesis.

When the gene expression of *Enterococcus mundtii* living in two regions of the gut (fore and hind) was compared, the only important enriched pathway belongs to lysine biosynthesis, which is seen to be upregulated in the hindgut as compared to the foregut (Fig. S5).

The genes involved in some of the important enriched pathways are discussed in detail in the next section (Figs. 4, 5).

Survival Strategies of *E. mundtii* in the Gut of *S. littoralis*

The differentially expressed genes that we identified are related to the adaptive strategies of *E. mundtii* in the fore- and hindguts of the larvae. We further classified these strategies in three broad categories: extracellular interactions, stress responses and metabolism, based on the results of the enriched pathways obtained in the previous section. All the genes and their fold changes mentioned below are listed in supplementary sheet S6.

Extracellular Interaction between *E. mundtii* and the Gut Epithelial Layer of *S. littoralis*

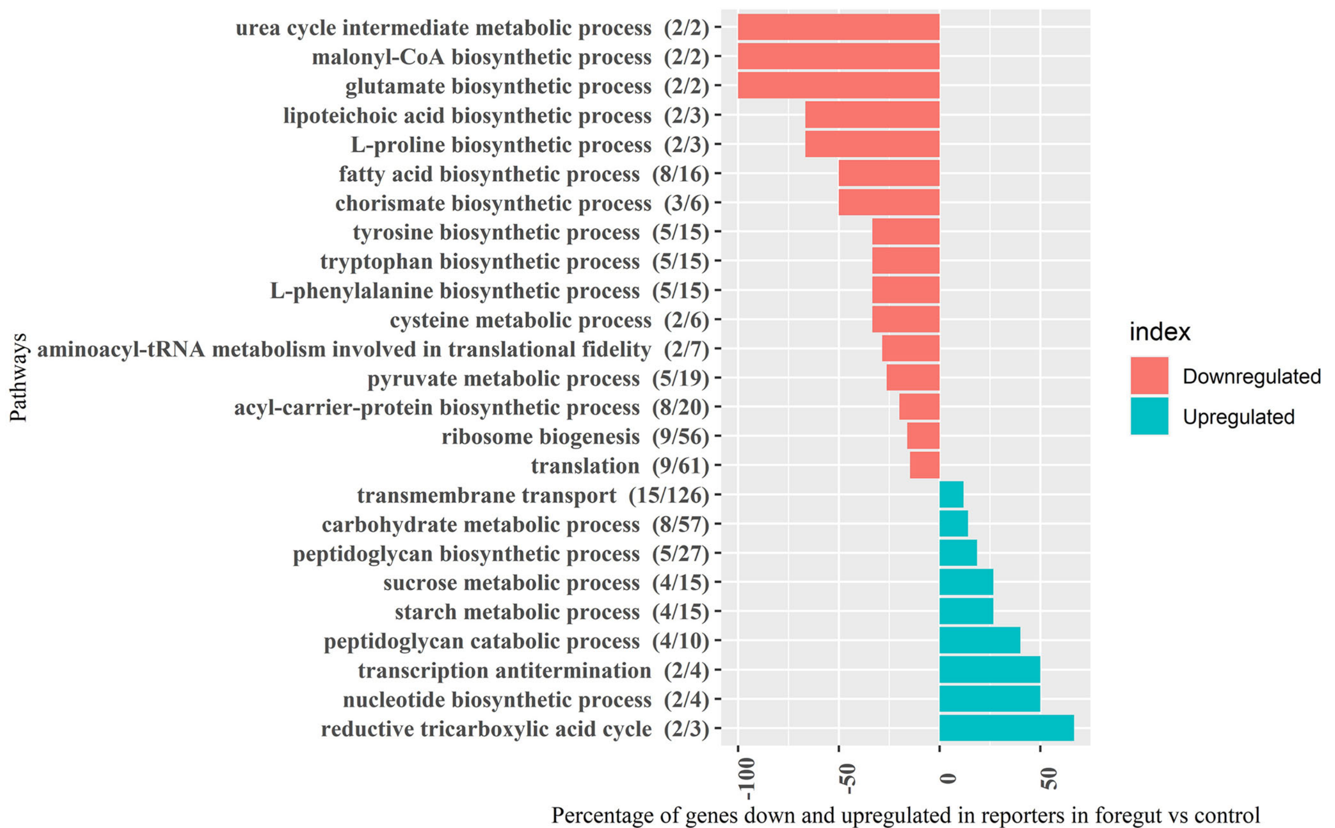
The biological process category of gene ontology showed enrichment in the pathway of cell adhesion (Fig. 3). This motivated us to look deeper into the genes that control adherence to the host gut. Various well-characterized surface-associated proteins with conserved motifs and domains contribute to the ability of *E. mundtii* to attach itself to the gut epithelial tissue of its host. C-terminal conserved LPxTG motifs (EMQU_1297: 33-and 124-fold in the fore- and hindgut, respectively, a slight upregulation of *fms3*) and WXL domains (EMQU_0541:30- and 8-fold in the fore-and hindgut, respectively, and EMQU_0539:383-fold in the foregut). The *lysM* domain that helps in biofilm formation by is upregulated (EMQU_0157: up to 3-fold in the fore- and hindgut, respectively). The sticky matrix helps *E. mundtii* deal with stress efficiently (Otto 2014;Voronina et al. 2016).

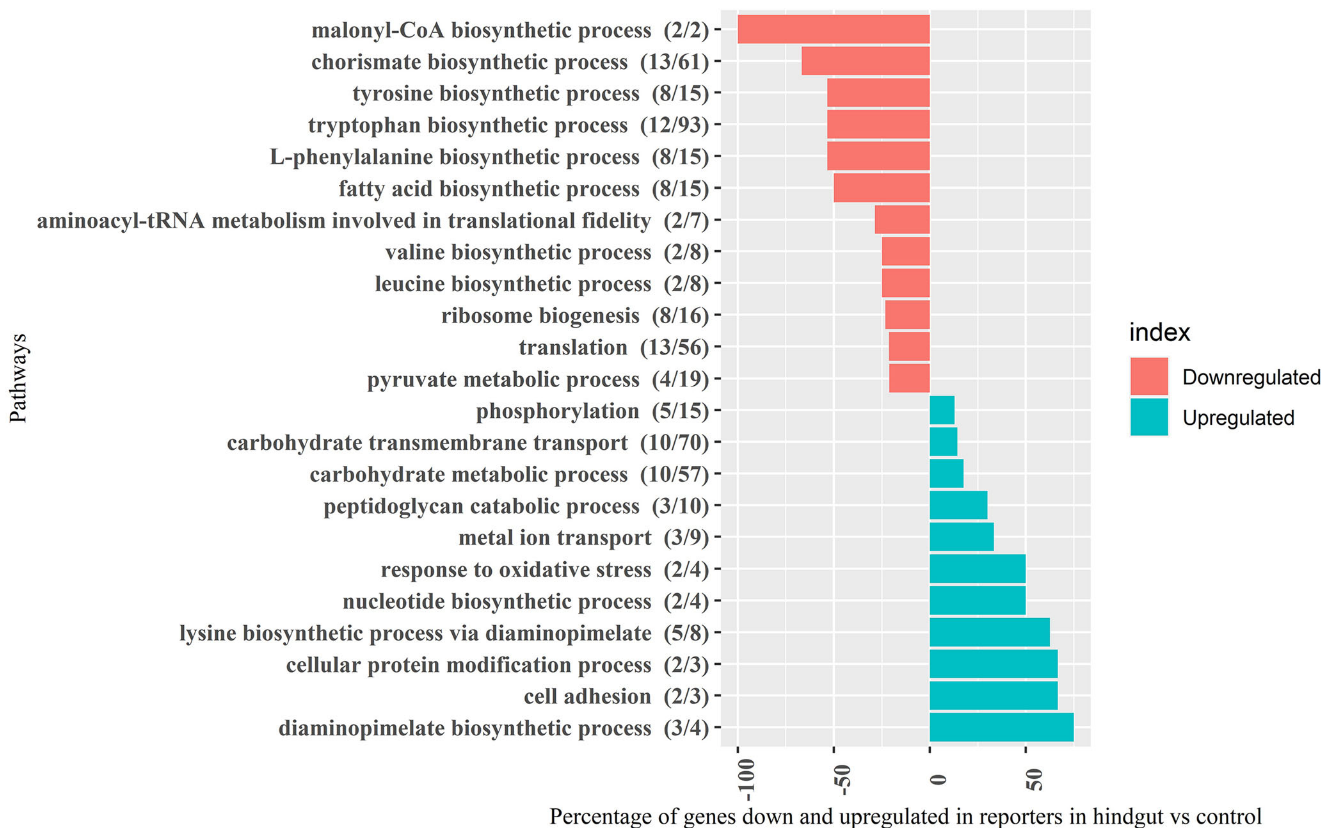
Genes for chitin-binding proteins form a class of surface-associated proteins that provide adhesive properties to lactic acid bacteria so that these can adhere to the N-acetyl glucosamine component of chitin present in insects' gut epithelial cells, especially the cells lining the midgut (Tellam et al. 1999). Two of these proteins show levels as high as EMQU_0940:47- and 138-fold and EMQU_1285:25- and 69-fold, in the fore- and hindgut, respectively. Lipoproteins are placed in defined subcellular spaces formed by the plasma membrane. Their position is convenient for capturing incoming nutrients or elements such as iron. In addition, lipoproteins have been shown to help bacteria adhere to host cells (Hancock et al. 2014). EMQU_0428 is upregulated 5- and 4-fold in the fore- and hindgut, respectively. EMQU_2743 is upregulated 7-fold in the hindgut. Both are zinc transporter lipoproteins (Fig. 4(a), supplementary S6).

Stress Responses of *E. mundtii* Dwelling in the Gut of *S. littoralis*

E. mundtii seems to be modulating their gene expression in response to the various stresses as was also seen with the upregulation of oxidative stress response in the hindgut (Fig. 3). Accordingly, they upregulate several antioxidant enzymes: superoxide dismutase (13- and 8-fold in the fore- and hindgut,

Fig. 3 Summary of gene ontology classification in the category of biological processes, after an enrichment test (*p* value cut-off = 0.05). The graph shows both up- and downregulation of the assembled genes of *E. mundtii,* with functional annotations, classified into enriched pathways, obtained from foregut and hindgut as, compared to genes of the control. The percentages of each pathway refer to the percentage of genes of that particular pathway that are enriched in *E. mundtii.* The fractions of the same are denoted next to the pathways on y-axis (supplementary S9, 10: sheets 1 and 2)


urea cycle intermediate metabolic process (2/2)
malonyl-CoA biosynthetic process (2/2)
glutamate biosynthetic process (2/2)
lipoteichoic acid biosynthetic process (2/3)
L-proline biosynthetic process (2/3)
fatty acid biosynthetic process (8/16)
chorismate biosynthetic process (3/6)
tyrosine biosynthetic process (5/15)
tryptophan biosynthetic process (5/15)
L-phenylalanine biosynthetic process (5/15)
cysteine metabolic process (2/6)
aminoacyl-tRNA metabolism involved in translational fidelity (2/7)
pyruvate metabolic process (5/19)
acyl-carrier-protein biosynthetic process (8/20)
ribosome biogenesis (9/56)
translation (9/61)
transmembrane transport (15/126)
carbohydrate metabolic process (8/57)
peptidoglycan biosynthetic process (5/27)
sucrose metabolic process (4/15)
starch metabolic process (4/15)
peptidoglycan catabolic process (4/10)
transcription antitermination (2/4)
nucleotide biosynthetic process (2/4)
reductive tricarboxylic acid cycle (2/3)
Pathways
-100
-50
0
50
Percentage of genes down and upregulated in reporters in foregut vs control
index
Downregulated
Upregulated


malonyl-CoA biosynthetic process (2/2)
chorismate biosynthetic process (13/61)
tyrosine biosynthetic process (8/15)
tryptophan biosynthetic process (12/93)
L-phenylalanine biosynthetic process (8/15)
fatty acid biosynthetic process (8/15)
aminoacyl-tRNA metabolism involved in translational fidelity (2/7)
valine biosynthetic process (2/8)
leucine biosynthetic process (2/8)
ribosome biogenesis (8/16)
translation (13/56)
pyruvate metabolic process (4/19)
phosphorylation (5/15)
carbohydrate transmembrane transport (10/70)
carbohydrate metabolic process (10/57)
peptidoglycan catabolic process (3/10)
metal ion transport (3/9)
response to oxidative stress (2/4)
nucleotide biosynthetic process (2/4)
lysine biosynthetic process via diaminopimelate (5/8)
cellular protein modification process (2/3)
cell adhesion (2/3)
diaminopimelate biosynthetic process (3/4)
Pathways
-100
-50
0
50
Percentage of genes down and upregulated in reporters in hindgut vs control
index
Downregulated
Upregulated

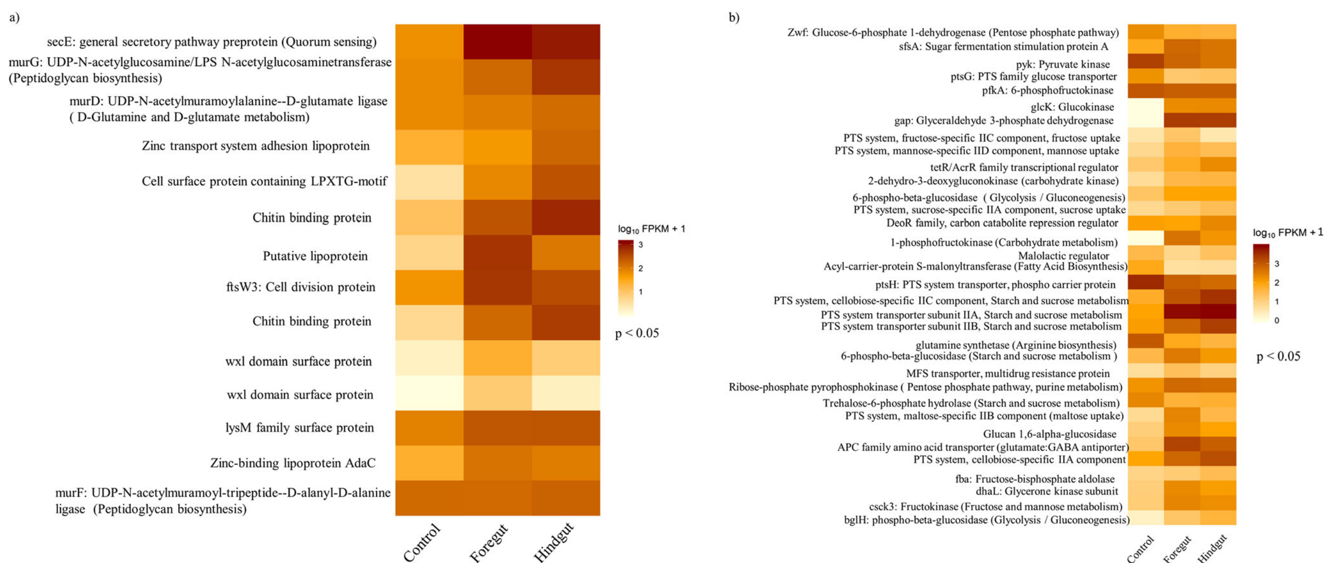


Fig. 4 **a** Heatmap showing the regulation of certain genes helping in the attachment of *E. mundtii*, when these bacteria are in the fore- and hindgut of *Spodoptera littoralis*. **b** Heatmap showing the regulation of some genes in *E. mundtii* is involved in metabolism when they are in the fore- and hindgut of *Spodoptera littoralis*

respectively), catalase (EMQU_0568: 4- and 10-fold in the fore- and hindgut, respectively), NADH oxidase- peroxidase cycleEMQU_0335, 0459, 1279: up to 4-fold in the hindgut), organic hydro peroxide resistance family protein (EMQU_1453: 6-fold in the fore- and hindgut), and peptide-methionine (R)-S-oxide reductase (EMQU_0165: 3-fold in the hindgut) (Tomusiak-Plebanek et al. 2018).

The *agr* two-component systems that bring about quorum sensing in bacteria show upregulation in both the fore- and the hindgut. Levels of *agrA* are upregulated about 3-fold in the hindgut and for *agrB,* about 5- and 8-fold in the fore- and hindgut, respectively.

Genes for general stress proteins (*glsB*: 32- and 97-fold; *glsB1*: 10- and 7-fold; *gls33*: 6-and 22-fold, in the fore- and hindgut, respectively) and universal stress proteins (USPs) (*uspA2*: 54-and 11-fold in the fore- and hindgut, respectively) are upregulated in *E. mundtii* in response to environmental conditions such as the presence of salt, oxygen or oxidative stresses, and toxic substances, and nutrient starvation. The expression of USPs may depend on the increased bacterial density brought about by quorum sensing (Kim et al. 2012).

Intracellular trafficking, secretion, and vesicular transport include *secE* (22- and 16- folds in the fore- and hindgut, respectively) needed for cell viability, and *virD4* (EMQU_1288: 47-and 46-fold in the fore- and hindgut, respectively) components of the type IV secretion system, all of which are upregulated (Rao et al. 2014).

Also upregulated: genes for repair proteins, such as *mutS* (EMQU_2803) and *recA* (EMQU_2752: 3-fold in the foregut) conferring DNA mismatch repair and its protection from oxidative stress; *recF* (2- and 3-fold in the fore- and hindgut, respectively) for recombination repair, whose general role is the maintenance of DNA; DNA alkylation repair protein (*alkD*) (upregulated 3-fold in the fore- and hindgut); *radA* (3-fold in the fore- and hindgut) and *radC* (3- and 6- folds in the fore- and hindgut, respectively), proteins helping in DNA repair and recombination (Ivanov and Haber 1997); *yafQ* (EMQU_3002) and DNA damage-induced protein J (EMQU_3001, 33- and 4- folds in the fore- and hindgut, respectively), which constitute a toxin-antitoxin system that plays a role in biofilm formation (Kurasz et al. 2018) (Fig. 5(a), supplementary S6).

Iron Homeostasis and Alkaline Stress Iron homeostasis in *E. mundtii* is important, especially in environments that are iron depleted owing to the presence of compound 8-HQA. These bacteria have upregulated their *fetC* permease gene (7- fold in the foregut and 11- fold in the hindgut) to increase their ferric uptake and the FUR family transcriptional regulator (EMQU_1067: 4- fold in the foregut), to maintain iron homeostasis. Adaptation that is mediated through FUR and iron uptake is common in iron-deprived environments (Fig. 5(a)), supplementary S6) (Haas 2012; Ho and Ellermeier 2015). The highly alkaline pH characteristic of the larval foregut in particular is a challenge to bacteria in general but also to *E. mundtii* specifically. For example, alkaline pH has been proven to unwind the double helical structure of DNA (Ageno et al. 1969). In addition, high expression levels of

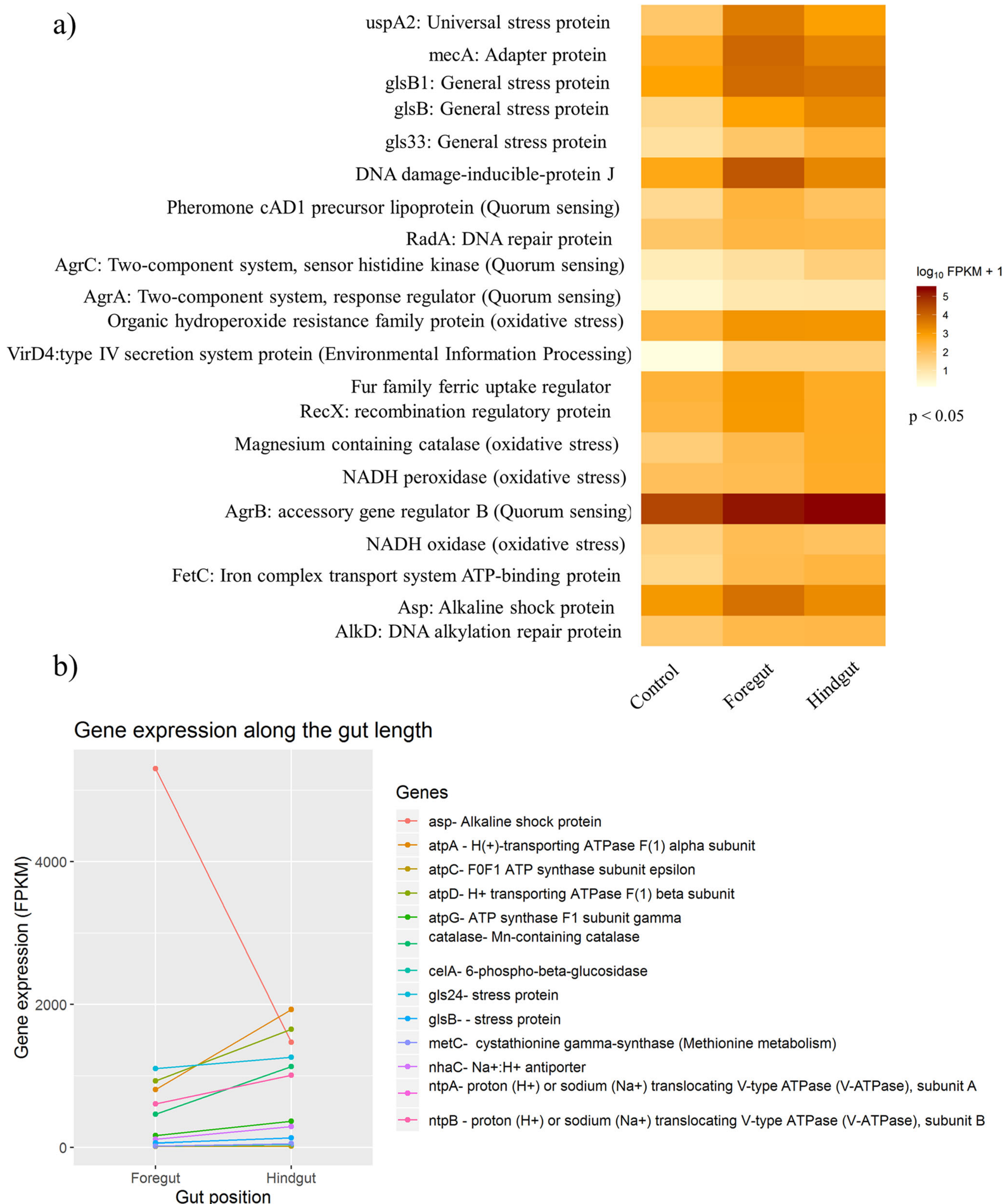


Fig. 5 **a** Heatmap showing the regulation of certain genes that help in the stress tolerance of *E. mundtii*, when these bacteria are in the fore- and hind gut of *S. littoralis*. **b** Graph showing the regulation of certain pH-related genes in *E. mundtii* living in the fore- and hindgut of *S. littoralis* larvae

the alkaline stress protein have been found in *E. mundtii* living in the alkaline foregut (5- folds), whereas its expression decreases in the neutral conditions of the hindgut (Fig. 5(b)).

Metabolism Carried Out by *E. mundtii* When They Are in the Gut of *S. littoralis*

Facultative anaerobes can switch between respiration and fermentation, based on oxygen availability. The expression of most glycolytic genes – for example, glucokinase (*glcK*), 1-phosphofructo kinase (*fruK*), 6-phospho-beta-glucosidase (*bglP, bglB, bglG*) phosphofructokinase A (*pfkA*) and glucose-6-phosphate isomerase in *E. mundtii* dwelling in the gut does not change much compared to the expression of genes in *E. mundtii* growing under control conditions, suggesting the glycolysis pathway is active. The same trend holds true for pyruvate dehydrogenase entering the citric acid cycle in aerobic conditions, along with lactate dehydrogenase (*ldhA* EMQU_2453). The protein that stimulates the fermentation of sugar (SfsA-EMQU_0871) under anaerobic conditions is upregulated 9- and 6-fold in the fore- and hindgut, respectively. Some alcohol dehydrogenases are upregulated to convert acetaldehyde to ethanol in the fermentation pathway (EMQU_1129:2-fold in the fore- and hindgut; EMQU_ 0525: 5- fold in the fore- and hindgut; and EMQU_0315: 3- and 4- folds in the fore- and hindgut, respectively). The acetyl CoA produced by pyruvate dehydrogenase does not significantly contribute to the production of fatty acids and amino acids, because both pathways are downregulated (Fig. 3, S4).

Phosphotransferase systems (PTSs), which take up alternative source of sugars such as sucrose, ascorbate, mannose and, most important, cellobiose, are upregulated in *E. mundtii* in both the fore- and hindgut (Kotrba et al. 2001). Cellobiose mostly comes from the plant products on which the host is fed. The genes of at least 13 PTS cellobiose transporter-subunits are upregulated; EMQU_0876, a particular cellobiose-specific IIA component, is upregulated as high as 78- and 88-fold in the fore- and hindgut, respectively. Ascorbate is mostly taken up in the hindgut. On the other hand, fructose and lactose do not seem to be a popular source of energy (supplementary S6).

Upregulation in starch and sucrose metabolism (Fig. 3, S4) is brought about by an increase in the sucrose-specific PTS transporter (EMQU_2136: 2- and 5- fold in the fore-and hindgut, respectively) and sucrose 6-phosphate dehydrogenase (*scrB*: 2-folds in the hindgut); and the alpha-amylase enzyme neopullanase (EMQU_1435: 52- and 30-fold in the fore- and hindgut, respectively).

Although *E. mundtii* do not seem to invest energy in synthesizing fatty or amino acids, they seem to produce lysine in the hindgut via the diaminopimelate pathway (Pavelka and Jacobs 1996) (supplementary S4, S5, Fig. 3).

Metabolism and the transport of nucleotides in *E. mundtii* living in the gut increases, as are also seen in Fig. 3.

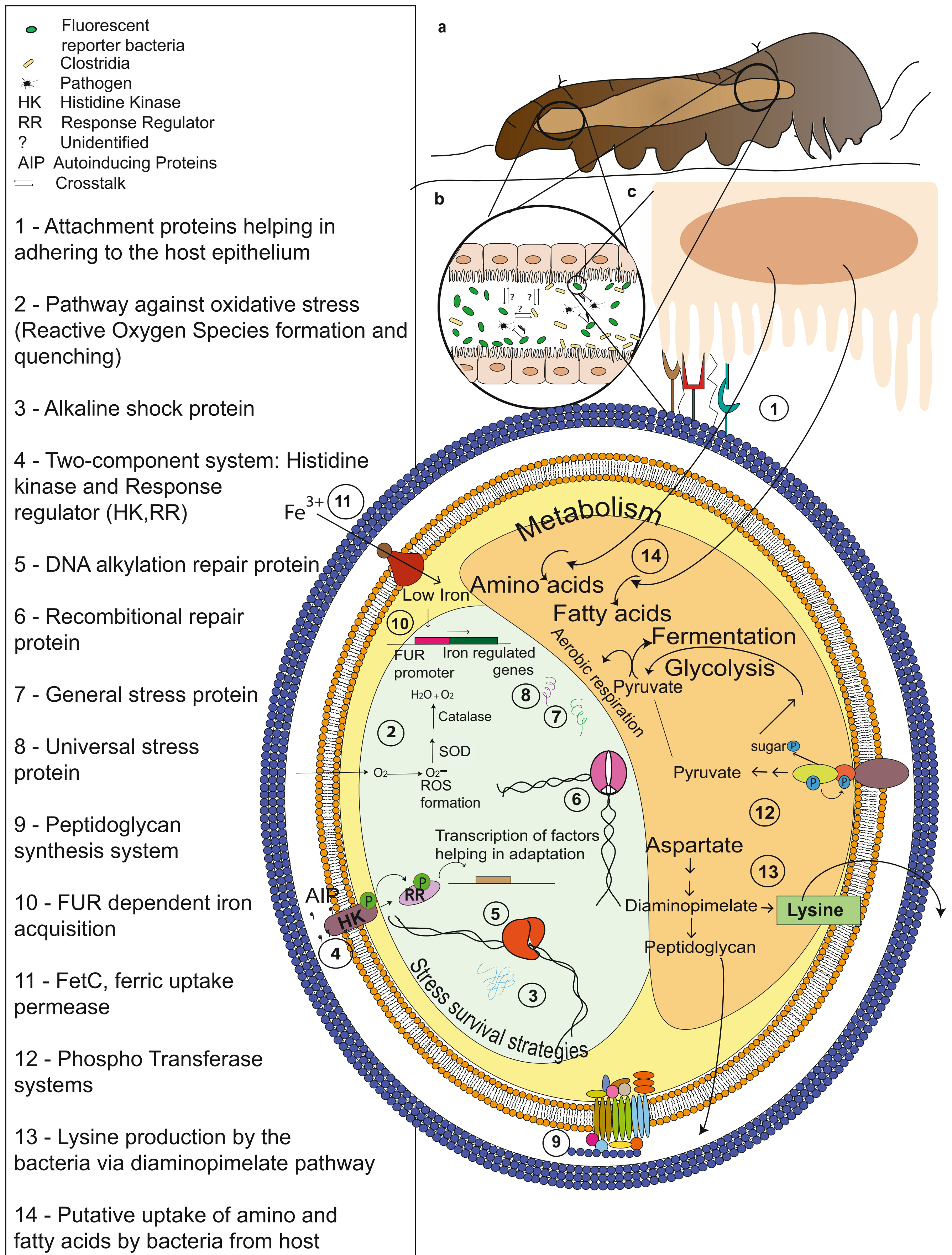
Regarding glycerol metabolism: the *glpF* gene required for glycerol uptake is downregulated (4-fold in the foregut), whereas the genes for metabolism – *glpO, dhaKL, glpQ*– are also expressed, suggesting these bacteria have an alternate way of obtaining glycerol (Ran et al. 2015) (Fig.4(b), supplementary S6).

Discussion

This work focuses on the survival strategies of *E. mundtii* in the larval gut of *S. littoralis*, an environment threatened by stressful conditions, namely high pH, low iron content and oxidative stress. This makes it a good system to study adaptation by the symbionts in the larval gut. By introducing the GFP-tagged reporter *E. mundtii* (Teh et al. 2016) to the larval gut, we were able to study how this dominant bacterium adapted to its new environment. The fluorescent bacteria were later retrieved from the fore- and hindguts of the larvae using flow cytometry (supplementary S1, Fig. S1). To prevent any metabolic changes from occurring between the individual experimental steps of larval dissection and FACS sorting, we used RNAlater and RNAprotect reagents. Comparing the gene expression profiles of these retrieved reporters with the profiles of *E. mundtii* grown under optimal culture conditions, we were able to obtain a snapshot of the genes and the pathways that help these symbionts to survive in and adapt to the gut of *S. littoralis* larvae. The transcriptional changes found in these bacteria are an amalgamation of these factors which illustrates how *E. mundtii* is responding to stress and colonizing its host gut (Fig. 6).

For bacteria to successfully colonize the host gut, they must prevent themselves from being flushed out of the system; adherence to gut tissue ensures they survive epithelial turnover (Otto 2014) (Fig. 4(a)). Biofilms, which are composed of adhering proteins, were first seen by FISH imaging (Tang et al. 2012). LPxTG is a sortase-dependent site for anchoring proteins covalently attached to the peptidoglycan (Navarre and Schneewind 1994). Lipid-anchored proteins or lipoproteins, which constitute another class of covalently associated adhesion proteins (Sengupta et al. 2013), are upregulated in *E. mundtii*. Wxl domains and LysM, or lysine-dependent motifs binding to the peptidoglycan, form non-covalent

Fig. 6 A snapshot of interactions between *Spodoptera littoralis* and its resident gut symbiont, *E. mundtii*. **a** An illustration of *S .littoralis* with its longitudinal gut **b** *E. mundtii* dominates in the gut along with Clostridia and keeps pathogens at bay by producing mundticiin KS. Unknown interactions occur among these two symbionts and the host gut. **c** Pathways and stress survival strategies of *E. mundtii* in the gut of *S. littoralis*


Fluorescent reporter bacteria
Clostridia
Pathogen
HK Histidine Kinase
RR Response Regulator
? Unidentified
AIP Autoinducing Proteins
Crosstalk
1 - Attachment proteins helping in adhering to the host epithelium
2 - Pathway against oxidative stress (Reactive Oxygen Species formation and quenching)
3 - Alkaline shock protein
4 - Two-component system: Histidine kinase and Response regulator (HK,RR)
5 - DNA alkylation repair protein
6 - Recombitional repair protein
7 - General stress protein
8 - Universal stress protein
9 - Peptidoglycan synthesis system
10 - FUR dependent iron acquisition
11 - FetC, ferric uptake permease
12 - Phospho Transferase systems
13 - Lysine production by the bacteria via diaminopimelate pathway
14 - Putative uptake of amino and fatty acids by bacteria from host
a
b
c
Fe^{3+}
Metabolism
Amino acids
Fatty acids
Fermentation
Glycolysis
Aerobic respiration
Pyruvate
Low Iron
FUR promoter
Iron regulated genes
$H_2O + O_2$
Catalase
SOD
O_2
O_2^-
ROS formation
sugar
Pyruvate
Transcription of factors helping in adaptation
Aspartate
Diaminopimelate
Lysine
Peptidoglycan
AIP
HK
RR
P
Stress survival strategies

associations with the peptidoglycan (Boekhorst et al. 2006; Voronina et al. 2016). Such associations occur in *Enterococcus fecalis* (Brinster et al. 2007). Chitin, a major part of the peritrophic matrix, lines the midgut epithelium of the host (Campbell et al. 2008). Chitin-binding proteins in *E. mundtii* also promote adherence to the host gut. Several bacteria, such as *L. monocytogenes*, adherent *E. coli* and *V. cholerae,* were found to initiate adhesion in the host gut by using their chitin-binding proteins (Tran et al. 2011). Peptidoglycan turnover is a sign of active cell division (Fig. 3). Peptidoglycan biosynthetic and catabolic processes show upregulation in both the fore- and hindgut. The N-acetylmuramoyl-L-alanine amidase enzyme in the foregut helps in cell separation during division. It also aids in cell motility and establishing a symbiotic association with the host (Vermassen et al. 2019).

E. mundtii dwelling in the gut employ various strategies to survive adverse conditions (Fig. 6). Reactive oxygen species (ROS) result from the reduction of oxygen. Thereafter, the dismutation product of the superoxide anion (O_2^-) is hydrogen peroxide (H_2O_2). O_2^- and H_2O_2, along with the hydroxyl radical, are potent oxidants that can remove electrons from DNA, proteins, lipids, other macromolecules, which can damage both the invading and resident symbionts (Paes et al. 2001). Lactobacilli employ enzymes such as NADH oxidase/peroxidase, superoxide dismutase and manganese-dependent catalase to counteract ROS, as was also true for *E. mundtii* (Tomusiak-Plebanek et al. 2018).

Universal stress proteins are found in many bacteria; these proteins aid the adaptation of bacteria to stresses such as extreme temperature, oxidative loss, nutrient starvation and toxic agents (Kim et al. 2012). In *E. coli*, stress proteins were first reported in fungi, archaea, plants and flies (Nyström and Neidhardt 1992). In *Burkholderia glumae*, genes that regulate universal stress protein are controlled by quorum sensing (Kim et al. 2012). The bacteria rely on quorum sensing as a survival strategy, aggregating on the host epithelia and forming a biofilm in the host gut. That *agrABCD* forms a two-component system and brings about quorum sensing has already been established in the Firmicutes *Staphylococcus aureus* and *Streptococcus pneumoniae* (Cvitkovitch et al. 2003). AgrC (histidine kinase), the sensor for autoinducing proteins produced and transported out by AgrD and AgrB respectively, transduces the signal to AgrA (response regulator), which in turn leads to expression of factors required for adaptation of bacteria (Le and Otto 2015). The upregulation of this two-component system in *E. mundtii* living in the gut is evident for their mechanism of quorum sensing. The adherence properties of *E. mundtii* may help it to form a biofilm layer on the gut wall. Thus, these two inter-related phenomena of quorum sensing and biofilm formation help bacteria to adapt to altered environments.

8-HQA is an iron chelator, and the larvae's ability to produce it may help them survive in an iron-depleted environment. The FetC iron complex transport permease and FUR family of transcriptional regulators may act in similar ways. FetC was found to be involved in iron homeostasis in *Apergillus fumigatus* (Haas 2012). A FUR-dependent iron-acquisition system was upregulated when *Clostridium difficile* tried to infect hamsters in iron-depleted conditions (Ho and Ellermeier 2015). It interacts with iron to determine its intercellular levels, hence bringing a halt to processes of iron dependant oxidative damage. FUR is also a major regulator of adaptation of bacteria to various hosts. They not only regulate iron homeostasis, but also mediate key adaptive responses as stress resistance, quorum sensing and biofilm formation. We presume, this could be very much the case with *E. mundtii* trying to adapt to the new living conditions of *S. littoralis* gut (Porcheron and Dozois 2015).

Alkaline shock proteins help the bacteria to adapt to extreme stress conditions (Balaji and Krishnan 2008). Owing to the alkaline environment of the foregut (Funke et al. 2008), the *E. mundtii* living there express alkaline shock proteins as protection (Funke et al. 2008). Such is also the case in *Staphylococcus aureus* (Kuroda et al. 1995). Previous studies reported several genes differentially expressed in *E. faecalis* grown under alkaline conditions; similar expression patterns characterize *E. mundtii,* if alkalinity is the only factor taken into consideration. For example, we found a downregulation of methionine transport and synthesis systems, Na^+H^+ antiporter (NhaC family, 1-fold downregulation), upregulation of adenosine and cytidine deaminases (upto 19-fold), purine and pyrimidine metabolism. The expression levels of Cation/H^+-related F and V-type antiporters (atp and ntp family proteins) are reduced under alkaline conditions (Fig.4(d), Supplementary S6) (Ran et al. 2015) .

As facultative anaerobes, *E. mundtii* potentially initiates fermentation inside the host gut. *E. mundtii* is found in the vicinity of the host gut surface only when some amount of oxygen is present, and that no *E. mundtii* is found in the inner layers of the anaerobic gut wall (Tang et al. 2012) highlights the low oxygen levels that characterize the gut lumen of most insects (Johnson and Barbehenn 2000). As pathway analysis clearly shows, the white-bean-based artificial diet that the host is fed on favors starch and sucrose uptake through PTS transporters and metabolic systems (Fig. 3, S4). PTS transporters help all bacteria survive environments with different levels of sugars (Kotrba et al. 2001). Enriched nucleotide metabolism suggests that *E. mundtii* are striving to colonize the gut of *S. littoralis*. Previous studies with mice models showed that *E. coli* enriched their metabolism of purine and pyrimidine when colonizing the intestines of mice (Vogel-Scheel et al. 2010). Although *E. mundtii* likes to reduce the energy they expend on their fatty acid and amino acid metabolism, their lysine metabolism is upregulated by bacteria living in the

hindgut (Russell et al. 2014). Whether *S. littoralis* is obtaining lysine from their symbiotic *E. mundtii* is a matter for further research. Pathway analysis shows lysine synthesis is enriched via the diaminopimelate pathway. Diaminopimelate also plays roles in peptidoglycan synthesis (Pavelka and Jacobs 1996) (Fig. 3, S4, S5).

Our data on *Enterococcus mundtii* are in agreement with several examples of how symbionts function in their respective host guts. Colonization of symbionts by extracellular interaction between the gut cells and the symbiont, by overcoming various stresses induced by hosts, and by changing metabolism to fit the nutrient-limiting conditions in the gut was also seen in *Snodgrasella alvi* in the gut of honey bees. Genes for biofilm formation, facing oxidative stress, fluctuating pH and repair proteins were upregulated in the symbiont *in vivo* (Powell et al. 2016). The microbiota of cockroach mid-gut is also engaged in digestion of complex carbohydrates with the help of amylase enzymes, along with responding to oxidative stress by upregulating genes involving peroxidase and catalase (Zhang et al. 2016). Likewise, gut microbiota hold a record of digesting recalcitrant carbohydrates in plant or wood-feeding insects. Termites form a classic example where the cellulolytic activity of bacteria residing in the hindguts of higher termites was detected (Tokuda and Watanabe 2007). Aerotolerant intestinal symbiont *Bacteroides fragilis*, upon facing an oxidative environment, immediately react to it to prevent the immediate effects of reactive oxygen species, and also regulate their biosynthetic processes accordingly (Sund et al. 2008).

High-throughput transcriptome sequencing from tiny quantities of starting material has revealed the strategies used by *E. mundtii* to survive the gut of *S. littoralis*. Our methods can be used to study interactions between any host and its symbiont. For example, fluorescently tagged bacteria can be introduced into the insect guts in which the 8-HQA-producing gene has been knocked out. A similar method will allow us to study the behavior of the retrieved bacteria and shed light on the mechanisms of survival that underlie the exchanges between symbionts and their genes.

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Author Contributions TM, BT and AM conducted all the experiments under the supervision of WB. WS and YS collaborated for the Bioinformatics and the flow cytometry parts, respectively. WB and HV helped edit the manuscript.

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Compliance with Ethical Standards

Conflict of Interest Authors BT and AM were PhD and master's students, respectively, at the department of Bioorganic Chemistry, Max Planck Institute for Chemical Ecology, during the time of the research. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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