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BACTERIAL AND FUNGAL PATHOGENESIS - RESEARCH PAPER

QseC sensor kinase modulates the human microbiota during enterohemorrhagic *Escherichia coli* O157:H7 infection in the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®)

Karine Melchior[1] · Mateus Kawata Salgaço[2] · Katia Sivieri[2] · Cristiano Gallina Moreira[1]

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Abstract
Enterohemorrhagic *Escherichia coli* (EHEC) is an important gastrointestinal pathogen known for its ability to cause hemorrhagic colitis and induce hemolytic-uremic syndrome. The inner membrane QseC histidine kinase sensor has shown to be an important regulator of the locus of enterocyte effacement (LEE) island, where important EHEC key virulence genes are located. However, the QseC role during EHEC infection in human microbiota remains unknown. Herein, using the *Simulator of the Human Intestinal Microbial Ecosystem* (SHIME®), we investigated whether the QseC sensor has a role in human microbiota modulation by EHEC in a dynamic model. Our data demonstrated that the QseC sensor modulates human microbiota during EHEC infection, and its absence leads to an increase in *Lactobacillaceae* and *Bifidobacterium* genus predominance, although non-effect on *Bacteroides* genus by EHEC strains was observed. In co-culture, the *Lactobacillus acidophilus* has affected EHEC growth and impaired the EHEC growth under space-niche competition, although no growth difference was observed in the QseC sensor presence. Also, differences in EHEC growth were not detected in competition with *Bacteroides thetaiotaomicron* and *EHEC* strains did not affect *B. thetaiotaomicron* growth either. When investigating the mechanisms behind the SHIME results, we found that *hcp-2* expression for the type 6 secretion system, known to be involved in bacterial competition, is under QseC sensor regulation beneath different environmental signals, such as glucose and butyrate. Our findings broaden the knowledge about the QseC sensor in modulating the human microbiota and its importance for EHEC pathogenesis.

Keywords Enterohemorrhagic *Escherichia coli* · QseC · O157:H7 · Human intestinal microbiota

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) is an important gastrointestinal pathogen known for its ability to cause hemorrhagic diarrhea, and due to its Shiga toxin production, it can lead to the development of uremic hemolytic syndrome (HUS) [1] that is known for inducing severe consequences in humans, such as kidney failure, being the leading cause of morbidity and mortality associated with outbreaks by this pathogen [2, 3]. Antibiotic treatments are not indicated for patients with EHEC infection since these drugs can induce through the SOS response, Shiga toxin production in EHEC, which is encoded by variants of the *stx* gene inserted into the EHEC chromosome via bacteriophage [1, 4]. Studying the microbiota to find probiotic competitors, as well as non-conventional antibiotic treatment, has emerged as a promising strategy to combat infections caused by EHEC.

EHEC has in its genome the *Locus of Enterocyte Effacement* pathogenicity island, which is activated by the Ler regulator for encoding proteins to the formation of the type III secretion system (T3SS) [5–7]. T3SS acts like a needle, injecting the Tir receptor into the host cell and binding to intimin, which is present in the bacterial cell membrane, thus promoting the close adhesion of the bacteria to the host cell [1]. The proteins inserted into the host promote changes in

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✉ Cristiano Gallina Moreira
cg.moreira@unesp.br

[1] Department of Biological Sciences, School of Pharmaceutical Sciences, São Paulo State University (UNESP), Araraquara, Brazil

[2] Department of Food and Nutrition, School of Pharmaceutical Sciences, State University of São Paulo (UNESP), Araraquara, SP, Brazil

the cytoskeleton leading to the *Attaching and Effacing* (A/E) lesion [1, 7].

To detect changes in the environment and establish cell–cell communication, EHEC can recognize small chemical molecules or signals through receptors in their membrane [8, 9]. The membrane sensor histidine kinase QseC is a two-component system with its cognate response regulator QseB that responds to the adrenergic hormones norepinephrine and epinephrine of mammals and to the autoinducer-3 produced by bacteria [10]. Through the detection of changes in the environment by these signals, the QseC sensor regulates virulence genes in EHEC, such as the LEE pathogenicity island for the formation of A/E lesion in epithelial cells [11–14]. Although it is established that the sensor QseC is involved in EHEC pathogenicity, little is known about its contribution or regulation during EHEC interactions with the microbiota.

The *Simulator of the Human Intestinal Microbial Ecosystem* (SHIME®) is an in vitro model used in microbiology due to its combination of dynamism and multi-compatibility [15]. To date, a few studies have been conducted with EHEC in dynamic models [16–18]. SHIME® is a system composed of 5 bioreactors that include all compartments of the gastrointestinal tract, from the stomach to the colon, mimicking the same conditions of these compartments. In the bioreactors mimicking the colon, the model is kept under a fermentation process, in which the microbiota from donors is inoculated and stabilized to study the changes in the intestinal microbial community under different conditions [15, 19].

Understanding how EHEC behaves in different environments is essential to know the main mechanisms used by this microorganism in its adaptation to the host. In vivo and in vitro models have been used to explain EHEC infection mechanisms. Mice are important study models, but they do not have the same conditions as those found in human gastrointestinal (GI) microbiota. Also, EHEC infects murine, but they do not develop classic symptoms of the disease [5, 20]. In vitro evaluation in dynamic colonic models has been used to simulate the conditions of the human GI tract, to analyze the response of microorganisms in these compartments, and to better understand the complex community/dynamism of the human microbiota [15, 19]. Herein, we investigated whether the QseC sensor has a role in human microbiota modulation by EHEC in the SHIME dynamic model to better understand how different conditions and distinct metabolites via the QseC sensor may modulate the human microbiota and its importance for EHEC pathogenesis.

Methodology

Strains and culture conditions

All the strains used in this study are described in Table 1. The EHEC 8624 strains and their isogenic mutants were grown aerobically in Luria–Bertani (LB) broth supplemented with 50 Ug/ml streptomycin at 37 °C, overnight in a shaker (250 rpm). *L. reuteri* and *L. acidophilus* were grown in De Man, Rogosa, and Sharpe (MRS) broth, anaerobically at 37 °C, under static conditions.

Simulator of the Human Intestinal Microbial Ecosystem (SHIME®).

The test was carried out as described by Bianchi et al., 2018. SHIME® (registered trade name of the University of Ghent and ProDigest) is a simulator of the human intestinal microbial ecosystem that mimics conditions such as pH, residence time, and temperature through software [21, 22]. For the experiment, five double-coated reactors were used, one for simulating the stomach, one for the duodenum, and a triplicate for the ascending colon. The five reactors were continuously stirred with a magnetic stirrer, and the temperature was maintained at 37 °C. The system was maintained anaerobically through the daily introduction of N_2 for 30 min. The colon pH (pH between 5.6 and 5.8) was

Table 1 Bacterial strains employed in the study

Strains	Resistance Marker	Source
E. coli TOP10 with pBADMychisA(+ *qseC*) construction	Ampicillin	This study
Bacteroides thetaiotaomicron	-	[27]
Enterohemorrhagic *E. coli* 0157:H7 8624 strain (wild-type (WT))	Streptomycin	[52]
8624—mutant Δ*qseC*	Streptomycin	[53]
Lactobacillus acidophilus 3258, ATCC 4356	-	André Tosello Foundation (http://fat.org.br)
Limosilactobacillus reuteri 3433, ATCC 23,272	-	André Tosello Foundation (http://fat.org.br)

automatically adjusted by adding 1 M NaOH or 0.1 M HCl [21, 22]. Each colon compartment received carbohydrate-based food that allows the adaptation of microorganisms to specific environmental conditions of the colon in terms of pH range, retention time, and available carbon sources, in volumes previously described. The stomach conditions and mimicking pancreatic juice (composed of Oxgall 6.0 g/L, $NaHCO_3$ 12.5 g/l, and pancreatin 0.9 g/l) were prepared as previously described [21, 22]. The colon reactors were inoculated with fecal microbiota samples from two donors, female and male healthy adults, ages between 20 and 26 years old, under a similar balanced diet and with no history of diarrhea or antibiotic use for at least 6 months prior to the trial, as previously described in the SHIME® usage [21, 22], and the absence of EHEC in the system was confirmed via the qRT-PCR assay prior to the infection. The feces were weighted, pooled, and diluted in 200 ml phosphate buffer containing Na_2HPO4 0.05 mol/l, NaH_2PO_4 0.05 mol/l, and Na-thioglycolate 0.1% (pH 6.5); stirred for 10 min in a homogenizer (Stirrer model 130, Norte Científica, São Paulo, BR.); and centrifuged at 3000 × g for 15 min. The supernatants were subsequently added (40 ml) to the three colon reactors. The experimental protocol included a control period of 2 weeks (without intervention) after inoculation of the supernatant in the three colon reactors to adapt the microbial community to the nutritional and physical–chemical conditions and stabilize the microbiota [21]. During this period, 200 ml of the SHIME® feed entered the system, and 200 ml were discarded from each column reactor twice a day for 2 weeks. Once stabilization was carried out, 10^9 per ml of CFUs from the EHEC WT or Δ*qseC* strains were introduced into the stomach-mimicking reactor and were distributed to the duodenum portion and added the pancreatic juice until they were introduced into the last colon-mimicking reactor. Prior to any infection, a wash-out period (period necessary for new microbiota stabilization and elimination of the WT strain) was carried out, in which the microbiota received 200 ml of SHIME® food twice a day for 1 week, and the PCR analysis was performed to confirm the elimination of the WT strain from the reactors, followed by inoculation of the Δ*qseC* strain, as an independent subsequent experiment. For both the WT and Δ*qseC* strains, samples of the stomach and duodenum were collected, and CFU counts were performed to determine the concentration of bacteria after dilution in the stomach contents and to perform the analysis of bacterial cell viability at the stomach pH and pancreatic juice after 1 h of exposure. Samples were collected before introduction into the system (day 0 or D0) and after 24 h of infection. Total RNA was extracted to analyze the gene expression of the WT and Δ*qseC* strains and determine the abundance of the intestinal microbiota. For this purpose, analyses were carried out on the phyla Firmicutes, Bacteroidetes, and γ-Proteobacteria and on the genera *Lactobacillaceae*, *Bifidobacterium*, *Prevotella*, and *Bacteroides*, in addition to the virulence genes *ler* and *stx-2* of the EHEC WT and Δ*qseC*.

Real-time qPCR

All RNA extractions were performed with Trizol and RiboPure-Bacteria RNA isolation kit (Ambion-Life), followed by the qRT-PCR technique. The total RNA was obtained at a concentration of 50 ng/μl per sample of the tests performed. For each reaction of 20 μl, Master Mix SYBR®, Multi-scribe® reverse transcriptase (Thermo Fisher Scientific), and RNAse inhibitor (Thermo Fisher Scientific) were used, in addition to 100 ng of sample RNA. The qRT-PCR reverse transcriptase reaction was performed in biological triplicates and experimental duplicates. The reactions were normalized with the RNA polymerase subunit A (rpoA) as an endogenous control to analyze the expression of virulence genes. As an endogenous control to identify the members of interest in the microbiota, eub338, a universal gene for Eubacteria, was used. QuantStudio3 equipment (Thermo Fisher Scientific) was used to carry out the reactions. The results obtained by the qRT-PCR assay were analyzed by the comparative critical threshold (ΔΔCT), as previously described (Walters and Sperandio, 2006). Error bars represent the standard deviation of the CT values. All primers used in this study are listed in Table 2.

In vitro analysis in high and low glucose in co-culture with *B. thetaiotaomicron*

The WT and isogenic mutant strains were grown overnight and inoculated in tubes in the proportion of 30:1 of high D-MEM medium (4.5 g/l) or low glucose (1.0 g/l) plus inoculum in the presence or absence of *B. thetaiotaomicron*. In co-cultures, the bacteria proportions used were 1:1 EHEC and *B. thetaiotaomicron*. The tests were performed in anaerobic and static conditions at 37 °C. The bacterial growth was measured via turbidity reading after 1 h, 2 h, 4 h, 6 h, and 8 h incubation, and growth curves were plotted after optical density measurement. The experiments were conducted in biological triplicates and experimental duplicates.

EHEC co-culture with *Lactobacillus acidophilus* or *Limosilactobacillus reuteri*

EHEC, *L. acidophilus*, and *L. reuteri* strains were grown overnight. After the measurement of the optical densities of the cultures, the strains were inoculated in LB + MRS broth at a ratio of 1:1 or 1:5 of EHEC strains and *L. acidophilus* or *L. reuteri*. In the single culture group, the strains were grown alone. The strains were kept in an incubator on static interaction at 37 °C for 8 h, then the samples were diluted, plated

Table 2 Oligonucleotides used in the study

Target	Primer set sequence 5′–3′		Source
	Forward	Reverse	
stx-2a	ACCCCACCGGGCAGTT	GGTCAAAACGCGCCTGATA	[54]
espA	TCAGAATCGCAGCCTGAAAA	GAAGGATGAGGTGGTTAAGCT	[14]
ler	CGACCAGGTCTGCCCTTCT	GCGCGGAACTCATCGAAA	[14]
hcp-2	GAACGTCAGGCAGTTTCCGT	GGCCACGCTATCTGGTGAAA	[30]
rpoA	GCGCTCATCTTCTTCCGAAT	CGCGGTCGTGGTTATGTG	[14]
Bacteroides (RNA 16S)	CGATGGATAGGGGTTCTGAGAGGA	GCTGGCACGGAGTTAGCCGA	[55]
Prevotella (RNA 16S)	CACCAAGGCGACGATCA	GGATAACGCCYGGACCT	[55]
Delta e Gamma proteobacteria (RNA 16S)	GCTAACGCATTAAGTRYCCCG	GCCATGCRGCACCTGTCT	[56]
Eubacteria (Eub – RNA 16S)	ACTCCTACGGGAGGCAGCAGT	ATTACCGCGGCTGCTGGC	[57]
Firmicutes (RNA 16S)	TGAAACTYAAAGGAATTGACG	ACCATGCACCACCTGTC	[56]
Bacteroidetes (RNA 16S)	CRAACAGGATTAGATACCCT	GGTAAGGTTCCTCGCGTAT	[35]
Bifidobacterium (RNA 16S)	TCGCGTC(C/T)GGTGTGAAAG	CCACATCCAGC(A/G)TCCAC′	[58]
Lactobacillus (*Lactobacillaceae*) (RNA 16S)	AGCAGTAGGGAATCTTCCA	CACCGCTACACATGGAG	[58]

on LB containing streptomycin for EHEC and MRS agar for *L. acidophilus* or *L. reuteri*, and the CFUs were counted. The experiments were conducted in biological triplicates and experimental duplicates.

Culture under sodium butyrate and glucose-mediated conditions

Bacterial cultures were grown in D-MEM low glucose or DMEM low glucose supplemented with But or NaCl until O.D. 1.0. Total RNA was extracted using trizol together with the RiboPure-Bacteria RNA isolation kit (Ambion-Life). After RNA extraction, gene expression analyses of virulence genes were performed. The experiments were conducted in biological triplicates and experimental duplicates.

Agar competition assay between EHEC and *L. acidophilus* or *L. reuteri*

The strains WT and Δ*qseC* and *L. acidophilus* and *L. reuteri* were grown overnight in LB and MRS media, respectively. The optical densities of the inoculum at 600 nm were measured. The lowest O.D. presented by the strains after 16 h of growth was used in the test. After adjusting the O.D., cultures were centrifuged at 7000 rpm for 2 min and resuspended in 300 µl PBS 1X. After this step, the ratio of 1:1 or 1:5 of the EHEC strains plus *L. acidophilus* or *L. reuteri*, respectively, was added in a new Eppendorf tube, centrifuged at 7000 rpm for 2 min, and resuspended in PBS 1X and 10 µl of the co-culture were inoculated on an LB + MRS (1:1) agar plates. In the single culture group, the strains were grown alone. After 16 h of interaction, the agar plates co-cultures were collected in tubes to perform serial dilutions and CFU counting on LB containing streptomycin for EHEC and MRS agar as a selective medium for *L. acidophilus* or *L. reuteri* (Peng et al., 2015). The experiments were conducted in biological triplicates and experimental duplicates.

Statistical analysis

The data were analyzed in the GraphPad Prism 8, and the statistical significance was determined by one-way analysis of variance (ANOVA). *P* values ≤ 0.05 were considered statistically significant.

Results

QseC modulates the intestinal fitness and microbiota shift promoted by EHEC infection

The sensor kinase QseC is an important bacterial communication sensor that helps EHEC to sense the environment and respond properly to its changes, modulating virulence genes accordingly with the niche inserted [23]. To evaluate whether the QseC sensor is involved in microbiota modulation by EHEC, we carried out an assay in the *Simulator of the Human Intestinal Microbial Ecosystem* (SHIME®) to evaluate the direct competition between the EHEC WT and Δ*qseC* strains with the human microbiota. EHEC absence was confirmed via *stx-2* and *ler* PCR prior to the infection (data not shown). The SHIME infection by EHEC has not been described in the literature, being an interesting and unprecedented work. Bacterial viability was controlled in the stomach and duodenum reactors. The strains were inoculated at the same rate in the stomach

reactor at 0 h on different days (Fig. 1A). At 1 h in the stomach, both WT and Δ*qseC* have shown high resistance to low pH (pH 2.5). Nevertheless, after 1 h in the duodenum, the WT has recovered its levels, whereas the Δ*qseC* has presented a 22% CFU reduction when compared with its initial CFUs at stomach 0 h.

Next, to further evaluate how the microbiota could be affected by the strains at phyla and genera levels, microbiota members were determined in reactors mimicking the ascending colon, which is one of the predicted niches for the initial EHEC infection [6, 24]. The samples were collected after 24 h of interaction between EHEC strains and

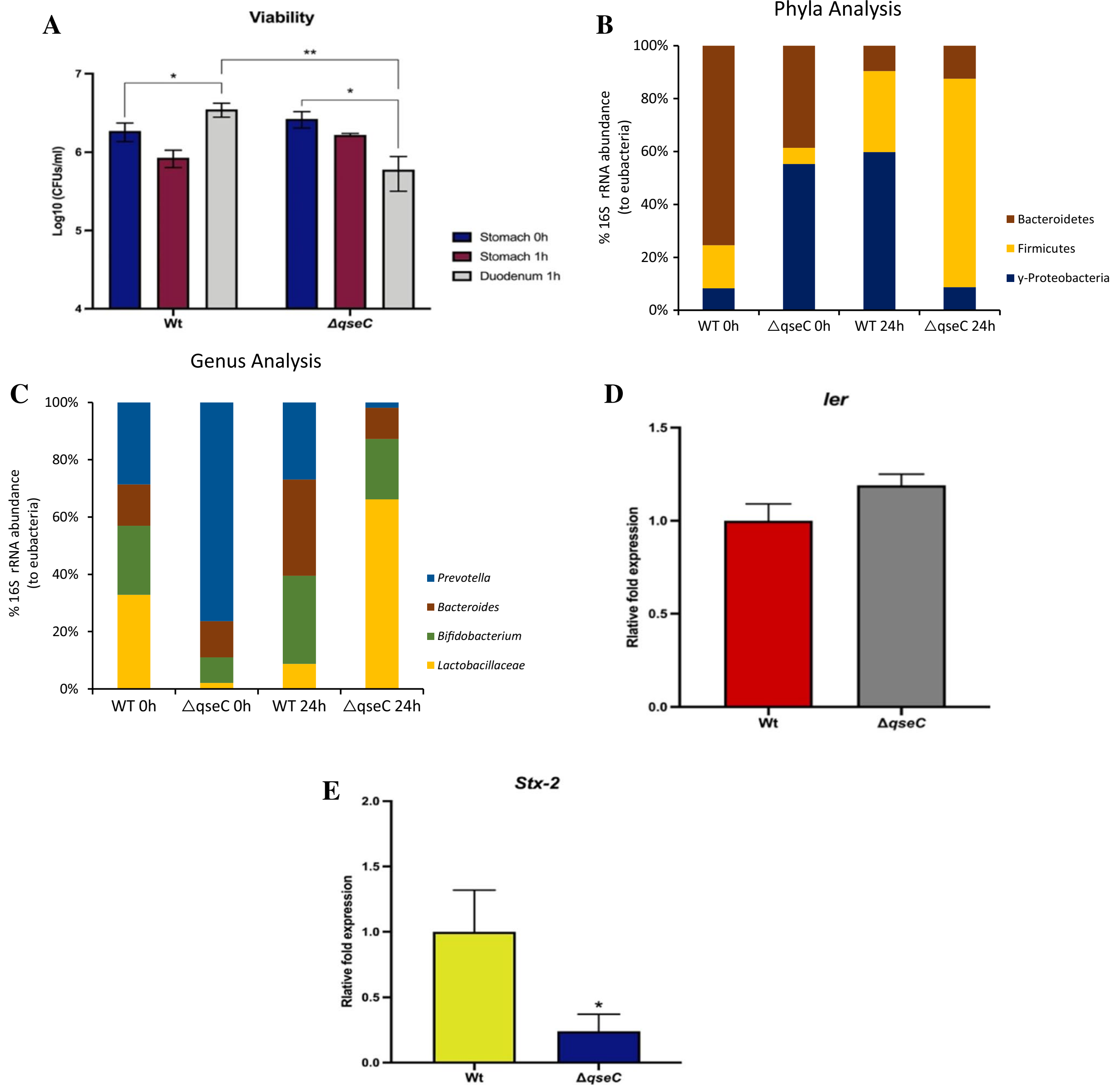

Fig. 1 Microbiota and gene expression analyses from samples collected at SHIME before and after 24 h of EHEC WT and Δ*qseC* strain infection. **A** Analysis of the survival profile of EHEC WT and Δ*qseC* strains at SHIME. **B** The abundance of 16S rRNA to detect phyla ɣ-Proteobacteria, Bacteroidetes, Firmicutes, and **C** *Lactobacillaceae*, *Bifidobacterium*, *Bacteroides*, and *Prevotella* genus frequency was investigated via total RNA extraction for the RT-PCR assay analysis, 0 and 24 h after infection for the analysis. **D** and **E** The virulence genes expression analyses of *ler* and *stx-2* samples collected from SHIME after 24 h of EHEC strains infection were performed. Statistical significance compared to the wild strain (WT). Bars without an asterisk show no statistically significant difference, $p<0.05$ (*), $p<0.01$ (**)

the microbiota at SHIME®. This period was chosen to allow EHEC colonization during SHIME® infection, once these bacteria in vivo depend on the attachment and effacement to survive and colonize the gut. Upon WT strain infection in the SHIME® model, almost 60% of the phyla analyzed belong to γ-Proteobacteria, which includes the inoculated EHEC and other Enterobacteriaceae members. The Firmicutes and Bacteroidetes phyla are essential members of the human microbiota; nevertheless, after 24-h infection, the proportion was 30% and 10%, respectively, of the total microbiota evaluated. Unlike the WT strain, during the Δ*qseC* mutant infection, there was a significant increase in the phylum Firmicutes, around 60% of the composition, followed by Bacteroidetes and γ-Proteobacteria, which together composed only 20% of the microbiota, when compared to 0-h microbiota composition (Fig. 1B). To analyze at genera level, microbiota members known for their beneficial performance in the intestine were chosen, such as *Lactobacilliacea* and *Bifidobacterium* genus, and members that have been demonstrated to be increased in some inflammatory diseases such as *Prevotella* and *Bacteroides* [25, 26]. The WT strain infection led to a *Prevotella* and *Bacteroides* ratio of 60% of the microbiota analyzed. On the other hand, the *Lactobacillaceae* and *Bifidobacterium* genera corresponded only 40% of the composition. Interestingly, for the Δ*qseC* mutant, there was an increase in approximately 70% *Lactobacillaceae* proportion, followed by 20% of *Bifidobacterium*, whereas *Prevotella* and *Bacteroides* were only 10% of the microbiota composition analyzed, when compared to 0-h microbiota composition (Fig. 1C).

We have also evaluated the virulence gene expression of Shiga toxin (*stx-2*) and LEE island (*ler*) genes. There was a significant decrease in the *stx-2* gene for the Δ*qseC* strain compared with WT (Fig. 1D). On the other hand, *ler*, the master regulator of LEE island, had similar expression levels in the WT and Δ*qseC* strain (Fig. 1E). This result corroborates with the function of *ler* in EHEC, once this gene is important to perform A/E lesion to the intestinal wall, absent in this SHIME® model.

B. thetaiotaomicron does not impair EHEC growth under co-culture conditions

Previous studies indicated that *B. thetaiotaomicron* did not affect EHEC growth [27], and during SHIME® infection, there were no significant differences in the *Bacteroides* genus in the WT and Δ*qseC* strains. That way, we performed a co-culture assay to evaluate if EHEC and *B. thetaiotaomicron* has a direct impairment in EHEC growth. Also, the growth curve was performed under low and high-glucose conditions to evaluate whether the availability of carbon sources in different concentrations could trigger competition between the bacterial strains. The growth curves performed confirmed that *B. thetaiotaomicron* does not affect EHEC growth, even when the availability of sugar was decreased (Fig. 2).

Lactobacillus acidophilus directly affects EHEC growth under co-culture conditions

During the SHIME® infection, the most prominent genus modulated by the QseC absence was *Lactobacillaceae*; we next carried out co-culture assays to analyze whether EHEC could affect *Lactobacilli* growth under QseC sensor regulation. Two different species of *Lactobacillaceae* were employed, *Lactobacillus acidophilus* 3258 (LA 3258) and *Limosilactobacillus reuteri* 3334 (LR 3334). Also, since the *Lactobacillaceae* is found in large amounts in the gut, two different proportions of *Lactobacillaceae* were evaluated: same proportion of EHEC and *Lactobacillaceae* (1:1) and fivefold *Lactobacillaceae* in comparison to EHEC (1:5).

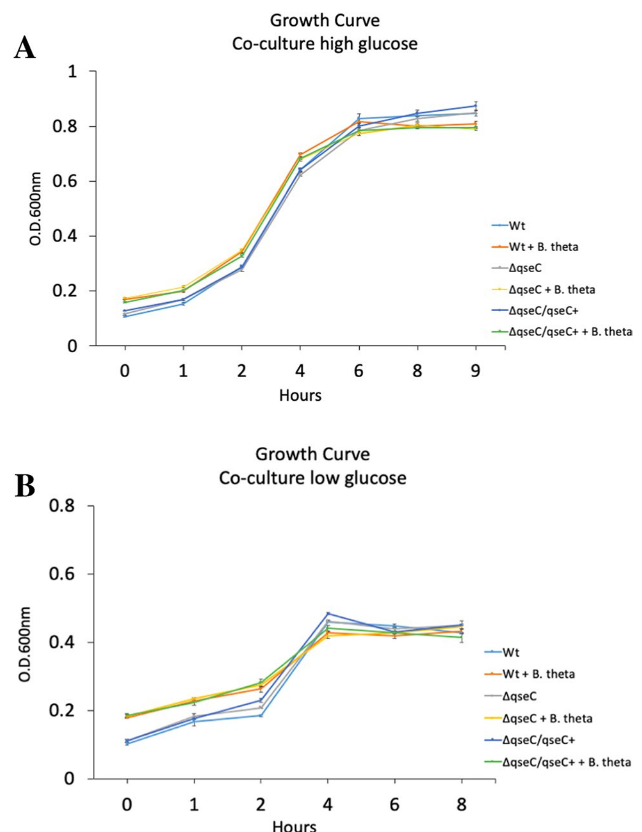

Fig. 2 Growth curve of EHEC strains in high or low glucose in the presence or absence of *B. thetaiotaomicron*. The WT, Δ*qseC*, and Δ*qseC/qseC*+strains were cultured anaerobically in DMEM high-glucose (**A**) or low-glucose (**B**) medium in the presence or absence of *Bacteroides thetaiotaomicron* (*B. theta*) at 37 °C for 8 h. The cultures were kept under the static condition at 37 °C, and each time interval of 1, 2, 3, 4, 6, and 8 h aliquots were taken to read the optical density (O.D. 600 nm). Bars without an asterisk showed no statistically significant difference

Different from the expected, *L. acidophilus* in both conditions (1:1 or 1:5) did not show a significant growth decrease when compared to its respective control group in a single culture. Conversely, *L. acidophilus* in co-culture with EHEC in both, 1:1 and 1:5, ratio caused a significant reduction in the EHEC growth when compared to their control group in the WT and Δ*qseC* strains single culture (Fig. 3A). Since at SHIME® there is a complex bacterial community, the WT and Δ*qseC* strains were also tested with *Limosilactobacillus reuteri* to evaluate whether we would observe a similar phenotype with *Lactobacillaceae* species. *L. reuteri* and EHEC strains demonstrated a similar growth profile in a single culture. Nevertheless, the 1:1 proportion led to a decrease in the EHEC growth in both WT and Δ*qseC* strains. In co-culture, in a 1:5 ratio with *L. reuteri*, EHEC strains' growth inhibition was higher (Fig. 3B). These results indicate that *L. reuteri* and *L. acidophilus* could interfere in the EHEC growth, more pronounced in the presence of *L. acidophilus*; however, EHEC did not seem to affect the growth of these *Lactobacillaceae* species in co-culture conditions, and the QseC role here did not seem evident.

The absence of glucose led to an increased gene expression of T6SS, and the sensor QseC is involved in the regulation of *hcp-2*

The gut environment has a high number of microorganisms struggling for nutrient availability in a niche where all cohabitants have evolved to compete for distinct nutrient sources. Bacteria have different mechanisms and forms to adapt to distinct conditions such as limited- and abundant-carbon sources, type secretion systems, and the production of antibacterial molecules to kill potential niche competitors [28]. The type VI secretion system works among

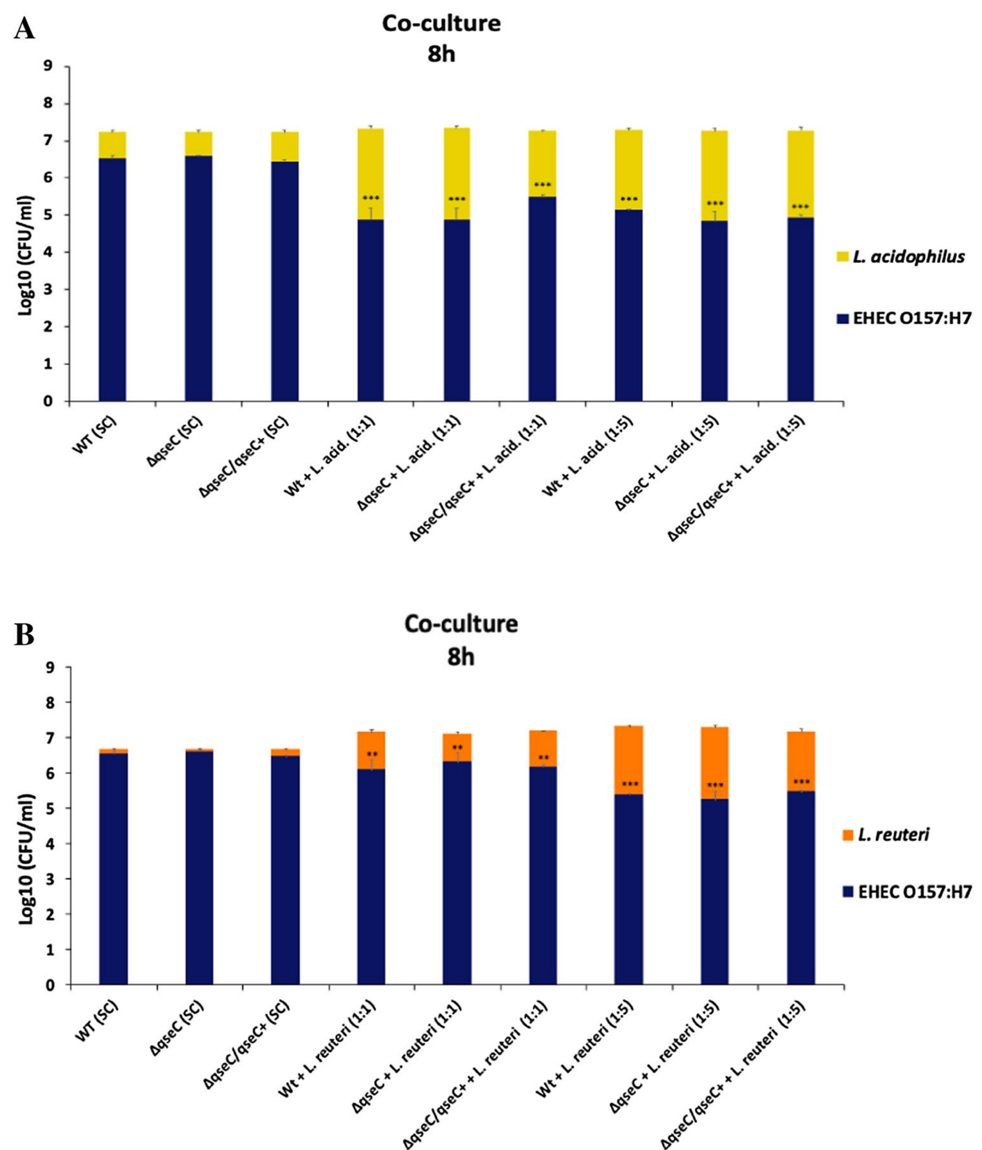

Fig. 3 Broth co-culture assay between (**A**) *Lactobacillus acidophilus* or (**B**) *Limosilactobacillus reuteri* and EHEC strains. UFCs count after 8 h of interaction. The strains WT, Δ*qseC*, Δ*qseC*/*qseC*+, and *L. acidophilus* or *L. reuteri* were grown as single culture (SC) in broth. The strains WT + L. acid. or *L. reuteri* and Δ*qseC* + L. acid. and Δ*qseC*/*qseC*+ + L. acid or *L. reuteri* were grown in broth co-culture at the ratio of 1:1 or 1:5 of EHEC plus *L. acidophilus* or *L. reuteri*, respectively. Bars without an asterisk showed no statistically significant difference, $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****) (two-way ANOVA)

bacterial communities through the introduction of bacteriolytic effectors into cells, usually involved in the pathogenic mechanisms of various pathogens [29, 30]. Due to the differences in the microbiota induced by WT and Δ*qseC* strains at SHIME®, we next investigated whether EHEC T6SS via QseC sensor kinase regulation could be involved in the results found. Thus, the assay was carried out in high- (4.5 g/l) and low-glucose (1 g/l) medium, and the *hcp-2* gene expression levels from T6SS were determined in both conditions to analyze if the variability of carbon sources that could happen at the SHIME® model would be involved directly in the T6SS regulation. The *stx-2* was also evaluated since it is a key virulence factor in EHEC. The low-glucose level led to an increased expression of the *hcp-2* gene both in WT and Δ*qseC* strains, respectively eightfold and sixfold. In this condition, the absence of the QseC sensor has shown a distinct *hcp-2* gene expression when compared to WT levels. Furthermore, the QseC sensor complementation restored the *hcp-2* expression similar to the WT strain under the same conditions (Fig. 4A). Seemingly, the glucose levels interfere in the regulation of the *hcp-2* gene, and the QseC sensor has a role in its regulation. Notably, the *stx-2* gene was 35-fold more expressed in WT and 25-fold in the Δ*qseC* strains compared to the control group. Also, the QseC sensor complementation returned the *stx*-2 expression to the levels of the WT strain (Fig. 4B). Moreover, these results suggest that an environmentally scarce source of carbon is sufficient to activate gene expression of a bacterial competition system that is under QseC sensor regulation in EHEC.

B. thetaiotaomicron inhibits the *hcp-2* gene expression induction by low-glucose conditions and did not affect EHEC growth

EHEC did not affect *Bacteroides* genus predominance at SHIME® conditions, and *B. thetaiotaomicron* did not impair EHEC growth during co-culture; we tested whether *hcp-2* gene expression would still be highly expressed under low glucose in co-culture with *B. thetaiotaomicron*, since T6SS modulation by bacteria is environmental and specie-dependent. Thus, *hcp-2* and *stx-2* gene expression analyses were performed. There were no significant differences observed in the *hcp-2* expression in both WT and Δ*qseC* strains in low glucose + *B. theta* when compared to the high glucose + *B.theta* conditions. These results indicate that the presence of *B. thetaiotaomicron* was sufficient to decrease the level of *hcp-2* expression induced by low glucose. Also, this commensal bacterium may not be a direct competitor to induce T6SS activation in EHEC. When the QseC sensor kinase was restored in the ∋¨¨*qseC* strain, the *hcp*-2 expression levels were unchanged in high but upregulated in low glucose (Fig. 5A), possibly due to the multiple copy complementation strategy in the ∋¨¨*qseC/qseC* + strain. In both

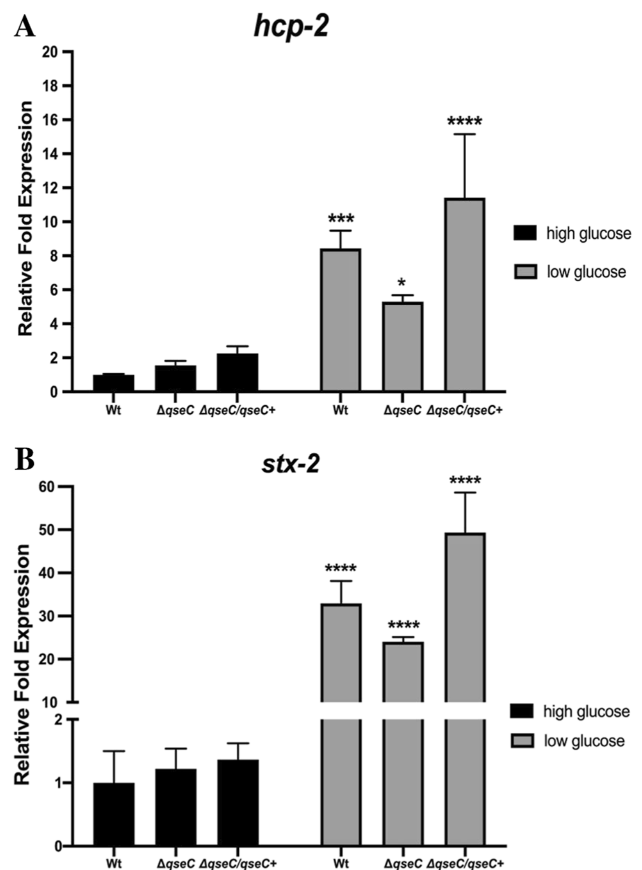

Fig. 4 qRT-PCR analysis of the virulence genes expression of the EHEC strains in high and low glucose. The WT, Δ*qseC*, and Δ*qseC/qseC* + strains were cultured in low or high glucose in DMEM at 37 °C for 4 h. Then, the qRT-PCR assay was performed with the following genes: **A** *ler* and **B** *stx-2*. Statistical significance compared to the wild-type strain in high-glucose D-MEM medium. Bars without an asterisk showed no statistically significant difference, $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****) (two-way ANOVA)

low and high glucose + *B. theta* conditions, there was no change in *stx-2* gene expression (Fig. 5B), which is a different result from that obtained for this gene in the absence of *B. thetaiotaomicron* in low-glucose conditions (Fig. 4A). These data strongly indicate that *B. thetaiotaomicron* is not a direct competitor, even when the availability of sugar was decreased, corroborating with the results obtained from SHIME®.

Different environmental signaling is involved in the *hcp-2* gene expression in EHEC, and the QseC sensor has a role in its regulation

Considering the vast cues present in a complex environment such as the gut, several intestinal metabolites such as short fatty acids are known to be key compounds produced by the microbiota that can modulate responses in pathogens [31]. We hypothesized whether fatty acids could have a

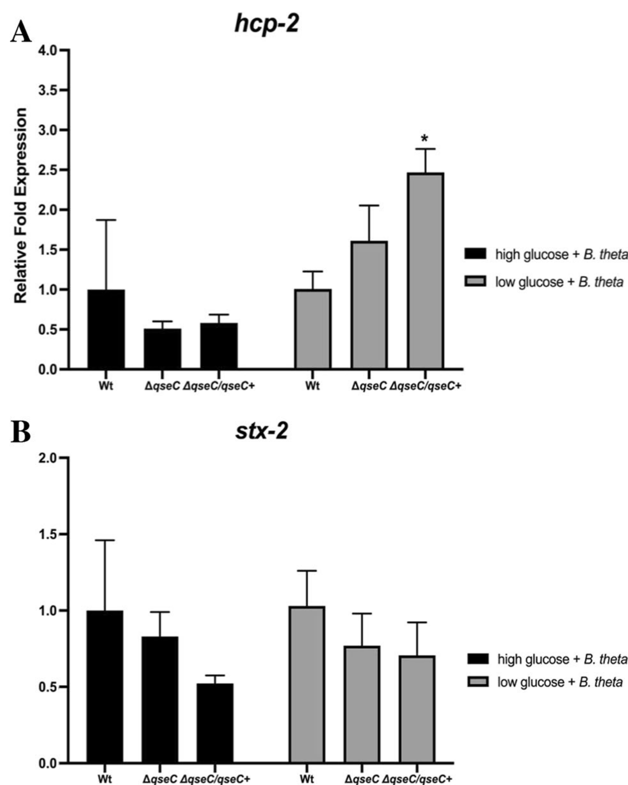

Fig. 5 qRT-PCR analysis of virulence gene expression of EHEC strains in low and high glucose in the presence of *Bacteroides thetaiotaomicron*. The WT, Δ*qseC*, and Δ*qseC/qseC*+strains were cultured in low glucose or high-glucose DMEM, anaerobically in the presence of *Bacteroides thetaiotaomicron* (*B. theta*) at 37 °C for 4 h. Then, the qRT-PCR assay was performed with the following genes: **A** *ler* and **B** *stx-2*. Statistical significance compared to the wild-type strain in high glucose + *B.theta*. Bars without an asterisk showed no statistically significant difference, $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) (two-way ANOVA)

role in the QseBC signaling in the EHEC T6SS system and would be involved in the SHIME® microbiota modulation by EHEC. Since recent studies have shown that butyrate is a short fatty acid capable of inducing optimal virulence gene expression in EHEC at 20 mM concentration [32], we decided to evaluate the gene expression in media supplemented with this short fatty acid. Expression analyses of *hcp-2* and *stx-2* genes from strains cultured in the presence of 20 mM butyrate sodium (But) were evaluated. Sodium chloride (NaCl) at 20 mM was used as the osmolarity control for the assays. The gene *hcp-2* showed no expression differences in the NaCl treatment nor WT or Δ*qseC* strains. On the other hand, there was a significant reduction in its expression by But in the Δ*qseC* strain (Fig. 6A). Corroborating with the *hcp-2* results in the NaCl treatment, the *stx-2* gene did not show any differences in its expression for both strains; however, there was a slight increase in the expression of this gene in the presence of But for the WT strain (Fig. 6B). Since SHIME® has a complex microbial

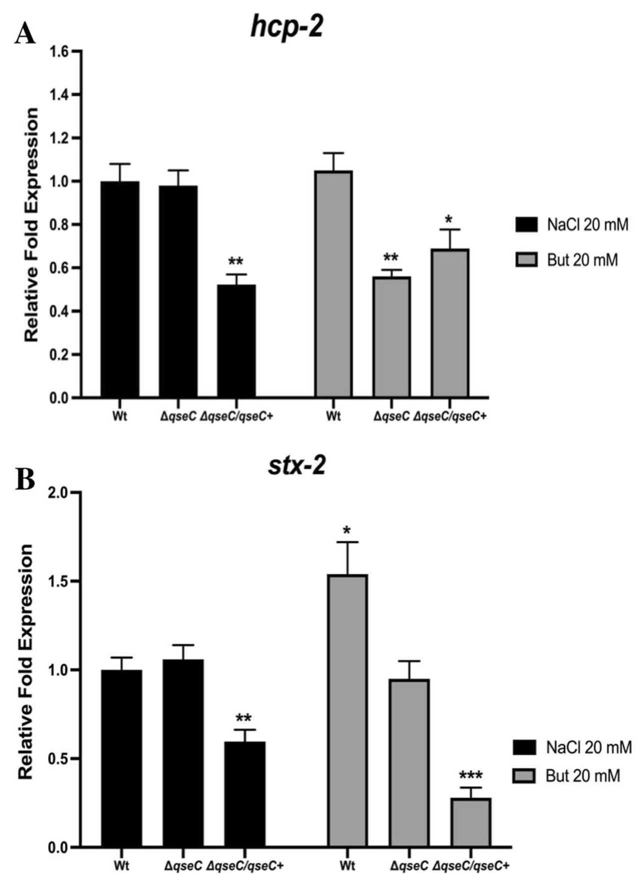

Fig. 6 qRT-PCR analysis of the virulence gene expression in the presence of sodium chloride (NaCl) or sodium butyrate (But). The wild-type WT, Δ*qseC*, and Δ*qseC/qseC*+strains were cultured in low-glucose DMEM medium at 37 °C in the presence of 20 mM NaCl or But up to O.D of 1. Then, the RT-PCR assay was performed with the following genes: **A** *ler* and **B** *stx-2*. Statistical significance compared to the wild-type strain in the presence of NaCl. The bars without an asterisk showed no statistically significant difference, $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) (two-way ANOVA)

community under different environmental signals, and the *hcp-2* gene was decreased under sugar and short fatty acids signals in Δ*qseC* strain, the absence of QseC sensor could affect the ability of EHEC to broadly senses environmental signals to activate a system that might be involved its competition with the microbiota and may be involved in the differences observed between the WT and Δ*qseC* strains at SHIME®.

L. acidophilus induces considerable EHEC growth inhibition when disputing for the same niche

Studies have shown that bacteria such as *Vibrio cholerae*, *Salmonella typhimurium*, and *Shigella sonnei* used their T6SS against commensal bacteria to clear their niche of inhibitory competitors [33–35], and for an efficient

T6SS mediate killing, studies have shown that contact between bacteria is necessary [36]. Considering that the *hcp-2* gene was downregulated in the Δ*qseC* strain under different environmental signals, significant differences were observed in the *Lactobacillaceae* modulation between WT and Δ*qseC* at the SHIME®. To further investigate whether growth inhibition in *L. acidophilus* and *L. reuteri* by EHEC would be different in directing surface interaction, an agar growth competition assay was performed between EHEC strains and *Lactobacillaceae* species. Individually plated, all EHEC strains without *Lactobacillaceae* species contact presented constant and lower growth than *Lactobacillaceae* in the control group. Also, no growth differences were observed between WT and the mutant strains. However, when co-cultured in direct contact with the agar surface in a 1:1 ratio with *L. acidophilus*, all the EHEC strains significantly inhibited their growth after 16 h of growth. Moreover, when the proportion of *L. acidophilus* was 1:5, EHEC inhibition was even higher. Thus, the Δ*qseC* mutant tended to be more affected by the increased ratio of *L. acidophilus* than the WT strain. Different from what was observed at SHIME®, *L. acidophilus* did not have its growth significantly affected by EHEC (Fig. 7A). On the other hand, when plated with *L. reuteri* at 1:1 and 1:5 ratios, all EHEC strains presented similar growth and smaller inhibition (Fig. 7B) compared to demonstrated in co-culture (Fig. 3A). Moreover, the different inhibition observed for EHEC is dependent on the species of *Lactobacillaceae* present, since *L. reuteri* did not induce the same inhibition levels in EHEC growth as observed for *L. acidophilus*. Also, the two species tested did not have their growth impaired by EHEC, which might explain the differences observed at SHIME®.

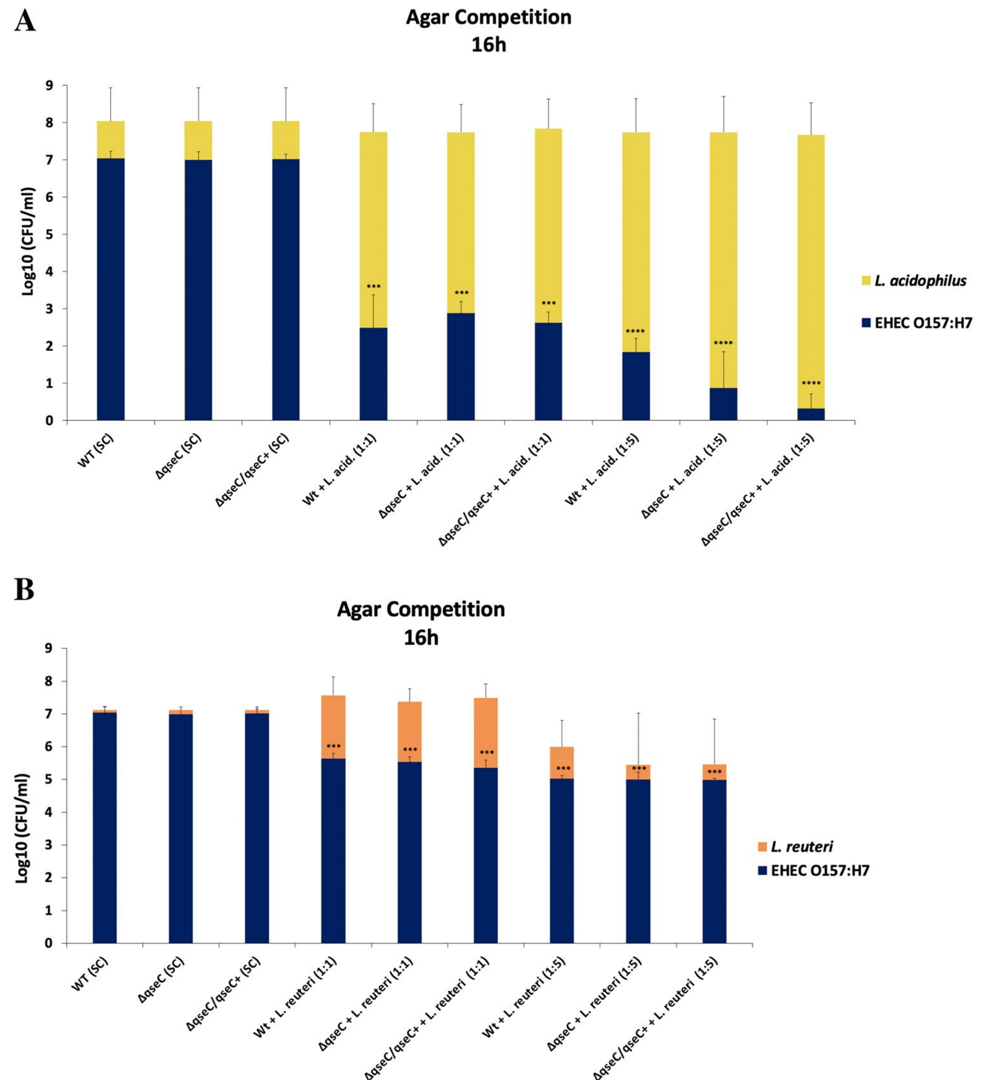

Fig. 7 Agar surface competition assay between *Lactobacillus acidophilus* (**A**) or *Limosilactobacillus reuteri* (**B**) and EHEC strains. The WT, Δ*qseC*, Δ*qseC*/*qseC*+, and *L. acidophilus strains* or *L. reuteri* in single culture (SC) were directly added to the agar surface. Then, the strain was mixed in co-culture (WT + *L. acid* or *L. reuteri*. and Δ*qseC* + *L. acid* or *L. reuteri*. and Δ*qseC*/*qseC*+ + *L. acid* or *L. reuteri*) at the ratio of 1:1 or 1:5 of EHEC plus *L. acid* or *L. reuteri*, respectively, and added to the agar surface. After 16 h, the growth halo from the single-culture and co-culture plates was collected, diluted, plated, and counted. Bars without an asterisk showed no statistically significant difference, $p < 0.001$ (***), $p < 0.0001$ (****) (two-way ANOVA)

Discussion

EHEC is an important human gastrointestinal pathogen with great clinical importance associated with food outbreaks, mainly due to undercooked contaminated meat, since EHEC can colonize the gastrointestinal tract of cattle being the main reservoir for these pathogens [1]. To date, the dynamic models to understand how EHEC shapes the microbiota or its behavior in the different compartments of the gastrointestinal tract still demand further studies [16–18]. The human intestinal microbiota is composed of a complex microbial community estimated at 10^{14} microorganisms that offer a variety of benefits for the host, such as integrity and reshape of the intestinal epithelium, immunity regulatory response, vitamins, energy molecules, resistance against pathogens, and assistance to maintaining the gastrointestinal homeostasis [37]. Since microbiota inhibits several niches and competes for energy sources directly with pathogens, it promotes a process called "resistance to colonization," which helps the host in the protection against infections [38]. Furthermore, the microbiota is essential for the host's health, and changes in this microbial community, called dysbiosis, have been associated with susceptibility to infections and various inflammatory diseases [38]. Studies have shown that individuals with Crohn's disease and ulcerative colitis have a relative increase in Bacteroidetes and a decrease in the abundance of Firmicutes phyla [39]. Also, pathogens that cause gastroenteritis may play a role in the initiation and/or exacerbation of inflammatory bowel diseases [40], so intestinal dysbiosis induced by infectious processes may directly impact the host's health.

EHEC has a high resistance to low pHs [41], which contributes to its low infectious dose of around 50 to 100 CFUs [1]. Also, EHEC is resistant to bile acids, and studies in a bovine host model showed that the EHEC O157:H7 strain could grow around 15% in bile [42]. Etienne-Mesmin et al. (2011) employed a novel probiotic *Saccharomyces cerevisiae* strain in the TNO gastrointestinal tract model (TIM, Zeist, Netherlands) multicompartmental system that mimics the human upper gastrointestinal tract, and EHEC was able to grow in the distal portions of the digestive tract model followed by an increase in viability after 1 h in the duodenum [15]. Thus, under favorable conditions, such as neutral pH and dissolution of bile salts in the ileum, there was a significant increase in EHEC growth [15, 43]. Previous studies in the single-step dynamic model of the human colon (ARCOL) and TIM models demonstrated that in general, EHEC is particularly resistant to the gastrointestinal environment [17, 18, 43], and Bacteroidetes and Firmicutes were the most upregulated groups. Therefore, the *Bacteroides* genus was upregulated when compared to the *Lactococcus/Pediococcus/Leuconostoc* genus, but there was a different modulation on the microbiota induced among the three donors tested [17].

Herein, our study has shown at SHIME® that even after 1 h of exposure to the pH 2.5, the WT and Δ*qseC* strains remained viable and presented a minor reduction compared to the initial inoculum (stomach 0 h). During the duodenum reactor passage, when the pancreatic juice started to be added to the system, the WT and Δ*qseC* strains presented differences in their UFCs recovery, which the WT seemed to replicate; however, the same was not observed for the Δ*qseC* strain (Fig. 1A). Therefore, the QseC sensor may have a role in activating signals to promote replication after stress conditions, such as acid conditions in the stomach. Moreover, the QseC sensor showed to be involved in microbiota modulation by EHEC and the absence of this sensor favored the Firmicutes phylum (Fig. 1B), suggesting that EHEC may have a considerable impact on the microbiota, and the QseC sensor kinase is directly involved in sensing the intestinal environment to regulate gene expression.

Distinct sugar levels lead to the expression of virulence genes in EHEC [9, 44], and bacteria compete for similar nutrients to survive and colonize the gut [28]. When EHEC was grown solely as monoculture, the low-glucose condition led to an increase in the T6SS gene expression in EHEC, but when *B. thetaiotaomicron* was added as co-culture under the same conditions, these differences were no longer observed (Figs. 4A and 5A). The analysis with *B. thetaiotaomicron* demonstrated *hcp-2* regulation is also dependent on bacteria in the environment. These results agree with previous studies that indicate *B. thetaiotaomicron* and EHEC in co-culture did not have their growth impaired, inferring that these two bacteria are not direct competitors [27]. Moreover, the results obtained at SHIME® supported these previous data since the *Bacteroides* did not show to be affected by the EHEC WT strain in the dynamic model used in the study (Fig. 1C). Besides the SHIME® data, here, we unraveled that the differences in the availability of glucose may lead to an increase in the T6SS *hcp-2* gene expression under QseC regulation since this gene was downregulated in the Δ*qseC* in comparison to WT strain (Fig. 4A and B). During the SHIME® passage, the large amounts of microorganisms promoted an environment with low-carbon sources available for EHEC; in this way, the decrease in *hcp-2* in the Δ*qseC* strain might be involved in the differences in the microbiota modulation observed between the WT and Δ*qseC* strains. Moreover, these results could suggest that under QseC regulation, in an environment that mimics conditions closer to the intestinal epithelium layer, the T6SS system

may be active in EHEC whether to induce cytotoxicity to the host cell epithelium or to inhibit possible competitors of this carbon source. Lastly, the *stx-2* gene had an overexpression in the low-glucose assay and was decreased in the Δ*qseC* strain, which corroborated with the differences in *stx-2* expression between the WT and Δ*qseC* observed at SHIME® infection.

The *Lactobacillaceae* was the bacterial group analyzed most affected by the absence of the QseC sensor (Fig. 1C). When investigating whether it may be a direct competitor with EHEC, our results suggest that the differences in the *Lactobacillaceae* members at SHIME® might not be due to direct competition between EHEC and *Lactobacillaceae*. EHEC growth was inhibited during *Lactobacillaceae* strains co-culture experiments (Figs. 3A, B and 7A, B); however, during SHIME® microbiota analyses, the absence of the QseC sensor kinase led to a significant increase of the *Lactobacillaceae*. Studies have demonstrated that the *Lacticaseibacillus casei* (*Lactobacillus casei*) LC2W inhibited the colonization of EHEC in mice [45]. Similarly, the administration of *L. reuteri* before and during infection by EHEC in germ-free mice resulted in the improvement of the disease symptoms and increased protection from EHEC infection in mice [46]. Thus, *Lactobacillaceae* species can produce a broad range of bacteriocins such as helveticin and lactocillin against different bacteria [47], and some gram-positive bacteria species, such as *Bacillus* and *Listeria*, possess the wall-associated protein A (WapA) that seems to be contact-dependent to promote bacterial growth inhibition [48, 49]. Also, previous studies have demonstrated that *L. acidophilus* through the production of bioactive molecules could impair EHEC virulence [50], and the high production of linoleic acid by *L. casei* limited the growth, survival, and virulence of EHEC and *Salmonella* typhimurium [51], which corroborates to our data here. Herein, the results suggest that *L. acidophilus* may have a competition niche system that is contact-dependent to inhibit EHEC growth, but the QseC sensor does not seem to help EHEC to survive in this condition. Furthermore, the differences observed for *Lactobacillaceae* between the strains during the in vitro co-culture and SHIME® analysis may be due to the dynamic model; there is a pool of *Lactobacillaceae* species that is possibly affected by EHEC, and it was not evaluated under co-culture conditions here performed. Also, the absence of the QseC sensor could lead to ineffective competition between the Δ*qseC* strain and other members of the microbiota that may help the *Lactobacillaceae* family at SHIME®. Nevertheless, the significant large inhibition promoted by *L. acidophilus* and the modulations observed at SHIME® opens a perspective that members of the *Lactobacillaceae* genus might be an important competitor for EHEC. Additionally, short fatty acids seem to be involved in the T6SS system regulation promoted by the QseC sensor (Fig. 6A and B), indicating the broad regulatory signals that may be under the control of this sensor and its role in EHEC pathogenesis.

Conclusions

The QseC sensor kinase modulates the gut microbiota within the conditions here tested, and its regulation under distinct glucose and butyrate concentrations is crucial to T6SS gene-encoding factors in EHEC. Therefore, this study brings new insights into the QseC sensor role that impacts the activation of LEE virulence factors and the direct competition of EHEC with the microbiota, contributing to a broad response to different metabolites and signals in the intestinal environment by EHEC. Our gut microbiome results have shown that the QseC sensor inhibition has an important impact on pathogen virulence, supporting the idea that QseC-blocking could be an interesting target during the gut microbiota competition for novel therapies. Further studies will help to understand how the microbiota modulation and intestinal metabolites under QseC sensor control contribute to EHEC colonization, as well as the benefits of an anti-virulence approach against pathogens as an alternative conventional therapeutic approach.

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Author contribution K.M. and C.G.M. designed the study. KM performed the research. K.M. and C.G.M. performed the data analysis. M.K.S., S.V., and K.M. performed and designed the SHIME assay. K.M. wrote the manuscript. C.G.M. supervised the research.

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Data availability All data and materials are available under request.

Code availability Not applicable.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication All authors consent for publication.

Competing interests The authors declare no competing interests.

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