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Probiotics Affect Virulence-Related Gene Expression in *Escherichia coli* O157:H7[▽]

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The attachment of enterohemorrhagic *Escherichia coli* O157:H7 (EHEC O157) to host intestinal epithelial cells is essential for the development of hemorrhagic colitis and hemolytic-uremic syndrome in humans. Genes involved in attachment are carried within a pathogenicity island named the locus of enterocyte effacement (*LEE*), known to be directly activated by quorum sensing (QS). In the present study, we investigated autoinducer-2 (AI-2) production and the expression of several virulence-related genes in EHEC O157 grown in the absence and presence of a *Lactobacillus acidophilus*-secreted molecule(s). Transcription of important EHEC O157 virulence-related genes was studied by constructing promoter-reporter fusions and reverse transcriptase PCR. Shiga toxin (Stx) production was assayed by an enzyme immunoassay. When EHEC O157 was grown in the presence of chromatographically selected fractions of *L. acidophilus* La-5 cell-free spent medium, we observed a significant reduction of both extracellular AI-2 concentration and the expression of important virulence-related genes, although no significant difference in Stx production was observed. We show here that *L. acidophilus* La-5 secretes a molecule(s) that either acts as a QS signal inhibitor or directly interacts with bacterial transcriptional regulators, controlling the transcription of EHEC O157 genes involved in colonization.

Enterohemorrhagic *Escherichia coli* serotype O157:H7 (EHEC O157) is a clinically important food- and waterborne pathogen. EHEC O157 colonizes the human colon epithelium, where it induces acute colonic inflammation and can lead to severe complications, such as hemolytic-uremic syndrome (HUS). Renal damage caused by HUS is believed to be linked to Shiga toxin (Stx) production. Shiga toxin-producing *E. coli* strains of serotype O157:H7 belong to a group of enteric pathogens that induce cytoskeletal rearrangements in infected epithelial cells leading to the formation of attaching and effacing (AE) lesions (19). EHEC O157 adheres to the apical membrane of colonic epithelial cells, signals the epithelial cells through a type III secretion system (T3SS) (15), and causes the AE lesions (16). The genes responsible for the AE lesion formation are contained within a chromosomal pathogenicity island (PAI) identified as the locus of enterocyte effacement (*LEE*) (17, 23). *LEE* and other potential virulence-associated factors, such as genes encoding the expression and assembly of flagella, motility, and chemotaxis, are influenced by the bacterial cell-to-cell signaling mechanism known as quorum sensing (QS) through the LuxS system (2, 33). EHEC O157 uses the QS regulatory system to sense its milieu and activate genes essential for intestinal colonization (14, 33, 34). It has been shown that EHEC O157 produces a signaling molecule referred to as autoinducer-2 (AI-2) used for bacterial interspecies communication (29, 34); this AI-2 was first described as a furanosyl borate diester (10), and subsequently it was found that the enzyme responsible for its synthesis was encoded by the *luxS* gene in EHEC O157 (41). Therefore, AI-2 was thought to be the enzyme responsible for the QS regulation of the *LEE* PAI (33, 34). In *E. coli*, knockout of the *luxS* gene affects the synthesis of another uncharacterized signaling molecule named autoinducer-3 (AI-3), which cross-communicates with the catecholamine host signaling system, and which was shown to be the one responsible for the regulation of the *LEE* PAI and flagellum-dependent motility in EHEC O157 (36, 43). Intestinal colonization by EHEC O157, which has a very low infectious dose, is primarily influenced by signaling molecules from nonpathogenic *E. coli* present in the normal intestinal microbiota (33) and/or by the mammalian catecholamines epinephrine and norepinephrine (NE) present in the gastrointestinal (GI) tract (7, 18, 36). Thus, QS acts as a global regulatory mechanism for basic physiological functions in EHEC O157, as well as for the control of key virulence factors (34).

EHEC O157 inhabits the large intestine, which contains large amounts of resident microbiota, making the interspecies communication system essential for the bacterium's survival and infectivity. Probiotic lactic acid bacteria (LAB) are an important part of the microbial ecosystem of the human GI tract due to their protective roles against diseases (1, 22, 27, 30, 37, 44). These protective roles include the prevention and amelioration of intestinal infections (31, 37, 38, 39). Mechanisms of interference by probiotics include direct action against pathogens through adherence competition at coloniza-

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TABLE 1. Bacterial strains, plasmids, and constructs used in this study

Strain, plasmid, or *E. coli* construct	Serotype	Relevant genotype or characteristic(s)	Source or reference[a]
Strains			
E. coli			
DH5α		λ$^-$ ϕ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	Promega
ATCC 43888	O157:H7	Stx1$^-$ and Stx2$^-$; isolated from human stool in Washington	CRIFS stock
ATCC 43894	O157:H7	Stx1$^+$ and Stx2$^+$; isolated from human stool in Michigan	CRIFS stock
L. acidophilus			
La-5		Probiotic LAB	CRIFS stock
V. harveyi			
BB170		*luxN*:Tn*5* (sensor AI-1$^-$ AI-2$^+$)	4
BB152		*luxM*::Tn*5* (AI-1$^-$ AI-2$^+$)	4
Plasmids			
pVS232Z		*LEE1-lacZYA* Amp^r Kan^r	33
pVS21		*LEE2-lacZYA* Amp^r Kan^r	33
pSB377		*luxCDABE* gene cluster, Amp^r	45
pVSLEE1-lux		*LEE1-luxCDABE* Amp^r Kan^r	This study
pVSLEE2-lux		*LEE2-luxCDABE* Amp^r Kan^r	This study
E. coli constructs			
ATCC 43888	O157:H7	Stx$^-$, *LEE1*::*lux*	This study
ATCC 43888	O157:H7	Stx$^-$, *LEE2*::*lux*	This study
ATCC 43894	O157:H7	Stx1$^+$ and Stx2$^+$, *LEE1*::*lux*	This study
ATCC 43894	O157:H7	Stx1$^+$ and Stx2$^+$, *LEE2*::*lux*	This study

[a] CRIFS stock, strains deposited in the Canadian Research Institute for Food Safety culture collection.

tion sites (1, 9, 11, 13, 21, 28, 32), antibacterial effects (6, 12, 20, 42), and stimulation of the epithelial cell host-acquired immune response (8, 13, 24, 25, 26, 44). The mode of action and molecular basis of probiotic effects are not yet fully understood but are likely to be multifactorial and strain specific. The increase of antimicrobial resistance has motivated the interest in therapeutic approaches other than antibiotics, focusing on the capacity of probiotics to inhibit attachment of certain pathogens. Determining the molecular basis and mechanisms of action of probiotics on bacterial pathogens is a promising new approach to protecting and controlling human infectious diseases.

If QS through the LuxS signaling system allows bacteria in the GI tract to communicate, it is possible that intestinal bacteria of other genera that also use QS as a main regulatory system may influence EHEC O157 gene expression. This regulatory system, apart from being able to control genes involved in pathogenesis (flagellation and motility) (35), regulates genes involved in bacterial metabolism, DNA repair, cell growth, and nucleotide and protein synthesis (34, 35). The objectives of this study were to investigate the role of a potential probiotic, *Lactobacillus acidophilus* La-5, in the control of EHEC infection. Here we report the investigation of chromatographically selected fractions of *L. acidophilus* La-5 cell-free spent medium (CFSM) on the expression of selected virulence factors and the secretion of AI-2 by EHEC O157.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. *Escherichia coli* strains were grown aerobically at 37°C in Luria-Bertani (LB) broth (BD Diagnostic Systems) and in Dulbecco's minimal essential medium (high glucose). *Lactobacillus acidophilus* strain La-5 was grown under anaerobic conditions at 37°C in modified DeMann, Rogosa, and Sharpe medium (mMRS; 10 g peptone from casein, 8 g meat extract, 4 g yeast extract, 8 g D-(+)-glucose, 2 g dipotassium hydrogen phosphate, 2 g diammonium hydrogen citrate, 5 g sodium acetate, 0.2 g magnesium sulfate, and 0.04 g manganese sulfate in 1 liter distilled water). *Vibrio harveyi* strains BB152 and BB170 were a kind gift from Bonnie Bassler (Department of Molecular Biology, Princeton University). These strains were grown at 30°C on an orbital shaker at an agitation rate of 125 rpm in marine broth 2216 (BD Diagnostic Systems) or autoinducer bioassay (AB) medium (17.5 g NaCl, 12.3 g $MgSO_4$, 2 g Casamino Acids in 1 liter distilled water adjusted to pH 7.5, supplemented after autoclaving with 10 ml/liter 1.0 M potassium phosphate [pH 7.0], 20 ml/liter 50% glycerol, and 10 ml/liter 0.1 M L-arginine; B. Bassler, personal communication).

Preparation of CFSM fractions of *L. acidophilus*. *L. acidophilus* La-5 was grown in mMRS medium overnight and was diluted 1:100 in fresh medium. When the culture grew to an optical density at 600 nm (OD_{600}) of 1.6 (1.0×10^8 cells/ml), the cells were precipitated by centrifugation at 6,000 × *g* for 10 min at 4°C. The supernatant was sterilized by filtering through a 0.2-μm-pore-size filter (Millipore, Bioscience Division, Mississauga, Ontario, Canada). The spent medium was then freeze-dried for concentration. Size exclusion chromatography (SEC) was carried out on a Bio-Gel P2 column [exclusion, 100 to 1,800 Da; 92 by 2.5 cm; Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario, Canada]. The column was eluted with 18-Ω water at a gravity flow rate of 0.8 ml/min, and 5-ml fractions were collected. The fractions collected were freeze-dried and resuspended in 1 ml 18-Ω water for preliminary screening against *LEE1*, *LEE2*, and AI-2 production. Initially, EHEC O157 *LEE* promoter activity and AI-2 production were determined after supplementation with 10% (vol/vol) of all *L. acidophilus* fractions collected (fractions 1 through 80). Fractions showing a strong inhibitory activity against AI-2 production and *LEE* expression were used separately in all subsequent experiments (Fig. 1 and 2).

Plasmid reporter constructs. The *LEE-lac* fusion plasmids (32), pVS232Z (*LEE1-lac*) and pVS21 (*LEE2-lac*), were provided by James B. Kaper (Center for Vaccine Development and Department of Microbiology and Immunology, University of Maryland School of Medicine). The coding region of the *lac* operon, consisting of structural genes *lacZYA*, was removed from the plasmids by restriction enzyme digestion. The plasmid pSB377 (45), with the *luxCDABE* gene cluster of *Xenorhabdus luminescens*, was provided by Phil Hill (Division of Food Sciences, School of Biosciences, University of Nottingham, England). The gene cluster was amplified with *Pwo* polymerase (Roche Diagnostics Canada, Laval, Quebec, Canada) by using forward primer *luxCE*-for (AGCGGGATCCATGA CTAAAAAAATTTCATTCATTATTAACGGCC) and reverse primer *luxCE*-rev (ACGCGTCGACCTAACTATCAAACGCTTCGGTTAAGC) and cloned into the *lac*-removed plasmids pVS232Z and pVS21 digested with SalI and

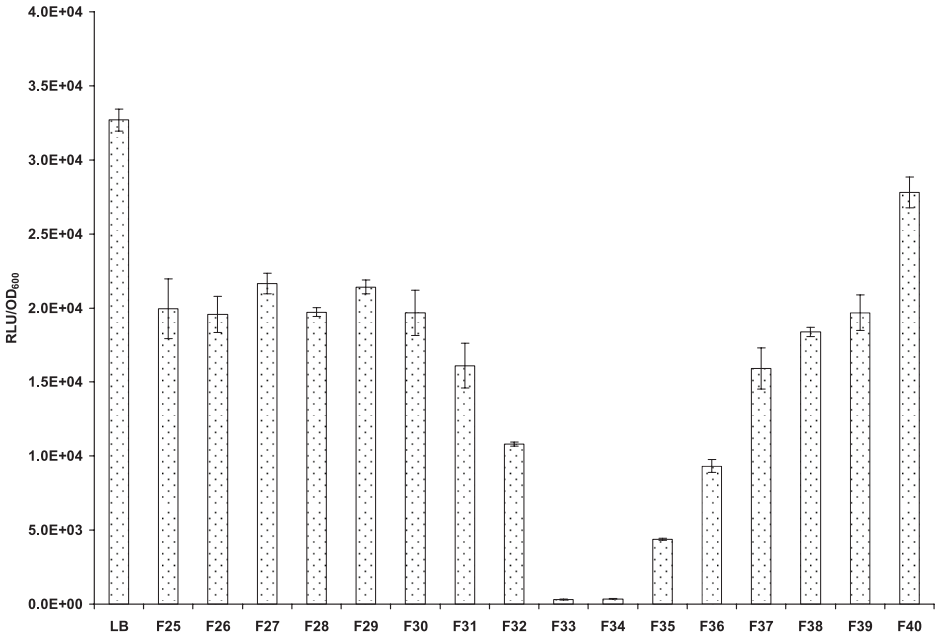

FIG. 1. AI-2 bioassay conducted three times with the same samples. EHEC O157 ATCC 43894 was grown for 16 h in LB broth alone (LB) or supplemented with 10% of *L. acidophilus* La-5 CFSM fractions (F25 through F40). The CFSM from these cultures was collected as described in Materials and Methods. Results were expressed as relative light units (RLU) defined as counts min^{-1} and adjusted to OD_{600} (RLU/OD_{600}). The data are the means ± the standard deviations of the results of three independent replicate trials.

BamHI, generating plasmids pVSLEE1-*lux* and pVSLEE2-*lux*, respectively. Expression of the *lux* genes was under the control of promoters of the *LEE* operons. The extent of gene expression could be measured by monitoring light emission from the cultures grown in LB broth as described below. Transformation of the new fusion plasmids into EHEC O157 cells was performed by electroporation using the protocols recommended by the instrument manufacturer (Gene Pulser II; Bio-Rad). Transformants were screened on the selective LB agar supplemented with the antibiotics ampicillin (Ap) and kanamycin (Km) at a concentration of 50 μg/ml each. The promoter$_{LEE}$::*luxCDABE* fusion constructs were confirmed by restriction analysis and DNA sequencing (data not shown). Two

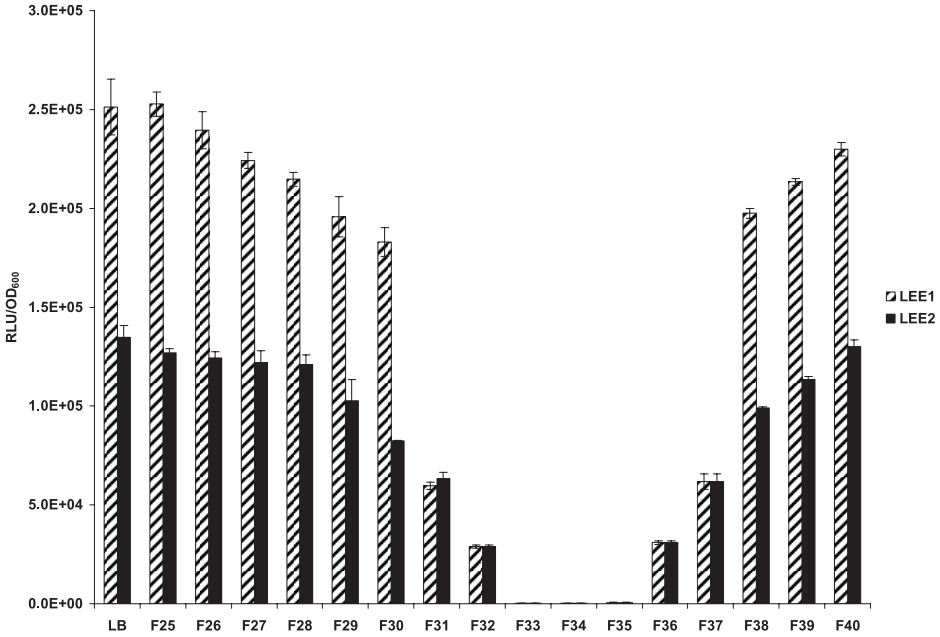

FIG. 2. Luminescence activity of *LEE*::*luxCDABE* fusion constructs in EHEC O157 (ATCC 43894) grown in LB broth alone (LB) or in LB broth supplemented with 10% of *L. acidophilus* La-5 CFSM fractions (F25 through F40). *LEE1*, EHEC O157 *LEE1*::*luxCDABE* constructs. *LEE2*, EHEC O157 *LEE2*::*luxCDABE* constructs. Data were collected after 16 h of growth. Results were expressed as relative light units (RLU) defined as counts min^{-1} and adjusted to OD_{600} (RLU/OD_{600}). The data are the means ± the standard deviations of the results from three independent replicate trials.

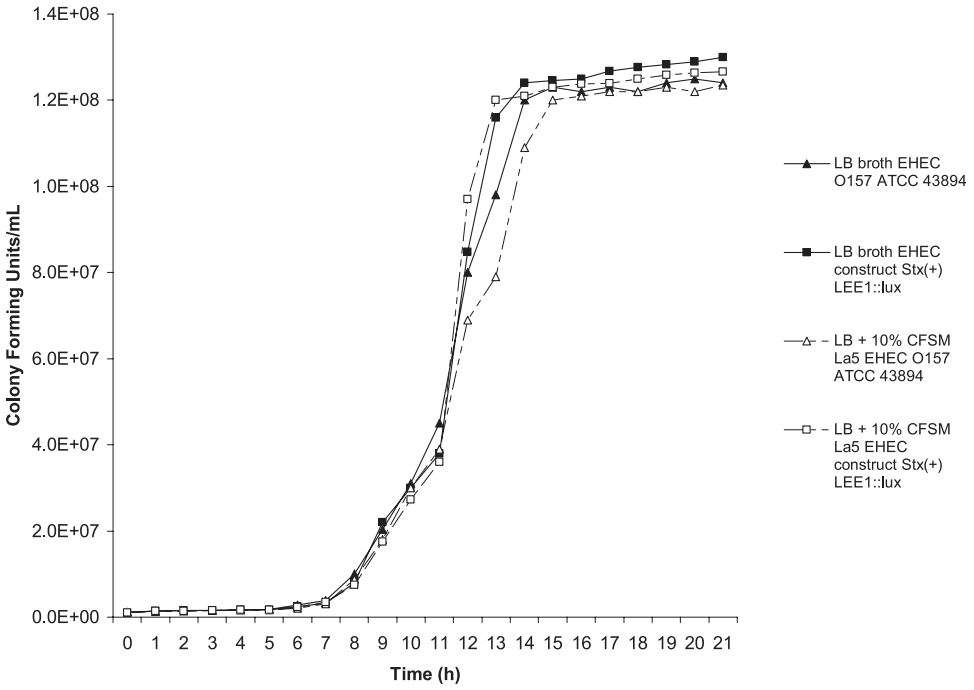

FIG. 3. EHEC O157 ATCC 43894 growing phases, showing the effect of *L. acidophilus* La-5 CFSM fraction 34. The solid lines represent EHEC 0157 grown in LB broth (LB). The dotted lines represent *E. coli* 0157:H7 grown in LB broth supplemented with 10% *L. acidophilus* La-5 CFSM fraction 34 (LB + 10% CFSM La5).

EHEC O157 constructs were selected from strain ATCC 43888 and two from strain ATCC 43894. Plasmid and construct details are summarized in Table 1.

Growth curves, monitored as plate counts and OD_{600}, of promoter$_{LEE}$::*luxCDABE* EHEC O157 fusion constructs grown in LB broth and supplemented LB broth were similar (Fig. 3), confirming that the molecules present in the *L. acidophilus* La-5 fractions were not affecting the primary metabolism of EHEC O157. This phenomenon was observed for constructs reporting both *LEE1* and *LEE2* expression.

Influence of *L. acidophilus* CFSM fractions on *LEE* promoter activity measured by plasmid reporter constructs. For preliminary screening of *L. acidophilus* fractions, EHEC O157 *LEE* constructs were grown for 18 h at 37°C in selective LB broth and then were diluted 1:100 in fresh medium with and without supplementation with 10% of all the *L. acidophilus* fractions. Two hundred microliters of each sample was distributed in triplicate into wells of a sterile, opaque 96-well plate (Corning No. 3610; Fisher Scientific Canada, Ottawa, Ontario, Canada) and incubated at 37°C. Luminescence and cell density were measured every hour with a Victor multilabel counter (Wallac, PerkinElmer Life Sciences Canada, Woodbridge, Ontario, Canada). Luminescence counts were expressed as counts min^{-1}. After the selection of specific *L. acidophilus* CFSM fractions, EHEC O157 constructs grown overnight in LB broth were diluted 1:100 in 10 ml fresh medium with and without supplementation with 10% of the selected *L. acidophilus* La-5 fractions (F33 to F35). Sample tubes were shaken at 250 rpm at 37°C for 16 h. Luminescence and cell density were measured with a Victor multilabel counter as described above.

Effect of *L. acidophilus* CFSM fractions on EHEC O157 AI-2 production. EHEC O157 CFSM was tested for the presence of AI-2 by inducing luminescence in the *V. harveyi* BB170 (*luxN*::Tn*5*) reporter strain (4). The assay was performed as described by Surette and Bassler (40). Briefly, BB170 was grown for 16 h at 30°C with aeration in AB medium and then diluted 1:5,000 into fresh AB medium. One hundred eighty microliters was distributed in triplicate into wells of a sterile, opaque 96-well plate (Corning No. 3610; Fisher Scientific Canada). CFSM of EHEC O157 strain 43894 grown in LB broth with and without supplementation with 10% (vol/vol) of all the *L. acidophilus* fractions collected was added to a final concentration of 10% (vol/vol) to each well. Positive controls contained 10% (vol/vol) CFSM from BB152 while negative controls contained 10% (vol/vol) CFSM from *E. coli* DH5α and sterile LB broth. Additional control wells contained 10% (vol/vol) *L. acidophilus* CFSM fractions 33 and 34. Plates were shaken at 175 rpm at 30°C. Luminescence and cell density were measured every 30 min for 6 h with a Victor multilabel counter. Luminescence counts are expressed as counts min^{-1}. After the selection of specific *L. acidophilus* fractions, CFSM of EHEC O157 grown in LB broth supplemented with 10% of the selected *L. acidophilus* La-5 fractions (F33 and F34) was tested as described above with the addition of another control. Briefly, BB170 grown overnight in AB medium was diluted 1:5,000 into fresh AB medium and into AB medium conditioned with 10% *L. acidophilus* CFSM fraction 34. Twenty microliters of EHEC O157 CFSM grown in LB broth and in LB broth supplemented with the addition of the selected *L. acidophilus* La-5 fractions (F33 and F34) was then tested for AI-2 production.

Influence of *L. acidophilus* CFSM fractions on transcription of selected virulence factors. For RNA isolation, EHEC O157 strain 43894 was grown in Dulbecco's minimal essential medium (high glucose) alone and supplemented with 10% of the selected *L. acidophilus* fractions separately to an OD_{600} of 1.0 (early stationary phase). Samples were taken after 16 h of growth. Two-milliliter samples were mixed with 4 ml of RNAprotect bacterial reagent (QIAGEN Inc., Mississauga, Ontario, Canada) to stabilize the total RNA. Cell pellets were lysed and RNA was selectively extracted using TRIzol according to the manufacturer's instructions (Invitrogen Canada Inc., Burlington, Ontario, Canada). For each experiment, four 1-ml samples were extracted. Following precipitation, the RNA pellets were air dried for 20 min at room temperature. Dried RNA pellets were resuspended in molecular-grade water (Sigma-Aldrich Ltd., Oakville, Ontario, Canada). The resuspended RNA was then mixed with a solution containing 10 μl of 0.1 M dithiothreitol, 3 μl of RNase Out RNase inhibitor, 10 μl of DNase (Promega Corp., Madison, WI), and 3 μl of DNase buffer solution (Promega) and incubated for 50 min at 37°C. Following DNase digestion, samples were mixed with 0.5 ml of acidic phenol-chloroform in a 5:1 dilution, pH 4.5, and centrifuged at 9,300 × *g* for 10 min for phase separation. The top phase containing pure RNA was removed and precipitated with 0.1 volume of 2.5 M sodium acetate (pH 5.2) and 2.5 volumes of cold (−10°C) 100% ethanol. Samples were placed at −20°C for 20 min, followed by centrifugation at 21,000 × *g* for 20 min to pellet the RNA. For amplification by reverse transcriptase PCR (RT-PCR), a purified, dried RNA pellet was resuspended in 30 μl of molecular-grade water (Sigma-Aldrich). The concentrations and the quality of the RNA in each sample were determined by measuring their absorbance at 260 and 280 nm (Beckman DU-520; Beckman Coulter Inc., Fullerton, CA), and the RNA integrity was assessed by running a denaturing agarose gel stained with ethidium bromide (data not shown). The remaining volume was used for RT-PCR. Ad-

TABLE 2. Oligonucleotide primers used in this study

Target gene	Function	Sequences[a]
tufA	Housekeeping gene	F: 5′ACTTCCCGGGCGACGACACTC′ R: 5′CGCCCGGCATTACCATCTCTAC′
qseA	QS *E. coli* regulator A	F: 5′CGCGGATCCCGTTGGCACAGGTTTGTACA′ R: 5′CGCGGATCCCGTTGGCACAGGTTTGTACA′
luxS	AI-2 synthesis	F: 5′GATCATACCCGGATGGAAG′ R: 5′AGAATGCTACGCGCAATATC′
ler	*LEE E. coli* regulator	F: 5′TTTCTTCTTCAGTGTCCTTCA′ R: 5′TGCGGAGATTATTTATTATGA′
eaeA	Intimin	F: 5′CCCGAATTCGGCACAAGCATAAGC′ R: 5′CCCGAATCCGTCTCGCCAGTATTCG′
hlyB	Enterohemolysin B	F: 5′TCCCCTCTGTTGTGAAATAC′ R: 5′TGTCATTGCAACCAGATTTA′
fliC	Flagellin	F: 5′TACCATCGCAAAAGCAACTCC′ R: 5′GTCGGCAACGTTAGTGATACC′
tir	Translocated intimin receptor	F: 5′ACTTCCAGCCTTCGTTCAGA′ R: 5′TTCTGGAACGCTTCTTTCGT′
espA	EspA protein	F: 5′CGGTTATTTACCAAGGGATA′ R: 5′TGGATACATCAAATGCAACA′
espD	EspD protein	F: 5′TAAATTCGGCCACTAACAAT′ R: 5′CTAAAGCGCTGGAGAATAAA′
$stxA_2$	Shiga toxin fraction 2A	F: 5′TTTACGATAGACTTCTCGAC′ R: 5′CACATATAAATTATTTCGCTC′
$stxB_2$	Shiga toxin fraction 2B	F: 5′AGATGTTTATGGCGGTTTTA′ R: 5′TTAAACTGCACTTCAGCAAA′

[a] F, forward; R, reverse.

ditional molecular-grade water was added as necessary to dilute the RNA extracts to equivalent absorbance readings. The extracted standardized RNA (1.30 μg/μl) was serially diluted (undiluted, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) using molecular-grade water prior to RT-PCR in order to compare band patterns from equivalent RNA extracts from the different conditions. First-strand cDNA synthesis was performed in a total volume of 20 μl, where RNA was reverse transcribed using 200 units of Superscript II RNase H− reverse transcriptase (Invitrogen), 1 μl of the reverse primer (20 pmol/μl), and 40 units RNase inhibitor (RNase Out; Invitrogen). The appropriate no-RT control was prepared to confirm the absence of contaminating DNA. Amplification conditions consisted of 1 cycle at 94°C for 3 min followed by 35 cycles of 94°C for 45 s, 54°C for 1 min, and 72°C for 1 min. A final extension step of 7 min at 72°C was included. Amplification products were visualized by separation on a 1% agarose gel, stained with ethidium bromide, and visualized by UV transillumination. Band intensity profiles of the diluted and undiluted amplified RNA were quantified using Gel Doc 2000 (Bio-Rad Laboratories) equipped with the MultiAnalyst software (Bio-Rad Laboratories). Sequences for primers used in this study are given in Table 2. An endogenous housekeeping gene (*tufA*) was used as a control for the reverse transcription. Band intensity profiles of the housekeeping gene *tufA* of the treated and the nontreated samples were used as indicators of EHEC O157 metabolism after the addition of the *L. acidophilus* La-5 fractions.

Effect of *L. acidophilus* CFSM fractions on Stx production. Stx2 was assayed using an immunoassay modified for quantification of Stx2 by using a standard curve established with purified Stx2 as described previously (3). EHEC O157 strain 43894 was grown in LB broth with and without supplementation with 10% of the selected fractions of *L. acidophilus* CFSM. One-milliliter samples were taken at 16 h, and simultaneously appropriate dilutions were plated to determine the cell density (CFU ml^{-1}). Cells were then pelleted by centrifugation at 6,000 × *g* for 5 min, and supernatants were filtered through a 0.22-μm-pore-size filter and stored at −80°C until used. EHEC O157 strain 43888 (*stx*-negative strain) CFSM was used as a negative control. One hundred microliters of each sample was added into the wells of capture antibody-precoated enzyme-linked immunosorbent assay (ELISA) plates in duplicate. Plates were read with a microplate reader (EL 312; BioTek Instruments, Winooski, VT) at dual wavelengths of 450 and 630 nm, blanking to air. The means ± 2 standard deviations of the negative control wells were calculated, and the samples with absorbance above this value were considered positive for the presence of toxin.

Statistical analysis. All results in this study are the means ± the standard deviations of the results of three independent trials between test and control groups. Student's *t* test was used when necessary to assess the statistical significance of the differences between test and control groups, where a *P* value of <0.05 was considered significant.

RESULTS AND DISCUSSION

Fraction selection of *L. acidophilus* La-5 CFSM by SEC. Communication among bacterial cells relies on signal molecules called autoinducers that accumulate in the extracellular space. These autoinducers allow EHEC O157 to regulate the expression of virulence-related genes. Preliminary findings demonstrated that EHEC O157 grown in the presence of CFSM of *L. acidophilus* strain La-5 showed significant inhibition in the expression of the *LEE* genes and AI-2 production by EHEC O157 (data not shown). Due to the high complexity of the *L. acidophilus* CFSM, it was not possible to exclude metabolic interference by other nonsignaling molecules present. Therefore, the CFSM mediator(s) of these effects could not be identified without further characterization. As a preliminary step, we fractionated the CFSM by SEC. We collected 80 fractions and identified fractions 33, 34, and 35 as having the greatest inhibitory activity on AI-2 production and *LEE* expression (Fig. 1 and 2). These fractions were selected for further investigation. It is important to point out that MRS medium had to be modified due to its interference with light detection. After determining that certain components of MRS medium masked luminescence detection, we prepared a series of modified MRS media and evaluated their effect on *L. acidophilus* growth. We found one mMRS medium that supported the growth of the probiotic LAB but did not inhibit luminescence detection.

Extracellular AI-2 activity is decreased by the addition of *L. acidophilus* La-5 CFSM. The *V. harveyi* BB170 bioassay was used to detect whether exogenous AI-2 signal molecules were present in the CFSM of EHEC O157 grown in the presence of

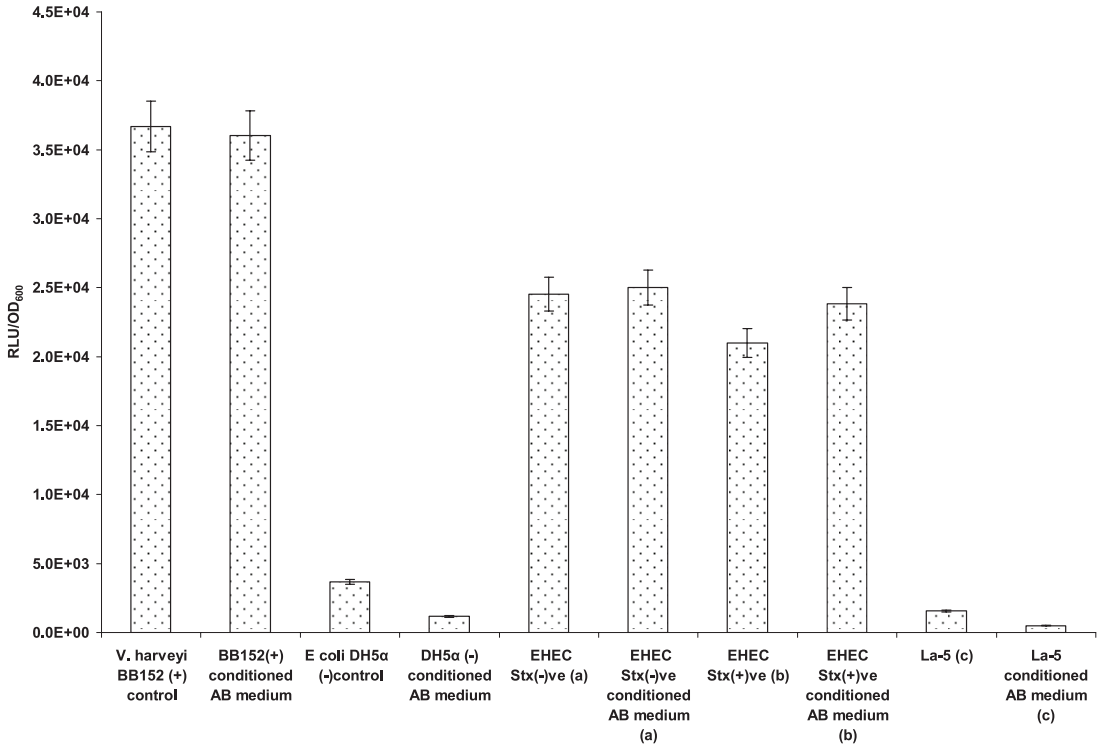

FIG. 4. EHEC O157 AI-2 signaling molecule production as detected by the *V. harveyi* AI-2 bioassay. The assay was performed in AB medium alone and in AB medium supplemented with *L. acidophilus* La-5 CFSM fraction 34 (conditioned AB medium). Positive and negative controls were *V. harveyi* strain BB152 (+) and *E. coli* DH5α (−), respectively. (a) EHEC O157 ATCC 43888. (b) EHEC O157 ATCC 43894. (c) EHEC O157 ATCC 43894 grown in LB broth supplemented with 10% *L. acidophilus* La-5 CFSM fraction 34. Results were expressed as relative light units (RLU) defined as counts/min^{-1} and adjusted to OD_{600} (RLU/OD_{600}). The data are the means ± the standard deviations of the results from three independent replicate trials with each sample.

L. acidophilus La-5 CFSM fractions. The sensor strain BB170 responds to the presence of exogenous AI-2 by induction of bioluminescence. As shown in Fig. 2, both EHEC O157 ATCC 43888 and ATCC 43894 produced significant amounts of AI-2 when grown in LB broth, but the amount of AI-2 signal molecules decreased when EHEC O157 was grown in LB broth supplemented with *L. acidophilus* La-5 CFSM fraction 34 (Fig. 4). These results indicated the presence of a molecules(s) in the *L. acidophilus* La-5 CFSM that is able to inhibit EHEC O157 AI-2 production or its detection by the *V. harveyi* sensor strain. The mechanism of action is not clear, but we suggest the possibility that *L. acidophilus* La-5 is somehow capable of quorum quenching EHEC O157. Although it is claimed that the LuxS/AI-2 QS system is not related to the activation of *LEE* genes in enterohemorrhagic *E. coli*, its absence in supernatant from EHEC treated with *L. acidophilus* La-5 coincides with downregulation of genes present in the *LEE* PAI (Table 3). This could be due to inhibition of the *luxS* gene, whose inactivation indirectly affects the synthesis of another autoinducer molecule named AI-3. AI-3 is responsible for activation of EHEC O157 virulence-related genes (42). If inhibition of *luxS* is affected in some way by a molecule(s) present in the CFSM of *L. acidophilus* strain La-5, then these results demonstrate that LuxS is involved in the regulation of important ecological attributes of EHEC O157.

***L. acidophilus* La-5 negatively regulates *LEE* expression.** Since the addition of *L. acidophilus* La-5 greatly decreased

TABLE 3. Band intensity profiles of the amplified EHEC O157 RNA samples

Gene and sample[a]	Band-adjusted vol (intensity · mm^2)[b]
luxS control	860 ± 275**
luxS La-5	320 ± 220**
qseA control	507 ± 186*
qseA La-5	79 ± 33*
ler control	697 ± 154*
ler La-5	210 ± 83*
espA control	1173 ± 186**
espA La-5	409 ± 141**
espD control	1188 ± 418*
espD La-5	402 ± 288*
tir control	293 ± 45**
tir La-5	60 ± 17**
eaeA control	696 ± 256*
eaeA La-5	244 ± 120*
fliC control	1564 ± 311**
fliC La5	557 ± 175**
hlyB control	1264 ± 187*
hlyB La5	521 ± 159*
$stxA_2$ control	376 ± 51
$stxA_2$ La-5	382 ± 31
$stxB_2$ control	837 ± 94
$stxB_2$ La-5	633 ± 35

[a] Samples were grown in LB broth alone (control) or in LB broth plus 10% (vol/vol) of *L. acidophilus* La-5 CFSM fraction 33 (La-5).

[b] Data are the means ± the standard deviations of the results of three replicate experiments. The expression levels of the studied genes were normalized according to the expression level of the housekeeping gene *tufA*. *, significant value ($P = 0.01$ to 0.05 [Student's *t* test]); **, very significant value ($P = 0.001$ to 0.01 [Student's *t* test]); no asterisk, not significant.

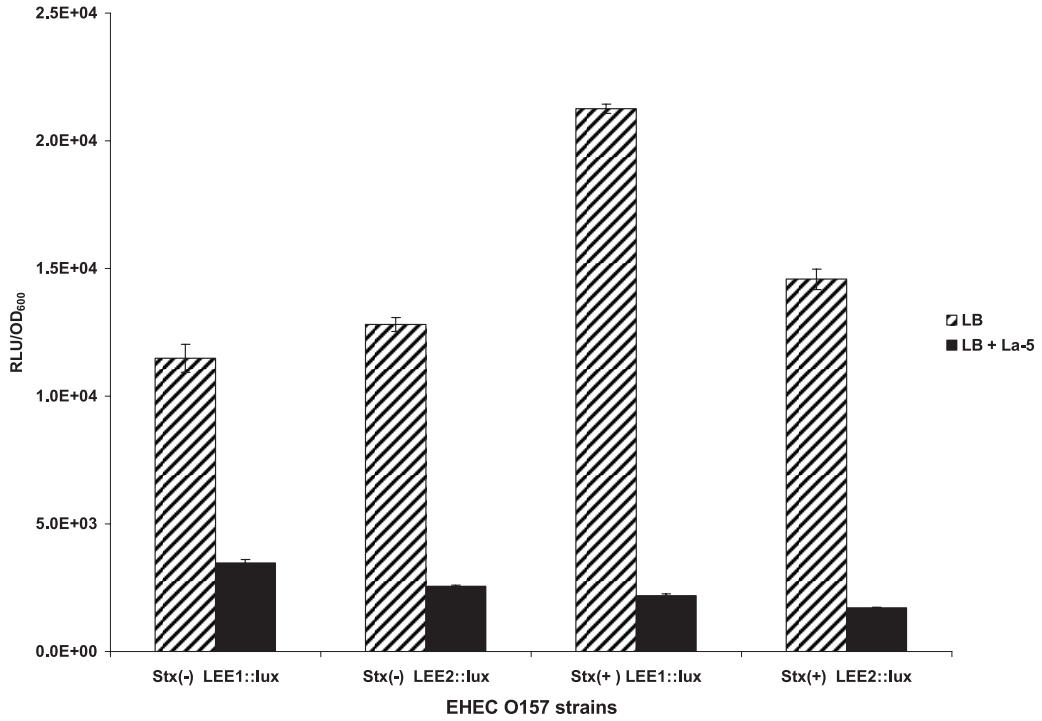

FIG. 5. Luminescence activity of *LEE*::*luxCDABE* fusion constructs in EHEC O157 when grown in LB broth (LB) and LB broth supplemented with 10% *L. acidophilus* La-5 CFSM fraction 34 (LB + La-5). EHEC O157 constructs were grown for 16 h and samples were measured as described in Materials and Methods. Results were expressed as relative light units (RLU) defined as counts min^{-1} and adjusted to OD_{600} (RLU/OD_{600}). The data are the means ± the standard deviations of the results from three independent replicate trials with each strain.

extracellular AI-2 activity in EHEC O157, we tested whether QS regulation of *LEE* genes was also affected. Fusions of *luxCDABE* were constructed to monitor the regulation of the *LEE1* and *LEE2* operons, which bear genes responsible for the formation of the AE lesion in intestinal epithelial cells. *LEE1* and *LEE2* were normally expressed when the constructs were grown in fresh LB broth, but light induction was significantly inhibited when the constructs were grown in LB broth supplemented with 10% of *L. acidophilus* La-5 CFSM fraction 34 (Fig. 5). These results demonstrate that the presence of the probiotic *L. acidophilus* CFSM negatively affects expression of the *LEE* operons. As measured by RT-PCR, the expression of important virulence-related genes of EHEC O157 was reduced in the presence of 10% *L. acidophilus* La-5 CFSM fraction 34 (Table 3). After RT-PCR analysis, the genes that showed statistically significant downregulation were *tir* ($P = 0.001$), *espA* ($P = 0.002$), *fliC* ($P = 0.005$), *espD* ($P = 0.012$), *luxS* ($P = 0.015$), *eaeA* ($P = 0.016$), *ler* ($P = 0.023$), *hlyB* ($P = 0.023$), and *qseA* ($P = 0.04$). The expression levels of Shiga toxin genes were not statistically different in both control and treated samples: $stxA_2$ ($P = 0.901$) and $stxB_2$ ($P = 0.27$).

***Lactobacillus acidophilus* strain La-5-secreted molecule(s) did not inhibit EHEC O157 Stx2 production.** EHEC O157 (ATCC 43894) grown in both fresh LB broth alone and in media containing *L. acidophilus* La-5 CFSM produces Stx, as demonstrated by the Stx2 ELISA (verotoxin 2 values [pg/cell] of 7.02E−08 ± 1.76E−08 and 5.33E−08 ± 2.60E−08, respectively; values are the means ± the standard deviations of the results from three replicate experiments; means were not significantly different [$P > 0.05$ by Student's *t* test]; Stx2 concentration was adjusted to μg/[CFU/ml] of each sample). Using RT-PCR, it was determined that the expression levels of $stxA_2$ and $stxB_2$ were not substantially different (Table 3). Shiga toxins are key virulence factors for EHEC and are encoded in the genomes of lysogenic lambdoid bacteriophages. In the human intestine, many factors contribute to induction of prophages and, thus, toxin production. These results indicated that the secreted compound(s) from *L. acidophilus* La-5 failed to prevent EHEC O157 Stx production. This information agrees with the fact that EHEC O157 uses an independent system to regulate Stx production and that QS might not be directly involved in Stx production. Although *L. acidophilus* La-5 does not reduce Stx production by EHEC O157, it clearly plays an important role in possibly reducing the ability of EHEC to colonize its host.

Conclusions. The present study provides evidence that *L. acidophilus* La-5 possesses strategies that interfere with QS regulation of food-borne pathogens such as EHEC O157. It is apparent that specific fractions of *L. acidophilus* La-5 CFSM substantially reduce the production of extracellular AI-2 molecules by EHEC O157, with a consequential reduction in EHEC O157 *LEE* expression. The mechanisms of action are currently unknown, but they may involve the production of a low-molecular-weight compound by the LAB that either binds to autoinducers (AI-2 or AI-3) or acts directly to prevent transcription of the *luxS* gene and important EHEC O157 virulence-related genes. Taken together, these observations suggest a model in which *L. acidophilus* La-5 acts to prevent EHEC O157 colonization. Such a model, if correct, has important clinical implications. If probiotic bacteria such as *L. acidophilus* La-5 can inhibit EHEC colonization, then it is

reasonable to consider them as a novel therapeutic strategy for EHEC treatment where antibiotic therapy is contraindicated. The need for new approaches is underscored by the fact that many antibiotics commonly used to treat diarrhea are known to induce bacteriophages and are associated with increased morbidity in patients with established EHEC infections (5, 46).

From this investigation, our current view is that this approach allows discrimination among *L. acidophilus* La-5-secreted compounds in terms of their ability to regulate the EHEC O157 QS system. It must be emphasized that this study represents only the first step in the identification of the *L. acidophilus* La-5 molecule(s) of interest; further characterization of the active molecule(s) is essential. Clearly, it is also important to consider data from tissue culture assays and animal models in order to study the effect of *L. acidophilus* La-5 on EHEC's ability to adhere to and cause AE lesions on mammalian cells in vitro and in vivo. This work is currently being undertaken.

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REFERENCES

1. **Alvarez-Olmos, M. I., and R. A. Oberhelman.** 2001. Probiotic agents and infectious diseases: a modern perspective on a traditional therapy. Clin. Infect. Dis. **32:**1567–1576.
2. **Anand, S. K., and M. W. Griffiths.** 2003. Quorum sensing and expression of virulence in *Escherichia coli* O157:H7. Int. J. Food Microbiol. **85:**1–9.
3. **Atalla, H. N., R. Johnson, S. McEwen, R. W. Usborne, and C. L. Gyles.** 2000. Use of a Shiga toxin (Stx)-enzyme-linked immunosorbent assay and immunoblot for detection and isolation of Stx-producing *Escherichia coli* from naturally contaminated beef. J. Food Prot. **63:**1167–1172.
4. **Bassler, B. L., E. P. Greenberg, and A. M. Stevens.** 1997. Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. J. Bacteriol. **179:**4043–4045.
5. **Bennish, M. L., W. A. Khan, M. Begum, E. A. Bridges, S. Ahmed, D. Saha, M. A. Salam, D. Acheson, and E. T. Ryan.** 2006. Low risk of hemolytic uremic syndrome after early effective antimicrobial therapy for *Shigella dysenteriae* type 1 infection in Bangladesh. Clin. Infect. Dis. **42:**356–362.
6. **Bernet-Camard, M. F., V. Lievin, D. Brassart, J. R. Neeser, A. L. Servin, and S. Hudault.** 1997. The human *Lactobacillus acidophilus* strain LA1 secretes a nonbacteriocin antibacterial substance(s) active in vitro and in vivo. Appl. Environ. Microbiol. **63:**2747–2753.
7. **Burton, C. L., S. R. Chhabra, S. Swift, T. J. Baldwin, H. Withers, S. J. Hill, and P. H. Williams.** 2002. The growth response of *Escherichia coli* to neurotransmitters and related catecholamine drugs requires a functional enterobactin biosynthesis and uptake system. Infect. Immun. **70:**5913–5923.
8. **Buts, J. P., N. De Keyser, and L. De Raedemaeker.** 1994. *Saccharomyces boulardii* enhances rat intestinal enzyme expression by endoluminal release of polyamines. Pediatr. Res. **36:**522–527.
9. **Chauvière, G., M. H. Coconnier, S. Kernéis, J. Fourniat, and A. L. Servin.** 1992. Adhesion of human *Lactobacillus acidophilus* strain LB to human enterocyte like Caco-2 cells. J. Gen. Microbiol. **138:**1689–1696.
10. **Chen, X., S. Schauder, N. Potier, A. V. Dorsselaer, I. Pelczer, B. L. Bassler, and F. M. Hughson.** 2002. Structural identification of a bacterial quorum-sensing signal containing boron. Nature **415:**545–549.
11. **Coconnier, M. H., M. F. Bernet, S. Kernéis, G. Chauvière, J. Fourniat, and A. L. Servin.** 1993. Inhibition of adhesion of enteroinvasive pathogens to human intestinal Caco-2 cells by *Lactobacillus acidophilus* strain LB decreases bacterial invasion. FEMS Microbiol. Lett. **110:**299–306.
12. **Coconnier, M. H., V. Lievin, M. F. Bernet-Camard, S. Hudault, and A. L. Servin.** 1997. Antibacterial effect of the adhering human *Lactobacillus acidophilus* strain LB. Antimicrob. Agents Chemother. **41:**1046–1052.
13. **Dahan, S., G. Dalmasso, V. Imbert, J. F. Peyron, P. Rampal, and D. Czerucka.** 2003. *Saccharomyces boulardii* interferes with enterohemorrhagic *Escherichia coli*-induced signaling pathways in T84 cells. Infect. Immun. **71:**766–773.
14. **DeLisa, M. P., C. F. Wu, L. Wang, J. J. Valdes, and W. E. Bentley.** 2001. DNA microarray-based identification of genes controlled by autoinducer 2-stimulated quorum sensing in *Escherichia coli*. J. Bacteriol. **183:**5239–5247.
15. **DeVinney, R., M. Stein, D. J. Reinscheid, A. Abe, S. Ruschkowski, and B. B. Finlay.** 1999. Enterohemorrhagic *Escherichia coli* O157:H7 produces Tir, which is translocated to the host cell membrane but is not tyrosine phosphorylated. Infect. Immun. **67:**2389–2398.
16. **Donnenberg, M. S., S. Tzipori, M. L. McKee, A. D. O'Brien, J. Alroy, and J. B. Kaper.** 1993. The role of the *eae* gene of enterohemorrhagic *Escherichia coli* in intimate attachment in vitro and in a porcine model. J. Clin. Investig. **92:**1418–1424.
17. **Elliott, S. J., L. A. Wainwright, T. K. McDaniel, K. G. Jarvis, Y. K. Deng, B. P. MacNamara, M. S. Donnenberg, and J. B. Kaper.** 1998. The complete sequence of the locus of enterocyte effacement (LEE) from enteropathogenic *E. coli* E2348/69. Mol. Microbiol. **28:**1–4.
18. **Freestone, P. P., P. H. Williams, R. D. Haigh, A. F. Maggs, C. P. Neal, and M. Lyte.** 2002. Growth stimulation of intestinal commensal *Escherichia coli* by catecholamines: a possible contributory factor in trauma-induced sepsis. Shock **18:**465–470.
19. **Kaper, J. B., S. J. Elliott, V. Sperandio, N. T. Perna, G. F. Mayhew, and F. R. Blattner.** 1998. Attaching-and-effacing intestinal histopathology and the locus of enterocyte effacement, p. 163–182. *In* J. B. Kaper and A. D. O'Brien (ed.), *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* strains. ASM Press, Washington, DC.
20. **Lievin-Le Moal, V., R. Amsellem, A. L. Servin, and M. H. Coconnier.** 2002. *Lactobacillus acidophilus* (strain LB) from the resident adult human gastrointestinal microflora exerts activity against brush border damage promoted by a diarrhoeagenic *Escherichia coli* in human enterocyte-like cells. Gut **50:**803–811.
21. **Mack, D. R., S. Michail, S. Wei, L. McDougall, and M. A. Hollingsworth.** 1999. Probiotics inhibit enteropathogenic *E. coli* adherence *in vitro* by inducing intestinal mucin gene expression. Am. J. Physiol. Gastrointest. Liver Physiol. **276:**G941–G950.
22. **Maldonado Galdeano, C., and G. Perdigón.** 2006. The probiotic bacterium *Lactobacillus casei* induces activation of the gut mucosal immune system through innate immunity. Clin. Vaccine Immunol. **13:**219–226.
23. **McDaniel, T. K., K. G. Jarvis, M. S. Donnenberg, and J. B. Kaper.** 1995. A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. Proc. Natl. Acad. Sci. USA **92:**1664–1668.
24. **Neeser, J.-R., D. Granato, M. Rouvet, A. Servin, S. Teneberg, and K.-A. Karlsson.** 2000. *Lactobacillus johnsonii* La1 shares carbohydrate-binding specificities with several enteropathogenic bacteria. Glycobiology **10:**1193–1199.
25. **Pothoulakis, C., C. P. Kelly, M. A. Joshi, N. Gao, C. J. O'Keane, I. Castagliuolo, and J. T. Lamont.** 1993. *Saccharomyces boulardii* inhibits *Clostridium difficile* toxin A binding and enterotoxicity in rat ileum. Gastroenterology **104:**1108–1115.
26. **Reid, G., D. Charbonneau, S. Gonzalez, G. Gardiner, J. Erb, and A. W. Bruce.** 2002. Ability of Lactobacillus GR-1 and RC-14 to stimulate host defences and reduce gut translocation and infectivity of *Salmonella* Typhimurium. Nutraceut. Food **7:**168–173.
27. **Reid, G., D. Charbonneau, J. Erb, B. Kochanowski, D. Beuerman, R. Poehner, and A. W. Bruce.** 2003. Oral use of *Lactobacillus rhamnosus* GR-1 and *L. fermentum* RC-14 significantly alters vaginal flora: randomized, placebo-controlled trial in 64 healthy women. FEMS Immunol. Med. Microbiol. **35:**131–134.
28. **Rigothier, M. C., J. Maccario, and P. Gayral.** 1994. Inhibitory activity of *Saccharomyces* yeasts on the adhesion of *Entamoeba histolytica* trophozoites to human erythrocytes *in vitro*. Parasitol. Res. **80:**10–15.
29. **Schauder, S., K. Shokat, M. G. Surette, and B. L. Bassler.** 2001. The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum sensing signal molecule. Mol. Microbiol. **41:**463–476.
30. **Schrezenmeir, J., and M. de Vrese.** 2001. Probiotics, prebiotics, and synbiotics—approaching a definition. Am. J. Clin. Nutr. **73**(Suppl.)**:**361S–364S.
31. **Servin, A. L., and M. H. Coconnier.** 2003. Adhesion of probiotic strains to the intestinal mucosa and interaction with pathogens. Best Pract. Res. Clin. Gastroenterol. **17:**741–754.
32. **Sherman, P. M., K. C. Johnson-Henry, H. P. Yeung, P. S. C. Ngo, J. Goulet, and T. A. Tompkins.** 2005. Probiotics reduce enterohemorrhagic *Escherichia coli* O157:H7- and enteropathogenic *E. coli* O127:H6-induced changes in polarized T84 epithelial cell monolayers by reducing bacterial adhesion and cytoskeletal rearrangements. Infect. Immun. **73:**5183–5188.
33. **Sperandio, V., J. L. Mellies, W. Nguyen, S. Shin, and J. B. Kaper.** 1999. Quorum sensing controls expression of type III secretion gene transcription and protein secretion in enterohemorrhagic and enteropathogenic *Escherichia coli*. Proc. Natl. Acad. Sci. USA **96:**15196–15201.
34. **Sperandio, V., A. G. Torres, J. A. Giron, and J. B. Kaper.** 2001. Quorum

sensing is a global regulatory mechanism in enterohemorrhagic *Escherichia coli* O157:H7. J. Bacteriol. **183:**5187–5197.
35. **Sperandio, V., A. G. Torres, and J. B. Kaper.** 2002. Quorum sensing *Escherichia coli* regulators B and C (QseBC): a novel two-component regulatory system involved in the regulation of flagella and motility by quorum sensing in *Escherichia coli*. Mol. Microbiol. **43:**809–821.
36. **Sperandio, V., A. G. Torres, B. Jarvis, J. P. Nataro, and J. B. Kaper.** 2003. Bacteria-host communication: the language of hormones. Proc. Natl. Acad. Sci. USA **100:**8951–8956.
37. **Sullivan, A., and C. E. Nord.** 2002. The place of probiotics in human intestinal infections. Int. J. Antimicrob. Agents **20:**313–319.
38. **Sullivan, A., and C. E. Nord.** 2002. Probiotics in human infections. J. Antimicrob. Chemother. **50:**625–627.
39. **Sullivan, A., and C. E. Nord.** 2005. Probiotics and gastrointestinal diseases. J. Intern. Med. **257:**78–92.
40. **Surette, M. G., and B. L. Bassler.** 1998. Quorum sensing in *Escherichia coli* and *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **95:**7046–7050.
41. **Surette, M. G., M. B. Miller, and B. L. Bassler.** 1999. Quorum sensing in *Escherichia coli*, Salmonella typhimurium, and *Vibrio harveyi*: a new family of genes responsible for autoinducer production. Proc. Natl. Acad. Sci. USA **96:**1639–1644.
42. **van de Guchte, M., S. D. Ehrlich, and E. Maguin.** 2001. Production of growth-inhibiting factors by *Lactobacillus delbrueckii*. J. Appl. Microbiol. **91:**147–153.
43. **Walters, M., M. P. Sircili, and V. Sperandio.** 2006. AI-3 synthesis is not dependent on *luxS* in *Escherichia coli*. J. Bacteriol. **188:**5668–5681.
44. **Wilson, K. H., and F. Perini.** 1988. Role of competition for nutrients in suppression of *Clostridium difficile* by the colonic microflora. Infect. Immun. **56:**2610–2614.
45. **Winson, M. K., S. Swift, P. J. Hill, C. M. Sims, G. Griesmayr, B. W. Bycroft, P. Williams, and G. S. Stewart.** 1998. Engineering the *luxCDABE* genes from *Photorhabdus luminescens* to provide a bioluminescent reporter for constitutive and promoter probe plasmids and mini-Tn5 constructs. FEMS Microbiol. Lett. **163:**193–202.
46. **Wong, C. S., S. Jelacic, R. L. Habeeb, S. L. Watkins, and P. I. Tarr.** 2000. The risk of the hemolytic-uremic syndrome after antibiotic treatment of *Escherichia coli* O157:H7 infections. N. Engl. J. Med. **342:**1930–1936.