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The effect of probiotics and organic acids on Shiga-toxin 2 gene expression in enterohemorrhagic *Escherichia coli* O157:H7

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Abstract

Probiotics are known to have an inhibitory effect against the growth of various foodborne pathogens, however, the specific role of probiotics in Shiga-toxin-producing *Escherichia coli* (STEC) virulence gene expression has not been well defined. Shiga toxins are members of a family of highly potent bacterial toxins and are the main virulence marker for STEC. Shiga toxins inhibit protein synthesis in eukaryotic cells and play a role in hemorrhagic colitis and hemolytic uremic syndrome. STEC possesses Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2), both of which have A and B subunits. Although STEC containing both Stx1 and Stx2 has been isolated from patients with hemorrhagic colitis, Stx2 is more frequently associated with human disease complications. Thus, the effect of *Lactobacillus*, *Pediococcus*, and *Bifidobacterium* strains on *stx2A* expression levels in STEC was investigated. Lactic acid bacteria and bifidobacteria were isolated from farm animals, dairy, and human sources and included *L. rhamnosus* GG, *L. curvatus*, *L. plantarum*, *L. jensenii*, *L. acidophilus*, *L. casei*, *L. reuteri*, *P. acidilactici*, *P. cerevisiae*, *P. pentosaceus*, *B. thermophilum*, *B. boum*, *B. suis* and *B. animalis*. *E. coli* O157:H7 (EDL 933) was coincubated with sub-lethal concentrations of each probiotic strain. Following RNA extraction and cDNA synthesis, relative s*tx2A* mRNA levels were determined according to a comparative critical threshold (Ct) real-time PCR. Data were normalized to the endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the level of *stx2A* expression between treated and untreated STEC was compared. Observed for all probiotic strains tested, *stx2A* was down-regulated, when compared to the control culture. Probiotic production of organic acids, as demonstrated by a decrease in pH, influenced *stx2A* gene expression.

Keywords: *Escherichia coli* O157:H7; Shiga-toxin; Probiotics; Organic acids; Quantitative real-time PCR

1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) serotype O157:H7, also known as Shiga toxin-producing *E. coli* (STEC), are responsible for foodborne outbreaks and sporadic diarrhea. Exposure to STEC in some patients can lead to hemorrhagic colitis and haemolytic uremic syndrome (HUS; (Kaper, 1998), both of which may lead to death. EHEC O157:H7 is estimated to cause 73,000 cases of disease per year in the United States (Mead et al., 1999). The majority of outbreaks attributed to *E. coli* O157:H7 have been associated with water and food. As antibiotic therapy in patients with STEC infections is regarded as controversial, probiotic therapy has increasingly been investigated as an alternative treatment.

Probiotics have been examined for their effectiveness in the prevention and treatment of antibiotic-associated diarrhea, as well as infectious bacterial and viral diarrhea. Although the mechanism of action is poorly understood, probiotics, such as *Lactobacillus* and *Bifidobacterium* strains, are known to exhibit an inhibitory effect against the growth of various enteric foodborne pathogens, including *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus*, *Clostridium perfringens*, *C. difficile*, and pathogenic *E. coli* (Meurman et al., 1995; Silva et al., 1987). Also a member of the lactic acid bacteria (LAB) group, pediococci are commonly associated with plants and their products (cabbage, sauerkraut, cucumbers), and have been used as starter cultures within the dry sausage industry. Recently, pigs displaying clinical and microbiological signs of *S. enterica* serovar Typhimurium

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infection were administered a mixture of five LAB strains, including *Pediococcus pentosaceus* (Casey et al., 2007). Animals treated with the LAB mixture had a reduced incidence, severity, and duration of diarrhea, illustrating the potential use of *Pediococcus* strains as a probiotic treatment. Acetic and lactic acid are major metabolites of LAB and have been reported to be responsible for their antimicrobial activity against *E. coli* in the intestine (Sinha, 1986). Ogawa et al. (2001) revealed that due to lactic acid production, probiotic *Lactobacillus* strains were able to inhibit *in vitro* growth of STEC. However, the specific role of probiotics in modulation of STEC virulence has not been well defined. A primary virulence marker in STEC, Shiga toxins (Stx1 and Stx2) inhibit protein synthesis in eukaryotic cells and play a role in hemorrhagic colitis and HUS (Robinson et al., 2006). Shiga toxins are composed of one enzymatic A subunit (*N*-glycosidase catalytic activity) and five receptor-binding B subunits. In most of the STEC strains isolated to date, the genes *stxAB* encoding the two subunits of Shiga toxin are located in the genomes of prophages of the lambda family. Although STEC containing both Stx1 and Stx2 has been isolated from patients with hemorrhagic colitis, Stx2 is more frequently associated with human disease complications. Previous DNA microarray analysis in our laboratory investigated the global transcriptional changes in *E. coli* O157:H7 EDL 933 gene expression induced by coincubation with *Lactobacillus rhamnosus GG* (LGG) (Kostrzynska et al., 2006). Results indicated that coincubation of *E. coli* O157:H7 EDL 933 with sub-lethal doses of LGG resulted in the down-regulation of *stx* genes. Consequently, the effect of *Lactobacillus*, *Pediococcus*, and *Bifidobacterium* strains from animal, dairy and human sources on *stx2* expression levels in STEC was investigated. The objective of this study was to investigate, using comparative real-time PCR, the effect of various lactic acid bacteria and bifidobacteria, as well as organic acids, on *stx2A* gene expression in *E. coli* O157:H7 EDL 933.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The strain of *E. coli* O157:H7 EDL 933 (ATCC 700927) was originally isolated from raw hamburger meat during a hemorrhagic colitis outbreak and produces both Stx1 and Stx2. Lactic acid bacteria and bifidobacteria were isolated from farm animals, dairy, and human sources and included *L. rhamnosus* GG (ATCC 53103), *L. curvatus* (FRP 15), *L. plantarum* (FRP 16), *L. jensenii* (ATCC 25258), *L. acidophilus* (FRP 728), *L. casei* (FRP 137), *L. reuteri* (ATCC 23272), *P. acidilactici* (ATCC 8081), *P. cerevisiae* (FRP 240), *P. pentosaceus* (FRP 243), *B. thermophilum* (ATCC 25525), *B. boum* (ATCC 27917), *B. suis* (27533) and *B. animalis* (719). FRP designates the Guelph Food Research Centre culture collection of Agriculture and Agri-Food Canada.

A single colony of each probiotic strain isolated on modified Mann-Rogosa-Sharpe (mMRS; 0.2g sodium carbonate, 0.1 calcium chloride in 1L distilled water and 5% (w/v) filter sterilized L-cysteine HCl monohydrate) agar plate was sub-cultured overnight at 37 °C in mMRS broth without agitation in a 5% CO_2 atmosphere. Similarly, a single colony of *E. coli* O157:H7 EDL 933 was grown overnight at 37 °C in LB broth with continuous agitation. Each probiotic strain and *E. coli* O157:H7 EDL 933 were sub-cultured (1/100) into fresh LB:mMRS (9:1) broth and incubated at 37 °C in 5% CO_2 atmosphere until reaching an optical density at 600 nm (OD_{600}) of 0.8 and 0.3, respectively. Each probiotic strain (5 ml) and *E. coli* O157:H7 EDL 933 (10 ml) were inoculated simultaneously in 20 ml total of fresh LB:mMRS (9:1) broth and coincubated for 2 h at 37 °C in 5% CO_2 atmosphere. *E. coli* O157:H7 EDL 933 incubated without probiotic bacteria was used as a control. *Bifidobacterium* species were grown and coincubated with *E. coli* under an anaerobic atmosphere using GasPak™ EZ Anaerobe Container System Sachets (Becton Dickinson and Company, Maryland, U.S.A.) in the GasPak™ EZ Incubation Container (Becton Dickinson and Company). Dry anaerobic indicators strips (Becton Dickinson and Company) were used to verify that an anaerobic atmosphere was attained. Samples were serially diluted in 0.1% (w/v) peptone water before and after the coincubation experiments and plated on mMRS agar (*Lactobacillus, Pediococcus* and *Bifidobacterium*) and Sorbitol-MacConkey (SMAC; *E. coli* 0157:H7) agar plates.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted using a silica gel-based membrane according to the manufacturer's instructions for Gram-negative bacteria (RNeasy RNA isolation mini kit, Qiagen, Mississauga, Ontario, Canada). RNA from lactic acid bacteria and bifidobacteria, which are Gram-positive, was not isolated using this protocol. In brief, 3.0 ml of each sample was incubated at room temperature for 5 min with 6.0 ml of RNAprotect Bacteria Reagent (Qiagen). Samples were centrifuged for 10 min at 4 °C at 5000 g. The pellet was resuspended in 100 µl of 1 mg/ml lysozyme (in TE buffer) and incubated at room temperature for 5 min. Following a vigorous vortex in 350 µl of RLT Buffer, the sample was centrifuged. The supernatant was mixed thoroughly with 100% ethanol and the lysate was applied to the RNeasy mini column. Following two washes with Buffer RW1 (700 µl), the column containing the lysate was washed with 500 µl of Buffer RPE. Residual ethanol was removed by centrifugation and the total RNA was eluted from the column with 45 µl of RNase-free water.

Contaminating DNA was removed from each RNA preparation using the Turbo DNA-free™ Kit (Ambion, Cambridge, UK), according to the manufacturer's instructions for rigorous DNase treatment. RNA quantity (A_{260}) and quality ($A_{260/280}$ ratio) were measured spectrophotometrically in molecular grade water and 10 mM Tris–HCl (pH 7.5), respectively.

Synthesis of cDNA was conducted using the SuperScript™ II Reverse Transcriptase kit (Invitrogen, Burlington, Ontario, Canada). In brief, 2.5 µg of RNA was reverse transcribed with 1 µg/µl random hexamer primers (Amersham, NJ, USA), 10 mM each dNTP (Invitrogen), 4 µl 5× First Strand Buffer (Invitrogen), 2 µl of 0.1 M DTT (Invitrogen), 40U RNasin

(Fisher), and 200U SuperScript II reverse transcriptase (Invitrogen). For each DNase treated RNA sample, a no-reverse transcriptase control (NRTC) was included. cDNA synthesis was performed in a DNA Engine Peltier Thermal Cycler (model PTC-200, BioRad Laboratories, Toronto, Ontario, Canada) with the following cycling conditions: 65 ˚C for 5 min, 25 ˚C for 2 min, 25 ˚C for 10 min, 42 ˚C for 50 min and followed by 70 ˚C for 15 min to inactivate the enzyme.

2.3. Real-time PCR

The cDNA (1 μl) was used as template for real-time PCR amplification in 25-μl final volume, containing 12.5 μl of Power SYBR® Green PCR Master Mix (Applied Biosystems, Toronto, Ontario, Canada), 500 nM of each primer, 0.25U AmpErase® Uracil *N*-glycosylase (UNG; Applied Biosystems) and nuclease-free water. Forward and reverse s*tx2A* primers were 5′-TTGCTGTGGATATACGAGGGC-3′ and 5′-TCC-GTTGTCATGGAAACCG-3′, respectively. Forward and reverse GAPDH primers were 5′-TCCGTGCTGCTCAGAA-ACG-3′ and 5′CACTTTCTTCGCACCAGCG-3′, respectively. Real-time PCR conditions included: 1 cycle at 50°C for 2 min to activate UNG, 1 cycle at 95°C for 10 min and 40 cycles at 95°C for 15 s; 63°C for 1 min; 72°C for 30 s, followed by melt curve analysis which involved heating the products to 95°C for 1 min, followed by cooling to 55°C and slowly heating to 95°C while monitoring fluorescence. Fluorescence data were collected at the end of each cycle on a MX4000® Multiplex Quantitative PCR System (Stratagene, La Jolla, CA). Relative mRNA levels were determined according to the comparative critical threshold (Ct) real-time PCR (separate tube), as described by Applied Biosystems User Bulletin No. 2 (P/N 4303859). Treated and untreated controls were amplified in separate wells. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize input amounts of RNA and the level of *stx2A* expression between treated and untreated STEC was compared.

2.4. Organic acids and effect of pH

To evaluate the effect of acetic acid, lactic acid and hydrochloric acid on s*tx2A* expression, *E. coli* O157:H7 was grown for 2h in LB:mMRS (9:1) with varying concentrations (freshly prepared) of each acid. To evaluate the effect of pH on *stx2A* expression, reagent grade glacial acetic acid (A.S.C. 99.7%, Fisher Scientific) was used to adjust the pH of LB: mMRS (9:1) media, covering a pH range of 4.0–5.4. Adjusted media were freshly prepared and filter sterilized. Plate counts were obtained on SMAC agar plates. RNA extraction, RT- and real-time PCR were performed as described above. Additionally, the supernatant pH was measured using an Accumet® Research AR15 pH meter (Fisher Scientific) prior to and following coincubation with each lactic acid bacterium.

2.5. Statistical analysis

Coincubation of *E. coli* O157:H7 EDL 933 with each lactic acid bacterium was investigated by three independent trials. Real-time RT-PCR analysis was conducted in duplicate for each trial. Statistical significance between treatment and control conditions was assessed by the Student's *t* test. A significant difference was defined as a P value of <0.05.

3. Results

3.1. Real-time PCR controls

Real-time PCR was used to assess s*tx2A* gene expression, using GAPDH as the endogenous control. Ct values were not significantly different between the control and treatment procedure for each lactic acid bacterium ($P>0.05$) and similar amplification efficiencies (close to 1) were observed between *stx2A* and GAPDH (data not shown). No Ct values were obtained after 40 cycles of PCR (data not shown) for both no template control (NTC) and no-reverse transcriptase control

Table 1
E. coli O157:H7 EDL 933 and probiotic bacteria plate counts (cfu/ml; mean±SD; $n=3$) with and without coincubation treatment with selected probiotic strains

Probiotic	Control *E. coli*[a] (T_0)[b]	Control *E. coli* (T_2)[c]	Coincubation STEC (T_2)
B. animalis	$(1.09\pm1.12)\times10^8$	$(1.05\pm5.82)\times10^8$	$(8.53\pm3.83)\times10^7$
B. boum	$(4.11\pm3.24)\times10^7$	$(1.03\pm1.32)\times10^8$	$(7.70\pm3.73)\times10^7$
B. suis	$(5.27\pm3.61)\times10^7$	$(8.40\pm3.60)\times10^7$	$(7.03\pm5.21)\times10^7$
B. thermophilum	$(8.48\pm5.46)\times10^7$	$(1.16\pm7.33)\times10^8$	$(5.10\pm2.78)\times10^7$
L. acidophilus	$(2.95\pm1.49)\times10^7$	$(1.58\pm2.83)\times10^8$	$(8.65\pm3.61)\times10^7$
L. casei	$(1.64\pm7.44)\times10^7$	$(8.93\pm1.39)\times10^7$	$(4.05\pm6.21)\times10^7$
L. curvatus	$(1.17\pm9.18)\times10^7$	$(1.12\pm5.40)\times10^8$	$(2.63\pm2.75)\times10^7$
L. jensenii	$(7.24\pm4.90)\times10^7$	$(1.26\pm1.12)\times10^8$	$(8.27\pm7.41)\times10^7$
L. plantarum	$(4.82\pm5.78)\times10^7$	$(1.30\pm2.35)\times10^8$	$(3.27\pm1.84)\times10^7$
L. reuteri	$(3.30\pm3.71)\times10^7$	$(8.63\pm8.00)\times10^7$	$(3.73\pm3.45)\times10^7$
L. rhamnosus GG	$(1.98\pm1.32)\times10^7$	$(1.14\pm1.24)\times10^8$	$(1.68\pm1.31)\times10^8$
P. acidilactici	$(1.07\pm4.13)\times10^8$	$(7.97\pm8.16)\times10^7$	$(7.17\pm2.47)\times10^7$
P. cerevisae	$(9.42\pm4.79)\times10^7$	$(7.63\pm8.36)\times10^7$	$(1.90\pm1.45)\times10^7$
P. pentosaceus	$(9.42\pm4.79)\times10^7$	$(7.63\pm8.36)\times10^7$	$(7.00\pm3.61)\times10^6$

[a] *E. coli* O157:H7 EDL 933.
[b] T_0 represents *E. coli* O157:H7 EDL 933 plate count (cfu/ml) prior to coincubation with LAB or bifidobacteria.
[c] T_2 represents *E. coli* O157:H7 EDL 933 plate count (cfu/ml) after 2-h coincubation with LAB or bifidobacteria.

(NRTC). As well, primer dimers were not generated with either GAPDH or *stx2A* primer sets, as demonstrated by the NTC SYBR Green dissociation curves (results not shown).

3.2. Effect of Lactobacillus, Pediococcus and Bifidobacterium strains on E. coli O157:H7 EDL 933 stx2A expression

The inhibitory effect of each probiotic bacterial strain on the growth of *E. coli* O157:H7 EDL 933 was evaluated by coincubating each strain with STEC, with subsequent plating on mMRS and SMAC agar plates, respectively. Plate counts (Table 1) between treated and untreated *E. coli* O157:H7 EDL 933 were not significantly different ($P>0.05$), suggesting *stx2A* down-regulation was attributed to an inhibitory effect of probiotics and not cell death.

All *Lactobacillus* (Fig. 1A), *Pediococcus* (Fig. 1B), and *Bifidobacterium* (Fig. 1C) strains tested down-regulated *stx2A*, however, the probiotic effect on s*tx2A* expression was strain specific. The greatest *stx2A* down-regulation (mean±SD) was observed following a 2-h coincubation of *E. coli* O157:H7 with *L. rhamnosus* GG (LGG; 0.405±0.048; $P=0.002$), *B. thermophilum* (0.424±0.260; $P=0.016$) and *P. pentosaceus* (0.428±0.170; $P=0.028$).

3.3. Effect of pH and acid on stx2 expression

The effect of pH on s*tx2A* expression was determined by real-time PCR. The pH of coincubated samples was significantly lower ($p<0.05$) than the control culture for *B. thermophilum*, *L. casei, L. curvatus L. plantarum*, *L.* reuteri, *LGG*, *P. acidilactici*,

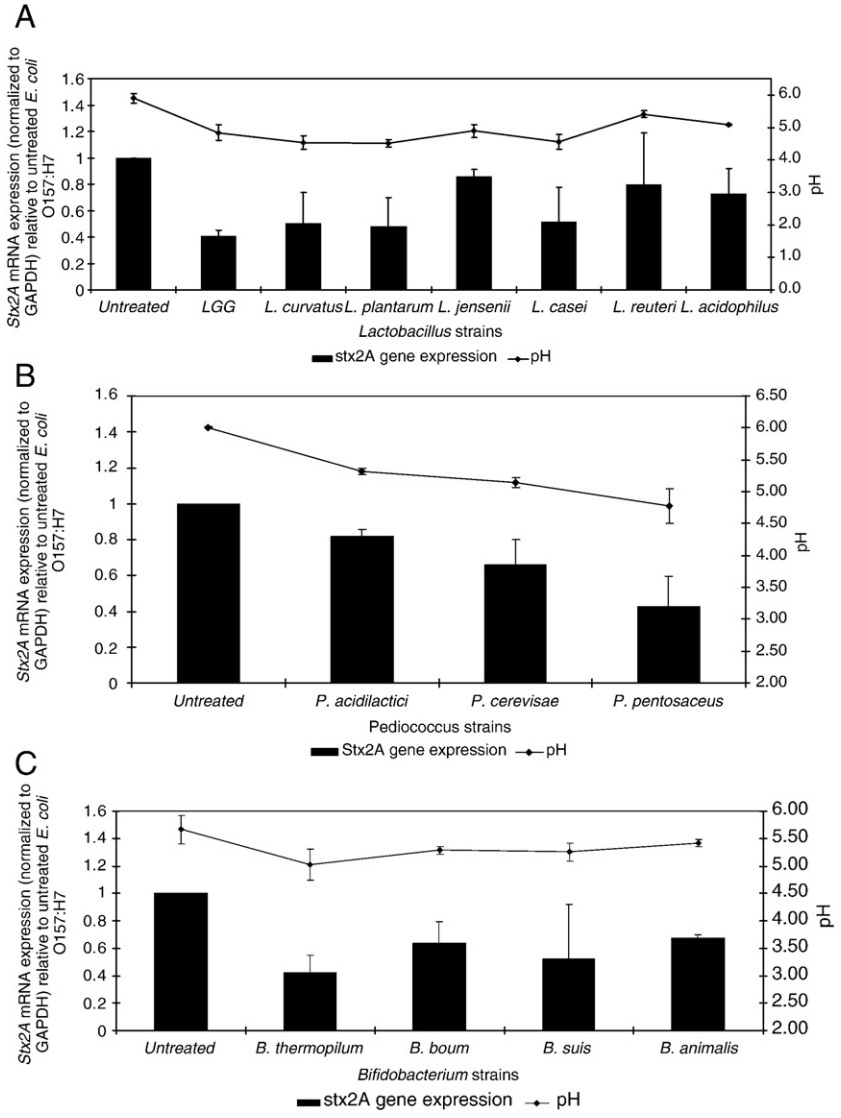

Fig. 1. The effect of *Lactobacillus* (A), *Pediococcus* (B) and *Bifidobacterium* (C) strains on *E. coli* O157:H7 EDL 933 *stxA2* gene expression using comparative real-time PCR. The housekeeping gene GAPDH was used to normalize input amounts of RNA and the level of *stx2* expression between treated and untreated STEC was compared. Corresponding pH values following the 2-h coincubation are indicated for each strain. Error bars shown represent the standard deviations from triplicate replicates of each sample.

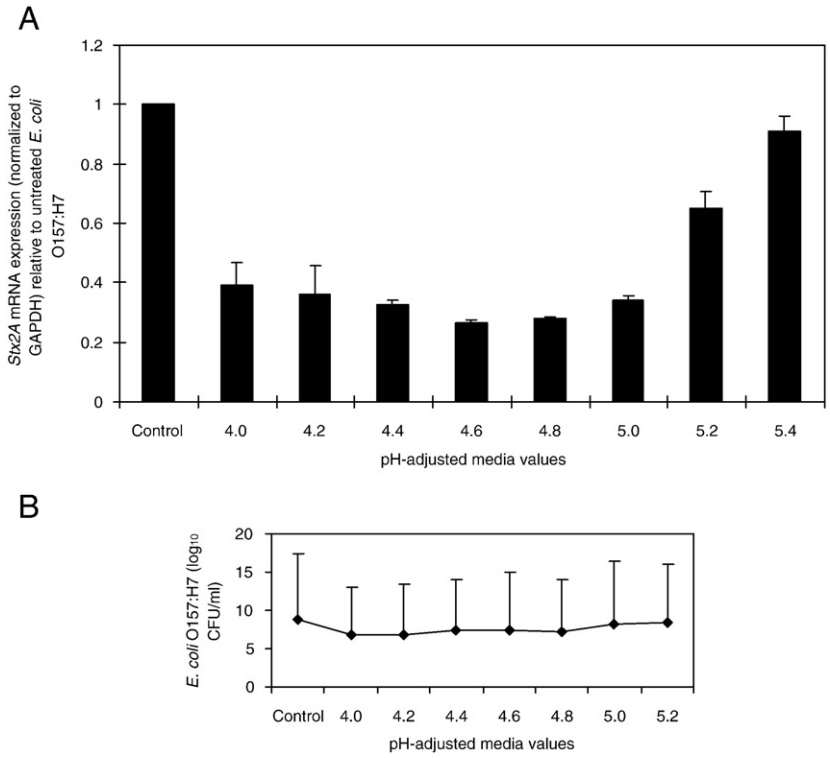

Fig. 2. (A) The effect of acetic acid pH-adjusted media on *E. coli* O157:H7 EDL 933 *stx2A* gene expression using comparative real-time PCR. The housekeeping gene GAPDH was used to normalize input amounts of RNA and the level of *stx2A* expression between treated and untreated STEC was compared. (B) The effect of acetic acid pH-adjusted media on *E. coli* O157:H7 EDL 933 plate counts (CFU/ml). Plate counts obtained on SMAC are indicated for each pH treatment. Error bars shown represent the standard deviations from triplicate replicates of each sample.

P. cerevisae, and *P. pentosaceus*. Co-incubation of *E. coli* O157:H7 with *L. plantarum*, *B. thermophilum* and *P. pentosaceus* resulted in the greatest *stx2A* down-regulation and caused the greatest decrease in media pH, as compared to the control culture (Figs. 1A–C).

The effect of acetic, lactic and hydrochloric acid on *stx2A* expression and *E. coli* O157:H7 plate counts were evaluated. In general, *stx2A* gene expression decreased as the concentration of organic acid increased and the greatest down-regulation was observed with acetic acid at a concentration of 10 mM (Fig. 3). Compared to the untreated control, *E. coli* O157:H7 cell growth was reduced for all three acids investigated, beginning at 5 mM concentration. The greatest reduction in cell growth occurred with 10 mM acetic acid (1.47 log).

To confirm the contribution of lowered pH on *stx2A* expression, LB:mMRS media was pH-adjusted with glacial acetic acid. Significant ($P<0.05$) *E. coli* O157:H7 EDL 933 *stx2A* down-regulation corresponded to pH measurements between 4.4 and 5.0 (Fig. 2), whereby the greatest down-regulation was observed for *E. coli* O157:H7 EDL 933 grown in media with lower pH values.

4. Discussion

Various studies have suggested that probiotic bacteria have an inhibitory effect on the growth of EHEC (Duncan et al., 1998; Hirano et al., 2003; Zhao et al., 1998), however, the role of probiotic bacteria on *E. coli* O157:H7 *stx* expression has not been well defined. In the present study, the effect of *Lactobacillus*, *Pediococcus*, and *Bifidobacterium* strains on *stx2A* expression levels in STEC was investigated.

Sensitive methods are required to effectively measure *stx* expression under various conditions. Comparison of a Stx-specific ELISA, conventional PCR and real-time PCR for the detection of STEC in human stool specimens indicated that both molecular methods were significantly more effective than ELISA (Pulz et al., 2003). Consequently, due to the increased sensitivity and specificity, real-time PCR was used in this study. Quantitative real-time PCR monitors the fluorescence emitted by accumulating PCR products following each cycle and as such, the signal intensity is proportional to the concentration of target in the sample. Reverse-transcriptase PCR has previously been used to study *stx* expression in viable STEC (McIngvale et al., 2002). Quantification of mRNA expression levels using real-time reverse transcription PCR (RT-PCR) has increasingly been used for more detailed analysis and confirmation of DNA microarray results (Li et al., 2002; Wang et al., 2006), as the method is amenable to high throughput analysis with a shorter turn around time, and represents a more accurate and sensitive method to investigate gene expression. Compared to commercial and home brewed microarray platforms (3–4 orders of magnitude; (Shippy et al., 2004; Stefano et al., 2005; Tan et al., 2003), quantitative real-time PCR has a larger dynamic range (6–8 orders of magnitude; (Yang et al., 2004; Zhao et al., 2005),

thereby offering increased sensitivity in detecting fold changes in gene expression. As such, our study was based on initial DNA microarray analysis to screen multiple *E. coli* O157:H7 gene targets. Results indicated that pre-incubation of *E. coli* O157:H7 EDL 933 with sub-lethal doses of LGG resulted in the down-regulation of *stx* genes (Kostrzynska et al., 2006). These results were confirmed in the present study by a comparative critical threshold (C_T) method, whereby LGG significantly down-regulated *E. coli* O157:H7 EDL 933 *stx2A* expression (0.405 ± 0.048; $P = 0.006$).

Acidic pH and short chain fatty acids, particularly lactic acid and acetic acid, have been used as food preservatives to prevent the growth of contaminating organisms (Abdul Raouf et al., 1993). Consequently, probiotic bacteria have been used to mitigate the effects of foodborne pathogens. For instance, lactobacilli are believed to interfere with pathogens by various mechanisms, including the production of antimicrobial compounds, such as organic acids, dihydrogen peroxide, or bacteriocin-like substances (McGroarty and Reid, 1988). Studies have demonstrated that lactic acid is able to cause sub-lethal injury to *E. coli* (Roth and Keenan, 1971) and similar properties have also been observed with acetic acid (Przybylski and Witter, 1979). While many investigations have focused on lactobacilli inhibition of *E. coli* adherence and growth *in vitro* (Lee et al., 2003; McGroarty and Reid, 1988), few studies have evaluated the effect of lactobacilli on *E. coli* virulence factors, such as Shiga toxins. In a recent study by Medellin-Pena et al. (2007), EHEC O157 virulence expression was evaluated in response to chromatographically selected fractions of *Lactobacillus acidophilus* La-5 cell-free spent medium (CFSM). Shiga toxins were among the virulence factors investigated. As measured by densitometry of reverse-transcribed PCR products (using the housekeeping gene *tufA*), expression s*tx2A* and s*tx2B* were not significantly different from the control samples. An enzyme immunoassay also indicated that the CFSM did not inhibit Stx2 production in EHEC O157.

Takahashi et al. (2004) evaluated an alternative probiotic, the butyric-acid producing *Clostridium butyricum* MIYAIRI strain 588, on EHEC O157:H7 infection *in vitro* and *in vivo* using gnotobiotic mice. Flow-cytometric analysis indicated that pre-incubation with *C. butyricum* inhibited EHEC O157:H7 adhesion to Caco-2 cells. As well, gnotobiotic mice prophylactically treated with *C. butyricum* resulted in significantly lower Stx1 and Stx2 titres from fecal samples two days after infection with EHEC, as compared to EHEC mono-associated mice ($P < 0.01$). Results indicated that *C. butyricum* had preventative and therapeutic effects on EHEC O157:H7 infection in gnotobiotic mice. The inhibitory effect on EHEC growth may be due to the *C. butyricum* production of short chain fatty acids. The antibacterial effects of butyric and lactic acid were demonstrated in a dose-dependent manner *in vitro*, whereby the antibacterial effect of lactic acid was demonstrated at low pH and butyric acid at low pH and pH 7.0. In our study, *stx2A* expression was down-regulated in dose dependent manner with acetic acid. These results indicate the importance of organic acids in Stx production and *stx2* gene expression. Consequently, further research investigating the production of organic acids by probiotics selected in this study would be beneficial to fully understand the probiotic mechanism(s) attributing to *stx2* down-regulation.

Possible mechanisms responsible for antagonistic effects between bacteria within the intestinal ecosystem include competition for nutrients or adhesions sites, production of bacteriocins or volatile fatty acids. The differing degrees of *stx2A* expression observed in our study may be attributed to the type and amount of organic acid produced by each probiotic strain. Organic acids, such as acetic acid, citric acid and lactic acid, have been reported to possess a higher bactericidal activity than inorganic acids, such as hydrochloric acid (Brocklehurst and Lund, 1990; Eklund, 1983). Additionally, bactericidal activity of organic acids depends primarily on the undissociated form of the acid (Eklund, 1983). Ogawa et al. (2001) revealed that not all *Lactobacillus* species exerted a growth inhibitory and bactericidal activity on STEC, which is likely a consequence of the pH value and concentration of undissociated acid. Similarly, Yuk and Marshall (2005) demonstrated that verotoxin production depended on both pH and type of organic acid since the amounts of verotoxin produced by organic-acid adapted *E. coli* O157:H7 differed at equivalent initial pH values. Brocklehurst and Lund (1990) and Eklund (1983) reported that short chain fatty acids, particularly acetic, lactic and citric acid, possessed greater bactericidal activity then inorganic acids, such as hydrochloric acid. The antibacterial effect of organic acids is due to the ability of undissociated molecules to pass through the cell membrane and ionize to release protons in the cytoplasm, thereby depressing intracellular pH and inhibiting metabolism (Diez-Gonzalez and Russell, 1997).

Bifidobacteria have recently been studied for their efficacy in the prevention and treatment of various animal and human gastrointestinal disorders (Farnworth et al., 2007; Leahy et al., 2005; Servin and Coconnier, 2003). Feeding probiotic bifidobacteria to experimental animals has previously been observed to prevent Gram-negative bacterial infections (Nader de Macias et al., 1992; Shu et al., 2000; Silva et al., 1999). In particular, Asahara et al. (2004) used a fatal mouse STEC infection model and determined that the anti-infectious activity of probiotic bifidobacteria against STEC O157:H7 was related to the combinatory effect of high acetic acid concentration and low pH. In our study, addition of organic acids (acetic and lactic acid) resulted in a greater decrease *stx2A* gene expression, then inorganic acid (HCl). In general, s*tx2A* gene expression decreased as the concentration of organic acid increased and the greatest down-regulation was observed with acetic acid at a concentration of 10 mM (Fig. 3).

Acid tolerance is likely an important component of *E. coli* virulence. While studies have investigated acid tolerance of *E. coli* O157:H7 at low pH (Conner and Kotrola, 1995) or survival in acidic foods, few studies have looked as the effect of low pH conditions, particularly as a result of organic acids, on *stx* expression. As previously mentioned, the anti-bactericidal effect of probiotics is believed to be related to the production of organic acids. An *in vitro* study by Ogawa et al. (2001), revealed that certain *Lactobacillus* strains, exhibited an inhibitory effect on STEC. The authors suggested that the bactericidal effect of

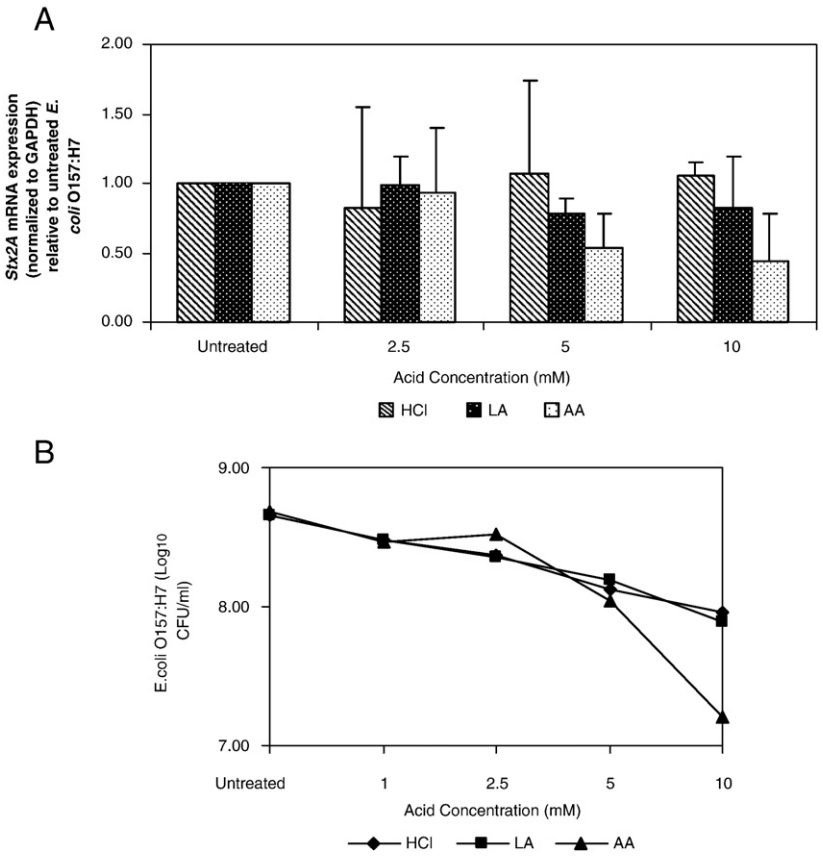

Fig. 3. The effect of lactic acid (LA), acetic acid (AA) and hydrochloric acid (HCl) on *E. coli* O157:H7 *stx2A* gene expression (A) and plate counts (B). Untreated samples were cultured in LB:mMRS without the addition of organic acids. $N=3$.

Lactobacillus on STEC depended on lactic acid production and a pH reductive effect. In particular, this study revealed that *L. casei* strain Shirota and *L. acidophilus* YIT 0070 exerted growth inhibition and a bactericidal effect when coincubation with STEC for 14 h. Additionally, the pH value and undissociated lactic acid concentration of the culture medium of STEC coincubated with *L. casei* or *L. acidophilus* was dramatically lower when compared with those of the control culture. Similar results were observed in our study, whereby a larger decrease in s*tx2* gene expression was related to those probiotics causing a larger drop in pH, as compared to the control. Conner and Kotrola (1995) demonstrated that *E. coli* O157:H7 growth inhibition varied depending on the temperature and type of acid tested. In general, growth at 25 and 37 ˚C in TSB with 0.6% yeast extract, the acids were less inhibitory, as compared to lower pH values with the 10 ˚C storage temperature. In general, the inhibitory pH values were considerably lower at higher temperatures, such that *E. coli* O157:H7 growth inhibition at 37 ˚C occurred at pH 4.0 and 4.5 for lactic and acetic acid, respectively. In our study, compared to the control, *E. coli* growth inhibition in media adjusted with acetic acid had not occurred at pH 4.5. Previous research showed that some strains of *E. coli* are able to survive at pH values as low as 2.5 (Benjamin and Datta, 1995; Small et al., 1994), but it does not grow at pH values less than 4.4 (Lin et al., 1995).

In conclusion, all *Bifidobacterium, Lactobacillus* and *Pediococcus* strains tested down-regulated *stx2A*, however, the probiotic effect on s*tx2A* expression was strain specific. Results indicated that the population of lactic acid bacteria and bifidobacteria used in this study did not elicit a bactericidal effect on *E. coli* O157:H7, however, at this population level a sub-lethal effect was observed indicating potential use as a prophylactic treatment against *E. coli* O157:H7. The greatest *stx2A* down-regulation was observed following a 2-h coincubation of *E. coli* O157:H7 with *L. rhamnosus* GG, *B. thermophilum* and *P. pentosaceus*. Results suggest that *stx2A* down-regulation may be related to pH effect as a result of organic acids produced by the probiotic bacteria. Consequently, a more in depth analysis of the specific organic acids produced by above-mentioned probiotic bacteria is needed to fully understand the probiotic mechanism responsible for the inhibitory effect on *stx2* expression in STEC.

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