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ORIGINAL ARTICLE

Survival and expression of acid resistance genes in Shiga toxin-producing *Escherichia coli* acid adapted in pineapple juice and exposed to synthetic gastric fluid

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Abstract

Aims: The aim of this research was to determine the ability of non-O157 Shiga toxin-producing *Escherichia coli* (STEC) serogroups to survive with exposure to synthetic gastric fluid (SGF) after adaptation to pineapple juice (PJ) at room and refrigerated temperatures compared to *E. coli* O157:H7 and to examine the relative transcriptional expression of acid resistance (AR) genes, *rpoS*, *gadA* and *adiA*.

Methods and Results: Resistant and sensitive strains belonging to five different STEC serogroups (O26, O103, O104, O111 and O157; $n = 10$) were used in this study. All strains were adapted in PJ (pH 3·8) stored at 4 and 20°C for 24 h, and then the relative transcription levels of genes in all strains were quantified using a real-time quantitative-PCR assay. After adaptation in PJ, the STEC strains were exposed to SGF (pH 1·5 and 2·0) at 37°C for 2 h. Generally, the STEC adapted in PJ at 4°C displayed enhanced survival compared to acid adaptation in PJ at 20°C and nonadapted controls with exposure to SGF ($P < 0·05$). Moreover, resistant strains exhibited higher survival rates compared to sensitive strains ($P < 0·05$). Overall, adaptation at 4°C resulted in significantly ($P < 0·05$) enhanced gene expression levels in PJ, and transcript levels of *gadA* were higher than those of the *rpoS* and *adiA* genes.

Conclusions: The up-regulation of AR genes due to adaptation in PJ at low temperature may increase STEC survival in acidic environments such as the gastrointestinal tract. Some non-O157 STEC strains, including serotypes O103:H2 and O111:H8, showed relatively high AR levels similar to those of STEC O157:H7.

Significance and Impact of the Study: Induction of AR genes in acidic fruit juice, and potentially in other acidic foods may increase the risk of foodborne illness by non-O157 STEC serogroups.

Introduction

Acidified foods have a low pH due to the addition of acidic ingredients to produce a final equilibrium pH of 4·6 or below. Foods that have a natural pH of 4·6 or below are classified as acid foods (Food and Drug Administration 2000). Acid or acidified products are considered safe because of their low pH; however, they might pose a health risk due to the presence of acid-resistant foodborne pathogens (Weagant *et al.* 1994; Breidt *et al.* 2004; Yuk and Marshall 2004; Oh *et al.* 2009). Reports of outbreaks from acid-resistant *Escherichia coli* O157:H7 in

fruit juice have increased (Vojdani *et al.* 2008), and there is evidence that the organism can utilize cellular and genetic mechanisms to promote its survival in acidic environments (Abee and Wouters 1999; Foster 2000).

Recently, an increasing incidence of non-O157 Shiga toxin-producing *E. coli* (STEC)-associated outbreaks has been observed (Free *et al.* 2012). Non-O157 STEC can induce illnesses similar to those caused by O157:H7, including diarrhoea and haemorrhagic colitis, as well as haemolytic uremic syndrome (Smith and Fratamico 2012). In addition, a few studies have shown that non-O157 STEC serogroups have an ability to survive in fruit juice or the gastrointestinal tract similar to *E. coli* O157:H7 (Bergholz and Whittam 2007; Usaga *et al.* 2014). There have been studies examining acid resistance (AR) of *E. coli* O157:H7 in fruit juice and the gastrointestinal tract; however, there is little of information concerning the survival of non-O157 STEC serogroups when exposed to various acidic conditions.

Pasteurization will generally eliminate pathogens in juices, but possible scenarios for outbreaks caused by contaminants include consumption of improperly or non-pasteurized juice or juice contaminated after pasteurization (Beuchat 1996). *Escherichia coli* O157:H7 can adapt to acidic conditions in the presence of organic acids in fruit juices, allowing survival during storage. Subsequently, the acid-adapted cells might have a greater ability to survive conditions in the human gastrointestinal tract and proliferate in the intestines to levels sufficient for causing illnesses (Yuk *et al.* 2008).

Escherichia coli O157:H7 possesses three well-described AR systems, which allow the pathogen to survive in acid environments, including the gastrointestinal tract (Foster 2000). These include an oxidative AR system (AR1), which is associated with the production of acid-shock proteins and the alternative stress sigma factor, RpoS (sigma factor; σ^s) and the σ^s-independent systems, which are the glutamate (AR2) and arginine (AR3) decarboxylase AR systems that involve removal of excess protons from the cytoplasm using glutamate and arginine respectively (Yuk *et al.* 2008). Price *et al.* (2004) reported that the σ^s-dependent system is more important for survival of *E. coli* O157:H7 in apple cider but that the σ^s-independent systems are required for its survival in the bovine gastrointestinal tract. The mechanisms used by non-O157 STEC to resist acid environments likely are similar to those used by O157:H7 strains. However, one study demonstrated that some non-O157 STEC strains utilize a chaperone-based acid stress response (HdeA and HdeB) to combat acidic conditions, which is lacking in *E. coli* O157:H7 (Smith and Fratamico 2012).

Therefore, it is critical to thoroughly examine the physiological differences, as well as gene expression levels between O157:H7 and non-O157 STEC serogroups that may affect their survival in acidic foods and the gastrointestinal tract. The aim of this research was to determine the survival of O157:H7 and non-O157 STEC serotypes exposed to synthetic gastric fluid (SGF) at different pH values after adaptation to pineapple juice (PJ) and to quantify the relative transcription levels related to AR genes in multiple serotypes. Data from this study will determine if acid-adapted non-O157 STEC strains have an ability to survive exposure to SGF comparable to that of O157:H7 strains.

Materials and methods

Strains preparation

We chose acid-resistant (O26:H11 B444, O103:H2 B458, O104:H4 B469, O111:H8 B478, O111:H$^-$ B472 and O157:H7 B493) and sensitive (O26:H11 B447, O103:H25 B459, O104:H4 B466 and O157:H7 B492) STEC strains belonging to five serogroups/serotypes, totalling 10 strains, showing a high (<1 log CFU reduction) and low (≥1 log CFU reduction) level of survival with exposure to acetic acid solution as defined in our previous study (Kim *et al.* 2015). All resistant and sensitive strains selected showed reductions in the range from 0·31 to 0·91 and 3·69 to 5·02 log reduction CFU ml^{-1} respectively. *Escherichia coli* O111:H$^-$ B472 was the most sensitive strain out of seven serogroup O111 strains tested showing 0·82 log reduction in CFU ml^{-1}, and the other O111 strain (B478) evaluated in this study showed a reduction of 0·31 CFU ml^{-1} in acetic acid solution. Kim *et al.* (2015) reported that the O111 serogroup strains had greater overall AR compared to the other serogroups tested.

The strains were from the culture collection at the USDA Agricultural Research Service Eastern Regional Research Center (Wyndmoor, PA). Prior to use, working cultures of all strains were prepared in tryptic soy broth (TSB; BD Biosciences, San Jose, CA) at 37°C with two consecutive transfers after 18 h of growth to obtain stationary-phase cells. Each culture was washed twice in phosphate buffered saline (PBS; Daejung Chemical, Daejueon, Korea; pH 7·2), centrifuged (3000 ***g***, 10 min, 4°C) and finally suspended in PBS. Actual starting concentrations were confirmed by plating serial dilutions onto tryptic soy agar (TSA; BD Biosciences).

Sample preparation

For the preparation of PJ, fresh pineapples were purchased from a local market in Chuncheon, South Korea, and stored at 4°C until used. After peeling, the pineapples were then blended, the mixture was prefiltered using

Whatman paper (Whatman International Ltd., Brentford, UK), and then re-filtered using a 0·22 μm syringe filter (Fisher Scientific, Pittsburgh, PA). The sterile PJ had a pH of 3·8 measured using a pH meter (ISTEK, Seoul, Korea) and was stored at 4°C until use.

Survival in PJ

Three different conditions, including PJ at 4°C (pH 3·8), PJ at 20°C (pH 3·8) and PBS (pH 7·2) at 4°C were evaluated for determining survival of STEC strains. A 0·1 ml aliquot of cells at 10^9 CFU ml^{-1} was transferred into 9·9 ml of each solution in a sterile 15 ml screw cap tube to achieve approx. 10^7 CFU ml^{-1}. After inoculation, the tubes were vortexed for 30 s. The inoculated test tubes were incubated at 4 and 20°C for 15 days under static conditions. A 0·1 ml sample was taken from each test tube at 0 h and 1, 3, 5, 7, 10 and 15 days. After making serial dilutions, samples were plated onto TSA and incubated at 37°C for 24 h to enumerate colonies. Inoculated PJ at 0 h served as a control.

Survival in SGF

The SGF (pH 1·5 and 2·0) was prepared as described (Beumer *et al.* 1992), and included per litre: 8·3 g proteose-peptone (Difco, Detroit, MI), 3·5 g D-glucose (Sigma Chemical Co., St. Louis, MO), 2·05 g NaCl (Fisher Scientific), 0·6 g KH_2PO_4 (Sigma), 0·11 $CaCl_2$ (Fisher Scientific), 0·37 g KCl (Sigma), 0·05 g bovine bile (Sigma), 0·1 g lysozyme (Sigma) and 13·3 mg of pepsin (Sigma). The final pH was adjusted to 1·5 and 2·0 with HCl. Solutions were autoclaved separately except for lysozyme and pepsin, which were filter-sterilized, before being combined with the rest of the SGF components. The SGF was prepared as a single batch and was stored at 4°C for 30 min. Previous experiments showed that storage of the SGF at 4°C for up to 7 days did not result in significant changes in bactericidal properties (Tamplin 2005). *Escherichia coli* cells were pretreated in PJ (acid adapted) or in PBS (pH 7·0, control) for 24 h, and 1 ml aliquots containing approx. 10^7 CFU ml^{-1} were added to bottles containing 99 ml of SGF, prewarmed to 37°C. Survival was monitored at intervals for 30 min up to 120 min by withdrawing 1 ml portions of the SGF-cell suspensions, serially diluting in 0·85% saline, plating onto TSA, and then incubating at 37°C for 24 h, prior to enumeration. The detection limit was 1 log CFU ml^{-1}.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from the cell pellets using the High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) with DNase I treatment (Roche Diagnostics) according to the manufacturer's instructions for Gram-negative bacteria. The total RNA concentration was determined by measuring the A_{260} using a NanoDrop 2000 UV spectrophotometer (Thermo Scientific, Wilmington, DE). The RNA quality was determined to be acceptable if the $A_{260/280}$ ratio was between 1·8 and 2·0. RNA was reverse transcribed using the Power cDNA Synthesis Kit (iNtRO Biotechnology, Sungnam, Kyunggi, Korea) according to the manufacturer's instructions. In brief, 1 μg of total RNA was added to 9·5 μl of sterile water in an RNase-free microfuge tube and was reverse transcribed with 1 μl of a random primer (included with the kit) at 75°C for 5 min, and then spun briefly to collect the solution at the bottom of the tube. After the samples were placed on ice for at least 1 min, the following reagents were added in the following order: 1 μl RNase inhibitor, 4 μl 5× RT buffer, 2 μl dNTP, 2 μl DTT and 0·5 μl AMV RT enzyme. cDNA synthesis was performed in a MyGenie 32 Thermal Block (Bioneer, Dajeon, Korea) with the following cycling conditions: 42°C for 60 min followed by 70°C for 5 min to terminate the reaction.

Real-time quantitative polymerase chain reaction

The expression of AR genes in O157 and non-O157 STEC strains in response to exposure to PJ at pH 3·8 for 24 h at 4 and 20°C was evaluated using Real-time quantitative polymerase chain reaction (RT q-PCR) conducted using the StepOne™ Real-Time PCR System (Applied Biosystems, Foster city, CA), based on SYBR Green. Each 25 μl reaction contained 3 μl of reverse-transcribed cDNA, 12·5 µl of Power SYBR Green PCR Mix, 1 μl (0·5 mol l^{-1}) of each primer, and nuclease-free water. The Power SYBR Green PCR Master Mix contained SYBR Green I Dye, AmpliTaq Gold DNA polymerase LD, dNTPs with a dUTP/dTTP blend, passive reference dye (ROX), and optimized buffer components, as described by the manufacturer. The sequences of the primers used in this study are given in Table 1. Thermal cycling conditions were as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 60 s. A final extension of 72°C for 5 min was employed. All PCR reactions were performed in triplicate. A melting curve analysis was performed at the end of the PCR run over the range 60–95°C, increasing the temperature stepwise by 0·5°C every 30 s. The baseline, threshold cycle (C_T), and relative quantification (RQ) were automatically determined using the STEPONE Software (ver. 2.0; Applied Biosystems). The analysis for relative AR gene expression levels for O157:H7 and non-O157 STEC strains was based on the comparative C_T ($2^{-\Delta\Delta C_T}$) method

Table 1 PCR primers used in real-time q-PCR gene expression assays

AR system	Target	Primer pair (5′–3′)	Amplicon size	References
Reference gene (housekeeping gene)	16S rRNA	F: GAATGCCACGGTGAATACGTT R: ACCCACTCCCATGGTGTGA	302	Parry-Hanson *et al.* (2010)
Oxidative or glucose-depressed	*rpoS*	F: GAATAGTACGGGTTTGGTTCATAAT R: GCGTTGCTGGACCTTATC	498	Parry-Hanson *et al.* (2010)
Glutamate decarboxylase	*gadA*	F: GGACCAGAAGCTGTTAACGGATTT R: GCGATAGTAGAAATGGCCTTTGC	180	Bergholz and Whittam (2007)
Arginine decarboxylase	*adiA*	F: CGAGAGCTCACCATGGAAGTATTAATTGTTG R: CGCGGTACCTTACGCTTTCACGCACAT	232	Giles and Graham (2007)

(Schmittgen and Livak 2008). Expression levels for *rpoS*, *gadA*, and *adiA* genes were measured for cells held at 4 and 20°C at pH 3·8 in PJ for 24 h. Relative gene expression levels were normalized using expression of 16s rRNA as the endogenous control (Wong and Medrano 2005).

Statistical analysis

The mean values and standard deviations for CFU ml^{-1} were calculated from triplicate experiments with duplicate determinations. All survival data were compared with *t*-test and one- or two-way ANOVA followed by Tukey's *post hoc* test. The RT q-PCR data were log-transformed in order to obtain normally distributed residuals. For all statistical analyses, $P < 0{\cdot}05$ was considered statistically significant.

Results

Survival of non-O157 STEC with exposure to PJ

To examine the survival of non-O157 STEC strains at different temperatures in PJ, one sensitive and one resistant non-O157 STEC strain were chosen based on acetic acid challenge studies (Kim *et al.* 2015). PJ was selected as an acidic food model because of its naturally low pH, and PJ has been linked to infections by pathogenic *E. coli* O157:H7 (Uljas and Ingham 1998; Vojdani *et al.* 2008).

As shown in Fig. 1, the population of B478 (O111:H8, resistant strain) was reduced from 6·50 to 6·21 log CFU ml^{-1} after 15 days of storage in PJ (pH 3·8) at 4°C. However, the populations of strain B447 (O26:H11, sensitive strain) decreased from 6·59 to 5·64 log CFU ml^{-1} after 15 days of storage with about 0·95 log CFU ml^{-1} reduction. A greater effect on inactivation of the non-O157 STEC strains was observed when the PJ was incubated at 20°C. After 15 days of storage at 20°C, the populations of B478 and B447 changed from 6·40 to 4·64 and from 6·60 to 3·69 respectively. Both strains did not show a significant ($P > 0{\cdot}05$) difference among all storage conditions up to 7 days except for strain B447 in PJ at 20°C ($P < 0{\cdot}05$), which showed a 1·14 log CFU ml^{-1} reduction. After 10 days, differences were evident between strain B478 and B447 stored in PJ at 4°C, and storage in PJ at 4°C and in PJ at 20°C in strain B478 ($P < 0{\cdot}05$). Survival of both strains was greater at 4°C than 20°C ($P < 0{\cdot}05$). When we performed an acetic acid challenge on O111 and O26 serogroups,

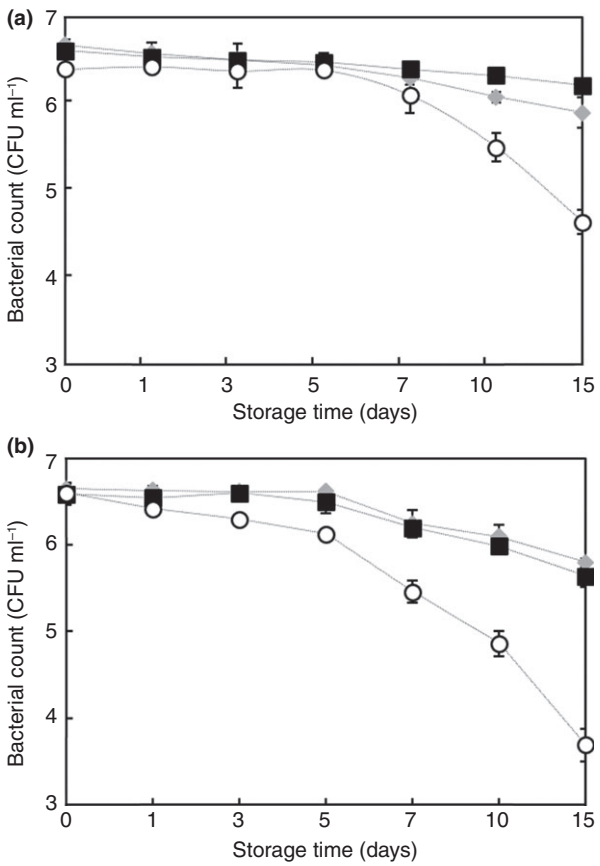

Figure 1 The survival curves of STEC B478 (O111:H8; acid-resistant; a) and B447 (O26:H11; acid-sensitive; b) in pineapple juice (PJ; pH 3·8) at 4°C (■), PJ at 20°C (○) and PBS (pH 7·2) at 4°C (◆) for 15 days. Each data point was represented by mean ± SD ($n = 3$).

including O111 B478 and O26 B447 at 20 and 30°C, the survival at lower temperature was significantly increased (Kim *et al.* 2015). Thus, these two strains showed enhanced survival at lower temperatures in both PJ and acetic acid solution.

Survival of PJ-adapted non-O157 STEC with exposure to SGF

The effect of acid adaptation on survival of five resistant STEC strains without O111:H$^-$ B472 strain following exposure to SGF is shown in Figs 2 and 3. Acid-resistant STEC strains PJ-adapted at 4°C showed approximately a 2 log reduction in CFU ml^{-1} after 90 min exposure to SGF at pH 1·5, except for O26:H11 B444, which had no viable cells (detection limit of 1 log CFU ml^{-1}; Fig. 2b). All STEC strains PJ-adapted at 20°C had less than 2·83 log CFU ml^{-1} reduction after 60 min exposure in SGF at pH 1·5 (Fig. 2c). In SGF pH 2·0 after 120 min, there was no significant difference ($P > 0·05$) between results from cells with prior exposure to PJ at 4 and 20°C (Fig. 3b,c). With exposure to SGF at pH 2·0, all STEC strains showed greater survival compared to exposure to SGF at pH 1·5. Pre-adaptation at low temperature in PJ had a significant ($P < 0·05$) effect on the survival of STEC strains compared to cells at room temperature (20°C) exposed to SGF regardless of serotype. In general, the same pattern of survival was found with two non-O157 STEC strains tested during storage in PJ (Fig. 1). Of note, O157:H7 B493 survived better compared to the other serotypes under all of the conditions, except for O103:H2 B458 non-adapted control exposed to SGF at pH 2·0, which showed higher survival than strain B493 with 3·45 and 3·00 log CFU ml^{-1} survival respectively (Fig. 3a).

The effect of acid adaptation on survival of sensitive STEC strains and O111:H$^-$ B472 strain following exposure to SGF is shown in Figs 4 and 5. The O111:H$^-$ B472 strain was included with sensitive STEC strains in Figs 4 and 5 because it showed sensitivity to SGF. Those STEC strains were inactivated rapidly; within 60 min of exposure to SGF at pH 1·5. The nonadapted controls of the sensitive STEC and O111:H$^-$B472 strains were inactivated within 30 min at pH 1·5 to undetectable cell populations (PBS at 4°C), except for O157:H7 B492, which showed a survival of 3·24 log CFU ml^{-1} at 30 min but was undetectable by 60 min (Fig. 4a). No viable cells of all strains PJ-adapted at 4 and 20°C were detected after 90 min of SGF exposure at pH 1·5 (Fig. 4a,b). However, STEC strains PJ-adapted at 4°C exposed to SGF showed enhanced survival compared to adaptation at 20°C ($P > 0·05$). No viable cells of O26:H11 B447 and O104:H4 B466 were detected after 30 min of SGF exposure at pH 1·5 and 2·0; these data were not shown in Figs 4 and 5. Of note, O111:H$^-$ B472 (O111 strain that was less resistant than O111:H8 B478) PJ-adapted at 20°C showed a survival of 1·92 log CFU ml^{-1} with exposure to SGF at pH 1·5 after 60 min, which was a higher survival than that of the sensitive strains (<1·00 for O103:H25 B459 and 1·06 for O157:H7 B492; Fig. 4c). On the other hand, O111:H$^-$ B472 showed a lower survival than resistant strains with a rate of >2·00 log CFU ml^{-1} (Figs 2c and

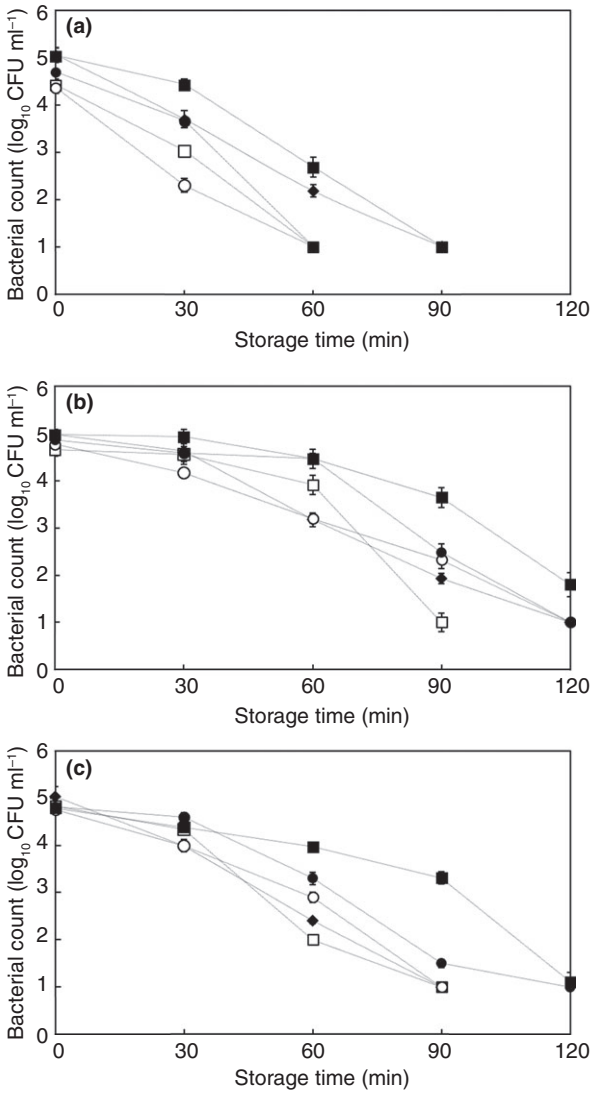

Figure 2 Survival curves of resistant STEC strains in PBS (nonadapted control; pH 7·2 at 4°C; a), pineapple juice-adapted at 4°C (b) and pineapple juice-adapted at 20°C (c) for 24 h exposure to simulated gastric fluid (pH 1·5). Each data point was represented by mean ± SD ($n = 3$). Detection limit was <1 log CFU ml^{-1}. O26:H11 B444 (□), O103:H2 B458 (♦), O104:H4 B469 (○), O111:H8 B478 (●), O157:H7 B493 (■).

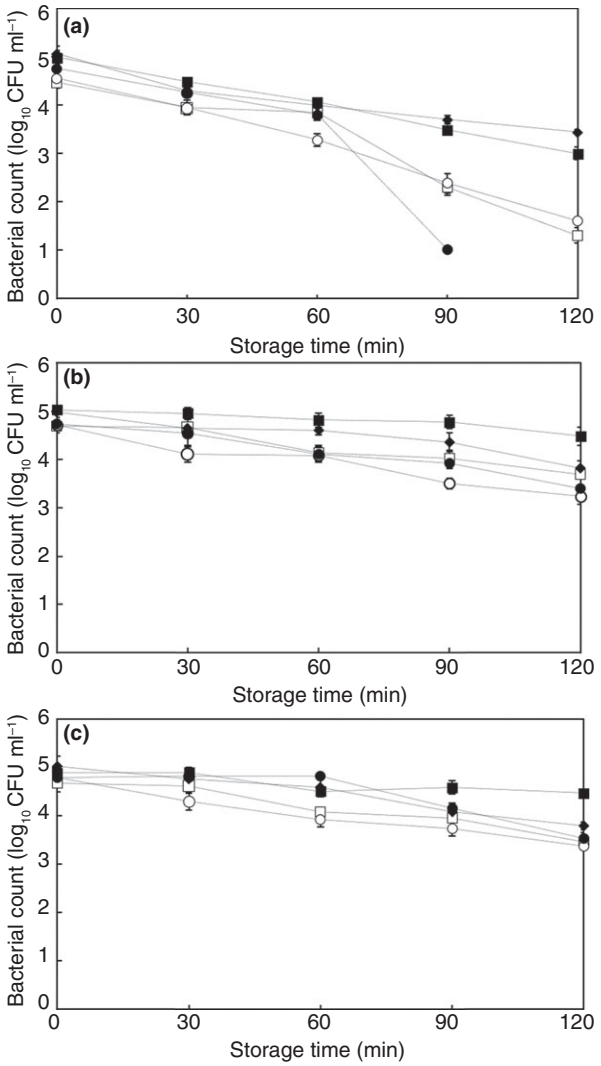

Figure 3 Survival curves of resistant STEC strains in PBS (nonadapted control; pH 7·2 at 4°C; a), pineapple juice-adapted at 4°C (b) and pineapple juice-adapted at 20°C (c) for 24 h exposure to simulated gastric fluid (pH 2·0). Each data point was represented by mean ± SD (n = 3). Detection limit was <1 log CFU ml^{-1}. O26:H11 B444 (□), O103:H2 B458 (♦), O104:H4 B469 (○), O111:H8 B478 (●), O157:H7 B493 (■).

4c). A comparison of both strains of serogroup O111 showed a significant ($P < 0·05$) difference in survival with exposure to SGF at pH 1·5 for 60 min (1·92 and 3·30 log CFU ml^{-1} for O111:H^{-} B472 and O111:H8 B478 respectively). Therefore, although these O111 strains both were resistant with exposure to acetic acid showing a decrease in log CFU ml^{-1} of <1·00, exposure to PJ that contains citric and malic acids, may have resulted in differences in adaptation with resultant tolerance to SGF, pH 1·5, being different between the two strains.

Relative quantification of AR gene expression

Transcript levels of AR genes (*rpoS*, *gadA* and *adiA*) following exposure of the cells to PJ were measured using RT q-PCR, and results are illustrated in Figs 6, 7, and 8. RQ values were expressed as the fold change of expression levels ($\log_2$) of STEC adapted with PJ compared to O104:H4 B466 as the nonadapted control, which had the lowest survival in SGF, pH 1·5 and 2·0. The results demonstrated that STEC cells PJ-adapted at 4°C had a significant ($P < 0·05$) increase in the expression of *rpoS*, *gadA* and *adiA* compared to the nonadapted control and cells that were PJ-adapted at 20°C. The transcript levels of *gadA* were higher than those of *rpoS* and *adiA* genes in cells PJ-adapted to 4°C. Furthermore, all of the resistant STEC strains tested in SGF had greater transcription levels of *rpoS* and *gadA* than sensitive STEC strains.

Specifically, the RQ values of *rpoS* ranged from 1·21 to 2·62 in PJ at 4°C and were higher than in cells exposed to the other conditions. O111:H^{-} B472 had greater relative levels of transcription in PJ at 4°C with a mean RQ of 2·62; however, no significant difference in RQ values was observed with acid-resistant strains tested with SGF ($P > 0·05$). With respect to the RQ values of *gadA*, they ranged from 2·14 to 12·33 and a significant induction was observed for O157:H7 B493 and O103:H2 B458 in PJ at 4°C compared to the other strains and conditions with a mean RQ value of 12·33 and 10·60 respectively; however, there was significant difference between those trains ($P < 0·05$). The expression levels of *adiA* showed a similar trend to those of *rpoS* and *gadA* genes, but the levels were lower compared to *rpoS* and *gadA*. The RQ values of the *adiA* gene ranged from 1·24 to 2·01 in PJ at 4°C. O103:H2 B458 showed the highest expression of *adiA* compared to other strains, with an RQ value of 2·01; however, no significant difference of RQ values was observed with all STEC serotypes ($P > 0·05$). The expression of *adiA* was repressed in both cells treated with PJ at 20°C and the un-treated control. The results revealed a significant decrease in the expression of *rpoS*, *gadA*, and *adiA* genes in nontreated cells and with the PJ at 20°C treatment ($P < 0·05$).

Discussion

This study evaluated differences in storage temperature and serotype as factors affecting AR of non-O157 STEC in PJ and SGF. To observe the effect of storage temperature on AR, each serotype was inoculated into PJ and stored at refrigerated (4°C) and room temperature (20°C) for 24 h to induce acid adaptation under the acidic environment. Adaptation in PJ at 4°C increased AR greater than in PJ at 20°C and non-adapted controls

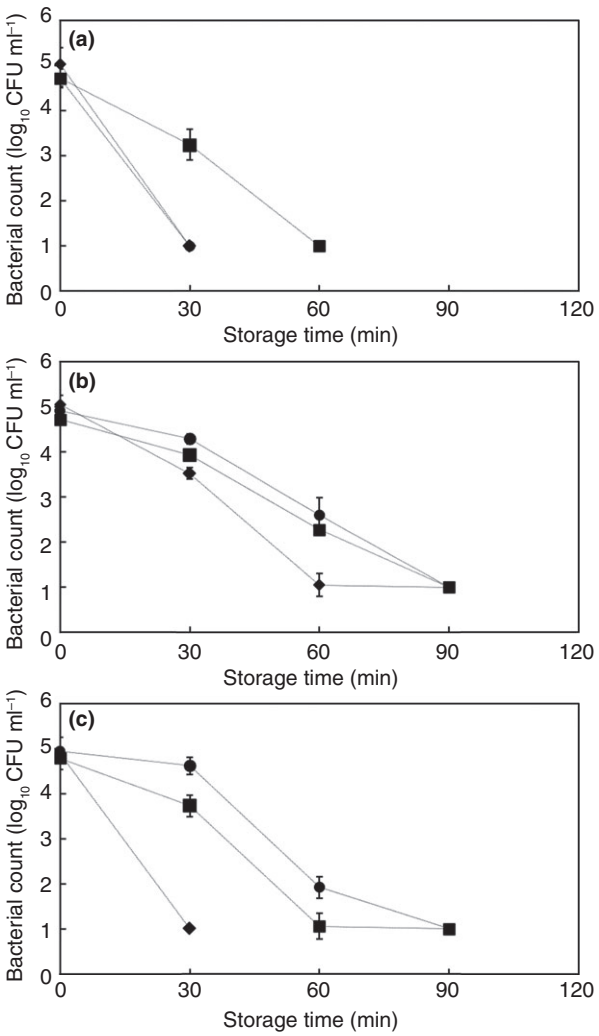

Figure 4 Survival curves of sensitive STEC strains (O103:H25 B459; ●, O157:H7 B492; □) and O111:H$^-$ B472 (■; resistant with exposure to acetic acid solution) in PBS (nonadapted control; pH 7·2 at 4°C; a), pineapple juice-adapted at 4°C (b) and pineapple juice-adapted at 20°C (c) for 24 h exposure to simulated gastric fluid (pH 1·5). No viable cells of O26:H11 B447 and O104:H4 B466 were detected after 30 min of SGF exposure at pH 1·5 and 2·0; these data were not shown. Each data point was represented by mean ± SD (n = 3). Detection limit was <1 log CFU ml^{-1}.

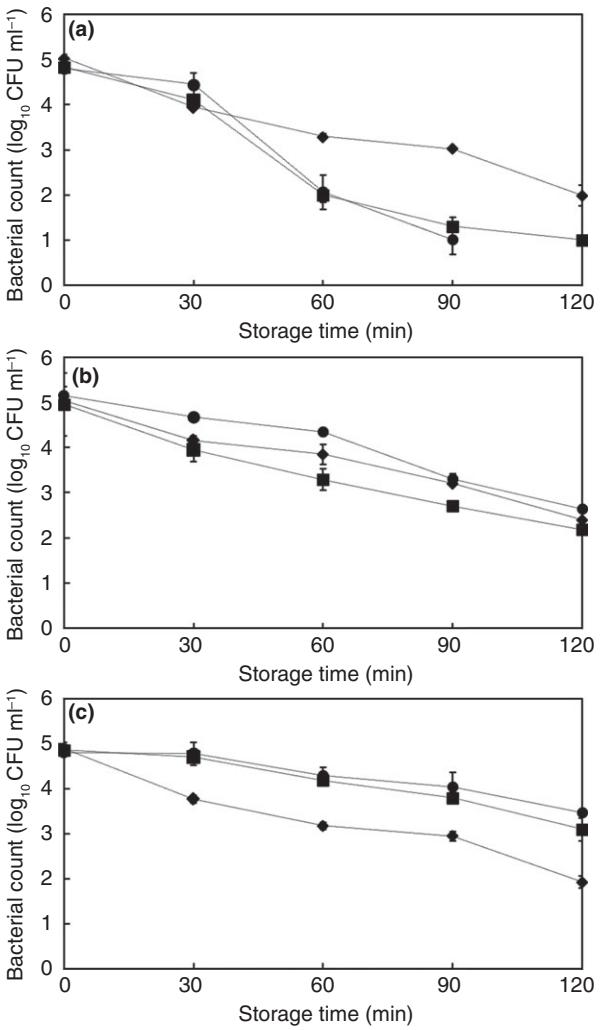

Figure 5 Survival curves of sensitive STEC strains (O103:H25 B459; ●, O157:H7 B492; □) and O111:H$^-$ B472 (■; resistant with exposure to acetic acid solution) in PBS (nonadapted control; pH 7·2 at 4°C; a), pineapple juice-adapted at 4°C (b) and pineapple juice-adapted at 20°C (c) for 24 h exposure to simulated gastric fluid (pH 2·0). No viable cells of O26:H11 B447 and O104:H4 B466 were detected after 30 min of SGF exposure at pH 1·5 and 2·0; these data were not shown. Each data point was represented by mean ± SD (n = 3). Detection limit was <1 log CFU ml^{-1}.

(PBS at 4°C). These results indicated that survival in PJ was affected by storage temperature. Han and Linton (2004) reported the survival of *E. coli* O157:H7 in strawberry juice (pH 3·6) at 4 and 37°C. The populations of *E. coli* O157:H7 decreased approx. 0·27 log CFU ml^{-1} at 4°C after 3 days of storage. However, when held at 37°C after 3 days of storage, no surviving bacteria were detected using a surface plating method (detection limit of <1 log CFU ml^{-1}) on TSA. Examining the effect of fruit juice and temperature on non-O157 STEC, Molina *et al.* (2005) found that exposure of STEC O91:H21, O111:H$^-$ or O157:H7 to apple juice at pH 3·2 at 37°C resulted in a reduction of approx. 1·0 log CFU ml^{-1} after 9 h. The data obtained by Kataoka *et al.* (2011) showed that the survival of seven STEC serogroups (O26, O45, O103, O111, O121, O145 and O157:H7) after 72 h exposure at 22°C in lemon (pH 2·5) and lime (pH 2·5) juices decreased up to 6 log CFU ml^{-1} in all of the STEC strains with both juices. Along with the results of these study, these observations demonstrate that O157:H7 and non-O157 STEC serogroup strains are more tolerant to acids at low temperature and could be more easily

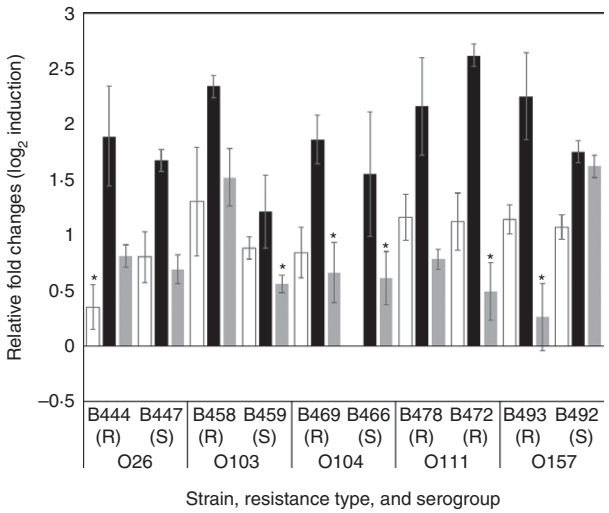

Figure 6 Effect of acid-adaption exposure to pineapple juice (pH 3·8) for 24 h on *rpoS* expression of O157:H7 and non-O157 STEC strains. The *rpoS* expression levels were determined by qRT-PCR analyses and expressed as the relative fold changes (log_2) of STEC adapted with PJ compared to O104:H4 B466 as the nonadapted (PBS) control, which had the lowest survival in SGF, 1·5 and 2·0. The relative levels of expression of *rpoS* are the mean ± SD of results from three independent experiments. * Indicates significant at $P > 0·05$ as compared to the control. (□) PBS at 4°C; (■) PJ at 4°C; (▒) PJ at 20°C. R, acid-resistant strain. S, acid-sensitive strain.

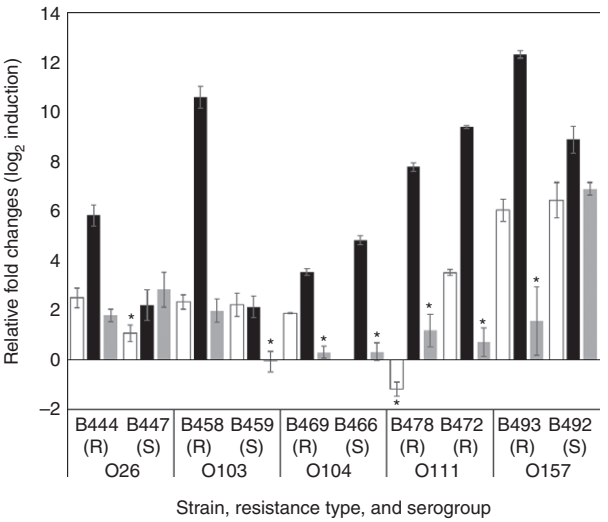

Figure 7 Effect of acid-adaption exposure to pineapple juice (pH 3·8) for 24 h on *gadA* expression of O157:H7 and non-O157 STEC strains. The *gadA* expression levels were determined by qRT-PCR analyses and expressed as the relative fold changes (log_2) of STEC adapted with PJ compared to O104:H4 B466 as the nonadapted (PBS) control, which had the lowest survival in SGF, 1·5 and 2·0. The relative levels of expression of *gadA* are the mean ± SD of results from three independent experiments. * Indicates significant at $P > 0·05$ as compared to the control. (□) PBS at 4°C; (■) PJ at 4°C; (▒) PJ at 20°C. R, acid-resistant strain. S, acid-sensitive strain.

injured at higher temperatures (Comi *et al.* 2000; Han and Linton 2004), and there is considerable variability in the susceptibility of STEC to acid environments. The difference in specific rates of inactivation between these studies may have resulted from differences in the specific food system, storage temperature and inoculum selection.

Gastric secretions are an early line of defence in the human digestive tract against enteric pathogens; however, *E. coli* O157:H7 has an ability to adapt to acid environments and survive the gastric fluid of the stomach (Mao *et al.* 2006). Prior exposure to mild acidity has the potential to increase the AR of *E. coli* upon exposure to higher acidity and thus enhance survival of bacterial cells during passage through the stomach (Bergholz and Whittam 2007). In addition, previous studies have proposed that non-O157 STEC strains colonize the ruminant gastrointestinal tract similarly to O157:H7 (Jacobsen *et al.* 2009), and the survival rates of O26:H11 and O111:H8 serotypes in ruminal fluid were comparable to O157:H7 (Free *et al.* 2012). SGF is a valuable tool for evaluating survival of bacterial cells in a gastric environment encountered after ingestion of a meal and was devised to address the criticism that some acid survival studies do not accurately account for the complexity of the gastric environment after eating (Bergholz and Whittam 2007). Therefore, to investigate those possibilities further, our goal in this study was to determine if exposure to acidic conditions in foods enhances the survival of non-O157 STEC strains in low pH SGF compared to serotype O157:H7.

Previous studies comparing survival of pathogenic *E. coli* in SGF have focused mainly on *E. coli* O157:H7 (Arnold and Kaspar 1995; Uljas and Ingham 1998). Arnold and Kaspar (1995) reported that four *E. coli* O157:H7 strains in stationary phase were shown to survive well for 3 h in SGF at pH 1·5, in contrast to the other serotypes of *E. coli*, which did not survive. Strain TB 226 was the most tolerant O157:H7 strain and showed the highest survival after 3 h in SGF (61% survivors). Bergholz and Whittam (2007) studied the effect of adaptation in apple juice on STEC strains O157:H7, O26:H11 and O111:H8. All strains were stored at 4 and 22°C in pH 3·5 apple juice for 24 h, and then inoculated into a model stomach system (pH 2·5), and the survival rate (log decrease in cell numbers per hour) was determined after 3 h at 37°C. The results indicated that the STEC O26/O111 strains were less resistant to gastric acid conditions than *E. coli* O157 and that prior storage of O157 and O26/O111 strains at 22°C decreased AR under gastric conditions (Bergholz and Whittam 2007).

However, in this study, B478 (O111:H8, resistant) and B458 (O103:H2, resistant) strains showed improved

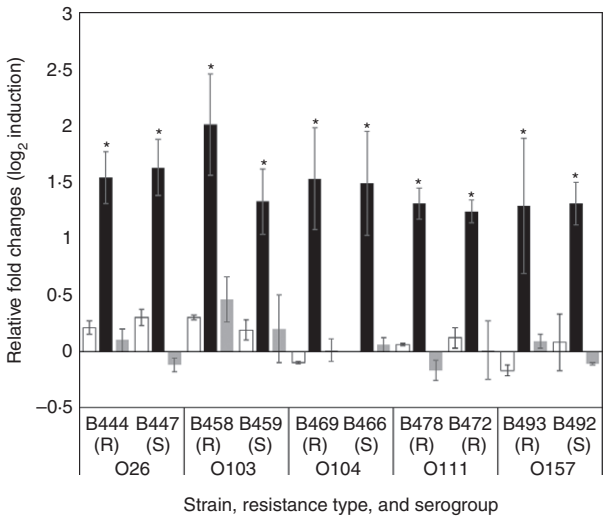

Figure 8 Effect of acid-adaption exposure to pineapple juice (pH 3·8) for 24 h on *adiA* expression of O157:H7 and non-O157 STEC strains. The *adiA* expression levels were determined by qRT-PCR analyses and expressed as the relative fold changes (log_2) of STEC adapted with PJ compared to O104:H4 B466 as the nonadapted (PBS) control, which had the lowest survival in SGF, 1·5 and 2·0. The relative levels of expression of are the mean ± SD of results from three independent experiments. * indicates significant at $P < 0·05$ as compared to the control. (□) PBS at 4°C; (■) PJ at 4°C; (▒) PJ at 20°C. R, acid-resistant strain. S, acid-sensitive strain.

survival in SGF after adaptation to PJ at 4°C (Figs 2b and 3b). Also, AR was found to differ between PJ-adapted cells and nonadapted cells. Bergholz and Whittam (2007) reported that four serogroup O26/O111 strains adapted to pH 3·5 had a survival rate of −0·38 compared with a survival rate of −0·89 for cells adapted to pH 7·0. Furthermore, both O157 and O26/O111 strains showed better survival in the model stomach system (pH 2·5) if they were adapted to low pH and temperature prior to exposure to gastric acid. This observation is consistent with our findings that the survival in the SGF after adaptation in PJ at 4°C was similar for the non-O157 STEC serotype strains and the O157:H7 strains. Results from these experiments suggest that storage in a refrigerated acidic fruit juice can, depending on the strain, result in good subsequent survival of non-O157 STEC in human gastric fluid. A possible explanation for enhancement of AR in cold temperature may be that the production of cold shock proteins induced by storage at 4°C led to cross-protection under extreme acid conditions (Smith and Fratamico 2012).

The AR1, 2 and 3 systems have been shown to provide AR in STEC O157:H7, O26:H11, O111:H8 and O121:H19 (Large *et al.* 2005) and are critical for survival of *E. coli* O157:H7 during passage through the gastrointestinal tract of calves and in apple cider (Price *et al.* 2004). In addition, prior exposure to acidic apple juice can increase the subsequent AR of non-O157 STEC serogroups upon exposure to the lower pH in SGF (Bergholz and Whittam 2007). We hypothesized that the difference in SGF survival between O157:H7 strains and non-O157 STEC serogroups could result from differences in the level of transcription of AR genes with exposure of the cells to fruit juice.

The transcription of *rpoS* in PJ-adapted cells at 4°C was higher in non-O157 STEC strains than at 20°C in this study. White-Ziegler *et al.* (2008) reported that the transcription of *rpoS* was 2·3-fold higher in *E. coli* K-12 cultivated at 23°C than when incubated at 37°C. Carey *et al.* (2009) reported that *rpoS* was slightly up-regulated when *E. coli* O157:H7 was inoculated onto Romaine lettuce and stored at 4°C; however, after prolonged storage at 15°C, *rpoS* down-regulation was observed. In general, all of the AR genes were up-regulated to a greater extent with storage of the cells at low temperature. This up-regulation due to pre-incubation in PJ at low temperature may increase survival in the gastrointestinal tract. Also, under conditions of starvation, exposure to stress, or as cells enter the stationary phase, the RpoS sigma factor is induced and may lead to resistance to a wide range of stresses (Battesti *et al.* 2011).

Bergholz and Whittam (2007) showed that the transcription levels of the AR2 genes, *gadA* and *gadB*, were higher in O157 STEC than in non-O157 STEC. In this study, the transcription level of *gadA* in B493 (O157:H7, resistant) and B458 (O103:H2, resistant) adapted in PJ at 4°C was higher than that of the other STEC strains. In addition, both strains were significantly more acid tolerant in SGF compared to other strains. The AR2 system is believed to be the most effective mechanism for the protection of enterohaemorrhagic *E. coli* from the acid conditions of the stomach (Bhagwat *et al.* 2005). With AR2, protons are removed from the bacterial cell by the decarboxylation of glutamate in the production of γ-aminobutyric acid. The γ-aminobutyric acid is then transported out of the cell in exchange for glutamate entering the cell. The incorporation of a proton by γ-aminobutyric acid production decreases the internal pH of the bacterial cell (Bhagwat *et al.* 2005; Smith and Fratamico 2012). In the present study, the AR2 system may explain the ability of non-O157 STEC to withstand gastric acidity as much as O157:H7 STEC strains. However, little information is available about the influence of growth temperature, especially low temperature, on non-O157 STEC. Our experiments indicated that there were significant differences among STEC serotypes in the transcription of AR genes; however, these levels were not highly correlated with survival with exposure to SGF, suggesting that other factors are also contributing to the AR and survival

ability in the SGF. Several studies reported that tolerance of acid or juice-adapted *E. coli* O157:H7 on subsequent exposure to low pH was influenced by the type of organic acid to which the cells had been exposed previously (Uljas and Ingham 1998; Deng *et al.* 1999; Ryu *et al.* 1999; Price *et al.* 2004; Yuk and Marshall 2005; Bergholz and Whittam 2007). Studies by Kim *et al.* (2015) and results of the present study suggest that the difference in the survival of O111:H$^-$ B472 in 400 mmol l^{-1} acetic acid solution at pH 3·2 at 30°C and with adaptation in PJ at pH 3·8 at 20°C may reflect the different acids involved, acetic acid *vs* citric acid and malic acid in PJ or hydrochloric acid in SGF. This result might indicate that the AR in O111:H$^-$ B472 was affected by the type of organic acid present, and further study is required to define the mechanisms.

We compared PJ-adapted STEC strains with non-adapted control cells to determine if there were differences in STEC resistance to SGF since foods such as acidic fruit juices can be contaminated with STEC. In conclusion, results of this study further demonstrated that AR genes are expressed during PJ exposure, and preadaptation in PJ at low temperature enhanced the survival of non-O157 STEC in SGF. The data with SGF exposure showed that non-O157 STEC strains adapted in PJ at 4°C had greater AR than non-adapted controls and cells adapted in PJ at 20°C. Both acetic acid-resistant and sensitive STEC strains showed better survival in SGF when they were adapted to PJ at 4°C than at 20°C. A comparative RT q-PCR assay was employed to evaluate expression of AR genes, *rpoS*, *gadA,* and *adiA* in O157:H7 and non-O157 STEC strains. The results indicated that refrigeration temperature resulted in up-regulation in gene expression with PJ exposure. This up-regulation due to preincubation in PJ at low temperature may increase survival in acidic environments such as the gastrointestinal tract, and some non-O157 STEC serotype strains showed high AR levels similar to STEC O157:H7. Therefore, the expression of AR genes, as well as survival of this pathogen in fruit juice exposed to low temperatures and mild pH levels warrants further investigation in order to develop control strategies.

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Conflict of Interest

No conflict of interests declared.

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