Acid Resistance and Molecular Characterization of *Escherichia coli* O157:H7 and Different Non-O157 Shiga Toxin-Producing *E. coli* Serogroups

Gwang-Hee Kim, Frederick Breidt, Pina Fratamico, and Deog-Hwan Oh

Abstract: The objective of this study was to compare the acid resistance (AR) of non-O157 Shiga toxin-producing *Escherichia coli* (STEC) strains belonging to serogroups O26, O45, O103, O104, O111, O121, and O145 with O157:H7 STEC isolated from various sources in 400 mM acetic acid solutions (AAS) at pH 3.2 and 30 °C for 25 min with or without glutamic acid. Furthermore, the molecular subgrouping of the STEC strains was analyzed with the repetitive sequence-based PCR (rep-PCR) method using a DiversiLab^{TM} system. Results for a total of 52 strains ranged from 0.31 to 5.45 log reduction CFU/mL in the absence of glutamic acid and 0.02 to 0.33 CFU/mL in the presence of glutamic acid except for B447 (O26:H11), B452 (O45:H2), and B466 (O104:H4) strains. Strains belonging to serogroups O111, O121, and O103 showed higher AR than serotype O157:H7 strains in the absence of glutamic acid. All STEC O157:H7 strains exhibited a comparable DNA pattern with more than 95% similarity in the rep-PCR results, as did the strains belonging to serogroups O111 and O121. Surprisingly, the DNA pattern of B458 (O103:H2) was similar to that of O157:H7 strains with 82% similarity, and strain B458 strain showed the highest AR to AAS among the O103 strains with 0.44 log reduction CFU/mL without glutamic acid. In conclusion, STEC serotypes isolated from different sources exhibited diverse AR and genetic subtyping patterns. Results indicated that some non-O157 STEC strains may have higher AR than STEC O157:H7 strains under specific acidic conditions, and the addition of glutamic acid provided enhanced protection against exposure to AAS.

Keywords: acid resistance, *E. coli* non-O157, DiversiLab^{TM} system, glutamic acid, repetitive sequence-based PCR

Introduction

In early May 2011, a large outbreak of diarrhea caused by enteroaggregative Shiga toxin-producing *Escherichia coli* (STEC) occurred in Germany and was associated with a high percentage of cases of hemolytic-uremic syndrome (HUS). At its official end in late July 2011, 782 cases of HUS (29 deaths) and 3128 non-HUS cases (17 deaths) were reported, making it the largest outbreak of HUS worldwide (Cheung and others 2011). Interestingly, the outbreak strain was serotyped as O104:H4, which historically had not been reported in outbreaks, and it has been associated with very few HUS cases (Bae and others 2006). Furthermore, there has been an observed increase in illness due to non-O157 STEC caused by serogroups O26, O45, O103, O111, O121, and O145 and there have been outbreaks attributable to STEC O26:H11, O111:H8, and O121:H19 (Taylor and others 2010). The Centers for Disease Control and Prevention (CDC) estimated that non-O157 STEC are responsible for over 100000 illnesses annually in the United States (Scallan and others 2011; Kalchayanand and others 2012) and more than 200 virulent non-O157 serotypes have been isolated from outbreaks in the United States, as well as other countries (Scallan and others 2011; Kalchayanand and others 2012). Mellmann and others (2011) reported that the European *E. coli* O104:H4 outbreak strain (LB226692) was significantly associated with high acid resistance (AR), which may facilitate survival in acidic food environments or the stomach, contributing to the high pathogenicity of the outbreak strain.

E. coli O157:H7 can survive at pH values below 3.0 for at least 4 h at 37 °C without loss of viability, and its low infectious dose corresponds to the ability to withstand acidic environments (Benjamin and Datta 1995). The association of *E. coli* O157:H7 outbreaks with acidic foods has led to concerns about the AR mechanisms of this organism. There are 3 well-studied AR systems in *E. coli*, which are required in order to survive environments of pH 2.0 to 2.5. AR system 2 (AR2) is glutamate dependent and involves the transport activity of GadC, a glutamate: 4-aminobutyrate antiporter with glutamate decarboxylase, (GadA/GadB); however, the induction of AR2 is affected by at least 11 regulatory proteins (Foster 2004). Protons are consumed in the cytoplasm resulting in an increased internal pH to levels that would not damage critical cell components.

Several investigators have reported on the survival of *E. coli* O157:H7 under acidic conditions encountered in various foods, in the stomach, and *in vitro* (Brudzinski and Harrison 1998; Large and others 2005; Breidt and others 2011). In contrast, there is a lack of research on AR of non-O157 STEC serogroups despite the relatively high incidence of infections caused by these pathogens worldwide (McCarthy and others 2001; Hedican and others 2009).

MS 20150631 Submitted 4/16/2015, Accepted 7/11/2015. *Authors Kim and Oh are with Dept. of Food Science and Biotechnology, Kangwon Natl. Univ. 1 Kangwondaehak-gil, Chuncheon-si, Gangwon-do, 200–701, South Korea. Author Breidt is with USDA, Agricultural Research Service, Food Science Research Unit, Dept. of Food, Bioprocessing and Nutrition Sciences, North Carolina State Univ., Raleigh, N.C. 27695, U.S.A. Authors Fratamico and Kim are with USDA, Agricultural Research Service, Eastern Regional Research Center, 600 East Mermaid Lane, Wyndmoor, Pa. 19038, U.S.A. Direct inquiries to author Deog-Hwan Oh (E-mail: deoghwa@kanagwon.ac.kr).*

Journal of Food Science A Publication of the Institute of Food Technologists

doi: 10.1111/1750-3841.12996

Rapid and reliable molecular genotyping or subtyping methods to epidemiologically trace *E. coli* O157:H7 and non-O157 STEC are important for outbreak investigations and for monitoring the presence of this pathogen in food. The recently introduced DiversiLab™ system is a typing technique based on repetitive-sequence-based PCR (rep-PCR) and an electrophoresis step using a Bioanalyzer (Agilent Technologies, Palo Alto, Calif., U.S.A.) (Fluit and others 2010). The analysis software allows the comparison of individual amplification product patterns (peak patterns), which enables easy interpretation, but a virtual gel image is also generated. The patterns can be stored in a database and used for comparison. An important advantage of DiversiLab™ is that a result can be obtained in 1 d starting from a pure culture (Shutt and others 2005; Fluit and others 2010).

Previous studies on the effect of organic acid on non-O157 STEC have shown strain or serotype differences (Brudzinski and Harrison 1998; Garren and others 1998; Oh and others 2009), but limited information has been reported comparing the AR of the different serotypes of *E. coli* isolated from different environments. A number of studies have defined growth and survival parameters for *E. coli* O157:H7 and some non-O157 strains in acidified foods (Breidt and others 2004; Oh and others 2009); however, there is a need for additional data with a greater diversity of STEC serotypes and strains. Therefore, the purpose of this research was to evaluate the survival of non-O157 STEC strains exposed to acetic acid solution (AAS) and compare the AR of non-O157 serogroups with O157:H7 under conditions typical of commercial acidified products, with glutamic acid (which can contribute to AR) or without glutamic acid under different temperatures. In addition, we analyzed the strains using the DiversiLab™ subtyping system.

Materials and Methods

Bacteria and growth media

The STEC strains used in this study are listed in Table 1. A total of 4 STEC *E. coli* O157:H7 strains and 48 non-O157 STEC strains, consisting of 7 serogroups O26, O45, O103, O104, O111, O121, and O145 from various sources including foods, animal carcasses and feces, and humans were kindly provided from the U.S. Food Fermentation Laboratory Culture Collection (USDA ARS, Raleigh, N.C., U.S.A.) and the USDA ARS Eastern Regional Research Center (Wyndmoor, Pa., U.S.A.). Bacterial strains were stored at –80 °C in tryptic soy broth (TSB, BD Biosciences, San Jose, Calif., U.S.A.) supplemented with 30% glycerol. Each culture was streaked from frozen stocks onto tryptic soy agar (TSA, BD Bioscience) and incubated at 37 °C for 24 h. To prepare cells for the acid challenge experiments, an overnight culture of each STEC strain was inoculated into 10 mL of TSB supplemented with 10 g/L glucose (Daejung Chemical, Daejueon, Korea) and incubated statically for 18 h at 37 °C to induce AR (Buchanan and Edelson 1996). Overnight cultures were washed twice by centrifugation at 3500 × *g* for 10 min at 4°C and finally suspended in physiological saline (8.5 g/L of NaCl; Daejung). The initial cell count was approximately 10^9 CFU/mL confirmed by standard plate count method using TSA.

Acid challenge studies

An AAS consisting of 400 mM acetic acid (Sigma Chemical Co., St. Louis, Mo., U.S.A.), pH 3.2, with an ionic strength of 0.34 was prepared as previously described (Breidt and others 2004; Oh and others 2009). For the pure culture experiments, aliquots of 0.2 mL of each bacterial cell suspension were exposed to 1.8 mL of AAS in a 12-well tissue culture plate (nontissue culture-treated flat-bottom plate, catalog no.32024; SPL Life Science, Pocheon-Si, Korea) with or without glutamic acid (2 mM; Sigma) and incubated at 30 °C for 25 min (Breidt and others 2004), aerobically. Prior to adding cells, the plates were incubated at 30 °C for approximately 1 h to ensure temperature equilibration. After incubation, the cells were immediately diluted at least 10-fold by adding 20 μL of the acid treated cell suspension into 180 μL of 0.1 M 3-(*N*-morpholino)-propane sulfonic acid (MOPS) buffer (pH 7.2; Sigma) with 0.85% saline in a 96-well tissue culture plate (SPL Life Science) to rapidly neutralize the pH prior to plating. The number of viable cells were determined after additional dilution and plating on TSA supplemented with 1% glucose. Plates were incubated for 24 h at 37 °C, and the colonies were enumerated.

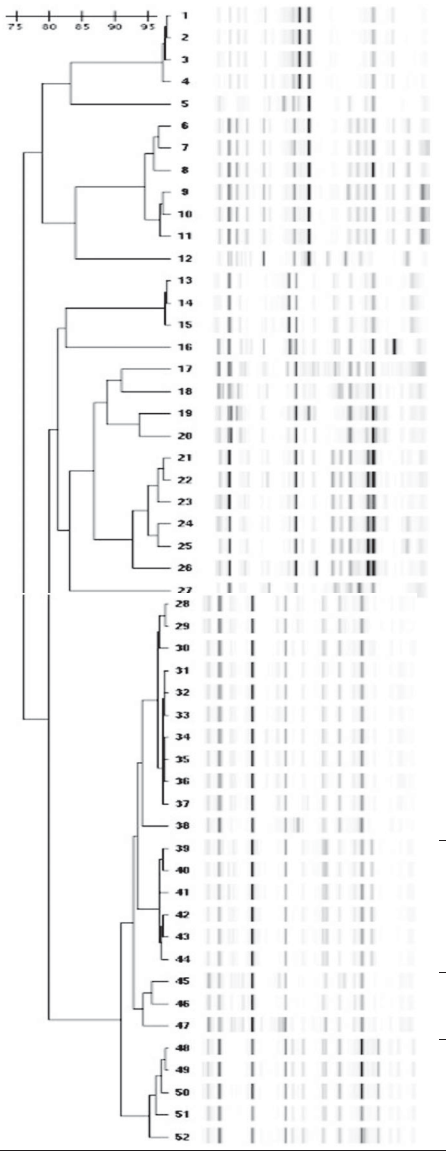
For the strain mixture study, equal volumes of bacterial cell suspensions of 4 to 7 strains of each serogroup (all of the strains in Table 1 were used in the mixtures) were pooled and mixed prior to the acid challenge studies. The procedure for the acetic acid challenge experiments for the mixed cultures was the same as that for individual strains, but additional conditions were tested, including different pH levels (pH 3.2 and 3.7), and different temperatures (20 °C and 30 °C) (Oh and others 2009).

Rep-PCR DNA fingerprinting of *E. coli* using DiversiLabTM

Rep-PCR DNA fingerprinting of *E. coli* using DiversiLab™ (Hyeon and others 2011; Chon and others 2012) was performed by using techniques described previously so that the results could be compared. A few modifications were made, and the methods are outlined briefly below.

A total of 52 STEC isolates (4 strains of *E. coli* O157:H7 and 48 strains of non-O157 STEC) were subcultured on nutrient agar (NA; BD Bioscience) for 24 h at 37 °C and 2 passages. The DNA template from each isolate was extracted using the UltraClean™ Microbial DNA Isolation Kit (MoBio Laboratories, Solana Beach, Calif., U.S.A.) according to the manufacturer's instructions. Genomic DNA samples were quantified with the NanoDrop 2000 UV spectrophotometer (Thermo Scientific, Wilmington, Del., U.S.A.) at 260 nm. All DNA samples and the positive and negative controls included in the DiversiLab™ *E. coli* kit (BioMěrieux, Durham, N.C., U.S.A.) were amplified for DNA fingerprinting. For PCR reactions, 2 μL of genomic DNA (approximately 25 ng/μL) was added to 23 μL of PCR mixture, which contained 0.5 μL (or 2.5 U) of AmpliTaq polymerase (Applied Biosystems, Foster City, Calif., U.S.A.), 2.5 μL of 10 × GeneAMP PCR buffer I (Applied Biosystems), 2 μL of kit-supplied primer mix, and 18 μL of the kit-supplied rep-PCR master mix (MM1) in the DiversiLab™ kit. The thermal cycling parameters were as follows: initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 70 °C for 90 s, and a final extension at 70 °C for 3 min. The rep-PCR products were separated and detected with a microfluidics chip using an Agilent 2100 Bioanalyzer (Agilent Technologies). All samples were analyzed simultaneously on a chip, and internal DNA standards of known size were added to each well to allow for normalization and efficient chip-to-chip comparisons (Chon and others 2012). Similarity of all samples was analyzed and compared using the Pearson coefficient with Web-based DiversiLab™ software (version 2.1.66, bioMérieux) to assess band position and intensity. The distance matrices and the

Table 1–Survival of *Escherichia coli* O157:H7 and non-O157 serotype strains in 400 mM acetic acid solution (pH 3.2 at 30 °C for 25 min) and DiversiLab™ system results (dendrogam and gel-like image).

Isolates				DiversiLab™		Reduction in log CFU/ml	
ID no.	Original ID no.	Serotype	Source (reference)	Dendrogram and gel-like image (% similarity)	Subgroups*	Without glutamic acid	With glutamic acid
B493	C7927	O157:H7	H		A	0.46 ± 0.12	0.06 ± 0.03
B492	Sakai	O157:H7	H			3.79 ± 0.13	0.05 ± 0.04
B494	FSIS 413-95	O157:H7	Gb			0.81 ± 0.12	0.11 ± 0.02
B495	380-94	O157:H7	H			2.14 ± 0.28	0.23 ± 0.16
B458	90-3128	O103:H2	H		A-1	0.44 ± 0.26	0.06 ± 0.04
B490	8235	O145:NM	H		B	1.63 ± 0.14	0.07 ± 0.03
B486	14728	O145:NM	H			3.88 ± 0.38	0.07 ± 0.03
B488	6383	O145:NM	H			1.08 ± 0.24	0.16 ± 0.10
B487	940941	O145:H-	H			1.09 ± 0.05	0.12 ± 0.03
B485	83-75	O145:NM	H			3.71 ± 0.19	0.02 ± 0.01
B491	6896	O145:NM	H			0.63 ± 0.20	0.07 ± 0.03
B456	1.2622	O45:H12	Co		B-1	2.26 ± 0.10	0.08 ± 0.06
B466	2009EL-2071	O104:H4	H		C	3.69 ± 0.28	1.25 ± 0.09
B465	2011C-3493	O104:H4	H			1.82 ± 0.13	0.33 ± 0.10
B469	2009EL-2050	O104:H4	H			0.72 ± 0.05	0.05 ± 0.03
B453	95.0941	O45:H2	H		C-1	0.88 ± 0.10	0.10 ± 0.04
B467	94-3024	O104:H21	H		D	0.77 ± 0.15	0.10 ± 0.01
B451	B8026-C1	O45:NM	Co (calf)			4.53 ± 0.20	0.28 ± 0.17
B460	97-3112	O103:H25	H			3.13 ± 0.17	0.24 ± 0.13
B459	03-2444	O103:H25	H			4.43 ± 0.42	0.11 ± 0.03
B455	10-2360	O45:H2	R		E	4.46 ± 0.38	0.09 ± 0.10
B452	85.0953	O45:H2	D			5.45 ± 0.47	3.67 ± 0.32
B457	B8227-C8	O45:H2	Co (calf)			0.71 ± 0.01	0.12 ± 0.09
B461	042446	O103:H2	H			2.61 ± 0.34	0.13 ± 0.07
B454	5.0623	O45:H2	Go			2.76 ± 0.13	014 ± 0.07
B463	04162	O103:H6	H		E-1	0.74 ± 0.25	0.05 ± 0.06
B489	BCL73	O145:NM	Co		DE-1	2.39 ± 0.18	0.10 ± 0.07
B464	14758	O103:H11	H		F	1.74 ± 0.11	0.06 ± 0.07
B462	13075	O103:H11	H			1.18 ± 0.09	0.02 ± 0.02
B444	10205	O26:H11	H			0.91 ± 0.12	0.08 ± 0.06
B478	14895	O111:H8	H			0.31 ± 0.11	0.11 ± 0.09
B476	8361	O111:H8	H			0.78 ± 0.24	0.05 ± 0.01
B473	96-3166	O111:NM	H			0.45 ± 0.02	0.09 ± 0.06
B475	TB226	O111:NM	H			0.38 ± 0.10	0.11 ± 0.05
B474	98-8338	O111:NM	H			0.46 ± 0.04	0.09 ± 0.03
B477	12893	O111:H8	H			0.74 ± 0.19	0.23 ± 0.02
B472	JB1-95	O111:H-	H			0.82 ± 0.01	0.02 ± 0.01
B445	12690	O26:H11	H			3.81 ± 0.07	0.11 ± 0.09
B483	9918	O121:NM	H		G	0.67 ± 0.17	0.08 ± 0.06
B482	11435	O121:H19	H			0.40 ± 0.14	0.13 ± 0.01
B484	10896	O121:NM	H			0.67 ± 0.09	0.11 ± 0.05
B481	03-4064	O121:NM	H			1.46 ± 0.41	0.06 ± 0.05
B479	DA-1	O121:NM	H			2.30 ± 0.29	0.20 ± 0.07
B480	97-3068	O121:H19	H			1.36 ± 0.15	0.23 ± 0.10
B471	1.2673	O104:H12	Co		H	3.75 ± 0.22	0.12 ± 0.09
B470	9.0124	O104:H2	Co (feces)			1.13 ± 0.14	0.33 ± 0.11
B468	6.0830	O104:H7	Ca			2.41 ± 0.06	0.11 ± 0.04
B449	93-3118	O26:H11	H		I	4.30 ± 0.27	0.09 ± 0.09
B448	96-0112	O26:H11	H			1.70 ± 0.14	0.11 ± 0.05
B450	10159	O26:H11	H			3.93 ± 0.25	0.13 ± 0.08
B446	H30	O26:H11	H			1.72 ± 0.17	0.15 ± 0.09
B447	94-0962	O26:H11	H			5.02 ± 0.03	2.57 ± 0.06

H; human, Gb; ground beef, Co; cow, R; reference strain, D; dog, Go; goat, Ca; carcass.
*Based on the rep-PCR banding patterns of genomic DNA from 52 isolates of STEC, the strains were divided into 9 diverse clusters, A to I, showing ≥ 95% similarity, although strains within D-cluster had only 88 % similarity: rep-PCR profiles A and D-cluster = 4 isolates; B, E, and G-cluster = 6 isolates; C and H-cluster = 3 isolates; F-cluster = 11 isolates, I-cluster = 5 isolates. Strains B458, B456, B453, B463, and B489 are classified into subsidiary-clusters according to nearest cluster by similarity of DNA patterns, including A-1, B-1, C-1, E-1, and DE-1-cluster, respectively.

unweighted pair group method with arithmetic mean (UPGMA) were determined to create dendrograms. According to Healy and others (2005), strains were considered to be indistinguishable if they had a high percentage of similarity (generally >97%) with no band difference.

Statistical analysis

Log reduction values (for 25 min) for viable cells were determined by the difference of the final count (N) from the initial number (N_0) [$\log_{10}(N_0/N)$]. Each experiment had at least 3 independent replicates and was repeated 3 times for each strain and for mixed strains, and data were grouped by serotype. Data analysis was done using a one-way analysis of variance with SPSS software (Statistical Package for the Social Science, Ver. 18.0 SPSS Inc., Chicago, Ill., U.S.A.). Differences ($P < 0.05$) between the means of the log reductions for the strains of the various serogroups were analyzed by using the least significant difference test.

Results and Discussion

Acid challenge studies of individual strains

AR was investigated on the STEC strains using sub-lethal pH conditions based on methods used in previous studies (Breidt and others 2004; Foster 2004). Usually, AR is strongly dependent on growth phase, and stationary-phase cultures are more resistant than their exponential-phase counterparts (Arnold and Kaspar 1995; Benjamin and Datta 1995; King and others 2010). In the natural environment, the bacteria are usually in the stationary phase, and cells in this phase exhibit pH-dependent acid tolerance (King and others 2010), which further increases AR. Our study is unique in characterizing the AR of stationary-phase non-O157 STEC cells with exposure to AAS, reflecting the potential physiological state of STEC in food manufacturing and processing systems (King and others 2010). The inhibitory effect of acetic acid was examined because it is the primary acidulant used in many acidified (pickled) products with a pH of 3.2 to 4.1 (Breidt and others 2004; King and others 2010). Individual and mixed cultures of *E. coli* O157:H7 strains have been reported to be more sensitive to acetic acid than other acids used in acidified foods, such as citric and malic (Lu and others 2011).

The survival of STEC O157:H7 and non-O157 strains, measured as log CFU/mL reduction of each individual strain exposed to AAS with or without glutamic acid is presented in Table 1. The mean value for viable cell reductions of the 7 O111 serogroup isolates after acetic acid treatment in the absence of glutamic acid was ca. 0.56 log CFU/mL. This serogroup showed the highest resistance compared with isolates belonging to other STEC serogroups (Table 1). The survival of STEC O111 isolates (strong AR phenotype) was significantly ($P < 0.05$) different from that of O26, O45, O103, O104, O121, O145, and O157 isolates (the mean reductions were 3.06, 3.01, 2.04, 2.04, 1.10, 2.06, and 1.80 log CFU/mL, respectively). There was no significant difference ($P > 0.05$) between O26 and O45 (highly sensitive AR phenotype) or among O103, O104, O145, and O157 (mid-sensitive AR phenotype). Surprisingly, the STEC O157:H7 strains included in the study were not the most resistant to AAS, although the variation in AR among O157:H7 strains was previously demonstrated (Oh and others 2009). Also, there were 7 strains: B458 (O103:H2), B473 (O111:NM), B474 (O111:NM), B475 (O111:NM), B478 (O111:H8), B482 (O121:H19), and B483 (O121:NM) showing high AR with less than 0.50 log CFU/mL reduction. This is in agreement with results of studies examining 4 non-O157 strains: 103C (O103:H11), 111B (O111:H8), 26B (O26:H2), and 26C (O26:H11) that had significantly greater survival than the O157:H7 strain in Luria-Bertani (LB)-HCl, pH 3.0 (Mand and others 2013). However, strain 111B had a significantly lower growth rate under all 5 acidified LB conditions and a lower maximum growth in 4 of the 5 low-pH conditions than the O157:H7 control strain. Thus, as also shown in this study, it is not surprising that some non-O157 strains survived better in the acidic conditions tested than the O157:H7 strains (Mand and others 2013).

All isolates showed a similar ($P < 0.05$) reduction in cell numbers in the presence of glutamic acid (<0.33 log CFU/mL), with the exception of strains B447 (O26:H11), B452 (O45:H2), and B466 (O104:H4). These strains were sensitive to AAS regardless of the presence of glutamic acid (Table 1).

Acid challenge studies using cocktails of each serogroup

The effect of AAS on the survival of cocktails of STEC O157:H7 and non-O157 strains at different pH values and temperatures in the absence of glutamic acid are shown in Figure 1. The resistance of the mixed strain cocktails of the 8 STEC serogroups to AAS without glutamic acid showed the same trend compared to mean values (data not shown) for each serogroup from the experiments with individual isolates.

When the cocktail of STEC serogroup O111 was exposed to the AAS without glutamic acid, the viable cell density was decreased by approximately 0.46 and 0.28 orders of magnitude at pH 3.2 and 3.7 at 30 °C, respectively. Strains of this serogroup had greater overall AR compared to the other serogroups tested. STEC serogroup O26 and O45 strains were more sensitive in AAS without glutamic acid compared to the other serogroups, with 2.03 and 2.21 log reduction in CFU/mL at pH 3.2 and 0.81 and 0.85 log reduction in CFU/mL at pH 3.7 at 30 °C, respectively. The sensitivity of the STEC to AAS was significantly decreased when the pH was increased from 3.2 to 3.7.

When the challenge test was performed at lower temperatures, the sensitivity of all of the STEC serogroups to AAS was significantly reduced. We found that decreasing the incubation temperature from 30 to 20 °C did not enhance the reduction of STEC cells in the absence of glutamic acid. At 20 °C, the AAS treatment reduced the viable cells of the O111 serogroup by approximately 0.34 and 0.12 log CFU/mL at pH 3.2 and 3.7, respectively. This serogroup showed high survival under all pH and temperature conditions compared with the other serogroups. On the other hand, STEC serogroups O26 and O45 were more sensitive to AAS exposure as compared with the other strains, with 0.65 and 0.81 log reductions CFU/mL at pH 3.2 and 0.45 and 0.50 log reductions CFU/mL at pH 3.7 at 20 °C, respectively.

Kalchayanand and others (2012) reported that spray treatment with peroxyacetic acid at 200 ppm reduced the population of non-O157 STEC strains by 0.9 to 1.5 log CFU/mL, with no difference in reduction among serogroups of O157:H7 and non-O157 STEC (O26, O45, O103, O121, and O145) strains. However, we found serogroup O111 strains to have significantly greater AR than the other STEC serogroups. Buchanan and Edelson (1999) demonstrated that serogroup O111 showed lower survival with exposure to 0.5% acetic acid at pH 3.0 than serogroup O157:H7 strains, which is not in agreement with the results of the current study using strain cocktails. Entani and others (1998) reported that the inactivation rates of 8 strains of EHEC O157:H7, O111:HNM, and O26:H11 were similar when exposed to diluted vinegar solution containing 2.5% acetic acid at pH 5.1 and 30 °C. However, an EPEC O111:K58:H$^-$ strain exhibited higher sensitivity to vinegar, being more quickly killed compared with other strains. Contrary to those results, serogroup O111 strains in the current study in experiments using individual strains showed a mean higher AR value than other serogroups. A wide range in AR among different isolates and strains of *E. coli* has been reported (Miller and Kaspar 1994; Buchanan and Edelson 1996); however, it is difficult to compare these data with our results because of differences in methodology. Differences in strains examined, physiological status of the organisms, growth media, and organic acid solutions used, pH, and incubation temperatures could be partially responsible for the different results (Lin and others 1995; Brudzinski and Harrison 1998). Thus, the AR of *E. coli* may increase or decrease due to these differences. Furthermore, AR studies on non-O157 STEC serogroups have not been reported sufficiently in the literature.

We determined the survival of O157:H7 and non-O157 STEC serogroups in AAS in the presence of glutamic acid as the carbon source (Figure 2). The survival was increased in the presence of glutamic acid compared to when glutamic acid was not added

(Figure 1). The AAS containing 2 mM glutamic acid showed minimal inhibitory effects on all of the *E. coli* serogroup cocktails, irrespective of pH values or temperature. There was no significant ($p > 0.05$) difference in acetic AR among the serogroups tested in the presence of glutamic acid.

We controlled 3 factors to evaluate the effect of AAS on each strain and serogroup, i.e., pH, temperature, and the presence or absence of glutamic acid, as described previously (Breidt and others 2004). However, there are many factors that affect acid sensitivity of bacteria, including: lowering intracellular pH, altering cell membrane permeability, depletion of the level of reducing agents available for electron transport systems and pKa values, and the concentration of protonated acid (Cherrington and others 1990). At pH values above 4.75, the acid will be mostly in the anion form, which can inhibit the acid from penetrating the cell, allowing a better chance for enhancing cell survival (Cherrington and others 1990; Brudzinski and Harrison 1998). However, acetic acid below the pKa (4.75) remains mostly undissociated, allowing it to penetrate through the cell membrane and eventually lower the internal pH of the cell. The inhibition of non-O157 STEC by acetic acid is affected by the concentration of the protonated form, that can diffuse across bacterial cell membranes, and pH, which are interdependent variables.

Previous studies found that the acid-adaptive response in *E. coli* O157:H7 in the presence of glutamic acid, a component of pickle brines, enhanced the pathogen's survival in acidified food such as fermented sausage at pH 5.6 and apple cider at pH 3.4. It can protect the *E. coli* strains from the specific effects of acetic acid (Brudzinski and Harrison 1998; Breidt and others 2004). According to these studies, *E. coli* grown in LB containing glucose was acid sensitive at pH 2.5 unless either glutamate or arginine was present in the acid challenge medium. In the current study, *E. coli* strains without the addition of glutamic acid were more sensitive to acid than cells in the presence of glutamic acid regardless of

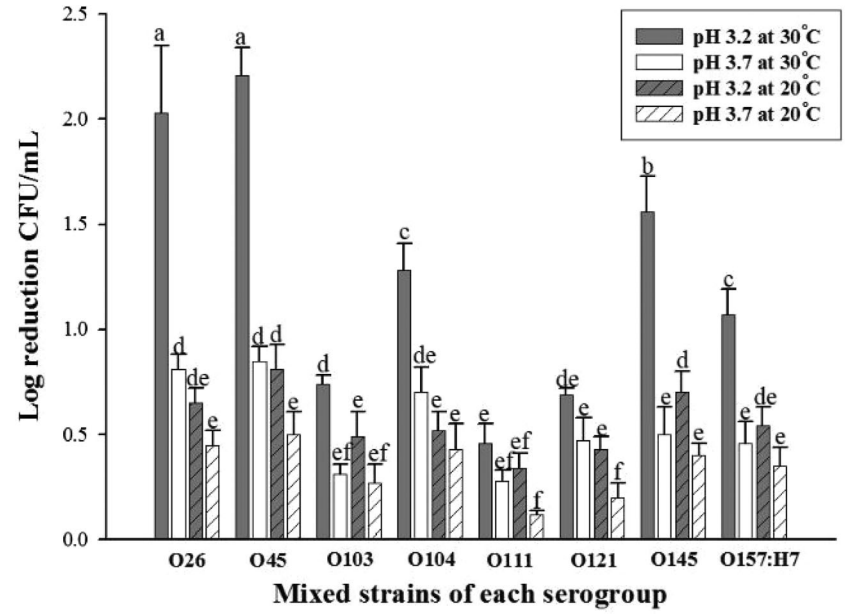


Figure 1–The effect of 400 mM acetic acid without glutamic acid on the survival of the mixtures of STEC strains in Table 1 at different pH values (pH 3.2 and 3.7) and temperatures (30 °C and 20 °C). All bars are expressed as mean ± standard deviation from triplicate replicates of each sample. Bars showing different letters (a–f) are significantly different ($P < 0.05$).

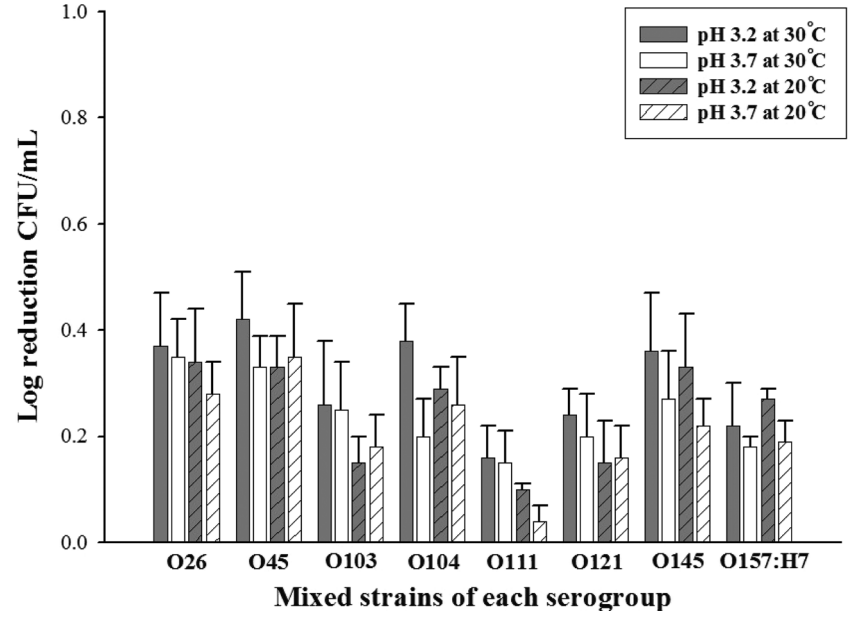


Figure 2–The effect of 400 mM acetic acid with glutamic acid on the survival of the mixtures of STEC strains in Table 1 at different pH values (pH 3.2 and 3.7) and temperatures (30 °C and 20 °C). All bars are expressed as mean ± standard deviation from triplicate replicates of each sample. Mean values did not differ significantly ($P > 0.05$).

serotype. However, a few serogroups showed a high survival rate without glutamic acid (Figure 1).

The glutamate (AR2) and arginine decarboxylase (AR3) AR systems afford acid-stress protection and require the decarboxylation of glutamate and arginine, respectively (Lin and others 1995). These amino acid-dependent AR systems consist of an amino acid decarboxylase and an end-product antiporter, which are necessary for acid protection. Richard and Foster (2003) reported that there is an increase in internal pH and a reversal of membrane potential from an internal negative to an internal positive charge in the presence of glutamate or arginine during acid challenge. Thus, one potential mechanism in this study is that the higher internal pH in the presence of glutamic acid compared to acid challenge in its absence may decrease the level of acid stress for the bacterial cell, resulting in increased survival.

There was no significant difference between the serogroups in the decarboxylate-dependent AR system (Table 1); however strains B447 (O26:H11), B452 (O45:H2), and B466 (O104:H4) apparently lacked the glutamate decarboxylase system or if present, it may have been partially or nonfunctional. *E. coli* O157:H7 strains lacking the glutamate decarboxylase system were also found by Bhagwat and others (2005) and Oh and others (2009).

Another AR system (AR1) involves the production of uncharacterized stress-response proteins dependent on or independent of RpoS that protect *E. coli* against multiple acid stresses. This AR system is repressed in the presence of glucose, and it was demonstrated that AR1 was less effective in protection against the combination of oxidative and acid stress than AR2 or AR3 (Bearson and others 2009).

Cheville and others (1996) demonstrated that resistance to acid challenges can be influenced by the incubation temperature of the *E. coli* O157:H7 prior to an acid challenge. It may be possible that *E. coli* either represses or synthesizes different temperature-related proteins at 30 °C versus 20 °C, which could partially explain the AR differences related to temperature observed with O157:H7 and non-O157 STEC in the current study. Temperature is a primary factor influencing organic acid activity, with increasing temperature typically resulting in increasing effectiveness of organic acid (Cherrington and others 1990; Brudzinski and Harrison 1998; Buchanan and Edleson 1999; Breidt and others 2004; Mellmann and others 2011). For each treatment, increasing temperature resulted in a reduced survival of all strains. These results are in agreement with previous studies on the relationship between temperature and the inhibitory effects of organic acids on food-borne pathogens (Entani and others 1998; Breidt and others 2004; Oh and others 2009; Mellmann and others 2011).

The acid tolerance response (ATR) can also induce cross-protection against other environmental stresses (Garren and others 1998). King and others (2010) reported that induction of acetic-ATR resulted in increased transcription levels of 6 genes (*yojI*, *yfdX*, *yqgA*, *yheM*, *aaeA* [*yhc*Q] and *yei*G) of the universal acid response, which was characterized by up-regulation of genes involved in the oxidative and cold shock stress resistance, envelope biosynthesis, and iron and manganese uptake in *E. coli* O157:H7 Sakai and K-12 MG1655 with adaptation with acetic acid. Likewise, in our studies, specific genes inducing AR might be induced in the STEC strains, which may have an effect on increasing their survival in an acetic acid environment, and potentially increasing resistance to other types of stresses, as well. In yet another study, a wide range of acid tolerances among different strains of *E. coli* O157:H7 and non-O157 STEC isolates was recorded (Brudzinski and Harrison 1998). Although these studies have contributed substantially to our knowledge of acid tolerance of pathogenic *E. coli*, a more detailed analysis aimed at dissecting individual AR pathways is needed (Bhagwat and others 2005).

Repetitive sequence PCR (rep-PCR) fingerprinting

An objective of the current study was to compare genotypic patterns obtained using the DiversiLab™ system and AR profiles for each of the STEC serogroups. In this work, we showed that the DiversiLab™ system classified the strains in subgroups and environmental sources and thus can be used for the preliminary fingerprinting of STEC strains. Rep-PCR has been proven to support high reproducibility of fragmentation patterns and successful discriminative ability for closely related isolates (Healy and others 2005).

A total of 52 STEC *E. coli* isolates were characterized by rep-PCR using a DiversiLab™ *E. coli* DNA fingerprinting kit. The dendrogram from rep-PCR patterns and computer-generated virtual gel images, as well as strain information are summarized in Table 1. The DNA fingerprints consisted of between 18 and 30 bands for a single strain. Our results showed a direct correlation between clusters constituted by DiversiLab™ types and O-serogroups. The O157:H7 strains and strains belonging to serogroups O111 and O121 clearly demonstrate similar banding profiles in term of band numbers and band size within the same serogroup with ≥ 98 % similarity level. However, some STEC serogroups exhibited diverse patterns unlike O157:H7, O111, and O121 strains. For example, B456, B453, and B451 strains belonging to serogroup O45 were different with E-cluster, showing 73%, 80%, and 86 % similarity, respectively. This serogroup consisted mainly of strains from animal sources. Similar to O45, the DNA patterns of serogroup O103 were the most diverse among the different serogroups, although strains of this serogroup were isolated from the same source, human. These results indicate that similarity in rep-PCR patterns may vary considerably within STEC serogroups; however, additional strains of each serogroup originating from different sources should be tested in order to draw further conclusions.

The DiversiLab™ technology has been successfully used to establish associations between DNA patterns and sources of many *E. coli* isolates, as well as other strains (Hyeon and others 2011; Chon and others 2012). In our study, the strains of serogroup O104 were divided into C- and H- clusters according to isolation source, showing 78% similarity between the 2 clusters. Furthermore, strain B489 in E-cluster isolated from cow was not within the B-cluster with O145 strains isolated from humans. These results showed that rep-PCR may have the ability to discriminate clusters with strains belonging to specific serogroups isolated from different sources. Dombek and others (2000) reported that the rep-PCR technique very successfully classified *E. coli* isolates into the correct source groups. All of the chicken and cow isolates and between 78% and 90% of the human, goose, duck, pig, and sheep isolates were correctly identified when the BOX A1R primer was used. Therefore, these data could indicate that the DiversiLab™ system may be a useful tool for the analysis of *E. coli* serogroups (Pitout and others 2009). Moreover, the DiversiLab™ system offers the following advantages: it is technically simple to perform and based on web-based software analysis, which provides the ability to standardize comparisons, and it allows completion of analysis of 12 samples in approximately 4 h, compared with 3 d for PFGE (Deplano and others 2011; Chon and others 2012).

With particular focus on the DNA patterns of each strain, one interesting result was that B458 (serotype O103) strain was more similar to serotype O157:H7 strains with 82% similarity than to the serotype O103 strains with 73% similarity in terms of the genetic subtyping pattern. Also, B458 and O157:H7 stains B493 and B494 showed a relatively high level of survival when exposed to AAS in the acid challenge study (Table 1). These results indicate that serogroup O103 strains may exhibit genetic and phenotypic diversity and that acid tolerance is likely not linked to serogroup.

It should be pointed out that rep-PCR banding patterns did not correlate with AR profiles in the present study. Oh and others (2009) reported that isolates from the same source having similar AR did not show an obvious trend based on PFGE pattern grouping. These results agree with findings from other authors, who found that rep-PCR analysis revealed that *E. coli* strains exhibited a high degree of genetic variability based on different environmental sources (Dombek and others 2000; MeLellan and others 2003), or on antimicrobial resistance (Hawser and other 2014), multidrug resistance (Deplano and others 2011), or various seasonal influences (Chandran and Mazumder 2015). However, comparisons between reports should be considered with caution because it is difficult to directly compare measures of diversity based on different methods used and conditions examined. Thus, conclusions regarding the genetic differences in the AR of non-O157 STEC strains will require examination of additional isolates from a variety of sources and belonging to different serotypes. The accurate determination of the survival of non-O157 STEC in acidic foods must take into account the biological variability of the microorganisms with respect to their AR and their ability to enhance their survival through the induction of physiological stress responses (Buchanan and Edelson 1999).

Conclusion

The results indicated that some STEC non-O157 serogroup strains can survive better under low pH conditions compared to STEC O157:H7 strains and the addition of glutamic acid provided enhanced protection against exposure to AAS. Temperature and pH are important factors to consider in establishing strategies for improving the safety of acidified foods against non-O157 STEC. The rep-PCR using the DiversiLab^{TM} system may be used to discriminate the source of isolates or serogroups of non-O157 STEC strains, although we were unable to distinguish between acid resistant and sensitive strains among the non-O157 serogroups. Additional research will be needed to determine the survival of O157:H7 and non-O157 STEC serogroups in specific acidified food products because many factors can affect survival of *E. coli* strains in actual foods. Based on our results, non-O157 STEC strains belonging to different serogroups strains should be investigated in research related to predictive microbiology and modeling under food manufacturing and storage conditions.

Acknowledgments

This study was supported by 2013 Research Grant from Kangwon National University (No. 120131286). We gratefully thank Dr. Kun-Ho Seo and laboratory members (Center for Food Safety and Animal Health, College of Veterinary Medicine, Konkuk Univ., Seoul, South Korea) for help with the DiversiLab^{TM} study.

References

Arnold K, Kaspar C. 1995. Starvation-and stationary-phase-induced acid tolerance in *Escherichia coli* O157: H7. Appl Environ Microbiol 61(5):2037–9.

Bae WK, Lee YK, Cho MS, Ma SK, Kim SW, Kim NH, Choi KC. 2006. A case of hemolytic uremic syndrome caused by *Escherichia coli* O104: H4. Yonsei Med J 47(3):437–9.

Bearson BL, Lee IS, Casey TA. 2009. *Escherichia coli* O157: H7 glutamate-and arginine-dependent acid-resistance systems protect against oxidative stress during extreme acid challenge. Microbiology 155(3):805–12.

Benjamin MM, Datta AR. 1995. Acid tolerance of enterohemorrhagic *Escherichia* coli. Appl Environ Microbiol 61(4):1669–72.

Bhagwat AA, Chan L, Han R, Tan J, Kothary M, Jean-Gilles J, Tall BD. 2005. Characterization of enterohemorrhagic *Escherichia coli* strains based on acid resistance phenotypes. Infect Immun 73(8):4993–5003.

Breidt F, Hayes J, McFeeters R. 2004. Independent effects of acetic acid and pH on survival of *Escherichia coli* in simulated acidified pickle products. J Food Prot 67(1):12–8.

Breidt F, Caldwell JM. 2011. Survival of *Escherichia coli* O157:H7 in cucumber fermentation brines. J Food Sci 76(3):M198–203.

Brudzinski L, Harrison MA. 1998. Influence of incubation conditions on survival and acid tolerance response of *Escherichia coli* O157:H7 and non-O157: H7 isolates exposed to acetic acid. J Food Prot 61(5):542–6.

Buchanan RL, Edelson SG. 1996. Culturing enterohemorrhagic *Escherichia coli* in the presence and absence of glucose as a simple means of evaluating the acid tolerance of stationary-phase cells. Appl Environ Microbiol 62(11):4009–13.

Buchanan RL, Edelson SG. 1999. pH-dependent stationary-phase acid resistance response of enterohemorrhagic *Escherichia coli* in the presence of various acidulants. J Food Prot 62(3):211–8.

Chandran A, Mazumder A. 2015. Pathogenic potential, genetic diversity, and population structure of *Escherichia coli* strains isolated from a forest-dominated watershed (Comox Lake) in British Columbia, Canada. Appl Environ Microbiol 81(5):1788–98.

Cherrington CA, Hinton M, Chopra I. 1990. Effect of short-chain organic acids on macromolecular synthesis in *Escherichia coli*. J Appl Microbiol 68(1):69–74.

Cheung M, Li L, Nong W, Kwan H. 2011. 2011 German *Escherichia coli* O104: H4 outbreak: whole-genome phylogeny without alignment. BMC Res Notes 4(1):533.

Cheville A, Arnold K, Buchrieser C, Cheng C, Kaspar C. 1996. rpoS regulation of acid, heat, and salt tolerance in *Escherichia coli* O157:H7. Appl Environ Microbiol 62(5):1822–4.

Chon JW, Kim JH, Lee SJ, Hyeon JY, Seo KH. 2012. Toxin profile, antibiotic resistance, and phenotypic and molecular characterization of *Bacillus cereus* in Sunsik. Food Microbiol 32(1):217–22.

Deplano A, Denis O, Rodriguez-Villalbos H, DeRyck R, Struelens MJ, Hallin M. 2011. Controlled performance evaluation of the DiversiLab repetitive-sequence-based genotyping system for typing multidrug-resistant health care-associated bacterial pathogens. J Clin Microbiol 49(10):3616–20.

Dombek PE, Johnson LK, Zimmerley ST, Sadowsky MJ. 2000. Use of repetitive DNA sequences and the PCR to differentiate *Escherichia coli* isolates from human and animal sources. Appl Environ Microbiol 66(6):25727.

Entani E, Asai M, Tsujihata S, Tsukamoto Y, Ohta M. 1998. Antibacterial action of vinegar against food-borne pathogenic bacteria including *Escherichia coli* O157: H7. J Food Prot 61(8):953–9.

Fluit A, Terlingen A, Andriessen L, Ikawaty R, VanMansfeld R., Top J, Stuart JC, Leverstein-van Hall M, Boel C. 2010. Evaluation of the DiversiLab system for detection of hospital outbreaks of infections by different bacterial species. J Clin Microbiol 48(11):3979–89.

Foster JW. 2004. Escherichia coli acid resistance: tales of an amateur acidophile. Nat Rev Microbiol 2(11):898–907.

Garren DM, Harrison MA, Russell SM. 1998. Acid tolerance and acid shock response of *Escherichia coli* O157:H7 and non-O157:H7 isolates provide cross protection to sodium lactate and sodium chloride. J Food Prot 61(2):158–61.

Hawser SP, Badal RE, Bouchillon SK, Hoban DJ, Hackel MA, Biedenbach DJ, Goff DA. 2014. Susceptibility of gram-negative aerobic bacilli from intra-abdominal pathogens to antimicrobial agents collected in the United States during 2011. J Infect 68(1):71–6.

Healy M, Huong J, Bittner T, Lising M, Frye S, Raza S, Schrock R, Manry J, Renwick A, Nieto JR. 2005. Microbial DNA typing by automated repetitive-sequence-based PCR. J Clin Microbiol 43(1):199–207.

Hedican EB, Medus C, Besser JM, Juni BA, Koziol B, Taylor C, Smith KE. 2009. Characteristics of O157 versus non-O157 Shiga toxin-producing *Escherichia coli* infections in Minnesota, 2000–2006. Clin Infect Dis 49(3):358–64.

Hyeon JY, Chon JW, Hwang IG, Kwak HS, Kim MS, Kim SK, Choi IS, Song CS, Park C. Seo KH. 2011. Prevalence, antibiotic resistance, and molecular characterization of *Salmonella* serovars in retail meat products. J Food Prot 74(1):161–6.

Kalchayanand N, Arthur TM, Bosilevac JM, Schmidt JW, Wang R, Shackelford SD, Wheeler TL. 2012. Evaluation of commonly used antimicrobial interventions for fresh beef inoculated with shiga toxin producing *Escherichia coli* serotypes O26, O45, O103, O111, O121, O145, and O157: H7. J Food Prot 75(7):1207–12.

King T, Lucchini S, Hinton JC, Gobius K. 2010. Transcriptomic analysis of *Escherichia coli* O157:H7 and K-12 cultures exposed to inorganic and organic acids in stationary phase reveals acidulant-and strain-specific acid tolerance responses. Appl Environ Microbiol 76(19):6514–28.

Large TM, Walk ST, Whittam TS. 2005. Variation in acid resistance among Shiga toxin-producing clones of pathogenic *Escherichia coli*. Appl Environ Microbiol 71(5):2493–500.

Lin J, Lee IS, Frey J, Slonczewski JL, Foster JW. 1995. Comparative analysis of extreme acid survival in *Salmonella typhimurium*, *Shigella flexneri*, and *Escherichia coli*. J Bacteriol 177(14):4097–104.

Lu HJ, Breidt F, Pérez-Díaz IM, Osborne JA. 2011. The antimicrobial effects of weak acids on the survival of *Escherichia coli* O157:H7 under anaerobic conditions. J Food Prot 74(6):893–8.

Lu HJ, Breidt F, Pérez-Díaz IM. 2013. Development of an effective treatment for A 5-log reduction of *Escherichia coli* in refrigerated pickle productions. J Food Sci 78(2):M264–9.

Lukinmaa-Åberg S, Horsma J, Pasanen T, Mero S, Aulu L, Vaara M, Siitonen A, Antikainen J. 2013. Applicability of DiversiLab Repetitive Sequence-Based PCR Method in Epidemiological Typing of Enterohemorrhagic *Escherichia coli* (EHEC). Foodborne Pathog Dis 10(7): 632–8.

Mand TD, Döpfer D, Ingham B, Ané C, Kaspar CW. 2013. Growth and survival parameter estimates and relation to RpoS levels in serotype O157:H7 and non-O157 Shiga toxin-producing *Escherichia coli*. J Appl Microbiol 114(1):242–55.

McCarthy TA, Barrett NL, Hadler JL, Salsbury B, Howard RT, Dingman DW, Brinkman CD, Bibb WF, Cartter ML. 2001. Hemolytic-uremic syndrome and *Escherichia coli* O121 at a lake in Connecticut, 1999. Pediatrics 108(4):e59.

McLellan SL, Daniels AD, Salmore AK. 2003. Genetic characterization of *Escherichia coli* populations from host sources of fecal pollution by using DNA fingerpringting. App Environ Microbiol 69(5):2587–94.

Mellmann A, Harmsen D, Cummings CA, Zentz EB, Leopold SR, Rico A, Prior K, Szczepanowski R, Ji Y, Zhang W. 2011. Prospective genomic characterization of the German enterohemorrhagic *Escherichia coli* O104:H4 outbreak by rapid next generation sequencing technology. PLoS One 6(7):e22751.

Miller LG, Kaspar CW. 1994. *Escherichia coli* O157:H7 acid tolerance and survival in apple cider. J Food Prot 57(6):460–4.

Oh, DH, Pan Y, Berry E, Cooley M, Mandrell R, Breidt F. 2009. *Escherichia coli* O157:H7 strains isolated from environmental sources differ significantly in acetic acid resistance compared with human outbreak strains. J Food Prot 72(3):503–9.

Pitout, JD, Campbell L, Church DL, Wang PW, Guttman DS, Gregson DB. 2009. Using a commercial DiversiLab semiautomated repetitive sequence-based PCR typing technique for identification of *Escherichia coli* clone ST131 producing CTX-M-15. J Clin Microbiol 47(4):1212–5.

Richard HT, Foster JW. 2003. Acid Resistance in *Escherichia coli*. Adv Appl Microbiol 52:167–86.

Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson M, Roy SL, Jones JL, Griffin PM. 2011. Foodborne illness acquired in the United States—major pathogens. Emerg Infect Dis 17(1):7.

Shutt CK, Pounder JI, Page SR, Schaecher BJ, Woods GL. 2005. Clinical evaluation of the DiversiLab microbial typing system using repetitive-sequence-based PCR for characterization of *Staphylococcus aureus* strains. J Clin Microbiol 43(3):1187–92.

Taylor EV, Nguyen TA, Machesky KD, Koch E, Sotir MJ, Bohm SR, Folster JP, Bokanyi R, Kupper A, Bidol SA, Emanuel A, Arends KD, Johnson SA, Dunn J, Stroika S, Patel MK, Williams I. 2010. Multistate outbreak of *Escherichia coli* O145 infections associated with romaine lettuce consumption. J Food Prot 76(6):939–44.