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Research Article

# Effects of Stresses on the Growth and Cytotoxicity of Shiga-Toxin Producing Escherichia coli in Ground Beef and Spinach

**Byong Kwon Yoo[1], Yanhong Liu[2], Vijay Juneja[1], Lihan Huang[1], and Cheng-An Hwang[1]***

**[1]Residue Chemistry and Predictive Microbiology Research Unit, Eastern Regional Research Center, Agricultural Research Service, United States Department of Agriculture, 600 East Mermaid Lane, Wyndmoor, PA 19038. USA.**
**[2]Molecular Characterization of Foodborne Pathogens Research Unit, Eastern Regional Research Center, Agricultural Research Service, United States Department of Agriculture, 600 East Mermaid Lane, Wyndmoor, PA 19038. USA.**

***E-mail:** andy.hwang@ars.usda.gov

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**Correspondence Author:** Cheng-An Hwang
**Address:** Residue Chemistry and Predictive Microbiology Research Unit, Eastern Regional Research Center, Agricultural Research Service, United States Department of Agriculture, 600 East Mermaid Lane, Wyndmoor, PA 19038. USA
Tel: (215) 233-6416; Fax: (215) 233-6581

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**Abstract:** The objectives of this study were to examine the effect of stresses on the growth and cytotoxicity of pathogenic Escherichia coli in beef and spinach. A mixture of three strains of Shiga toxin-producing E. coli (STEC) O157:H7 or 4 strains of non-O157 STEC, O26:H11, O103:H1, O104:H4, and O145:NM, was subjected to stress of 2 ppm chlorine, $a_w$ 0.97, pH 5, or 15-day starvation. Stressed or non-stressed STEC was inoculated into 5 g of irradiated ground beef or spinach. The cell populations during storage at 8, 12, or 16°C for 4 weeks were compared to evaluate the growth variation between O157 and non-O157 STEC. Supernatant from each sample after 24-h incubation at 22°C was used to determine Vero-cytotoxicity using [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, MTS] or lactate dehydrogenase (LDH) assay to evaluate the effects of stresses on the cytotoxicity exhibited by STEC. After one week at 8°C, the population of non-stressed non-O157 (3.1 log CFU/g) was significantly ($p<0.05$) higher than O157 (1.9 log CFU/g) in ground beef, and the difference in populations (3.9 vs. 1.7 log CFU/g, $p<0.05$) was also observed after 4 weeks. However, in spinach, the populations after 4 weeks at 8°C were not significant (3.9 vs. 3.3 log CFU/g). Starvation or chlorine stress induced higher growth in non-O157 than O157 in ground beef (4.5 vs. 1.2 log CFU/g) and spinach (4.7 vs. 2.5 log CFU/g). At 12 and 16°C, cell populations of stressed and non-stressed O157 or non-O157 STEC were not significantly ($p>0.05$) different in beef and spinach. MTS assay showed that stressed O157 and non-O157 STEC exhibited significantly ($p<0.05$) higher cytotoxicity than the non-stressed controls. The numbers of surviving Vero cells were 47-52% (stressed) vs. 65% (control) in beef and 20-30% (stressed) vs. 52-53% (non-stressed) in spinach. Similarly, LDH assay also indicated an increased cytotoxicity ($p<0.05$) in stressed O157 and non-O157 STEC than non-stressed controls in spinach. There was no significant difference among the four stresses in inducing the levels of cytotoxicity in O157 or non-O157 STEC. Results showed that STEC cells exposed to sub-lethal stresses might have increased cytotoxicity during subsequent growth in ground beef or spinach. The findings illustrate the importance of applying suitable control measures to eliminate the presence of stressed STEC in beef and spinach processing environment or their subsequent contamination in the products.

**Key words:** Pathogenic Escherichia coli, environmental stress, growth, cytotoxicity

## 1.Introduction

Cattle have been recognized as the reservoir of pathogenic Shiga-toxin producing Escherichia coli (STEC). Many outbreaks caused by STEC have been linked to the consumption of undercooked ground beef. Although E. coli O157:H7 is classified as an adulterant in beef products in U.S. market, non-O157 STEC are also presumed to be prevalent in finished ground beef (Bosilevac and Koohmaraie, 2011). To overcome this potential threat posed by STEC-contaminated meat, the meat industry has introduced hurdles to prevent the contamination (e.g., acid surface washing of meat) or growth of STEC (e.g., adding lactate and diacetate) in meat products. However, STEC that survived the hurdles is a concern to the manufacturers, because the pattern of their growth is not necessarily similar to those bacteria that are not exposed to the hurdles. In recent years, fresh produce has been emerging as a main source of STEC infections for its popularity among consumers, who regard produce as a healthy food. Cases of foodborne illnesses associated with fresh produce have increased as its consumption increased over the past two decades, probably due to the increased cattle production near produce production, increased import from countries with sub-standard sanitation, and greater numbers of immuno-compromised individuals (Beuchat, 2002). Since often consumed raw or minimally processed (Harris et al., 2003), produce contaminated with pathogenic STEC is a potential health hazard. Among the highly diverse produce categories, leafy greens are most frequently implicated in bacterial foodborne illnesses, and leafy greens contaminated with E. coli O157:H7 were linked to more than half of the outbreaks between 1998 and 2008 (Painter et al., 2013).

Microorganisms in food are constantly exposed to sub-lethal stresses from the farm to table. Microbial cells in the exponential growth phase, in which rapid cell division occurs, are more sensitive to inimical processes than in the stationary phase, in which cell division ceases. Common sub-lethal stresses that bacteria may encounter in the food processing environment include osmotic stress, acid stress, starvation, and bactericidal treatments. In some bacterial cells, virulence genes are under RpoS control so that cells in stationary phase may be more virulent. Additionally, physiological stresses in human intestines are known to induce the expression of virulence in colonizing pathogens (Zaborina et al., 2011). Exposure to sub-lethal stresses is also known to render bacterial cells more resistant to subsequent stresses and potentially increase the ability of cells to survive stresses (Allen et al., 2008).

Although STEC is a significant pathogen of concern for produce and meat products, there is still lack of information regarding the survival and pathogenicity of STEC in response to environmental stresses. We have reported that chlorine, water activity, acidity, or starvation stresses on STEC growth in lettuce and cantaloupe (Yoo et al., 2015). A question was raised whether stressed STEC would behavior the same in other food matrices. Therefore, the purpose of this study was to investigate the growth behavior and cytotoxicity of O157 STEC and non-O157 STEC in ground beef and spinach after they survived sub-lethal environmental stresses.

## 2.Materials and Methods

### 2.1Bacterial strains and inoculums preparations

Three strains of E. coli O157:H7 (ATCC 43888, 43889, and 43890) and four strains of E. coli non-O157 STEC including O26:H11 (05-6544), O103:H1 (04/62), O104:H4 (2009EL-2050), and O145:NM (03-4699) were obtained from the Molecular Characterization of Foodborne Pathogens Research Unit, Eastern Regional Research Center, Agricultural Research Service, United States Department of Agriculture (Wyndmoor, PA). Frozen stock cultures (-80°C) of each strain were streaked onto Sorbitol MacConkey Agar (SMAC; BD Biosciences, Sparks, MD) and incubated at 37°C for 24 h. A single colony from each strain was grown overnight in brain heart infusion broth (BHI; BD Biosciences) at 37°C on a shaker with shaking at 200 rpm. Each culture was centrifuged at 2800 ×g for 3 min at 4°C (Marathon 21000R, Fisher Scientific, Pittsburgh, PA). Cells were washed twice by removing supernatant and suspending the cell pellet in 10 ml of 0.1% peptone water.

### 2.2 Stress treatments

#### 2.2.1 Chlorine stress

Stock solutions of sodium hypochlorite (NaOCl; Fisher Scientific, Fair Lawn, NJ) were prepared on the day of the experiment by diluting 5% NaOCl in sterile distilled water (22°C). Ten milliliters of E. coli cell cultures grown overnight at 37°C were pelleted (2800 ×g, 3 min, 4°C), washed twice with phosphate buffered saline (PBS), and suspended in sterile distilled water. Cells of each strain at $10^7$ CFU/ml were exposed to 1, 2, or 5 ppm chlorine at 22°C for 24 h. After exposure, 1 ml of liquid from each tube was immediately transferred to test tubes containing PBS and filter-sterilized 2 µg/ml of sodium thiosulfate (Fisher Scientific) to neutralize the residual chlorine. Among the three tested concentrations, 2 ppm of chlorine was able to differentiate non-O157 strains from O157 strains based on cell populations. Therefore, STEC cells that survived the 2 ppm chlorine exposure were collected and subjected to another chlorine exposure for 24 h to select cells that were resistant to chlorine stress. After three exposures, the third generation of stressed STEC cells was collected. Cells of each generation and non-stressed control were stored and frozen in 15% (v/v) glycerol until they were used for growth/cytotoxicity studies in ground beef or spinach.

#### 2.2.2 Starvation stress

Ten milliliters of STEC grown overnight at 37°C were pelleted (2800 ×g, 3 min, 4°C), washed twice by PBS. The cells were suspended in sterile distilled water to a final concentration of

~ $10^8$ CFU/ml and stored at 4°C to simulate a nutrient-depleted (starvation) condition (Kolling and Matthews, 2007). After 15 d, STEC cells surviving the starvation condition were collected and subjected to another set of starvation stress for 15 d. After three consecutive exposures, the third generation of stressed cells was collected. Cells of each generation were stored and frozen in 15% (v/v) glycerol until they were used for growth/cytotoxicity studies in ground beef or spinach.

**2.2.3 Acidic stress**

Ten milliliters of STEC cultures grown overnight at 37°C were pelleted (2800 ×g, 3 min, 4°C), washed twice by PBS, and diluted in sterile distilled water to a final concentration of ~$10^8$ CFU/ml. PBS was acidified to pH 7, 6, 5, or 4 using 1 N hydrochloric acid (Fisher Scientific). The acidified PBS was sterilized using 0.22-µm Corning filter units (Costar, Corning, NY). The cells ($10^7$ CFU/ml) were exposed to the acidified PBS for 24 hr. Among the four pH, pH 5 was found to differentiate non-O157 from O157 strains based on cell populations. Therefore, after exposure for 24 h, cells surviving pH 5 were collected and subjected to another set of acidic stress (pH 5) for 24 h. After three consecutive exposures, the third generation stressed STEC cells were collected and cells of each generation were stored and frozen with 15% (v/v) glycerol until they were used for growth/cytotoxicity studies in ground beef or spinach.

**2.2.4 Osmotic stress**

Stock solutions of various water activities were prepared fresh on the day of experiment by using 8.5, 7, 5.25, and 3.5 g of NaCl (Mallinckrodt Baker, Phillipsburg, NJ) to obtain different levels of water activities ($a_w$ 0.95, 0.96, 0.97, and 0.98) in sterile distilled water (22°C). Water activity was measured using an AquaLab water activity meter (Decagon Devices, Pullman, WA) calibrated with saturated salt solutions of known water activity. Ten milliliters of STEC cells grown overnight at 37°C were pelleted (2800 ×g, 3 min, 4°C), washed twice in PBS, and diluted in sterile distilled water to reach a final concentration of ~$10^8$ CFU/ml. Among the four levels of water activity, $a_w$ of 0.97 was able to differentiate non-O157 strains from O157 strains based on cell populations. Therefore, after exposure for 24 h, cells surviving $a_w$ of 0.97 were collected and subjected to another set of osmotic stress ($a_w$ 0.97) for 24 h. After three consecutive exposures, the third generation of each stressed STEC cells was collected and cells of each generation were stored and frozen with 15% (v/v) glycerol until they were used for growth/cytotoxicity studies in ground beef or spinach.

**2.3 Sample inoculation and sampling**

Ground beef and baby spinach were purchased from a local grocery store and stored at -20°C (ground beef) or 4°C (spinach). Ground beef was irradiated (2 kGy) to eliminate background microflora. Spinach was soaked in 200-ppm chlorine solution for 10 min and cut into 2×2 cm pieces with a sterile knife. Each STEC cocktail mixture (O157 or non-O157 STEC and stressed or non-stressed control cells ($10^4$ CFU/g) at 50 µl was inoculated at 10 random spots on the surface of 5 g of ground beef or spinach. Samples were sealed in 100-ml sterile bags (Interscience, St Nom, France) and incubated at 8, 12, and 16°C for four weeks. At every 7-day sampling point, two samples were added with 5 ml PBS, mixed for 2 min in a Bag Mixer 400 stomacher (Interscience), and then serially diluted in sterile 0.1% peptone water. Duplicate 0.1 ml of appropriate dilutions were plated onto tryptic soy agar (TSA; BD Biosciences). The plates were incubated at 37°C, and, after 48 h, and colonies were counted by an IUL Flash & Go automated colony counter (Neutec Group Inc., Farmingdale, NY). The counts were expressed as log CFU/g ground beef or spinach.

**2.4 Sample inoculation and sampling**

**2.4.1 MTS assay**

Cytotoxicity was determined using Vero cells (ATCC CCL-81, African green monkey kidney epithelial cell line) by MTS assay kit (CellTiter 96® AQueous One Solution Cell Proliferation Assay; Promega, Madison, WI). Monolayer of subconfluent Vero cells in 96-well plates was seeded in 96-well plates. STEC strains ($G_3$ or control) grown overnight in BHI broth at late exponential or early stationary phase were collected and filter-sterilized to obtain supernatants that included the Shiga toxin released by the cells. Vero cells were exposed to filter-sterilized supernatants for 24 h or up to 48 h at 37°C in 5% $CO_2$ incubator. After incubation, cytotoxicity was measured based on the manufacturer's instructions. The cell viability was determined by transferring 100 µl of supernatant from each well to wells of a 96-well, flat bottomed microplates (Corning Inc., Corning, NY), adding 20 µl of assay reagent to wells, incubating at 37°C for 1-4 h in the dark, humidified, and 5% $CO_2$ atmosphere, and measuring the absorption at 490 nm with Saffire2 microplate reader (Tecan, Morrisville, NC). In the MTS assay, the percentage of cell viability was calculated using the optical density readings:

Cell viability (%) = [(treated – blank well) / (untreated control – blank well)] × 100

Measured cell viability was normalized by the cell population when the supernatants were collected. Each sample was tested in triplicates. At least three independent experiments were performed for each sample.

**2.4.2 LDH assay**

Cytotoxicity was also determined using Vero cells by lactate dehydrogenase (LDH) assay kit (Roche Diagnostics, Indianapolis, IN) in the same way as MTS assay. After incubation of Vero cells with supernatants of STEC, the release of LDH was measured according to the manufacturer's instructions. The spontaneous LDH release (=low control) was determined by adding 100 µl of assay medium that contains 1% fetal bovine serum without pyruvate to wells containing 100 µl of cells in assay medium. Maximum LDH release (=high

control) was determined by adding 100 µl of Triton X-100 (Sigma, St. Louis, MO) solution (2% Triton X-100 in assay medium) to each wells containing 100 µl of cells in assay medium. The amount of LDH released was determined by transferring 100 µl of supernatant from each well to wells of a 96-well microplates (Corning), adding 100 µl of reaction mixture to wells, incubating at room temperature for 30 min in the dark, and measuring the absorption at 492 nm using a reference wavelength of 620 nm with Saffire2 microplate reader (Tecan). In the cytotoxicity LDH detection assay, the percentage of cytotoxicity was calculated using optical density readings:

Cytotoxicity (%) = [(experiment value – low control) / (high control – low control)] × 100

Measured cytotoxicity was normalized by the cell population when the supernatants were collected. Each sample was tested in triplicates. At least three independent experiments were performed for each sample.

**2.5 Statistical Analysis**

Each experiment was conducted in duplicate and repeated three times. Comparison of means was tested using the Student's t test of the Statistical Analysis Software (SAS Institute Inc., Cary, NC). Significance of differences in growth in terms of cell populations between stressed and non-stressed O157 and non-O157 STEC strains were determined at $\alpha$=0.5.

## 3. Results and Discussion

### 3.1 Influence of stresses on the growth and survival of STEC in ground beef

The results of growth studies in ground beef are presented in Figure 1. In general, the populations of non-O157 STEC were higher than O157 STEC during the 4-week storage at 8°C (Fig. 1A). The cell populations of non-O157 STEC didn't change significantly from the initial levels during storage and ranged from 3.5 log CFU/g (non-stressed control and osmotic-stressed) to 4.5 log CFU/g (starvation-stressed) after 4 weeks. In comparison, the cell populations of O157 STEC were decreased and significantly lower at 1.2-1.9 log CFU/g during the same incubation period. Among non-O157 STEC, only starvation-stressed non-O157 had a significantly ($p<0.05$) higher population than osmotic-stressed strain after 4 days at 8 °C. For O157 STEC, no significant difference ($p>0.05$) in cell populations were observed between stressed and non-stressed cells or among cells subjected to difference stresses (Fig. 1A). At 12°C (Fig. 1B) and 16°C (Fig. 1C), the populations of both O157 and non-O157 increased during storage and there were not significant differences in cell populations between O157 and non-O157 STEC or stressed and non-stressed STEC. The results showed that non-O157 strains survived better than O157 only at 8°C, which is near the minimal growth temperature for STEC, and the four stresses did not significantly promote survival or growth of STEC. The result is in agreement with a recent study examining the influence of environmental factors on the adhesion of O157 and non-O157 STEC and their biofilm formation on the skeletal-muscle extracellular matrix proteins (Chagnot et al., 2014). The study reported that low temperatures might trigger an unknown mechanism to promote the adhesion of non-O157 STEC to the surface of ground beef leading to a better survival than O157 STEC.

**Fig. 1**

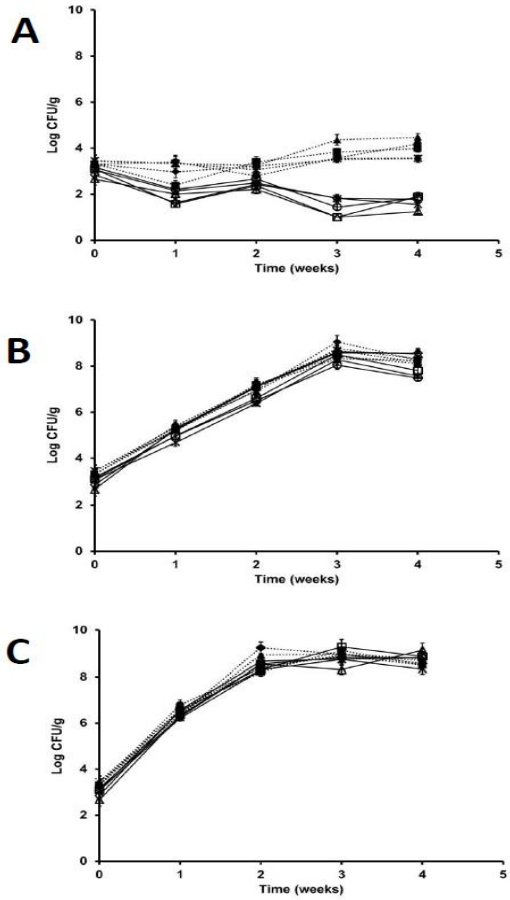


**Figure 1** Growth of non-stressed control (CON, diamond), chlorine-stressed (CHL, square), starvation-stressed (STV, triangle), acid-stressed (pH, circle), and osmotic-stressed ($a_w$, asterisk) O157 (solid line, empty) or Non-O157 (dotted line, filled) STEC in ground beef at three different temperatures; 8°C (A), 12°C (B), and 16°C (C) for 4 weeks. The data represent the average log CFU per gram of ground beef and data from three independent experiments are presented as means ± SE.

### 3.2 Influence of stresses on the growth and survival in spinach

Results of growth studies in baby spinach are presented in Fig. 2. In general, the populations of non-O157 STEC were higher than O157 STEC during the 4-week storage at 8°C (Fig. 2A). At

8°C, chlorine-stressed non-O157 STEC reached 4.7 log CFU/g after 4 weeks, which was significantly ($p<0.05$) higher than that of chlorine-stressed O157 STEC (2.5 log CFU/g). In ground beef, growth of non-stressed or stressed O157 STEC declined during 4 weeks at 8°C (Fig. 1A). However, in spinach, growth of O157 STEC remained at the inoculation level or slightly higher than the initial population despite of stress treatments (Fig. 2A). At 12°C (Fig. 2B) and 16°C (Fig. 2C), the growth patterns of O157 and non-O157 STEC were similar. In addition, there were no differences in growth of STEC subjected to different stresses. The growth of O157 or non-O157 STEC in spinach at 8°C was better than in ground beef. Better survival of pathogenic bacteria in fresh produce than ground beef at refrigeration temperatures has been reported. Fresh produce has higher water activity and more readily available nutrients at that support the growth of foodborne pathogens (Olaimat and Holley, 2012). E. coli O157 was observed to form biofilm on lettuce after 24 h at 10°C (Olmez and Temur, 2010) and on spinach after 24 h at 4°C (Niemira and Cooke, 2010) because it is able to attach to fresh produce by motility which facilitates its entry into produce tissue openings (Saldana et al., 2011).

**Fig. 2**

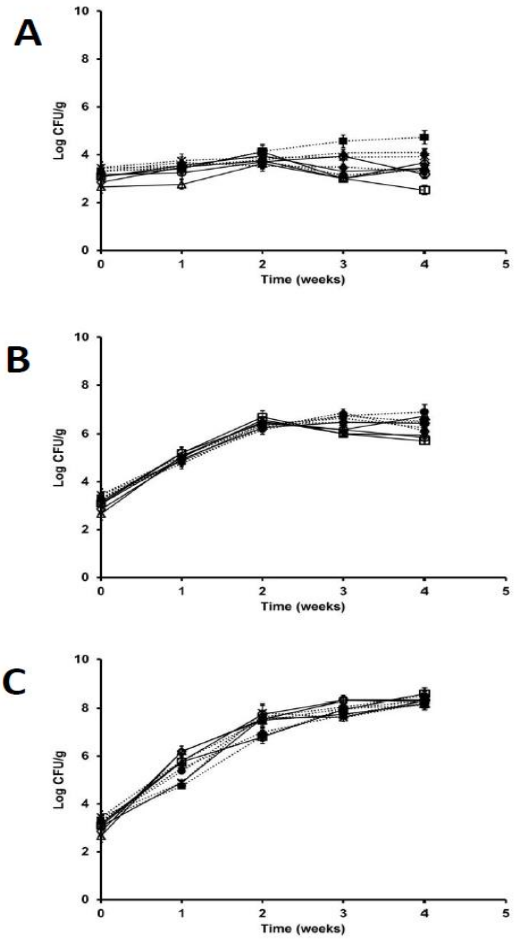


**Figure 2** Growth of non-stressed control (CON, diamond), chlorine-stressed (CHL, square), starvation-stressed (STV, triangle), acid-stressed (pH, circle), and osmotic-stressed ($a_w$, asterisk) O157 (solid line, empty) or Non-O157 (dotted line, filled) STEC in spinach at three different temperatures; 8°C (A), 12°C (B), and 16°C (C) for 4 weeks. The data represent the average log CFU per gram of spinach and data from three independent experiments are presented as means ± SE.

### 3.3 Influence of stresses on the cytotoxicity of STEC grown in ground beef and spinach

Two methods based on LDH release (LDH assay) and mitochondrial oxidoreductase activity (MTS assay) were used to assay the cytotoxicity produced by STEC. LDH release, taken as an indicator of host cell membrane integrity and cell viability, was measured, and compared to untreated cells. The cytotoxicity of stressed STEC grown in ground beef and spinach against Vero cells is shown in Figure 3. In ground beef, STEC produces high levels of indigenous lactate dehydrogenase, which interferes with LDH assay that is designed to measure the cytosolic LDH resulting from Vero cellular damage. When bacterial supernatant was collected from ground beef, its LDH level was too high to measure the basal level of healthy Vero cells. NADH-dependent mitochondrial oxidoreductase reduces MTS tetrazolium to formazan in metabolically active cells and its activity indicates the number of viable cells, in other words, cell viability. Cell viability reflected the inverse of cytotoxicity. Therefore, MTS assay was selected for a substitute as a way to quantify the cytotoxicity by measuring the cell viability of Vero cells that survived damages by Shiga toxin. Cell viabilities of Vero cells that were incubated with supernatant of STEC grown for 24 h in ground beef and spinach are shown in Figs. 3A and 3B, respectively, while Fig. 3C shows the cytotoxicity of Vero cells that were incubated with supernatant of STEC grown for 24 h in spinach. In ground beef, non-stressed O157 and non-O157 STEC showed higher cell viability and the two had similar levels of cell viability (65%). Chlorine-stressed and osmotic-stressed O157 and non-O157 STEC had lower cell viabilities at 52% and 50%, respectively. Starvation- and acidic-stressed non-O157 STEC had lower cell viabilities, compared with O157 STEC, however the difference was not significant (47% vs. 52% and 48% vs. 52%). In spinach, both MTS and LDH assays were used to investigate the difference among stresses and between O157 and non-O157 STEC. Stressed STEC had significantly lower cell viabilities (20-29%) than non-stressed STEC (52-53%). The cell viabilities were not different among the stresses or between O157 and non-O157 (Fig. 3B). Results from the LDH assay (Figure 3C) showed that the cytotoxicity from O157 STEC at 51-53% and acidic-stressed O157 STEC at 58%. In contrast, starvation-, acidic-, and osmotic-stressed non-O157 STEC showed 65-66% of cytotoxicity, which were significantly higher than 54% of non-stressed non-O157 STEC. It was noted that MTS assay had higher sensitivity than LDH

assay in that it had better resolution to distinguish the results of cytotoxicity between stressed and non-stressed STEC in spinach. It has also been reported that MTS assay had higher sensitivity that LDH assay (Arechabala et al., 1999; Fotakis and Timbrell, 2006). As MTS assay is dependent on the metabolism of MTS reagent into formazan by dehydrogenase enzymes (Buttke et al., 1993), they may have drawbacks including false positive results where cellular metabolisms are affected by the other source (Berridge et al., 2005) and inability to differentiate cell cycle inhibition and cellular death (Galluzzi et al., 2009). In contrast, LDH assay may underestimate the amount of dead cells that do not release LDH (Smith et al., 2011). A study using viable but non-culturable O157 STEC cells showed stresses can induce Shiga-toxin production by qRT-PCR and Vero-cytotoxicity assay (Liu et al., 2010). Another study showed that stresses could promote Shiga toxin release as well as biofilm formation (Villegas et al., 2013). In our study, stressed STEC had higher cytotoxicity than non-stressed STEC, and the increased release of toxin may also increase their survival under stressed environments.

Fig. 3

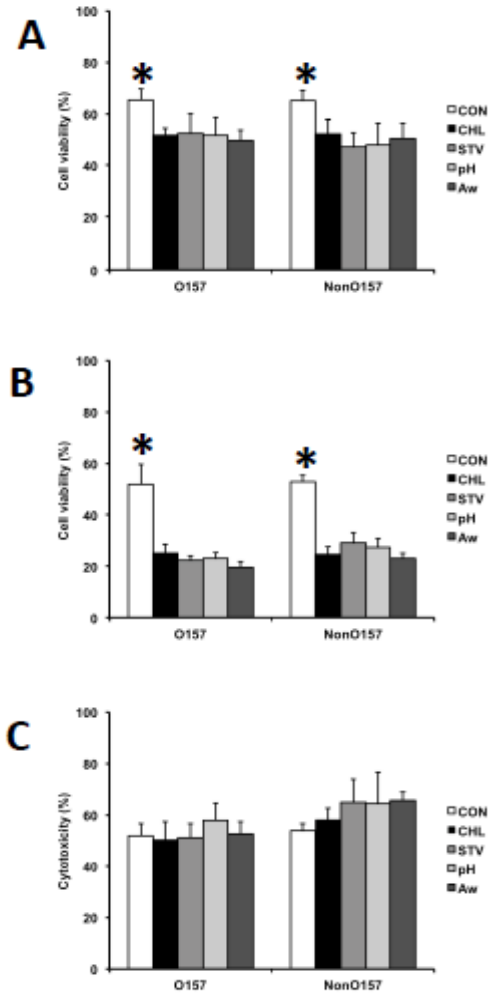


**Figure 3** Cytotoxicity of O157 or Non-O157 STEC in ground beef (A) or spinach (B, C) was determined. MTS assay (A, B) was used to measure the cell viability of non-stressed control (CON), chlorine-stressed (CHL), starvation-stressed (STV), acid-stressed (pH), and osmotic-stressed ($a_w$) O157 or Non-O157 STEC in ground beef using Vero cells inoculated with supernatant. Vero-cytotoxicity was assessed using LDH assay (C) of Vero cells treated with supernatants of STEC cells. Supernatants were collected after 24 h of growth in ground beef or spinach. Cell viability or Vero-cytotoxicity was determined and normalized by each cell density population. The results are the average of three independent experiments. *, $p<0.05$.

This study aimed to determine the influence of environmental stresses on the growth and cytotoxicity of STEC in food matrices. The results illustrated a potential of increased hazard from stressed STEC in beef and spinach and hence the importance of designing control measures to eliminate the presence of stressed STEC in beef and spinach processing environment and their contamination in the products.

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