Short title: Grape seed extract and non-O157 Shiga toxin producing *E. coli*

**Inhibitory effects of grape seed extract on growth, quorum sensing, and virulence factors of
CDC “top-six” non-O157 Shiga toxin producing *E. coli***

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ABSTRACT

Non-O157 Shiga toxin producing *Escherichia coli* (STEC) have become a growing concern to the food industry. Grape seed extract (GSE), a byproduct of wine industry, is abundant in polyphenols that are known to be beneficial to health. The objectives of this study were to evaluate the effect of GSE on the growth, quorum sensing, and virulence factors of Centers for Disease Control and Prevention (CDC) “top-six” non-O157 STEC. Minimal inhibitory concentration (MIC) of GSE was 2 mg/ml against *E. coli* O26:H11, and 4 mg/ml against the other non-O157 STEC tested. Minimal bactericidal concentration (MBC) was the same as MIC for all six non-O157 STEC tested. At 5×10^5 CFU/ml inoculation level, 4 mg/ml GSE effectively inhibited the growth of all tested strains, while 0.25-2 mg/ml GSE delayed bacterial growth. At a higher inoculation level (1×10^7 CFU/ml), GSE had less efficacy against the growth of the selected six non-O157 STEC. Its impact on bacterial virulence was then assessed at this inoculation level. Autoinducer-2 (AI-2) is a universal signal molecule mediating quorum sensing (QS). GSE at concentration as low as 0.5 mg/ml dramatically reduced AI-2 production of all non-O157 STEC tested, with the inhibitory effect proportional to GSE levels. Consistent with diminished QS, GSE at concentration of 0.125 mg/ml caused marked reduction of swimming motility of all motile non-O157 STEC tested. In agreement, GSE treatment reduced the production of flagella protein FliC and its regulator FliA in *E. coli* O103:H2 and *E. coli* O111:H2. Furthermore, 4 mg/ml GSE inhibited the production of Shiga toxin, a major virulence factor, in *E. coli* O103:H2 and *E. coli* O111:H2. In summary, GSE inhibits the growth of “top-six” non-O157 STEC at the population level relevant to food contamination. At higher initial population, GSE suppresses QS with concomitant decrease in motility, flagella protein

expression and Shiga toxin production. Thus, GSE has the potential to be used in food industry to control non-O157 STEC.

Keywords: non-O157 STEC, GSE, growth, quorum sensing, motility, flagella, Shiga toxin

1. Introduction

Shiga toxin producing *E. coli* (STEC) are important zoonotic foodborne pathogens. Aside from the well-recognized notorious serotype *E. coli* O157:H7, non-O157 STEC are of growing food safety concern to the food industry and increasing importance to human infection due to sporadic cases or outbreaks in the United States and globally (Hedican et al., 2009; Mathusa et al., 2010). Non-O157 STEC are estimated to cause more than 110,000 cases of illness annually throughout the United States, contributing to a larger portion of total STEC infections than *E. coli* O157:H7 (Scallan et al., 2011). Centers for Disease Control and Prevention (CDC) has designated six most common non-O157 STEC serogroups including O26, O45, O103, O111, O121 and O145 as the “top-six” non-O157 STEC, which together account for more than 70% of diseases caused by STEC that emerged from 1983 to 2002 (Brooks et al., 2005). Like notorious *E. coli* O157:H7, non-O157 STEC infection can cause symptoms ranging from diarrhea and hemorrhagic colitis, to fatal hemolytic uremic syndromes (HUS) and even death (Espie et al., 2008; Hedican et al., 2009; Johnson et al., 2006; Mathusa et al., 2010).

Due to the increasing public health impact, the USDA-FSIS declared these “top-six” non-O157 STEC serogroups as adulterants if present in non-intact raw beef products (Anonymous, 2011). In order to meet this requirement, meat industries are actively looking for effective interventions to inactivate non-O157 STEC. For example, high pressure processing was used on ground beef inoculated with the “top-six” non-O157 STEC cocktail; however, 5 Log reduction of

bacteria was achieved only when processed at 450 MPa for 15 min (Hsu et al., 2015). Spray treatment of non-O157 STEC inoculated beef subprimal surfaces with commonly used antimicrobials such as lactic acid and peroxyacetic acid only slightly reduced bacterial numbers after 14 days storage (Liao et al., 2015). In addition, individual non-O157 STEC might be more resistant to a particular intervention method. For example, *E. coli* O103 inoculated on fresh beef flanks is less sensitive to hot water spray compared with either other non-O157 STEC or *E. coli* O157:H7 strains (Kalchayanand et al., 2012). Therefore, developing effective intervention methods for non-O157 strains is of great necessity.

A wide variety of natural products have been explored for their antimicrobial effects on bacterial growth. Grape seed extract (GSE) is a byproduct of wine and juice industry and rich in polyphenolic compounds that have received increasing attention for their health-beneficial effects (Kaur et al., 2009; Vislocky, Fernandez, 2010; Wang et al., 2013; Yang et al., 2015). Meanwhile, GSE is known for its antimicrobial activity against both gram-positive and gram-negative bacteria including *Staphylococcus aureus* (Kao et al., 2010), *Listeria monocytogenes* (Bisha et al., 2010; Rhodes et al., 2006), *Vibrio parahaemolyticus* (Mahmoud, 2014) and *E. coli* O157:H7 (Ahn et al., 2007; Over et al., 2009). Previously, we reported that GSE inhibits the growth of *E. coli* O157:H7 (Zhu et al., 2015). Up to now, there is no information regarding efficacy of GSE in controlling non-O157 STEC. Here we assessed the effect of GSE on the growth of the CDC's "top-six" non-O157 STEC.

In addition to traditional approach targeting growth, interference of bacterial virulence provides an alternative strategy for controlling foodborne pathogens. Quorum sensing (QS) is an extracellular bacterial signaling that senses population density in the community through

excreting autoinducer (AI) molecules, which orchestrates important events related to bacterial pathogenesis (Sperandio et al., 2001). QS in *E. coli* O157:H7 regulates flagellar synthesis and motility (Sperandio et al., 2002a), Shiga toxin (Stx) production (Sperandio et al., 2001), and expression of the type III secretion system (Sperandio et al., 1999). Thus, QS has been suggested as a global regulator controlling virulence factors as well as basic physiological function of *E. coli* O157:H7 (Sperandio et al., 2001). As a result, QS molecules are novel intervention targets to control foodborne pathogens. Therefore, the efficacy of GSE against QS and the motility of “top-six” non-O157 STEC as well as flagella and Stx production were further evaluated in this study.

2. Materials and methods

2.1 Bacterial strains

CDC “top-six” non-O157 STEC strains used in this study were obtained from the STEC center at Michigan State University (Table 1). The non-O157 STEC strains were routinely grown in 3 mL LB broth or LB containing 0.5% (w/v) glucose (LBG) for quorum sensing assay in 10 mL glass test tubes at 37 °C under shaking at 250 rpm in MaxQ™ 4000 Incubator Shaker (Thermo Scientific, Marietta, OH). Basic media (Diez-Gonzalez, Russell, 1999) with 0.4% glucose (BMG) was then utilized in all experiments with GSE (Zhu et al., 2015). *Vibrio harveyi* was obtained from ATCC and grown in autoinducer bioassay (AB) medium at 30 °C with shaking at 250 rpm in MaxQ™ 4000 Incubator Shaker (Thermo Scientific).

2.2 Grape seed extract

GSE was purchased from Optipure (Los Angeles, CA) and it contains a minimum of 95% total flavonols and 82% of proanthocyanidin. GSE stocking (40 mg/ml) solution was prepared in

10% ethanol and microwaved for 5 min for the maximal extraction of phenolic compounds, and then filter sterilized (Zhu et al., 2015).

2.3 Minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC and MBC of GSE against all non-O157 STEC strains were determined as previously described (Sheng, Zhu, 2014). Briefly, GSE stocking was added to BMG to a final concentration of 16 mg/ml, which was then 2-fold serial diluted in 96 well polystyrene microtiter plates (Costar, Tewksbury, MA). The final tested GSE concentrations ranged from 0.03125 to 16 mg/ml. Each well was inoculated with 5×10^5 CFU/ml of non-O157 STEC. Cultures were incubated statically at 37 °C. MIC was determined as the lowest concentration of GSE that inhibited bacterial growth as measured by optical density (OD) values at 600 nm at 24 h incubation. Samples from wells without visible growth were spread on LB agar plates and incubated at 37 °C for 24 h. MBC was defined as the lowest concentration that inhibited bacterial growth on the LB agar plates (<10 CFU/plate was regarded as no growth). Experiments were repeated three times with twice activated fresh culture.

2.4 Growth curves

Overnight STEC cultures were diluted in BMG with variable GSE concentrations to achieve 1×10^7 or 5×10^5 CFU/ml, and 200 µl of bacterial suspension was added to 96 well microtiter plates (Costar), and incubated statically at 37 °C. One column per treatment, 6 inner wells of each column were inoculated with bacteria, while the two outside wells of each column were loaded with BMG with respective GSE concentration. OD values were read hourly at 600 nm using SM5 spectrometer (Molecular Device, Sunnyvale, CA). The experiments were repeated three times with fresh activated culture.

2.5 AI-2 assay

AI-2 assay was performed as described previously (Surette, Bassler, 1998). Briefly, non-O157 STEC at 1×10^7 CFU/ml were cultured in BMG with various concentrations of GSE for 5 h at 37 °C under shaking at 250 rpm. Then the bacterial cultures were sampled for CFU enumeration, and the remaining cultures were centrifuged at 20,000 ×g for 5 min. The resulting supernatant was harvested and incubated with *V. harveyi* in 96-well microtiter plates at 30 °C / 250 rpm for 5 h. *V. harveyi* was chosen as an indicator microorganism because it can express bioluminescence in response to AI-2 produced either by itself or by other bacteria (Bassler et al., 1997). Therefore, light production was measured using a BioTek microplate reader (Synergy H1, BioTek). *V. harveyi* incubated with various tested concentrations of GSE was used as negative control. Luminescence value of treatments was subtracted by corresponding luminescence value of negative control to rule out negative influence of GSE itself on *V. harveyi* AI-2 production. **Results were normalized to control.** This experiment was repeated three times with fresh activated culture.

2.6 Swimming motility assays

Swimming motility was measured according to the previously published method (Zhu et al., 2015). Briefly, overnight cultures of respective non-O157 STEC were concentrated 10 times, and then 1:1 mixed with various concentrations of GSE solutions. Then, 5 µL of respective non-O157 preparation was inoculated onto 0.25% LB soft agar plates containing 0, 0.125, 0.25 or 0.50 mg/ml GSE, which were then incubated at 37 °C for 6 h. Motility was measured as the diameter of the swimming zone in millimeters. A diameter of 2 mm is considered as “no motility” as that is the diameter of the initial inoculation. The experiments were performed three times with fresh activated culture.

2.7 Immunoblotting analysis

Overnight *E. coli* O103:H2 and *E. coli* O111:H2 cultures were subcultured at $\sim 1 \times 10^7$ CFU/ml in BMG with various GSE concentrations for 5 h at 37 °C under shaking at 250 rpm. Then cultures were sampled for CFU enumeration and protein sample preparation for analyzing Stx2A, FliA, and FliC according to published methods (Zhu et al., 2015). Briefly, the above bacteria preparation was 5:1 mixed with 6 × SDS-PAGE sample lysis buffer and boiled for 15 min to lyse the bacteria and denature protein. Immunoblotting was conducted as previously described (Zhu et al., 2015). Briefly, bacterial protein samples were separated by 10% (w/v) SDS polyacrylamide gels, transferred to nitrocellulose membrane, subjected to primary and secondary antibody incubation, and ECL incubation (GE Healthcare, Buckinghamshire, UK) for band visualization. Primary antibody against Stx2A (Toxin Tech. Inc., Sarasota, FL), FliA and FliC (BioLegend, San Diego, CA) were used at 1:1000 dilution. **The density of the target protein bands was normalized to that of control group without GSE treatment.** The relative values were compared among GSE treatments and reported. The experiment was repeated three times with fresh activated culture.

3. Results

3.1 GSE inhibited the growth of non-O157 STEC

MIC was determined to be 2 mg/ml GSE for *E. coli* O26:H11 and 4 mg/ml for the other non-O157 STEC tested (Table 1). MBC was the same with MIC for all the six non-O157 STEC (Table 1).

At an initial population of 5×10^5 CFU/ml, 4 mg/ml GSE completely inhibited growth of all six strains up to 16 h (Fig. 1A-F). For *E. coli* O26:H11, 0.125 and 0.25 mg/ml GSE prolonged bacterial lag phase in a dose dependent manner, and 0.5-4 mg/ml GSE inhibited bacterial growth for at least 17 h (Fig. 1A). For *E. coli* O45:H2 and *E. coli* O121:H19, 0.125-0.5 mg/ml GSE failed to show any inhibitory effect on bacterial growth while 1-4 mg/ml GSE inhibited bacterial growth in a dose dependent manner (Fig. 1B&E). Interestingly, GSE at concentrations between 0.125-1 mg/ml enhanced the growth of *E. coli* O103:H2 and *E. coli* O111:H2 (Fig. 1C&D). GSE at all concentrations inhibited the growth of *E. coli* O145:NT for at least 15 h (Fig. 1F).

At an initial population of 1×10^7 CFU/ml, the growth of *E. coli* O26:H11 was the most affected by GSE; as low as 0.5 mg/ml GSE significantly stunted bacterial growth (Fig. 2A). For the growth of *E. coli* O45:H2 and *E. coli* O145:NT, inhibitory effect of GSE increased proportionally to its concentrations (Fig. 2B&F). All tested concentrations of GSE exhibited similar effects on *E. coli* O103:H2 growth, with no inhibitory effect until 9 h incubation (Fig. 2C). For *E. coli* O111:H2, 0.5-1mg/ml GSE showed little effect on bacterial growth while 2 and 4 mg/ml GSE slightly inhibited bacterial growth (Fig. 2D). GSE had minimal inhibitory effect against *E. coli* O121:H19 (Fig. 2E)

3.2 GSE inhibited quorum sensing in non-O157 STEC

AI-2 is a universal QS signal used by a variety of bacteria for communication and is a global regulator of virulence factors in *E. coli* O157:H7 (Sperandio et al., 2001). GSE inhibited AI-2 production of selected “top-six” non-O157 STEC in a similar pattern and in a dose dependent manner (Fig. 3), while 0.5-4 mg/ml GSE had limited inhibitory effects on bacteria growth except *E. coli* O26:H11 at 5h of incubation (Fig. 3). QS of *E. coli* O26:H11 was the least

sensitive to GSE. 0.5 mg/ml GSE reduced the AI-2 production of *E. coli* O26:H11 to ~20% of the untreated cells (Fig. 3A), while to ~10% of the untreated cells for the other bacteria (Fig. 3B-F). AI-2 production in 1-4 mg/ml GSE treated groups was dramatically reduced for all non-O157 STEC tested (Fig. 3).

3.3 GSE inhibited flagella motility of non-O157 STEC

Flagella mediated motility is an important virulence factor for many foodborne pathogens, and it is essential for bacterial biofilm formation, adhesion to epithelial cells, and intestinal colonization (Duan et al., 2013). Motility is also of great importance to the food industry since it increases the ability of bacteria to infect a larger surface area, such as the surface of food processing equipment (O'May, Tufenkji, 2011). Thus, inhibitory effect of GSE against flagella motility was further assessed. Based on the motility halo size, *E. coli* O111:H2 is the most motile, while *E. coli* O26:H11 is the least motile (Fig. 4). GSE was effective in reducing swimming motility of all the tested non-O157 STECs in a dose dependent manner. Including 0.125 mg/ml GSE in a swimming agar plate completely inhibited motility of tested non-O157 STEC except *E. coli* O111:H2 (Fig. 4). 0.125, 0.25 and 0.5 mg/ml of GSE reduced the motility of *E. coli* O111:H2 by 30%, 65% and 100%, respectively.

3.4 GSE inhibited flagella protein and Stx production

Shiga toxin (Stx) is one of the major virulence factors of STEC causing bloody diarrhea and fatal hemolytic uremic syndrome (HUS) (Karmali et al., 1983). Flagella are mainly responsible for the swimming motility. Epidemiological studies have shown that *E. coli* O103:H2 and *E. coli* O111:H2 of “top-six” non-O157 serotypes are more frequently involved in

outbreaks (Johnson et al., 2006; Luna-Gierke et al., 2014). Therefore, they were chosen to further test effects of GSE on flagella and its major regulatory proteins as well as Stx2 production.

Consistent with stunted motility, GSE as low as 0.5 mg/ml reduced FliC production of both *E. coli* O103:H2 and *E. coli* O111:H2 to undetectable level (Fig. 5C&D). Furthermore, 0.5-4 mg/ml GSE inhibited FliA production of *E. coli* O103:H2 to under detection level (Fig. 5E). *E. coli* O111:H2 was less sensitive to GSE in FliA production. However, 0.5 mg/ml GSE significantly reduced FliA production of *E. coli* O111:H2, and this inhibitory effect strengthened with the increase of GSE concentrations (Fig. 5F).

In addition, 4 mg/ml GSE significantly reduced Stx2 production of both *E. coli* O103:H2 and *E. coli* O111:H2 (Fig. 6C&D). For *E. coli* O103:H2, 0.5-2 mg/ml GSE increased Stx2 production, but Stx2 production was not different among 0.5-2 mg/ml GSE treatments (Fig. 6C). For *E. coli* O111:H2, GSE reduced Stx2 production in all tested concentrations with 4 mg/ml GSE to be the most effective (Fig. 6D).

4. Discussion

GSE is a by-product of the wine industry, with abundant polyphenolic compounds known for their anti-inflammatory and anti-oxidative effects (Wang et al., 2011). A wide variety of studies examined their clinical potential, both in terms of disease prevention and treatment (Kaur et al., 2009; Vislocky, Fernandez, 2010; Wang et al., 2013; Yang et al., 2015). Meanwhile, GSE has shown inhibitory effect against a variety of pathogens including *E. coli* O157:H7 (Zhu et al., 2015), *L. monocytogenes* (Bisha et al., 2010), and *V. parahaemolyticus* (Mahmoud, 2014). No study has been conducted on non-O157 STEC thus far. Given that non-O157 STEC are of

growing concern for the food industries of both the United States and worldwide, the objective of this study was to assess the antimicrobial efficacy of GSE on the CDC's "top-six" non-O157 STEC.

Inhibitory effect of GSE against the growth of non-O157 STECs was population, concentration, and strain dependent. At the low inoculation level, 4 mg/ml of GSE showed great inhibitory effect against all the "top-six" non-O157 STEC, similar to the effect of GSE on *E. coli* O157:H7 (Zhu et al., 2015). Mechanism of this antimicrobial effect might include disruption of cytoplasmic membrane, inhibition of extracellular enzymes, binding of vital compounds for bacterial metabolism, and others (Walsh et al., 2003). Interestingly, lower concentrations of GSE promoted the growth of *E. coli* O103:H2 and *E. coli* O111:H2. Similar phenomenon was also seen in *E. coli* O157:H7 when GSE (Zhu et al., 2015), broccoli extract (Lee et al., 2011b), and honey (Lee et al., 2011a) were used at low concentrations. It is probably due to the competition between the growth promotion effect of nutrients and the growth inhibition effect of the polyphenols in GSE. At the high initial population, GSE showed limited effect against most bacterial growth.

Mediated by autoinducer molecules, QS is a crucial signaling regulating motility and virulence factors in pathogenic bacteria including *E. coli* O157:H7 (Sperandio et al., 2001). Thus, QS has emerged as a promising target for eliminating or alleviating bacterial virulence. However, the role of QS in non-O157 STEC virulence has not been uncovered, let alone QS regulation by natural antimicrobials including GSE. Our results, for the first time indicated that GSE effectively inhibited the "universal" signal molecule AI-2 levels of all six non-O157 STEC tested. In agreement with regulatory role of QS in motility of *E. coli* O157:H7 (Sperandio et al.,

2002b) and reduced QS observed in GSE treated bacteria, GSE effectively reduced swimming motility of all six non-O157 STEC tested. In line with that, polyphenolic compounds derived from algae and green tea, brominated furanone and epigallocatechin gallate, reduced AI-2 production and motility in *Campylobacter jejuni* (Castillo et al., 2015). Furthermore, 0.5% broccoli extract suppressed AI-2 production and motility of *E. coli* O157:H7 (Lee et al., 2011b).

QS regulates bacterial motility and pathogen virulence, both of which are critically important to the food industry. Flagella are mainly responsible for motility. In *E. coli*, the expression of flagella genes is in a hierarchical manner (Macnab, 2003). Induction of master flagella regulator FlhDC activates a transcription regulator FliA, which further regulates expression of genes encoding the key structural component of the flagellum filament FliC (Chilcott, Hughes, 2000; Macnab, 2003). Consistent with the reduced motility, GSE effectively reduced key the flagella protein FliC as well as its regulator FliA in both *E. coli* O103:H2 and *E. coli* O111:H2. In support of our finding, *E. coli* mutant strain defective in AI-2 production showed lower FliC gene expression compared with wild type strain (Soni et al., 2007), while addition of AI-2 restored the expression of FliA and FliC (Lee et al., 2008). These findings suggested that QS might play an important role in the motility as well as flagella production of non-O157 STEC. Furthermore, there is a strain dependent effect in response to GSE treatment among non-O157 STEC. *E. coli* O111:H2 was least sensitive to GSE in regards to swimming motility. Of note, GSE is rich in polyphenols, which are known to bind relatively strongly to proteins and to cause aggregation of proteins. Thus, the inhibition of motility might also due to binding of GSE to flagellar protein directly.

Shiga toxin (Stx) is one of the major virulence factors contributing to the pathogenicity of non-O157 STEC, causing symptoms from mild diarrhea to hemolytic uremic syndrome (HUS) and even death (Johnson et al., 2006). QS plays a positive role in Stx production of *E. coli* O157:H7 (Sperandio et al., 2001). Consistent with reduced AI-2 production in *E. coli* O103:H2, *E. coli* O111:H2, GSE at all tested concentration inhibited Stx2 production in *E. coli* O111:H2, with higher doses having more profound effects. In *E. coli* O103:H2, 4mg/ml of GSE reduced Stx2 production, which is in agreement with diminished AI-2 production. Interestingly, Stx2 production in *E. coli* O103:H2 was increased in response to 0.5-2mg/ml of GSE treatment, which is in contrast to decreased AI-2 production. These observations are similar to our findings on Stx2 level of *E. coli* O157:H7 upon low concentrations of GSE treatment (Zhu et al., 2015). Meanwhile, it highlights the complex nature of Stx2 production among non-O157 STEC, which may not be solely regulated by AI-2 production. Study also indicates that antimicrobial resistance in non-O157 is serogroup and Shiga toxin genotype dependent. For instance, in a study on antimicrobial efficacy of fosfomycin against 11 strains of O26 isolated from cattle, MIC was ranged from 2 to 32 mg/L depending on serotype; one strain was even resistant to fosfomycin (Sasaki et al., 2012). Therefore, more studies are needed to assess the effects of GSE on the growth and virulence of other non-O157 STEC not tested in this study.

In conclusion, GSE inhibits the growth of the selected CDC “top-six” non-O157 STEC at a population level of 5×10^5 CFU/ml. At higher population, GSE suppresses AI-2 production, motility and Stx production of the non-O157 STEC tested with minor effect on bacterial growth. Taken together, GSE is an effective antimicrobial to control non-O157 STEC tested via inhibiting both growth and virulence.

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310 **Table 1** MIC and MBC of grape seed extract for CDC top-six non-O157 Shiga toxin producing
 311 *E. coli* (STEC)

<i>E. coli</i> serotype	Isolate	MIC ²	MBC ³
<i>E. coli</i> O26: H11 ¹	97-3250	2 mg/ml	2 mg/ml
<i>E. coli</i> O45: H2 ¹	DA-21	4 mg/ml	4 mg/ml
<i>E. coli</i> O103: H2 ¹	MT#80	4 mg/ml	4 mg/ml
<i>E. coli</i> O111: H2 ¹	RD8	4 mg/ml	4 mg/ml
<i>E. coli</i> O121: H19 ¹	MT#2	4 mg/ml	4 mg/ml
<i>E. coli</i> O145: NT ¹	IH 16	4 mg/ml	4 mg/ml

312 ¹ The strain has human origin and was obtained from STEC center, Michigan State University,
 313 East Lansing, MI 48824; ²MIC: minimum inhibitory concentration; ³MBC: minimum
 314 bactericidal concentration

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Figure legends

Figure 1. Growth curves of the selected CDC "top-six" non-O157 Shiga toxin producing *E. coli* strains in basic growth media with 0.4% glucose under various GSE concentrations and 5×10^5 CFU/ml initial inoculation population at 37 °C. (A) *E. coli* O26: H11; (B) *E. coli* O45: H2; (C) *E. coli* O103: H2; (D) *E. coli* O111: H2; (E) *E. coli* O121: H19; (F) *E. coli* O145: NT. (Mean $\pm$ SEM, n=3).

Figure 2. Growth curves of the selected "top-six" non-O157 Shiga toxin producing *E. coli* strains in basic growth media with 0.4% glucose under various GSE concentrations and 1×10^7 CFU/ml initial inoculation population at 37 °C. (A) *E. coli* O26: H11; (B) *E. coli* O45: H2; (C) *E. coli* O103: H2; (D) *E. coli* O111: H2; (E) *E. coli* O121: H19; (F) *E. coli* O145: NT. (Mean $\pm$ SEM, n=3).

Figure 3. CFU enumeration and AI-2 production of six non-O157 STEC in BMG with varying concentrations of GSE at 37°C with initial inoculation population 1×10^7 CFU/ml after 5h incubation (Mean $\pm$ SEM; N=3). (A) *E. coli* O26: H11 (up: Log₁₀ CFU, down: AI-2 production); (B) *E. coli* O45: H2 (up: Log₁₀ CFU, down: AI-2 production); (C) *E. coli* O103: H2 (up: Log₁₀ CFU, down: AI-2 production); (D) *E. coli* O111: H2 (up: Log₁₀ CFU, down: AI-2 production); (E) *E. coli* O121: H19 (up: Log₁₀ CFU, down: AI-2 production); (F) *E. coli* O145: NT (up: Log₁₀ CFU, down: AI-2 production).

Figure 4. Swimming motility of selected “top-six” non-O157 STEC on 0.25% LB soft agar plates with various GSE concentrations. Swimming zones were measured 6 h post-incubation at 37 °C. (Mean $\pm$ SEM; n = 3).

Figure 5. Effect of GSE on FliA and FliC production in *E. coli* O103:H2 and *E. coli* O111:H2. Bacteria with initial inoculation population of 1×10^7 CFU/ml were incubated with GSE for 5 h in BMG and protein concentrations were analyzed by immunoblotting. (A) Log₁₀ CFU of *E. coli* O103:H2 after 5 h incubation in BMG with GSE. (B) Log₁₀ CFU of *E. coli* O111:H2 after 5 h incubation in BMG with GSE. (C) FliC production of *E. coli* O103:H2. (D) FliC production of *E. coli* O111:H2 (E) FliA production of *E. coli* O103:H2. (F) FliA production of *E. coli* O111:H2. (Mean $\pm$ SEM; n = 3).

Figure 6. Effect of GSE on Stx2 production of *E. coli* O103:H2 and *E. coli* O111:H2. Bacteria with initial inoculation population of 1×10^7 CFU/ml were incubated with GSE for 5 h in BMG and protein concentrations were analyzed by immunoblotting. (A) Log₁₀ CFU of *E. coli* O103:H2 after 5 h incubation in BMG with GSE. (B) Log₁₀ CFU of *E. coli* O111:H2 after 5 h incubation in BMG with GSE. (C) *E. coli* O103:H2 Stx2 production. (D) *E. coli* O111:H2 Stx2 production. (Mean $\pm$ SEM; n = 3).

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Figure1

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