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Antimicrobial efficacy of grape seed extract against *Escherichia coli* O157:H7 growth, motility and Shiga toxin production

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ABSTRACT

Escherichia coli O157:H7 produces Shiga toxin (Stx) which is heat stable and causes Hemolytic Uremic Syndrome (HUS), a serious disease associated with bloody diarrhea and even death. To ensure food safety, both live *E. coli* O157:H7 and its toxin production in food products need to be controlled. Natural ingredients with inhibitory effects on *E. coli* O157:H7 growth and toxin production are top choices of antimicrobials for the food industry. The objectives of this study were to evaluate efficacy of grape seed extract (GSE) against the growth, swimming motility and Stx production of *E. coli* O157:H7. The disc diffusion assay indicated that 3.2 mg GSE per disc resulted in an inhibition zone of 14.8 ± 0.21 mm. The minimal inhibitory concentration of GSE against *E. coli* O157: H7 was 4.0 mg/ml. At high inoculation level (1×10^7 CFU/ml), including GSE at 0.25–2.0 mg/ml reduced Stx production without inhibiting *E. coli* O157:H7 growth. At 5×10^5 CFU/ml inoculation level, 2.0 and 4.0 mg/ml GSE effectively inhibited the growth of *E. coli* O157:H7 for at least 72 h, however, a low level of GSE (0.125–1.0 mg/ml) enhanced *E. coli* O157:H7 growth and Stx2 production. At 4 mg/ml, GSE completely abolished Stx2 production in addition to it bactericidal effect against *E. coli* O157:H7. In addition, GSE at concentration as low as 0.125% blocked the swimming motility, which is important for *E. coli* O157:H7 surface adherence. In conclusion, GSE is effective in inhibiting the motility of *E. coli* O157:H7, GSE shows potential to be used as a natural antimicrobial to control *E. coli* O157:H7.

1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are important foodborne pathogens associated with enteritis, hemolytic uremic syndrome (HUS) and even death (Majowicz et al., 2014). *E. coli* O157:H7 is the most well-known pathogen among STEC, which is a major food safety threat that results in significant economic losses. Shiga toxin (Stx) is the major virulence factor of *E. coli* O157:H7, which causes bloody diarrhea and life threatening HUS in humans (Karmali, Petric, Steele, & Lim, 1983). The mortality associated with *E. coli* O157:H7 infection is mainly due to the production and release of Stx. A specific *E. coli* O157:H7 strain can produce either Stx1 or Stx2, or both; and strains producing Stx2 are most likely associated with HUS cases (Ostroff et al., 1989). It has been shown that Stx2 is 400 times more virulent than Stx1 in mouse studies (Tesh et al., 1993), with an oral lethality dose lower than 50 μg per mice (Rasooly, Do, Griffey, Vilches-Moure, & Friedman, 2010). Heat inactivation of Stx2 only occurs after the toxin is subjected to 100 °C for 5 min (Rasooly & Do, 2010), indicating that typical cooking temperature and duration associated with meat processing are insufficient to inactivate the toxin. Therefore, it is not only viable *E. coli* O157:H7 that poses a safety threat, but also Stx produced by *E. coli* O157:H7 constitutes a critical safety issue in foods if not controlled.

Currently studies on the intervention of *E. coli* O157:H7 in foods have been focused on live *E. coli* O157:H7, less attention has been paid to inhibiting or monitoring Stx production. In a recent study, three commonly used food preservatives including potassium sorbate, sodium benzoate and sodium propionate were compared for their effects on Stx production, and 5 mg/ml sodium benzoate or potassium sorbate reduced Stx production (Subils, Aquili, Ebner, & Balague, 2012). However, due to the rising health and safety concern about synthetic chemical additives, food industries are increasingly motivated to use natural

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antimicrobial such as essential oils and other plant derived natural extracts instead of synthetic antimicrobials. Grape seed extract (GSE), an inexpensive by-product from the wine and juice industry, is high in polyphenolic compounds and known for its anti-inflammatory, anti-oxidative, cardioprotective and other health benefits (Hogan et al., 2010; Terra et al., 2009; Wang et al., 2013). GSE has further gained attention in food industry due to its potential to reduce lipid oxidation and improve color and flavor stability (Ahn, Grun, & Mustapha, 2007; Kulkarni, DeSantos, Kattamuri, Rossi, & Brewer, 2011). In addition, GSE has antimicrobial properties. It is effective in reducing the titers of human enteric virus (Su & D'Souza, 2011) and inhibiting the growth of *Listeria monocytogenes* (Bisha, Weinsetel, Brehm-Stecher, & Mendonca, 2010; Rhodes, Mitchell, Wilson, & Melton, 2006). Dipping in 1.25 mg/ml GSE solution for 2 min resulted in a 2-log reduction of *L. monocytogenes* inoculated to tomato surfaces (Bisha et al., 2010). However, information about its antimicrobial efficacy against *E. coli* O157:H7 is sparse, neither has production of Stx2 nor motility been well evaluated. The objective of this study is to assess the antimicrobial efficacy of GSE at relative low concentrations against *E. coli* O157:H7 growth, motility and Stx2 production.

2. Materials and methods

2.1. Bacteria strains

E. coli O157:H7 strain EDL933, which produces both Stx1 and Stx2, was obtained from the STEC center at Michigan State University. *E. coli* O157:H7 was stored in Luria–Bertani (LB) medium containing 15% glycerol at −80 °C, which was routinely grown in LB medium at 37 °C overnight with aeration. To minimize interference of turbidity due to precipitation of medium ingredients with tannins in GSE, Basic Media with Glucose (BMG) (Diez-Gonzalez & Russell, 1999) was used in all experiments with GSE.

2.2. Grape seed extract (GSE)

Grape seed extract was purchased from Optipure (Los Angeles, CA), which contains a minimum of 95% total flavonols and 82% of proanthocyanidin. Electrospray ionization tandem mass spectrometry analysis in our lab further indicates that GSE is rich in dimer (35.8%) and trimmer (38.6%) (Wang, Yang, Liang, Zhu, & Du, 2014). Stocking GSE (40 mg/ml) solution was prepared with 10% ethanol and then microwaved for 5 min. The resulting liquid was filter sterilized with 0.45 μm filtration unit (Nalgene, Rochester, NY).

2.3. Disc diffusion assay

E. coli O157:H7 were activated in LB for 14 h at 37 °C and diluted to 1×10^6 CFU/ml with sterile phosphate buffered saline (PBS, pH7.4). 100 μL of the suspension was spread over the plates containing Mueller-Hinton agar. Sterile paper discs (Whatman no.5, 7 mm diameter) loaded with 0, 0.4, 0.8, 1.6 and 3.2 mg GSE were placed on the agar surface. The plates were left under BSL2 cabinet for 30 min at room temperature and then incubated at 37 °C for 24 h. The diameters of the inhibition zones were measured in millimeters. Experiments were repeated three times independently.

2.4. Minimal inhibitory concentration (MIC)

MICs of GSE against *E. coli* O157:H7 were determined as previously described (Sheng & Zhu, 2014). Briefly, BMG was used with GSE at concentrations varying from 0.0625 to 16 mg/ml in 96 well polystyrene (PS) microtiter plates (Costar, Tewksbury, MA). Each well contained 100 μL of BMG/GSE solution and 100 μL of *E. coli* suspension of 1×10^6 CFU/ml resulting in approximately 5×10^5 CFU/ml of *E. coli* O157:H7. Cultures were incubated statically at 37 °C for 24 h and optical density (OD) values at 600 nm were obtained at 0 and 24 h using SM5 spectrometer (Molecular Device, Sunnyvale, CA). MIC was determined as the lowest concentration of GSE that inhibited growth of *E. coli* O157:H7. Experiment was repeated three times independently.

2.5. Minimum bactericidal concentration (MBC)

MBC was determined per previous published method (Sheng & Zhu, 2014). Briefly, 100 μL sample was taken from wells of the MIC assay without visible growth, spread on LB agar plates, and incubated at 37 °C for 24 h. MBC was defined as the lowest concentration that inhibits bacterial growth on the LB agar plates (<10 CFU/plate was regarded as no growth) (Siddique, Shah, Shahid, & Yasmin, 2013). Experiment was repeated three times independently.

2.6. Growth curves

Growth curves were conducted in 96 well plates. Briefly, overnight *E. coli* O157:H7 culture was washed with PBS and diluted to approximately 5×10^5 or 1×10^7 CFU/ml in BMG broth containing various concentrations of GSE (0, 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 mg/ml). 200 μL of *E. coli* O157:H7 respective suspension was inoculated into the corresponding wells. The plates were incubated at 37 °C, and OD values at 600 nm were read hourly for 24 h and then every 12 h in an SM5 spectrometer (Molecular Device, Sunnyvale, CA). Each treatment has six replicates. Experiment was repeated three times independently.

2.7. Protein sample preparation

Overnight *E. coli* O157:H7 culture was sub-cultured at an inoculation level of 1×10^3, 1×10^4, 5×10^5, 1×10^6, or 1×10^7 CFU/ml into BMG containing different GSE concentrations (0, 0.125, 0.25, 0.5, 1, 2 or 4 mg/ml), and incubated at 37 °C with aeration for 6 h or 12 h, respectively, when BMG containing *E. coli* O157:H7 was sampled for the analyses of colony forming units (CFU) and protein content.

2.8. Immunoblotting analysis

Protein content was analyzed by immunoblotting according to procedures described previously (Harris et al., 2012). Briefly, protein extractions were separated by 10% (W/V) SDS polyacrylamide gels and transferred to nitrocellulose membranes for immunoblotting. Stx2A monoclonal antibody was purchased from Toxin Tech. Inc. (Sarasota, FL) and was used at 1:1000 dilution. RecA monoclonal antibody was purchased from Enzo Life Sciences. Inc. (Farmingdale, NY) and was used at 1:1000 dilution. Bands were visualized using ECL detection reagent (GE Healthcare, Buckinghamshire, UK).

2.9. Swimming motility assays

Swimming motility was performed according to the previously published method (Ryjenkov, Simm, Romling, & Gomelsky, 2006). Briefly, *E. coli* O157:H7 culture was mixed with GSE solution, then 5 μL of mixture was inoculated onto 0.25% swimming agar plates (0.25% Bacto agar, 0.5% NaCl, and 1% Bacto tryptone) containing 0,