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Short communication

Effectiveness of a spontaneous carvacrol nanoemulsion against *Salmonella* enterica Enteritidis and *Escherichia coli* O157:H7 on contaminated broccoli and radish seeds

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ABSTRACT

The incidence of foodborne illness associated with the consumption of fresh produce has continued to increase over the past decade. Sprouts, such as mung bean, alfalfa, radish, and broccoli, are minimally processed and have been sources for foodborne illness. Currently, a 20,000 ppm calcium hypochlorite soak is recommended for the treatment of sprouting seeds. In this study, the efficacy of an antimicrobial carvacrol nanoemulsion was tested against *Salmonella* enterica subspecies enterica serovar Enteritidis (ATCC BAA-1045) or EGFP expressing *Escherichia coli* O157:H7 (ATCC 42895) contaminated sprouting seeds. Antimicrobial treatments were performed by soaking inoculated seeds in nanoemulsions (4000 or 8000 ppm) for 30 or 60 min. Following treatment, surviving cells were determined by performing plate counts and/or Most Probable Number (MPN) enumeration. Treated seeds were sprouted and tested for the presence of pathogens. Treatment successfully inactivated low levels (2 and 3 log CFU/g) of *S.* Enteritidis and *E. coli* on radish seeds when soaked for 60 min at concentrations ≥4000 (0.4%) ppm carvacrol. This treatment method was not affective on contaminated broccoli seeds. Total sprout yield was not influenced by any treatments. These results show that carvacrol nanoemulsions may be an alternative treatment method for contaminated radish seeds.

1. Introduction

The incidence of foodborne illness associated with the consumption of fresh produce has continued to increase over the past decade. Between 1990 and 2005, there have been over 700 outbreaks, resulting in roughly 34,000 cases of foodborne illness (Sapers et al., 2009). One potential reason for the increase in outbreaks may be due to the change in social eating habits and the accessibility of fresh produce. For example, the per capita demand and consumption of fresh produce has dramatically increased compared to past decades (Garrett et al., 2003). Also, the produce industry has experienced a rapid globalization in its supply chain, making the implementation of universal protocols challenging (Garrett et al., 2003). Yet, these sociological and supply changes do not fully explain the increased outbreaks seen in fresh produce. The incidence of outbreaks in particular produce systems, such as leafy greens and sprouts, have increased 4- fold compared to the increase in consumption (Control and Prevention, 2008; Herman et al., 2008).

The demand for minimally processed, natural produce has continued to increase despite the inherent risk of foodborne illness. At the forefront of this resurgence are sprouts. Sprouts, such as mung bean, alfalfa, radish, and broccoli, are minimally processed and can be vectors for both *Salmonella* spp. and *Escherichia coli* O157:H7 (Mohle-Boetani et al., 2009). Prior to sprouting, seeds are generally soaked in lukewarm water (32–35 °C) for 2–4 h or allowed to soak at room temperature for 24 h (Schrader, 2002). Unfortunately, this necessary step not only triggers seed germination, but can also act as an enrichment step for any present human pathogen. To help minimize the potential of foodborne illness, a 2% calcium hypochlorite soak is recommended prior to sprouting (Thomas et al., 2003). However, rapid sequestering of free chlorine by organic load, inadequate pH adjustments, and seed topography

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limit its effectiveness (Food and Drug Administration, 1997; Schrader, 2002; White, 2010).

A potential alternative to current recommended methods is the use of emulsified essential oils (Landry et al., 2014). Essential oils are natural compounds that are isolated from various plant sources such as thyme, oregano, and basil, which demonstrate antimicrobial activity (Burt, 2004). One essential oil that has been shown to have promising antimicrobial properties against a variety of foodborne pathogens is carvacrol (Burt, 2004; Landry et al., 2014; Lu and Wu, 2010b; Pei et al., 2009). Essential oils, alone, have minimal solubility in water. The spontaneous emulsification of carvacrol, originally put forth by Chang et al. (2013), is simple to produce and requires minimal equipment and training (Date et al., 2010; McClements and Rao, 2011). The nanoemulsion has also been shown to be effective against foodborne pathogens in both *in vitro* and *in vivo* sprout settings (Landry et al., 2014). In a broth based system, the minimal inhibitory concentration for the spontaneous carvacrol nanoemulsion was found to be 500 ppm, with complete inactivation at concentrations greater than or equal to 4000 ppm. When applied to a sprout based system, a 60 min treatment in 4000 or 8000 ppm carvacrol nanoemulsion resulted in complete inactivation of both *Salmonella* Enteritidis and *E. coli* O157:H7 on both mung bean and alfalfa seeds (Landry et al., 2014). Based on these findings, the efficacy of the spontaneous carvacrol nanoemulsion at similar concentrations was tested against *Salmonella* Enteritidis and *E. coli* 0157:H7 contaminated radish and broccoli seeds. The influence of organic loads on the emulsions antimicrobial efficiency *in vitro* was also studied.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The bacterial strains used in the presented experiments were *Salmonella* enterica subspecies enterica serovar Enteritidis (ATCC BAA-1045) and an enhanced green fluorescent protein (EGFP) expressing *E. coli* O157:H7 (ATCC 42895) (Prachaiyo and McLandsborough, 2000). Stock cultures of each organism were stored at −80 °C in tryptic soy broth (TSB; BD Diagnostic Systems, Cat# DF0064-07-6) containing 25% (v/v) glycerol. Monthly, frozen stock cultures were transferred to working cultures by plating on tryptic soy agar (TSA; BD Diagnostic Systems, Cat# DF0370-075) slants/plates and incubating at 37 °C for 24 h. Following incubation, single colonies of *E. coli* O157:H7 were picked and transferred to Luria broth (Lennox, LB) (Fisher BioReagents Cat# BP9724-500) plates containing 500 μg/mL ampicillin (Fisher Scientific Cat# BP1760-5). The absorbance at 600 nm was used to determine cells numbers, with an absorbance of 0.5 equal to 1.0×10^8 CFU for both *E. coli* O157:H7 and *Salmonella* Enteritidis as determined by plate counts.

Periodically, working cultures were streaked on differential media to ensure purity. For *S.* Enteritidis, cultures were spread on xylose, lysine, deoxycholate (XLD) agar (Remel Cat# R459902). For *E. coli* O157:H7, cultures were spread on LB (Fisher BioReagents Cat# BP9724-500) plates containing 500 μg/mL ampicillin (Fisher Scientific Cat# BP1760-5) and 20 μg/mL IPTG (Thermo Scientific Cat# FERR0392) and observed under UV light. Cultures were incubated overnight in TSB at 37 °C on a rotary shaker set at 150 RPM. All cultures were diluted with TSB to the desired cell numbers.

2.2. Formation of antimicrobial nanoemulsions

The preparation of the antimicrobial nanoemulsion was based on a method previously reported by our group (Chang et al., 2013; Landry et al., 2014). Carvacrol (4 g) (Sigma–Aldrich, Cat# W224502-100G-K) was added to 6 g of medium chain triglyceride (MCT) oil (Miglyol 812, Witten, Germany) and thoroughly mixed for 5 min at 125 RPM. Once mixed, Tween 80® (10 g) (Sigma–Aldrich, Cat# P1754-500 mL) was added to the oil mixture and mixed for another 5 min at 125 RPM. The oil/Tween 80 mixture (20 g) was titrated, at a rate of 2 mL/min, into 80 g of 5.0 mM sodium citrate buffer (pH 3.5) containing a magnetic stirring bar set to 600 RPM and allowed to mix for an additional 15 min. The emulsion was filter sterilized through a sterile 0.45 μm syringe filter (Fisher Scientific Cat# 09-719-005) and stored in sterile 50 mL tubes at 2–5 °C for up to 3 weeks. Droplet size was measured using dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, UK) to ensure that the mean droplet diameter was ≈100 nm.

2.3. Effect of organic load on the effectiveness of the carvacrol nanoemulsion against Salmonella Enteritidis and E. coli 0157:H7 in vitro

Overnight growth (9 log CFU/mL) of either *S.* Enteritidis or *E. coli* O157:H7 was added to test tubes containing TSB (9 mL), carvacrol nanoemulsion (8000, 4000, 2000, 1000, and 500 ppm final carvacrol concentration), and various concentrations of organic load (2%, 10%, or 20% v/v) to give an initial level of approximately 6 log CFU/mL. The tubes were incubated at 37 °C for 24 h. A dilution series was created and plated on the appropriate media. For *S.* Enteritidis, dilutions were spread on XLD (Remel Cat# R459902). For *E. coli* O157:H7, dilutions were spread on LB (Fisher BioReagents Cat# BP9724500) plates containing 500 μg/mL ampicillin (Fisher Scientific Cat# BP1760-5) and 20 μg/mL IPTG (Thermo Scientific Cat# FERR0392) and observed under UV light. The absorbance at 600 nm was used to determine cells numbers, with an absorbance of 0.5 equal to 1.0×10^8 CFU for both *E. coli* O157:H7 and *S.* Enteritidis as determined by plate counts.

Organic loads were simulated with either horse serum (Thermo Scientific Cat# R55075) or homogenized mung bean sprouts purchased from a local supermarket. To produce the homogenized mung bean extract, 50 g of mung beans and 50 mL of sterile water were homogenized in an Oster Osterizer Classic® blender (Oster: Boca Raton, FL, USA) for 45 s.

2.4. Effectiveness of carvacrol nanoemulsion on contaminated seeds

All seeds used in this study were generously provided by Jonathan's Organics (Rochester, MA) and each treatment condition was tested and sprouted in triplicate. Seeds were inoculated and sprouted using a modified version of the method presented by Ye et al. (2010). Batches (20 g) of seeds were soaked in 50 mL of diluted *S.* Enteritidis or *E. coli* O157:H7 for 20 min resulting in final inoculums of 8, 5, 3, or 2 log CFU/g. The inoculated seeds were then transferred to a sterile glass petri dish containing sterile filter paper within a biological safety cabinet, and allowed to dry overnight at ambient temperature. The inoculated bean/seed batches were placed in 250-mL beakers and treated by soaking in 50 mL of nanoemulsion (4000 or 8000 ppm) with agitation (125 RPM) for 30 or 60 min. For the control, contaminated batches were soaked in 5.0 mM, pH 3.5 sodium citrate buffer. After treatment, the batches were rinsed once with 50 mL of sterile deionized water and transferred to a Whirl-Pack bag containing 50 mL of TSB. The Whirl-Pack bag was placed on a rotary shaker set to 50 RPM for 15 min. After agitation, a dilution series was created and plated on the appropriate media and incubated at 37 °C for 24 h. For samples with low *S.* Enteritidis or *E. coli* O157:H7 inoculation levels (2 and 3 log CFU/g), a three tube most probable number (MPN) assay was used in conjunction with spread plating according to the FDA's