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Antimicrobial efficacy of cinnamon oil against *Salmonella* in almond based matrices

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ABSTRACT

Reduced moisture enhances resistance of *Salmonella* and subsequently reduces the antimicrobial efficacy of thermal treatment. Alternative and supplementary non-thermal intervention methods are urgently needed. In this study, *Cinnamonum cassia* oil was tested for its antimicrobial effect against outbreak strains *Salmonella* Enteritidis PT30 and *S.* Tennessee K4643. Minimal inhibitory concentration and minimal bactericidal concentration for both strains were 0.05% (v/v) and 0.1% (v/v), respectively. Death curves showed that including 0.1% and 0.15% (v/v) *C. cassia* oil resulted in ~7 Log reduction of bacteria within 2 h and 1 h, respectively. However, the antimicrobial efficacy of *C. cassia* oil was reduced when *S.* Enteritidis PT30 existed in low moisture condition. When *S.* Enteritidis PT30 was established on almonds/paper discs, 0.4% *C. cassia* oil resulted in ~1.7 Log_{10} CFU/almond or 3.2 Log_{10} CFU/disc reduction within 2 h at room temperature, respectively. *S.* Enteritidis PT30 established on both almonds and paper discs were very stable, there was only a 0.80 Log_{10} CFU/almond and 1.20 Log_{10} CFU/disc reduction during 9-week and 7-week storage at room temperature, respectively. *C. cassia* oil intervention increased *S.* Enteritidis PT30 reduction on both almonds and paper discs during storage with more reduction on paper discs. 0.4% *C. cassia* oil treatment reduced *S.* Enteritidis PT30 on paper disc to undetectable level within 4 weeks, but only led to 2 Log_{10} CFU reduction on almonds, indicating a protection effect from the almond matrix or almond surface components. Additionally, *S.* Enteritidis PT30 established on paper disc coated with almond surface components exhibited higher resistance to desiccation and *C. cassia* oil treatment, further demonstrating the protection role of food matrix. In conclusion, *C. cassia* oil is effective against *S.* Enteritidis PT30 and *S.* Tennessee K4643, but its antimicrobial efficacy against the tested *Salmonella* was compromised by low moisture environment and food matrix.

1. Introduction

Salmonella is one of the most challenging foodborne pathogens in the food industry and contributes to more than 93.8 million cases of gastroenteritis annually (Majowicz et al., 2010). *Salmonella* is responsible for most of the outbreaks related to low moisture food products. Recently, tree nuts, such as almonds, pecans, and walnuts, have been recognized as potential contributors to foodborne illnesses related to low moisture food (Harris, Palumbo, & Danyluk, 2016). From 2000 to May 2016, there were 30 outbreaks related to tree nuts (Harris et al., 2016). All tree nut outbreaks were related to *Salmonella*, except one was due to *E. coli* O157:H7 in 2011 (Harris et al., 2016). Moreover, numerous tree nut recalls were due to *Salmonella* contamination (Palumbo, Beuchat, Danyluk, & Harris, 2016). *Salmonella* could survive under a low moisture environment for up to 15 months and cause gastroenteritis in humans (Finn, Condell, McClure, Amezquita, & Fanning, 2013; Kimber, Kaur, Wang, Danyluk, & Harris, 2012; Uesugi, Danyluk, & Harris, 2006). A study of salmonellosis suggested that *Salmonella* in low moisture foods has a low infectious dose of 10–100 cells (Finn et al., 2013).

Heat treatment is a widely used intervention method in the food industry for elimination of foodborne pathogens. However, *Salmonella* develops heat resistance in reduced moisture conditions (Santillana Farakos, Frank, & Schaffner, 2013), becoming a big threat to low moisture food products. There is an urgent need for alternative and supplementary effective intervention methods. *Cinnamonum cassia* is a well-known traditional Chinese medicine and widely used as a food additive in Chinese cuisine (Ooi et al., 2006). *C. cassia* oil is rich in cinnamaldehyde, which has antimicrobial effect against a broad range of microorganisms (Nabavi et al., 2015).

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C. cassia essential oil also has a broad antimicrobial activity against Gram-negative bacteria like *E. coli* O157:H7 (Friedman, Henika, & Mandrell, 2002; Sheng, Rasco & Zhu, 2016), non-O157 Shiga toxin producing *E. coli* strains (Sheng & Zhu, 2014), *Salmonella* Typhimurium (Melo et al., 2015; Zhou et al., 2007), Gram-positive bacteria like *Staphylococcus aureus* (Zhu, Du, Fox, & Zhu, 2016) and fungi (Ooi et al., 2006). However, its antimicrobial efficacy has not been evaluated on *S.* Enteritidis PT30, which caused salmonellosis in a foodborne outbreak linked to raw almond consumption in 2000–2001, or *S.* Tennessee K4643, which is linked to a peanut butter outbreak in 2006–2007 (Centers for Disease & Prevention, 2007; Isaacs et al., 2005). Neither was its effect against *Salmonella* under low moisture condition studied.

In this study, we aimed to evaluate antibacterial efficacy of *C. cassia* oil against disease outbreak *Salmonella* strains in media setting, low moisture condition and almond based food matrix condition. We hypothesized that *C. cassia* oil has antimicrobial effects against the selected *Salmonella* strains but its antimicrobial efficacy would be compromised in low-moisture condition or food matrices.

2. Materials and methods

2.1. Bacteria strains and growth conditions

Almond outbreak strain *S.* Enteritidis PT30 (Isaacs et al., 2005) and peanut butter outbreak strain *S.* Tennessee strain K4643 (Centers for Disease & Prevention, 2007), which were previously obtained from Dr. Linda Harris (University of California, Davis) and Dr. P. Michael Davidson (University of Tennessee, Knoxville, TN), respectively, were maintained at −80 °C in Trypticase Soy Broth (TSB, Becton, Dickinson and Company, Sparks, MD) supplied with 0.6% Yeast Extract (Fisher Scientific, Fair Lawn, NJ) (TSBYE) and 20% (v/v) glycerol. Bacteria were activated in TSBYE at 35 ± 2 °C for 8 h, statically. Eight-hour-activated bacteria cultures were 1:1000 transferred to TSBYE and activated at 35 ± 2 °C statically for additional 14 h. Three hundred microliter twice-activated *Salmonella* were plated on TSAYE plates (TSBYE with 1.5% agar) and incubated at 35 ± 2 °C for 24 h. *Salmonella* lawn was collected from TSAYE plate using sterile Phosphate Buffered Saline (PBS, pH7.4) and centrifuged at 8000× *g*, 4 °C for 15 min. The resulting pellet was resuspended in PBS to achieve 10^{10} CFU/mL for further inoculation.

2.2. Disc diffusion assay

C. cassia oil used in this study was purchased from Sigma (St. Louis, MO), which contains 60% trans-cinnamaldehyde (Sheng & Zhu, 2014). Disc diffusion was conducted as previously described (Sheng & Zhu, 2014). Briefly, twice-activated *S.* Enteritidis PT30 and *S.* Tennessee K4643 were adjusted to 1 × 10^5 CFU/mL with sterile PBS, and plated on the Mueller-Hinton agar (MHA) plates (Becton, Dickinson and Company, Sparks, MD). Five, ten and 20 μL of 4% (v/v) *C. cassia* oil (in 10% DMSO and 0.5% Tween 80, v/v) were loaded onto sterile paper discs (Whatman no.5, 7 mm dia) placed on MHA plates seeded with *S.* Enteritidis PT30 or *S.* Tennessee K4643. The paper discs loaded with 20 μL of PBS containing 10% (v/v) DMSO and 0.5% (v/v) Tween 80 were used as vehicle control. The MHA plates were incubated at 35 ± 2 °C for 24 h. The diameter of inhibition zones was measured and compared. Three replicates were used for each treatment in disc diffusion assay.

2.3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Two-fold microdilution broth method was used and performed in 96-well plates to determine the MIC of *C. cassia* oil against *S.* Enteritidis PT30 and *S.* Tennessee K4643 per our previous method (Sheng & Zhu, 2014). Mueller-Hinton broth with 0.15% agar (MHBA) was used to dilute *C. cassia* oil. Twice-activated cultures were diluted to 10^6 CFU/mL with sterile PBS. One hundred microliter diluted cultures was added into each well containing 100 μL MHBA with different concentrations of *C. cassia* oil (0.00625%–1.6%, v/v). The wells containing un-inoculated MHBA were used as negative control and the wells with inoculated MHBA without *C. cassia* oil were used as positive control. The 96-well plates were incubated at 35 ± 2 °C statically for 24 h. MIC was determined as the lowest concentration of *C. cassia* oil without visible growth (no turbidity was observed). A hundred microliter sample without turbidity from MIC assay was spread on Luria broth (LB) agar plate and incubated at 35 ± 2 °C for 24 h. MBC was defined as the lowest concentration that inhibits bacterial growth on LB agar plates (<10 CFU/plate was regarded as no growth) (Sheng & Zhu, 2014). Six replicates were used for each treatment in MIC and MBC assay. The experiment was repeated three times, independently.

2.4. Death curves

Twice-activated *S.* Enteritidis PT30 and *S.* Tennessee K4643 were first washed with sterile PBS and added into Luria broth (LB) with 0.15% (v/v) agar (LBA broth) containing *C. cassia* oil (0%, 0.05%, 0.1% and 0.15%, v/v) at 1 × 10^7 CFU/mL. Inoculated LBA was mixed and incubated in a 37 °C water bath for up to 120 min. The survival of *Salmonella* was enumerated at 0, 15, 30, 60, 120 min post-incubation in LBA broth with respective *C. cassia* oil concentrations. One hundred of appropriate diluted samples were plated on LB agar plates in duplicate and incubated at 35 ± 2 °C for 24 h. Four replicates were used for each treatment in death curve. The experiment was repeated three times, independently.

2.5. Almond inoculation, storage, treatment and survival enumeration

Inoculation and treatment: The whole almonds with skin were purchased from local store (COSTCO, Clarkston, WA). The whole almonds with sizes 23/25 (23–25 almonds per 28 g) were used in this study, which represents the typical size range of almonds (10 almonds/11 g). Almonds were placed in empty petri dishes and spot-inoculated with 10 μL of 10^{10} CFU/mL at the center that resulted in an inoculation level of 10^8 CFU/almond. The inoculated almonds were dried in a biosafety cabinet for 2 h and then equilibrated at environmental water activity ($a_w = 0.27$ to 0.3) for 24 h on the bench top. Ten microliters of 0.4% (v/v) *C. cassia* oil suspension in PBS containing 10% (v/v) DMSO and 0.5% (v/v) Tween 80 was applied at the same spots of almonds inoculated with *Salmonella*, and dried for additional 2 h. The PBS suspension without essential oil was used as a control. The inoculated almonds were subjected to room temperature (RT, ~22 °C) storage for up to 9 weeks and sampled weekly.

Enumeration: at each sampling day, 10 almonds per replicate were sampled and mixed with 100 mL sterile PBS in a stomach bag and incubated at RT for 10 min. After incubation, the stomach bags were shaken vigorously for 2 min by hand. The recovered *Salmonella* suspensions were serially diluted with sterile PBS, and appropriate dilutions were plated on LB agar plates in duplicate. Plates were incubated at 35 ± 2 °C for 24 h for survival enumeration.

2.6. Preparation of paper disc loaded with almond surface components

Almond washing solution (AWS) preparation: Almonds were