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The inhibitory effects of essential oil constituents against germination, outgrowth and vegetative growth of spores of *Clostridium perfringens* type A in laboratory medium and chicken meat

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ABSTRACT

C. perfringens type A is the causative agent of *C. perfringens* type A food poisoning (FP) and non-food-borne (NFB) human gastrointestinal diseases. Due to its ability to form highly heat-resistant spores, it is of great interest to develop strategies alternative to thermal processing to inactivate *C. perfrinegens*. Thus, in this study we evaluated the inhibitory effects of essential oil constituents (EOCs) (cinnamaldehyde, eugenol, allyl isothiocyanate (AITC), and carvacrol) against germination, outgrowth and vegetative growth of spores of *C. perfringens* FP and NFB disease isolates in laboratory medium and chicken meat. The cinnamaldehyde, eugenol and carvacrol, but not AITC, all at 0.05–0.1%, inhibited the germination of spores of all tested *C. perfringens* isolates in Tripticase-glucose-yeast extract (TGY) medium. Furthermore, all tested EOCs at 0.05–0.1% arrested the outgrowth and vegetative growth of *C. perfringens* spores in TGY, with AITC and carvacrol being the most effective. However, among four tested EOCs, only AITC (at 0.5%–2.0%) was able to inhibit the growth of *C. perfringens* spores in chicken meat and no such inhibitory effect was observed even with a 10-fold higher concentration (5%) of carvacrol. In conclusion, our current work identified AITC as an effective EOC to control spores and vegetative cells of *C. perfringens* isolates in laboratory medium and chicken meat. Further studies on evaluating the effectiveness of different combination of EOCs against *C. perfringens* spore growth in different meat products should establish an effective use of EOCs to control the risk of *C. perfringens*-mediated illnesses.

1. Introduction

Clostridium perfringens is a gram-positive, rod-shaped, anaerobic, endospore forming bacterium, which is a causative agent of histotoxic and gastrointestinal (GI) diseases in human and animals (McClane et al., 2013; Petit et al., 1999). *C. perfringens* is classified into 5 types (A-E) and these types are established based on the production of four major toxins (alpha, beta, epsilon, and iota) (McClane et al., 2013; Petit et al., 1999). A small group (~5%) of type A isolates, that produces *C. perfringens* enterotoxin (CPE), cause *C. perfringens* type A food poisoning (FP) and nonfood-borne (NFB) human GI illnesses such as antibiotic-associated diarrhea and sporadic diarrhea (Sarker et al., 1999). Previous studies have shown that *C. perfringens* isolates associated with FP typically carry the *cpe* on their chromosome (referred to in this study as FP isolates), whereas NFB human GI illnesses are linked to isolates carrying *cpe* on their large plasmid (referred to in this study as NFB isolates) (Collie and McClane, 1998; Cornillot et al., 1995; Sarker et al., 2000; Sparks et al., 2001). However, recent studies suggested that isolates carrying plasmid borne *cpe* also can be a causative agent for FP

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(Lahti et al., 2008; Lindström et al., 2011; Xiao et al., 2012).

Both FP and NFB isolates can produce metabolically dormant spores that are highly resistant to various stresses related to food preservation approaches (Li and McClane, 2006a, b; Paredes-Sabja et al., 2007). The remaining viable spores then germinate, outgrow and multiply in food products to a high level and cause disease after entering into GI tract through consumption of contaminated foods (McClane et al., 2013; Paredes-Sabja et al., 2008). Thus, one of the main goals of the food industry is to develop bacterial spore-inactivation strategies alternative to heat processing technologies, that meet consumer demand for ensured food safety, extended shelf life, and enhanced food quality. Previous studies in our laboratory demonstrated the inhibitory effects of polyphosphates, nisin, sorbate, benzoate, and chitosan against *C. perfringens* FP and NFB isolates (Akhtar et al., 2008; Alnoman et al., 2015, 2017; Udompijitkul et al., 2012). In laboratory conditions, these antimicrobial agents exhibited slight inhibition on spore germination and significant inhibition on spore outgrowth and vegetative growth of *C. perfringens* isolates. However, in meat model system, only polyphosphates and chitosan exhibited inhibitory activity against spore germination and outgrowth of *C. perfringens* isolates (Akhtar et al., 2008; Alnoman et al., 2017).

Alternative technologies also consider the use of natural antimicrobial compounds in foods such as spices and herbs (Sabah et al., 2003; Thippareddi et al., 2003; Valenzuela-Martinez et al., 2010). Essential oil constituents (EOCs) are natural, aromatic and volatile liquids extracted from plant material such as flowers, roots, seeds, leaves, peel and whole plant (Hyldgaard et al., 2012; Kuorwel et al., 2011). These are important for plant defense and considered to be secondary metabolites (Herman et al., 2013). Many EOCs have been accepted by the European Commission as flavoring agents in food products such as linalool, thymol, eugenol, carvone, cinnamaldehyde, vanillin, carvacrol, citral, and limonene. Also, the United States Food and Drug Administration (FDA) considers these EOCs and others such as, clove, oregano, thyme, nutmeg, basel, mustard, and cinnamon as 'generally recognized as safe (GRAS)' (Hyldgaard et al., 2012). Extensive research has been reported on the use of essential oils as antimicrobial agents against different spoilage bacteria (Burt, 2004; Oussalah et al., 2006; Smith-Palmer et al., 1998; Ultee et al., 2002) and their inhibitory activity has been assigned to a number of substituted aromatic molecules such as cinnamaldehyde, eugenol, allyl isothiocyanate (AITC), and carvacrol (Burt, 2004; Cha and Chinnan, 2004; Gutierrez et al., 2008; Kuorwel et al., 2011; Natrajan and Sheldon, 2000). Cinnamaldehyde, eugenol and AITC have been reported to inhibit the growth of *Clostridium botulinum, Staphylococcus aureus, Escherichia coli* 0157:H7 and *Salmonella enterica* serovar Typhimurium (Blaszyk and Holley, 1998; Burt, 2004; Cosentino et al., 1999). Carvacrol has been well examined for its antimicrobial activity against many food-borne pathogens such as *E. coli, Listeria monocytogenes, Bacillus cereus, Salmonella* spp, and *Lactobacillus sakei* (Nair et al., 2014; Natrajan and Sheldon, 2000; Smith-Palmer et al., 1998). Although two studies have been conducted on evaluating the inhibitory effects of EOC against growth of *C. perfringens* spores during abusive chilling of cooked ground beef and turkey (Junja et al., 2006, 2007), each of those studies was limited to *C. perfringens* type A FP laboratory strains. Therefore, a detailed study on the effect of EOCs against a collection of enterotoxigenic *C. perfringens* type A FP and NFB clinical isolates both in laboratory conditions and in meat products is warranted. Moreover, the fundamental knowledge of whether EOCs inhibits initiation of spore germination or prevents outgrowth of germinated spores remains unclear.

The objectives of this study were to evaluate (1) the inhibitory effects of EOC (cinnamaldehyde, eugenol, AITC, and carvacrol) against spore germination, outgrowth and vegetative growth of *C. perfringens* FP and NFB isolates in laboratory medium, and (2) the effectiveness of EOC against growth of *C. perfringens* spores in cooked chicken meat during storage at extremely abusive conditions. We found that while all tested EOCs were effective against spore outgrowth and vegetative growth of *C. perfringens* in laboratory medium, only AITC could control the growth of *C. perfringens* in cooked chicken meat during storage at abusive conditions.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Six isolates of enterotoxigenic *C. perfringens* type A were used in this study, including three FP (SM101, E13, and NCTC8239) and three NFB (F4969, B40, and NB16) isolates (Sarker et al., 2000). Cooked meat medium (Difco, BD Diagnostic Systems, Sparks, MD, USA) was used to maintain *C. perfringens* isolates in stock cultures stored at −20 °C. Bacterial growth was revived by inoculating 0.1 ml of cooked meat cultures into 10 ml fluid thioglycollate medium (FTG) (Difco) then incubating overnight at 37 °C. TGY broth (3% trypticase, 2% glucose, 1% yeast extract, and 0.1% L-cysteine) was used for vegetative growth (Paredes-Sabja et al., 2008).

2.2. Spore preparation and purification

Sporulating cultures of *C. perfringens* were prepared and purified as described previously (Akhtar et al., 2008; Paredes-Sabja et al., 2008). Briefly, 0.4 ml of FTG overnight grown culture was transferred to a new 10 ml FTG medium and grew for 8–12 h at 37 °C. Next, 0.4 ml of this FTG culture was transferred to sterile 10 ml Duncan-Strong (DS) sporulating medium (Duncan and Strong, 1968) and cultured 8–12 h at 37 °C. Spore formation was confirmed by using phase contrast microscope (Leica MDLS, Leica microsystems). Large amounts of spores were gained by scaling up the latter procedure and these spores were purified by repeated washing and centrifuging with cold sterile distilled water. After obtaining spore suspensions that were >99% free of cell debris, sporulating and germinating cells, as determined by phase-contrast microscopy, purified spores were adjusted to a final optical density at 600 nm (OD_{600}) of ~6.0 that corresponds to approximately 10^8 CFU/ml, and stored at −20 °C until used.

2.3. EOCs used in this study

EOCs used in this study were: cinnamaldehyde, eugenol, AITC and carvacrol. Cinnamaldehyde (M_W 132.16 g/mol), eugenol (M_W 164.21 g/mol), and AITC (M_W 99.16 g/mol) were purchased from Alfa-Aesar (Sunrise Valley Drive Reston, VA United States). Carvacrol (M_W 150.22 g/mol) was purchased from TCI America (9211 N Harborgate St, OR United States). EOCs were diluted in TGY broth to desired final % (vol/vol) concentration. Inclusion of EOCs did not affect the pH of TGY. All tested EOCs are slightly soluble in water.

2.4. C. perfringens spore germination in the presence of EOCs

Purified spore suspensions were heat activated at 80 °C, 10 min for FP spores and at 75 °C, 15 min for NFB spores as described previously (Paredes-Sabja et al., 2008, 2009), and then cooled in a water bath at room temperature for 5 min. Spore germination was measured by mixing heat-activated spore suspension (33 μl) with TGY (167 μl) alone or supplemented with various concentrations of EOC in 96-well microtiter plate, incubating at 37 °C for 60 min and then monitoring OD_{600} using a spectrophotometer (BioTek Instrument Inc., Winooski, VT). As observed in our previous studies