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ANTIBACTERIAL ACTIVITY OF CINNAMALDEHYDE AND SPORAN AGAINST *ESCHERICHIA COLI* O157:H7 AND *SALMONELLA*

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ABSTRACT

The *in vitro* antimicrobial effect of cinnamaldehyde and Sporan in combination with acetic acid against *Escherichia coli* O157:H7 and *Salmonella* was investigated in Luria-Bertani broth (7 log cfu/mL) containing cinnamaldehyde or Sporan (800 and 1,000 ppm) alone or in combination with 200 ppm acetic acid, and incubated at 37°C for up to 6 h. Surviving populations of test pathogens were determined by spiral plating on selective media. *E. coli* O157:H7 and *Salmonella* were undetectable after 1 h in the presence of 800 ppm cinnamaldehyde. A 1,000 ppm Sporan significantly reduced *Salmonella* and *E. coli* O157:H7 populations by 1.83 and 3.02 log cfu/mL within 2 and 4 h, respectively. Scanning and transmission electron microscopy of oil-treated bacterial cells revealed cell structural damage and leakage of cellular content. Cinnamaldehyde was highly effective against both *E. coli* O157:H7 and *Salmonella* whereas the effect of Sporan was dependent on its concentration, exposure time and pathogen.

PRACTICAL APPLICATIONS

Consumers' preference for fewer chemicals in food has led to research on potential use of natural antimicrobials in food for controlling spoilage and pathogenic microorganisms. The antimicrobial effect of essential oils has been studied earlier. This is the first report on the antimicrobial activity of Sporan, and the combined effect of acetic acid. The results of the present study indicate that the antimicrobial activity of cinnamaldehyde and Sporan against enteric pathogens could be useful in food processing and preservation.

INTRODUCTION

Foodborne illnesses caused by the consumption of food contaminated with pathogenic bacteria have been a concern to public health (Burt and Reinders 2003; Oussalah *et al.* 2007). From 2000 to 2008, *Salmonella* alone caused 380 deaths, 19,000 hospitalizations and 1 million illnesses in the United States. Although, the number of infections with *Escherichia coli* O157:H7 decreased, *E. coli* O157:H7 still remains a concern; it ranked fifth in causing 63,000 illnesses, 2,100 hospitalizations and 20 deaths in the United

States (Scallan *et al.* 2011). The battle against these bacteria remains challenging because they contaminate diverse foods; *Salmonella* can be found in poultry, eggs, ground meat, fruits, vegetables, and processed foods such as frozen pot pies and peanuts (CDC 2011a). *E. coli* O157:H7 has been detected from ground meat, poultry, fruits, leafy greens, dough and processed foods such as pizza (CDC 2011b).

The antimicrobial properties of some essential oils and their components have been described earlier (Shelef 1983; Nychas 1995). Recent studies have determined the

antimicrobial activity of cinnamaldehyde, cloves, thyme, and rosemary against *E. coli* O157:H7 and *Salmonella* (Hammer *et al.* 1999; Del Campo *et al.* 2000; Rhayour *et al.* 2003; Kim *et al.* 2004; Oussalah *et al.* 2007). Thyme and clove oils disintegrate the outer membrane of gram-negative bacteria releasing lipopolysaccharides and increasing the permeability of the cytoplasmic membrane (Lambert *et al.* 2001; Devi *et al.* 2010). Wendakoon and Sakaguchi (1995) reported that the carbonyl group of cinnamaldehyde binds to the proteins, preventing amino acid decarboxylase activity in *Enterobacter aerogenes*. Smid *et al.* (1996) observed the damage to cytoplasmic membrane of *Saccharomyces cerevisiae* when treated with cinnamaldehyde, leading to excessive leakage of metabolites and enzymes from the cell, and finally loss of viability. We evaluated the effects of cinnamaldehyde and Sporan, a proprietary blend of 10% clove, 18% rosemary, and 10% thyme oils against *E. coli* O157:H7 and *Salmonella* strains *in vitro*. The effect of these oils on *E. coli* and *Salmonella* cell structure was observed through the scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

MATERIALS AND METHODS

Bacteria and Essential Oils

Five nalidixic acid-resistant strains of *E. coli* O157:H7 and five *Salmonella* strains were used in the study. The strains of *E. coli* O157:H7 RM 4406, RM 4688 and RM 1918 (clinical isolates from lettuce outbreaks), RM 4407 (clinical isolate from spinach outbreak), and RM 5279 (clinical isolate, bagged vegetable isolate, outbreak) were used from our Environmental Microbial and Food Safety Laboratory culture collection. Five *Salmonella enterica* serovars, *S. Braenderup* (CDC clinical isolate), *S. Newport*, *S. Negev* 26 H (thyme isolates), *S. Thompson* 2051H and *S. Tennessee* 2053N, were used from our culture collection. A dam mutant *Salmonella* MT 2195 and *E. coli* O157:H7 B6914 strains were used for SEM and TEM studies.

Cinnamaldehyde (Sigma-Aldrich, St. Louis, MO) and Sporan (EcoSMART Technologies, Alpharetta, GA) were used to prepare 800 and 1,000 ppm cinnamaldehyde (800C, 1000C) and Sporan (800S, 1000S) in Luria-Bertani Broth (LB, Acumedia, Lansing, MI, supplemented with 50 ppm nalidixic acid for *E. coli*, LBN). The concentrations were selected based on the minimum inhibitory concentration of cinnamaldehyde (800 ppm). Additionally, these oils were used in combination with acetic acid (20%, Fleischmann's Inc., Baltimore, MD) as 800 ppm cinnamaldehyde + 200 ppm acetic acid (1000CV) and 800 ppm Sporan + 200 ppm acetic acid (1000SV).

Effect of Cinnamaldehyde and Sporan on the Viability of Nalidixic Acid-Resistant *E. coli* O157:H7 and *Salmonella*

Bacterial cells were grown overnight in LB or LBN at 37C. The actively growing overnight cultures were centrifuged ($7,500 \times g$, 10 min at 10C) and washed in 0.1 M sterile phosphate buffer solution (phosphate buffer saline [PBS], pH 7.0). Bacterial populations of each strain were estimated by measuring the optical density at 600 nm and cocktails of *Salmonella* and *E. coli* O157:H7 strains were prepared using equal volume of five strains. About 3 mL of the cocktail (~ 7 log cfu/mL) was transferred to 27 mL LB broth containing 800 and 1,000 ppm of cinnamaldehyde or Sporan alone or in combination with 200 ppm acetic acid, and incubated at 37C in shaker incubator (75 gpm) for 6 h. Samples (1 mL) pulled every hour were centrifuged at 12,000 rpm for 5 min, washed with PBS, and spiral plated on Sorbitol MacConkey media (Acumedia) supplemented with 0.05 mg/L of cefixime, 2.5 mg/L of potassium tellurite and 50 μ g/mL nalidixic acid (CTSMAC-N, Sigma-Aldrich) for *E. coli* O157:H7 and on XLT4 agar (Acumedia) for *Salmonella*, in duplicate. Cells suspended in LB or LBN without oils were used as control.

SEM

Actively growing *E. coli* O157:H7 B6914 and *Salmonella* MT 2195 cells were harvested by centrifugation as described above and washed with sterile water. About 5 mL of sterile Tryptic soy broth containing an aliquot of cinnamaldehyde, Sporan and Sporan + acetic acid was added to the cell pellet and incubated for 3 h at 37C. Following incubation, bacterial cells were washed three times with sterile water and observed under microscopy.

Low-temperature SEM observations were performed using an S-4700 field emission scanning electron microscope (Hitachi High Technologies America, Inc., Pleasanton, CA) equipped with a Quorum CryoPrep PP2000 (Quorum Technologies, Ltd., East Sussex, U.K.) cryo-transfer system. Bacteria were transferred onto filter paper (Whatman #1) attached with a thin layer of Tissue Tek (OCT Compound, Ted Pella, Inc., Redding, CA), which acted as the cryo-adhesive upon freezing to a flat specimen holder consisting of 16×30 mm copper plate. The samples were frozen conductively in a Styrofoam box by placing the plates on the surface of a precooled (-196°C) brass bar whose lower half was submerged in liquid nitrogen (LN_2). After 20–30 s, the holders containing the frozen samples were transferred to a LN_2 Dewar for future use or cryotransferred under vacuum to the cold stage in the prechamber of the cryotransfer system. Removal of any surface contamina-

FIG. 1. *ESCHERICHIA COLI* O157:H7 POPULATIONS IN LURIA-BERTANI BROTH TREATED WITH ANTIMICROBIALS AND INCUBATED AT 37°C FOR 6 H

The values are the means of three replications. 800C – 800 ppm cinnamaldehyde; 800S – 800 ppm Sporan; 1000C – 1,000 ppm cinnamaldehyde; 1000CV – 800 ppm cinnamaldehyde + 200 ppm acetic acid; 1000S – 1,000 ppm Sporan; 1000SV – 800 ppm Sporan + 200 ppm acetic acid.

tion (condensed water vapor) took place in the cryotransfer system by etching the frozen specimens for 10–15 min by raising the temperature of the stage to -90°C . Following etching, the temperature was lowered below -130°C , and a magnetron sputter head equipped with a platinum target was used to coat the specimens with a very fine layer of platinum. The specimens were transferred to a precooled (-140°C) cryostage in the SEM for observation. An accelerating voltage of 5 kV was used to view the specimens. Images were captured using a 4pi Analysis System (Durham, NC).

TEM

Bacterial cells were fixed for 1 h at room temperature by immersion in 3% glutaraldehyde/0.05 M Na cacodylate buffer (pH 7.0). This was followed by washing in a 0.05 M Na cacodylate buffer, rinsed three times over 1 h, postfixed in 2% buffered osmium tetroxide for 2 h, dehydrated in ETOH and infiltrated with Spurr's low-viscosity embedding resin. About 60–90-nm silver-gold sections of the tissue were cut on a Reichert/AO Ultracut microtome with a Diatome diamond knife and mounted onto 400 mesh Ni grids. They were stained with 4% uranyl acetate and 3% lead citrate and viewed in an HT-7700 Hitachi Microscope at 80 kV.

Statistical Analysis

The experiment was repeated three times for *in vitro* antimicrobial effect of cinnamaldehyde and Sporan at each exposure time. Surviving populations of *E. coli* O157:H7

and *Salmonella* were converted to log cfu/mL and were compared among antimicrobial-exposure time combinations by a three-way analysis of variance using “proc-mixed” and “Tukey” procedure (SAS 9.2, Cary, NC). The level of statistical significance was set at $P < 0.05$ in all cases.

RESULTS

Effect of Cinnamaldehyde and Sporan on the Viability of *E. coli* O157:H7 and *Salmonella*

The growth curves of nalidixic acid-resistant *E. coli* O157:H7 and *Salmonella* in LB broth containing cinnamaldehyde and Sporan are shown in Figs. 1 and 2.

E. coli O157:H7 populations in control LB broth increased from 5.72 to 8.38 log cfu/mL during the 6-h incubation at 37°C. All treatments reduced *E. coli* O157:H7 at 1 h. The effect of 800C, 1000C and 1000CV on *E. coli* O157:H7 was bactericidal within 1 h, the *E. coli* O157:H7 populations were undetectable in 1 h (detection limit 1 log cfu/mL). *E. coli* O157:H7 populations were reduced by ca. 1.25 log cfu/mL ($P > 0.05$) during the first 2 h of incubation followed by increase in their populations when treated with 800 ppm Sporan alone or in combination with acetic acid. Nevertheless, populations after 3 and 4 h in 800S-treated LB broth (5.06 and 5.71 log cfu/mL) and 1000SV-treated LB broth (5.11 and 5.74 log cfu/mL) were significantly lower than those in control (7.70 and 8.02 log cfu/mL), respectively. Significant reductions in *E. coli* O157:H7 populations were observed throughout the 6-h period when treated with

FIG. 2. *SALMONELLA* POPULATIONS IN LURIA-BERTANI BROTH TREATED WITH ANTIMICROBIALS AND INCUBATED AT 37°C FOR 6 H. The values are the means of three replications. 800C – 800 ppm cinnamaldehyde; 800S – 800 ppm Sporan; 1000C – 1,000 ppm cinnamaldehyde; 1000CV – 800 ppm cinnamaldehyde + 200 ppm acetic acid; 1000S – 1,000 ppm Sporan; 1000SV – 800 ppm Sporan + 200 ppm acetic acid.

FIG. 3. SCANNING ELECTRON MICROSCOPY OF *ESCHERICHIA COLI* B6914 CELLS AFTER A 3-H TREATMENT WITH CINNAMALDEHYDE, SPORAN AND SPORAN-ACETIC ACID

(A) Untreated cells; cells treated with (B) 0.4% cinnamaldehyde, (C) 0.4% Sporan and (D) 0.36% Sporan + 0.04% acetic acid. Scale bar = 2 μ m

1,000 ppm Sporan. *E. coli* O157:H7 populations in 1000S-treated LB broth were 4.83, 5.32, 4.99 and 4.46 log cfu/mL, which were significantly lower than their corresponding controls at 3, 4, 5 and 6 h, respectively.

Likewise, the populations of *Salmonella* in control LB broth increased from 6.23 to 8.48 log cfu/mL during the 6-h incubation period (Fig. 2). Similar to *E. coli* O157:H7 results, the effects of cinnamaldehyde at all concentrations were bactericidal within 1 h. Nevertheless, a marginal growth of *Salmonella* populations of 0.23–0.47 log cfu/mL was observed in cinnamaldehyde-treated LB broth at 5–6 h. *Salmonella* populations were increased in 800S- and 1000SV-treated LB broth after 2 h, the difference in *Salmonella* populations of 800S- and 1000SV-treated LB broth was not significant from those of control LB broth at 4–6 h. *Salmonella* populations were reduced by 1.83 cfu/mL within

2 h in 1000S-treated LB broth followed by an increase to reach the initial inoculum level (ca. 6.23 cfu/mL). *Salmonella* populations in 1000S-treated LB broth after 2, 3, 4 and 5 h (4.40, 4.75, 4.99 and 5.69 log cfu/mL), respectively, were significantly lower than their corresponding controls.

The effect of 800 ppm Sporan was insignificant irrespective of the presence of acetic acid.

SEM and TEM Investigation of the Effect of Cinnamaldehyde and Sporan on *E. coli* O157:H7 Cell Surface

SEM images of treated *E. coli* O157:H7 showed different surface patterns than the untreated control (Fig. 3). The untreated cells presented a wavelike structures, whereas the

FIG. 4. TRANSMISSION ELECTRON MICROSCOPY OF *ESCHERICHIA COLI* B6914 CELLS AFTER 3-H TREATMENT WITH CINNAMALDEHYDE, SPORAN AND SPORAN-VINEGAR

Images are viewed in HT-7700 Hitachi Microscope at 80 kV. (A) Untreated cells; cells treated with (B) 0.4% cinnamaldehyde, (C) 0.4% Sporan and (D) 0.36% Sporan + 0.04% acetic acid.

treated cells showed surface deformation. *E. coli* O157:H7 cells treated with cinnamaldehyde, Sporan and Sporan + acetic acid appeared wrinkled and shrunken, including morphological rod alterations. In addition, some cells treated with cinnamaldehyde or Sporan + acetic acid were transparent. Likewise, TEM image of *E. coli* B1914 (Fig. 4) of untreated cells showed no alterations of the internal structures whereas treated cells exhibited a granulated surface for some cells, deformation and even disruption of the outer membrane for other cells.

Similarly, SEM images of treated *Salmonella* cells showed morphological alterations (Fig. 5). When compared to untreated cells, all treated cells collapsed and appeared

empty of contents. On the other hand, images of TEM of *Salmonella* cells (Fig. 6) revealed a severe damage caused by the antimicrobial treatment. Cells treated with cinnamaldehyde, Sporan and Sporan + acetic acid showed either empty contents without visible changes in the outer membrane, disruption of the plasma membrane, or cell wall lysis.

DISCUSSION

Essential oils are the odorous volatile products of an aromatic plant's secondary metabolism, found in leaves, barks, and fruits, and when they occur in various organs in the same plant, they frequently have different composition pro-

FIG. 5. SCANNING ELECTRON MICROSCOPY OF *SALMONELLA* MT 2195 CELLS AFTER A 3-H TREATMENT WITH CINNAMALDEHYDE, SPORAN AND SPORAN-VINEGAR

Images were viewed in S-4700 Hitachi. (A) Untreated cells; cells treated with (B) 0.4% cinnamaldehyde, (C) 0.4% Sporan and (D) 0.36% Sporan + 0.04% acetic acid. Scale bar = 4 μ m.

files (Oussalah *et al.* 2007). Recently, antimicrobial effects of essential oils against bacteria, yeasts, fungi and viruses have been reported (Reichling *et al.* 2009). The major active essential oil components are phenols, terpenes and aldehydes (Ceylan and Fung 2004), which mainly damage the cell cytoplasmic membrane (Sikkema *et al.* 1995; Di Pasqua *et al.* 2007).

In this study, cinnamaldehyde exhibited strong antimicrobial properties on *E. coli* O157:H7 and *Salmonella*. Our results are in agreement with Kim *et al.* (2004) who observed complete inhibition of *E. coli* O157:H7 in LB containing 1,000 ppm of cinnamaldehyde after 2 h. Ali *et al.* (2005) observed 3.5 log reduction of *E. coli* O157:H7 in broth containing 1 ppm cinnamaldehyde after 75 min. Likewise, Helander *et al.* (1998) reported inhibition of *E. coli*

and *Salmonella* Typhimurium at 132–396 ppm cinnamaldehyde in LB broth. Cinnamaldehyde is a highly effective fungicidal agent (Smid *et al.* 1996) with minimal mammalian toxicity (Jenner *et al.* 1964). On the other hand, the inhibitions of bacterial cells by Sporan (blend of clove, rosemary and thyme oil) were dependent on its concentration, exposure time and test pathogen. Combination of acetic acid with Sporan to the treatment did not improve its antimicrobial effects.

SEM images of untreated *E. coli* O157:H7 and *Salmonella* cells revealed a surface with wrinkled protrusions, naturally present structures on the surface of living bacteria. Similar observations of intact cells were reported by Greif *et al.* (2010). In our study, the cell membranes of *E. coli* O157:H7 were severely affected by antimicrobial treatments rather

FIG. 6. TRANSMISSION ELECTRON MICROSCOPY OF *SALMONELLA* MT 2195 CELLS AFTER 3-H TREATMENT WITH CINNAMALDEHYDE, SPORAN AND SPORAN-VINEGAR

Images are viewed in HT-7700 Hitachi Microscope at 80 kV. (A) Untreated cells; cells treated with (B) 0.4% cinnamaldehyde, (C) 0.4% Sporan and (D) 0.36% Sporan + 0.04% acetic acid.

than its cellular content. This finding is in agreement with those of Di Pasqua *et al.* (2007) who stipulated that cinnamaldehyde and thymol caused structural alteration of the outer envelope in *E. coli* O157:H7. The *E. coli* O157:H7 cell envelopes of treated cells were transparent, indicating interactions of the antimicrobial compounds with the cell membranes. Helander *et al.* (1998) reported that cinnamal-

dehyde penetrated to the periplasm and to the cell interior of gram-negative bacteria through outer membrane-traversing porin proteins. *E. coli* O157:H7 cells treated with 625 ppm oregano oil collapsed, resulting in leakage of cellular contents (Burt and Reinders 2003). Cinnamaldehyde and Sporan damaged the outer membrane of *E. coli* O157:H7 cells in our study. We speculate that the antimicrobial action

of cinnamaldehyde and Sporan could be different from oregano oil, or the strain variation might have influenced differences in our results.

On the other hand, *Salmonella* seemed to be affected internally and externally. The penetration of antimicrobials in the cell envelope might have caused internal damage to the *Salmonella* cells. Our results are in agreement with Smid *et al.* (1996) who reported the disintegration of the cytoplasmic membrane of *Saccharomyces cerevisiae*, leading to excessive loss of viability. Cellular damage to *Salmonella* by cinnamaldehyde and Sporan was similar. Sporan has been reported to disrupt the cell membrane of fungal hyphae and spores (Anon 2008).

Most authors have suggested that the modes of action of essential oils depend on the type of microorganisms, mainly on their cell wall structure and to their outer membrane arrangement. They observed damages due to the significant differences in the outer membranes of gram-negative and gram-positive bacteria (Rhayour *et al.* 2003; Shan *et al.* 2007). In the light of our findings, SEM and TEM images showed us that essential oils could damage differently cells belonging to the same bacteria group. Despite these differences, the mechanism of action of cinnamaldehyde and Sporan involves the cell membrane. Some European countries have abandoned the use of hypochlorite for disinfection of foods; environmental friendly natural plant-derived antimicrobials with less mammalian toxicity could be better alternatives. This study shows that cinnamaldehyde and Sporan effectively eliminate or reduce bacterial cells. The effects of cinnamaldehyde and Sporan in reducing enteric pathogens on spinach leaves have been reported (Yossa *et al.* 2012). These antimicrobials can be further evaluated with different food (poultry, livestock food), water and irrigation water to control foodborne pathogens.

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