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Biofilm Formation by Environmental Isolates of *Salmonella* and Their Sensitivity to Natural Antimicrobials

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Abstract

The objective of this study was to determine reduction of *Salmonella* in biofilms by essential oils. Biofilm formation of 15 *Salmonella* isolates from conventional swine farm environment was evaluated by 96-well microtiter plate crystal violet and minimum biofilm eradication concentration (MBEC) assays. Only one of the 15 isolates was a strong biofilm producer as classified by crystal violet assay. All *Salmonella* isolates formed biofilm on MBEC assay. The curli expression was robust among strains S322 and S435 (*Salmonella* Infantis), S644, S777, S931, S953, and S977 (*Salmonella* Typhimurium) as observed by Congo red dye binding assay. The cell hydrophobicity varied with strains and growth phase of the strain; however, there was no significant difference in hydrophobicity of these strains. Natural antimicrobials were evaluated with MBEC assay for their bactericidal efficacy in reducing *Salmonella* in biofilms. Cinnamaldehyde and sporran at 1000 ppm significantly reduced *Salmonella* in biofilms. The bactericidal effect of these antimicrobials increased with their concentrations. *Salmonella* were reduced by 6 log CFU from their initial populations of 7–7.5 log CFU/cm^2 when 2000 ppm concentration of these antimicrobials were used. *Salmonella* were undetectable when 3000 ppm of cinnamaldehyde or sporran was used. Natural antimicrobials such as cinnamaldehyde and sporran can be used to reduce *Salmonella* in biofilms.

Introduction

In the United States, nontyphoidal *Salmonella* alone is responsible for 1 million foodborne illnesses, 19,000 hospitalizations, and 400 deaths each year (Scallan *et al.*, 2011). *Salmonella* is frequently associated with outbreaks of human illnesses due to consumption of fresh produce and meat (Pakalniskiene *et al.*, 2009; CDC, 2010; Scallan *et al.*, 2011). Environmental isolates of *Salmonella* can form biofilm on produce and abiotic surfaces as a defensive mechanism to overcome the adverse environmental conditions (Patel *et al.*, 2013; Yaron and Romling, 2014).

Biofilm is an organized group of bacterial cells that has ability to attach to biotic or abiotic surfaces (Costerton *et al.*, 1999). Cell surface hydrophobicity and specific appendages, including fimbriae, curli, and outer membrane proteins, can influence bacterial attachment to surface (Goulter *et al.*, 2009). Studies have shown association of curli expression, cellulose production, and bacterial hydrophobicity for effective attachment and biofilm production by *Escherichia coli* O157:H7 and *Salmonella enterica* on biotic and abiotic surfaces (Saldaña *et al.*, 2009; Patel *et al.*, 2011, 2013).

Biofilm forming *Salmonella* may persist for longer duration on food contact surfaces and subsequently cross-contaminate food. Furthermore, higher antimicrobial concentrations and effective treatment measures will be required to remove those biofilm forming *Salmonella* (Kroupitski *et al.*, 2009; Soni *et al.*, 2013). Ineffective antimicrobial concentrations may lead to development of antibiotic-resistant *Salmonella* that is even more hazardous from public health and food safety perspective. Therefore, it is important to evaluate the efficacy of alternative antimicrobials for removing *Salmonella* in biofilm.

Chlorine and other acid-based chemical sanitizers have been used to remove enteric pathogens on food equipment surfaces (Bodur and Cagri-Mehmetoglu, 2012; Wang *et al.*, 2012). Effectiveness of chlorine varies with its chemical and physical state, treatment conditions, and organic residues on fresh produce. Furthermore, reaction of chlorine with water containing organic matter results in harmful byproducts such

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as chloramines and trihalomethanes (Dychdala, 2001; Lopez-Galvez *et al.*, 2010). Chemical sanitizers such as peracetic acid, chlorhexidine gluconate, benzalkonium chloride, and other quaternary ammonium compounds have been used; however, 5–7 log reduction of enteric bacteria is difficult with these sanitizers (Wong *et al.*, 2010; Steenackers *et al.*, 2012; Corcoran *et al.*, 2014). Due to these limitations of sanitizers, advent of antimicrobial resistance of foodborne pathogens, and consumer preference for natural ingredients for their health benefits, there is a need for evaluation of natural antimicrobials as an alternative to chemical sanitizers (Lee Wong, 1998; Condell *et al.*, 2012). Sporran, a proprietary mixture of eugenol, thymol, and rosemary extract and cinnamaldehyde have significantly reduced *Salmonella* on spinach, lettuce, and in soil (Yossa *et al.*, 2011, 2012; Yossa et al., 2013). Zhang *et al.* (2014) reported inhibitory activity of natural antimicrobials against biofilm forming *Salmonella* in microtiter plate assay. In this study, we have used minimum biofilm eradication concentration (MBEC) assay to determine the bactericidal efficacy of antimicrobials in killing *Salmonella* in biofilms. The objective of this study was to mitigate the biofilm forming swine environmental isolates of *Salmonella* by cinnamaldehyde and sporran.

Materials and Methods

Salmonella *strains*

Fifteen *Salmonella* strains: *Salmonella* Derby (2), *Salmonella* Infantis (4), and *Salmonella* Typhimurium (9) of conventional swine farm environment (soil and lagoon) origin were obtained from North Carolina State University (Table 1). These strains were isolated as part of a longitudinal study conducted on 30 conventional farms in North Carolina. The details of isolation, antimicrobial susceptibility profiles, and their phenotypic and genotypic characterizations have been reported previously (Keelara *et al.*, 2013, 2014).

Biofilm formation, hydrophobicity, and curli expression

Salmonella strains were evaluated for biofilm formation by 96-well microtiter plate crystal violate assay using four growth media: full-strength and diluted (10%) Luria-Bertani (LB) and Tryptic Soy Broth (TSB) as described by Patel and Sharma (2010).

Table 1. *Salmonella* Serotypes Isolated From Conventional Swine Farm Environment

	Strain ID	*Serotype*	*Source*
1	S322	*Salmonella* Infantis	Soil
2	S421	*Salmonella* Typhimurium	Soil
3	S435	*Salmonella* Infantis	Lagoon
4	S481	*Salmonella* Typhimurium	Lagoon
5	S643	*Salmonella* Infantis	Lagoon
6	S644	*Salmonella* Typhimurium	Soil
7	S657	*Salmonella* Derby	Lagoon
8	S777	*Salmonella* Typhimurium	Lagoon
9	S931	*Salmonella* Typhimurium	Soil
10	S948	*Salmonella* Typhimurium	Lagoon
11	S953	*Salmonella* Typhimurium	Lagoon
12	S977	*Salmonella* Typhimurium	Lagoon
13	S1013	*Salmonella* Derby	Lagoon
14	S1214	*Salmonella* Typhimurium	Lagoon
15	S1238	*Salmonella* Infantis	Lagoon

Hydrophobicity assay was determined using bacterial adherence to hydrocarbons (BATH) assay as described by Li and McLandsborough (1999). Percent hydrophobicity was calculated as ratio of the absorbance of the bacterial assays to the control.

Overnight cultures of individual *Salmonella* strains grown in TSB were streaked on tryptone agar supplemented with Congo red (40 mg/mL) and Coomassie brilliant blue (20 mg/mL) (Romling *et al.*, 2003), and incubated at 22°C and 37°C for 48 h to determine curli expression. Curli expressing *Salmonella* were identified by typical red colonies on agar.

Effect of antimicrobials on Salmonella *in biofilms formed on MBEC pegs*

A 22 mL of overnight grown culture adjusted to 5.5 log CFU/mL cell density in 10% LB without salt (LBNS) was transferred to the trough of the MBEC® plate (Innovotech, Inc., Edmonton, Canada), and then, the trough plate was covered with the peg lid cover. Plates were incubated at 22°C for 48 h on rocking table with the angle of the rocking between 9° and 16° of inclination. After incubation, biofilm cells were determined by removing all eight pegs from first column of MBEC plate using sterile pliers and immersed in the respective wells of rinse plate containing PBS. Rinse plate was sonicated for 15 min in an ultrasonic cleaner (Branson Ultrasonic Corporation, Danbury, CT). After sonication, serially diluted aliquots of the biofilm rinsates were spot plated (8 spots of 10 μL each) on XLT4 agar. Colonies from each spot were counted following incubation at 37°C for 24 h.

A 96-well microtiter plate was set as an antimicrobial challenge plate to determine the antimicrobial effect of natural antimicrobials. The wells of last column were filled with 200 μL LB (control) and remaining column wells were filled with 200-μL of 1000, 2000, 3000, 4000, and 5000 ppm concentrations of cinnamaldehyde (Sigma-Aldrich, St. Louis, MO) and sporran (EcoSmart Tech, Alpharetta, GA), or 5 ppm of chlorine (Clorox, Oakland, CA). Biofilm pegs with *Salmonella* population were exposed to the challenge plate containing antimicrobials for 30 min at 22°C. After incubation, peg lids were rinsed in a 96-well rinse plate containing 200 μL PBS and then neutralized in a neutralizing agent medium for 2 min. After neutralization, *Salmonella* in biofilm pegs were dislodged by sonication for 15 min in a 96-well microtiter recovery plate containing 200 μL PBS. Serially diluted suspensions from wells of each column of recovery plate were spot plated (8 spots of 10 μL each) on XLT4 agar and incubated at 37°C for 24 h to determine surviving *Salmonella* populations in antimicrobial-treated biofilm pegs.

Statistical analysis

A split-plot analysis of variance (ANOVA) model was used for surviving *Salmonella* populations in biofilms (log CFU/cm^2) using a negative binomial distribution and logit link function (SAS 9.4, Cary, NC) with whole plot being plate and subplot being well within plate. The effects of *Salmonella* source of origin, *Salmonella* strains, and their combinations on biofilm formation were analyzed. Pairwise means comparisons used the Royen–Tukey–Kramer multiplicity adjustment to maintain experimentwise $\alpha = 0.05$ (SAS 9.2, Cary, NC).

TABLE 2. BIOFILM FORMATION OF *SALMONELLA* STRAINS BY CRYSTAL VIOLET ASSAY

	Growth medium			
Strains	*LB (1:10)*	*TSB (1:10)*	*LB*	*TSB*
S322	0.21 ± 0.03^{bcx}	0.24 ± 0.08^{bcdex}	0.24 ± 0.03^{cx}	0.26 ± 0.02^{bx}
S421	0.14 ± 0.02^{dex}	0.17 ± 0.00^{fx}	0.16 ± 0.01^{dx}	0.18 ± 0.01^{cx}
S435	0.23 ± 0.06^{bcy}	0.30 ± 0.07^{ax}	0.25 ± 0.03^{cy}	0.30 ± 0.04^{abx}
S481	0.18 ± 0.02^{cdy}	0.22 ± 0.04^{defy}	0.30 ± 0.06^{abcx}	0.30 ± 0.04^{abx}
S643	0.20 ± 0.02^{bcy}	0.28 ± 0.04^{abx}	0.29 ± 0.03^{abcx}	0.31 ± 0.04^{abx}
S644	0.09 ± 0.01^{efx}	0.11 ± 0.01^{gx}	0.11 ± 0.00^{efx}	0.14 ± 0.01^{cdx}
S657	0.33 ± 0.09^{ax}	0.25 ± 0.06^{abcdy}	0.29 ± 0.04^{abcxy}	0.35 ± 0.01^{ax}
S777	0.14 ± 0.02^{dey}	0.20 ± 0.01^{efx}	0.15 ± 0.01^{dexy}	0.16 ± 0.01^{cdxy}
S931	0.09 ± 0.01^{efy}	0.21 ± 0.02^{defx}	0.17 ± 0.06^{dx}	0.18 ± 0.04^{cx}
S948	0.07 ± 0.00^{fz}	0.11 ± 0.01^{gyz}	0.13 ± 0.01^{defxy}	0.16 ± 0.01^{cdx}
S953	0.20 ± 0.01^{bcz}	0.27 ± 0.02^{abcy}	0.32 ± 0.03^{abx}	0.34 ± 0.02^{ax}
S977	0.20 ± 0.01^{bcy}	0.22 ± 0.04^{cdefy}	0.33 ± 0.03^{ax}	0.31 ± 0.06^{abx}
S1013	0.08 ± 0.00^{fz}	0.12 ± 0.01^{gyz}	0.13 ± 0.01^{defxy}	0.18 ± 0.01^{cx}
S1214	0.20 ± 0.03^{bcy}	0.23 ± 0.03^{bcdefy}	0.31 ± 0.04^{abx}	0.31 ± 0.03^{abx}
S1238	0.24 ± 0.03^{by}	0.23 ± 0.03^{bcdefy}	0.27 ± 0.02^{bcxy}	0.32 ± 0.05^{ax}

Results are mean values and standard deviation of three replicates.
^{xyz}Means followed by different letters in a same row are significantly different ($p < 0.05$).
^{abcdef}Means followed by different letters in a same column are significantly different ($p < 0.05$).
LB, Luria-Bertani; TSB, Tryptic Soy Broth.

Results

Crystal violet biofilm assay

Most *Salmonella* strains formed biofilms on polystyrene 96-well microplates. A cutoff value (three standard deviations above the mean optical density of the negative controls) was used for classifying biofilm strength of *Salmonella* in growth media. *Salmonella* strains were classified as negative (2), weak (three strains), moderate (eight strains), and strong (one strain) biofilm producers according to classification suggested by Stepanovic *et al.* (2000). In general, these *Salmonella* strains formed stronger biofilms in full-strength LB and TSB media than in corresponding diluted media. Biomass produced by eight *Salmonella* strains grown in full-strength LB (0.13–0.33) was significantly higher ($p < 0.05$) than in diluted LB (0.07–0.20) (Table 2). Similarly, seven of 15 *Salmonella* strains grown in full-strength TSB (0.16–0.34) produced significantly more biomass than in diluted TSB (0.11–0.25). Biofilm formation varied with the *Salmonella* serotypes. Four of the nine *Salmonella* Typhimurium strains were either weak on nonbiofilm formers. All strains of *Salmonella* Infantis were moderate biofilm producers, whereas *Salmonella* Derby strain S657 was the only strong biofilm producer.

Hydrophobicity

Hydrophobicity was characterized by adherence of bacterial cells to hydrocarbons, which contributes to the ability of bacteria to attach to the biotic or abiotic surfaces. In this study, 12 out of 15 *Salmonella* strains were hydrophobic in nature. Percent hydrophobicity was diverse among strains and different phases of growth cycle (Table 3). There was no significant difference in percent hydrophobicity of *Salmonella* strains in their log or stationary growth phase except for strains S421, S481, S931, and S1214 (Table 3). Significantly higher hydrophobicity of log-phase cultures was observed with *Salmonella* strains S421 (20.2%) and S931 (20.1%) compared with other *Salmonella* strains (Table 3). In stationary phase, strain S1214 showed significantly higher hydrophobicity (20.4%) than S1238 (7.4%), S948 (3.9%), S931 (8%), S481 (4.2%), and S421 (9.1%).

Curli expression

Salmonella strains were evaluated for their ability to produce curli fimbriae on Congo red agar at two different temperatures (22°C and 37°C). Based on colony characteristics, strains were classified as negative (5, colorless colonies), weak curli producer (3, light pink colonies), and strong curli

TABLE 3. HYDROPHOBICITY OF *SALMONELLA* STRAINS BY BACTERIAL ATTACHMENT TO HYDROCARBON ASSAY

	% Hydrophobicity	
Strain ID	*Log phase*	*Stationary phase*
S322	10.61 ± 3.64^{bcdx}	5.66 ± 0.27^{cdex}
S421	20.21 ± 4.77^{ax}	9.12 ± 3.41^{bcy}
S435	9.51 ± 6.07^{cdx}	5.23 ± 5.22^{cdex}
S481	14.70 ± 3.76^{bx}	4.29 ± 3.68^{cdefy}
S643	10.13 ± 1.20^{bcdx}	5.21 ± 0.82^{cdex}
S644	10.32 ± 2.20^{bcdx}	12.80 ± 3.88^{bx}
S657	7.56 ± 6.17^{dex}	3.82 ± 2.11^{defx}
S931	20.16 ± 5.03^{ax}	8.09 ± 1.80^{bcdy}
S948	14.75 ± 4.09^{bx}	3.90 ± 1.24^{defy}
S1013	3.82 ± 2.68^{efx}	1.81 ± 0.78^{efx}
S1214	13.66 ± 3.31^{bcy}	20.48 ± 5.64^{ax}
S1238	13.24 ± 3.11^{bcx}	7.48 ± 1.20^{cdy}

Results are mean values and standard deviation of three replicates.
^{xy}Means followed by different letters in a same row are significantly different ($p < 0.05$).
^{abcdef}Means followed by different letters in a same column are significantly different ($p < 0.05$).

Table 4. Effect of Natural Antimicrobials on Reducing *Salmonella* on Minimum Biofilm Eradication Concentration Biofilm Pegs

				Treatment					
Source	*Serotype*	*Strain*	*Biofilm*	*Control (LB)*	*C 1000*	*C 2000*	*S 1000*	*S 2000*	*C 5*
Soil	*Salmonella* Infantis	S322	7.56 ± 0.25^{au}	5.87 ± 0.20^{bu}	4.72 ± 0.28^{cu}	2.09 ± 0.85^{du}	4.80 ± 0.09^{cu}	4.34 ± 0.21^{cu}	2.46 ± 0.40^{du}
Soil	*Salmonella* Typhimurium	S421	6.63 ± 0.08^{au}	5.79 ± 0.17^{au}	4.66 ± 0.17^{bcu}	3.66 ± 1.21^{du}	4.55 ± 0.43^{bcu}	1.03 ± 0.68^{ev}	3.43 ± 0.30^{cdu}
	Salmonella Typhimurium	S644	8.05 ± 0.10^{av}	5.77 ± 0.33^{bu}	4.66 ± 1.68^{cu}	0 ± 0.00^{ev}	4.89 ± 0.17^{bcu}	2.98 ± 1.53^{du}	2.03 ± 1.54^{dv}
	Salmonella Typhimurium	S931	6.60 ± 0.15^{au}	5.75 ± 0.08^{bu}	3.63 ± 0.10^{cu}	0 ± 0.00^{ev}	4.75 ± 0.29^{cu}	2.14 ± 1.22^{du}	1.72 ± 0.93^{dv}
Lagoon	*Salmonella* Derby	S657	6.86 ± 0.26^{au}	5.94 ± 0.17^{bu}	4.80 ± 0.29^{cu}	3.82 ± 0.19^{cdu}	3.61 ± 0.29^{dv}	2.27 ± 1.23^{eu}	2.14 ± 1.28^{eu}
	Salmonella Derby	S1013	6.72 ± 0.32^{au}	5.80 ± 0.12^{bu}	4.73 ± 0.18^{cu}	1.03 ± 0.60^{ev}	4.69 ± 0.13^{bcu}	2.72 ± 1.28^{du}	1.50 ± 1.01^{eu}
Lagoon	*Salmonella* Infantis	S435	7.07 ± 0.13^{au}	5.83 ± 0.24^{bu}	4.62 ± 0.19^{du}	0.72 ± 0.57^{ex}	4.70 ± 0.09^{cdu}	3.19 ± 0.64^{du}	1.98 ± 1.02^{eu}
	Salmonella Infantis	S643	7.91 ± 0.08^{au}	5.84 ± 0.17^{abu}	2.76 ± 0.20^{bcu}	3.33 ± 0.12^{cdu}	2.64 ± 0.24^{ev}	0.00 ± 0.00^{fv}	3.07 ± 1.33^{deu}
	Salmonella Infantis	S1238	6.72 ± 0.18^{au}	5.80 ± 0.39^{abu}	4.73 ± 0.24^{deu}	1.03 ± 1.08^{gv}	4.69 ± 0.09^{cdu}	4.46 ± 0.28^{efu}	1.50 ± 1.17^{gu}
Lagoon	*Salmonella* Typhimurium	S481	7.85 ± 0.46^{au}	5.83 ± 0.31^{bu}	3.56 ± 0.38^{dv}	0 ± 0.0^{ey}	4.83 ± 0.07^{cu}	2.72 ± 0.09^{dvx}	1.03 ± 0.68^{ex}
	Salmonella Typhimurium	S777	7.80 ± 0.23^{au}	6.27 ± 0.46^{bu}	4.77 ± 0.30^{cu}	5.04 ± 1.17^{evx}	3.53 ± 1.27^{dv}	1.72 ± 0.05^{exyz}	1.76 ± 1.15^{euvx}
	Salmonella Typhimurium	S948	7.95 ± 0.23^{au}	5.84 ± 0.07^{bu}	4.89 ± 0.06^{bu}	0 ± 0.00^{ey}	4.86 ± 0.13^{bu}	1.63 ± 1.06^{dyz}	3.00 ± 1.73^{cu}
	Salmonella Typhimurium	S953	6.87 ± 0.39^{au}	6.66 ± 0.38^{bu}	4.73 ± 0.28^{bcuv}	3.49 ± 1.87^{cdeu}	3.65 ± 0.28^{cdv}	2.46 ± 0.28^{evxy}	2.58 ± 1.32^{cdeu}
	Salmonella Typhimurium	S977	7.85 ± 0.23^{au}	5.78 ± 0.26^{bu}	4.84 ± 0.43^{bu}	1.63 ± 1.04^{dx}	4.81 ± 0.09^{bv}	2.77 ± 0.12^{cu}	2.27 ± 1.14^{cduv}
	Salmonella Typhimurium	S1214	7.70 ± 0.22^{au}	5.80 ± 0.10^{bu}	4.69 ± 0.41^{cuv}	3.00 ± 1.64^{duv}	2.82 ± 0.23^{dv}	2.46 ± 0.68^{ez}	1.33 ± 0.89^{evx}

Results are mean values and standard deviation of three replicates (log CFU/cm^2). LB = medium used as control for antimicrobial challenge; C1000, C2000 = cinnamaldehyde 1000 ppm and 2000 ppm concentration; S1000, S2000 = sporran 1000 ppm and 2000 ppm concentration; C5 = chlorine 5 ppm concentration.

^{uvxyz}Means followed by different letters in a same column within source and serotype are significantly different ($P < 0.05$).

^{abcdefg}Means followed by different letters in a same row are significantly different ($P < 0.05$).

CFU, colony-forming unit; LB, Luria-Bertani.

producer (7, dark pink colonies). All curli-negative strains, *Salmonella* Derby (2), *Salmonella* Infantis (2), and *Salmonella* Typhimurium (1), were isolated from lagoon. Strong curli expressing *Salmonella* Typhimurium (5) and *Salmonella* Infantis (2) were isolated from soil and lagoon. The difference in colony characteristics of these strains incubated at 22°C and 37°C was marginal except strain S1215 that was curli-negative at 37°C and exhibited weak curli expression at 22°C.

Antimicrobial activity of cinnamaldehyde and sporran against Salmonella *on MBEC biofilm pegs*

Salmonella population in biofilm recovered from MBEC pegs ranged from 6.63 to 8.05 log CFU/cm^2. Most *Salmonella* strains formed biofilm as evidenced by recovery of significantly higher bacterial populations attached to MBEC pegs (Table 4). There was no significant difference in biofilm formation by *Salmonella* when compared within source (soil and lagoon) and serotype except for *Salmonella* Typhimurium (S644) from soil, which produced significant biofilm (8.05 ± 0.10 log CFU/cm^2) among all the isolates.

Natural antimicrobials were used to remove *Salmonella* in biofilms formed on MBEC pegs. Cinnamaldehyde and sporran significantly reduced *Salmonella* populations in biofilm and their antimicrobial effect increased with concentrations. *Salmonella* were undetectable (<0.73 log CFU/cm^2) when 3000 ppm cinnamaldehyde or sporran were used (Table 4). Cinnamaldehyde and sporran at 2000 ppm reduced ($p < 0.05$) *Salmonella* in biofilm by 6 log CFU from their initial biofilm populations of 7–7.5 log CFU/cm^2. The antimicrobial effect of cinnamaldehyde varied with strains; four *Salmonella* strains (S481, S644, S931, and S948) were undetectable at 2000 ppm concentration of cinnamaldehyde. Cinnamaldehyde at lower concentration (1000 ppm) reduced ($p < 0.05$) all *Salmonella* strains in biofilms except strains S948 and S977. Likewise, a 1000-ppm sporran significantly reduced *Salmonella* strains S644, S948, S977, and S1013 in biofilms. Similar to natural antimicrobials, chlorine also significantly reduced *Salmonella* by 1–3.43 log CFU/cm^2 at low concentrations (5 ppm) (Table 4) and to undetectable levels at 25 and 50 ppm concentration (not shown).

Discussion

Bacteria in biofilm environment are resistant to commonly used sanitizers and can contaminate food product. Therefore, it is imperative to eradicate bacterial pathogens in biofilms. In this study, we evaluated biofilm formation by environmental isolates of *Salmonella* and subsequent removal of *Salmonella* in biofilms by cinnamaldehyde and sporran. Studies have reported that the farm environment plays an important role in persistence and dissemination of *Salmonella* all along the food chain (Keelara *et al.*, 2013; Holvoet *et al.*, 2014). In our study, all *Salmonella* strains of lagoon origin (S481, S948, S953, S977, S1013, and 1214) and three strains from soil origin (S322, S421, and S644) formed stronger biofilms ($p < 0.05$) in full growth media. However, it should be noted that these strains were weak or moderate biofilm formers as classified by Stepanovic *et al.* (2000). Our results are in agreement with previous studies where enteric pathogens formed biofilm when under stress or grown in media lacking essential nutrients (Solomon *et al.*, 2005; Patel *et al.*, 2011). Diluted growth media enhance the expression of promoter agfD, which is involved in *Salmonella* spp. biofilm formation (Romling *et al.*, 2000; Gerstel and Romling, 2001). We observed stronger *Salmonella* biofilm formation in TSB medium than in LB medium. Weak biofilm formation in LB medium could be attributed to interference of salt in expression of adhesive extracellular matrix as observed with *E. coli* (Romling, 2005; Patel *et al.*, 2011).

Most *Salmonella* strains were significantly hydrophilic at log phase compared with stationary phase as observed previously (Patel *et al.*, 2011). The relationship between hydrophobicity and biofilm formation is unclear. In the previous study, *E. coli* O157:H7 strains 4406 and 4407 were hydrophobic in nature but formed poor biofilm (Patel *et al.*, 2011). We observed strong biofilm by MBEC assay by those *Salmonella* isolates (S777, S953, and S977), which were hydrophilic in nature. Bacterial hydrophobicity had a minimum role in biofilm formation of Shiga-toxigenic *E. coli* (Rivas *et al.*, 2007). We suggest that biofilm formation by *Salmonella* in our study may rely on conditions such as nutrient availability, environmental stressors, and effectors for biofilm formation (Reisner *et al.*, 2006). Studies have reported the role of curli expression and relationship with biofilm formation in *E. coli* and *Salmonella* (Barnhart and Chapman, 2006; Saldaña *et al.*, 2009; Patel *et al.*, 2011, 2013). Curli production varied among *Salmonella* strains, categorized as strong, weak, and curli-negative isolates based on uptake of dye. This variation in curli expression has been associated with survival fitness of pathogens as observed in *E. coli* O157:H7 (Carter *et al.*, 2011; Macarisin *et al.*, 2012). In the previous study, a stronger curli expression was reported when environmental isolates of *E. coli* O157:H7 were grown at 22°C than at 37°C (Macarisin *et al.*, 2012, 2014). However, we did not find any significant difference in curli production by *Salmonella* isolates at different temperatures. In addition, studies have reported both positive (Boyer *et al.*, 2007; Patel *et al.*, 2011; Yaron and Romling, 2014) and negative correlation (Rivas *et al.*, 2007; Kim and Harrison, 2009) with hydrophobic nature of *E. coli* isolates and biofilm formation. Among all *Salmonella* strains, *Salmonella* Typhimurium exhibited strong curli production and hydrophobicity, which is alarming as these factors may influence biofilm formation in *Salmonella* Typhimurium, one of the most commonly associated serotypes of foodborne outbreaks (Medalla *et al.*, 2013; Keelara *et al.*, 2014).

Removal of *Salmonella* on equipment surfaces is challenging once it forms biofilm. Wong *et al.* (2010) evaluated the susceptibility of 3- and 7-day-old *Salmonella* biofilm on MBEC plate to chemical sanitizers. In their study, the age of *Salmonella* biofilm did not influence the efficacy of disinfectants. Sodium hypochlorite (500 mg/L) or benzalkonium chloride (0.2%) could not remove *Salmonella* biofilms in CDC biofilm reactor (Corcoran *et al.*, 2014). Chen *et al.* (2015) observed the synergistic antimicrobial effect of levulinic acid and SDS in removing up to 7.6 log CFU/mL of *Salmonella* in biofilms. A chlorinated alkaline detergent (Sanifoam®) was superior to 0.2% peracetic acid in removing biofilm-forming *Salmonella* on poultry processing surfaces (Ziech *et al.*, 2016). Natural antimicrobials such as cinnamaldehyde and sporran have shown antimicrobial activity against various pathogens, including *Salmonella* and *E. coli*

O157:H7 on biotic and abiotic surfaces (Jia *et al.*, 2011; Yossa *et al.*, 2013; Zhang *et al.*, 2014; Liu *et al.*, 2015). It is suggested that the mechanism of these antimicrobials is due to more than one mode of action. Cinnamaldehyde inhibited amino acid decarboxylase and ATPase in *Enterobacter aerogenes* and *Listeria monocytogenes*, respectively (Thoroski *et al.*, 1989; Wendakoon and Sakaguchi, 1995). Clove oil and thymol present in sporran inhibit amylase and protease activity in *Bacillus cereus* and increase ATP permeability resulting in lethal damage to bacterial cell (Gill and Holley, 2006). Role of carvacrol at sublethal concentration (20 mM) in disruption of *Salmonella* biofilm formation has been reported (Knowles *et al.*, 2005). At lower concentrations, essential oil may interfere with bacterial attachment to equipment surface by reducing flagellar production or by inhibiting quorum sensing (Nostro *et al.*, 2007; Burt *et al.*, 2014). In preliminary studies, we observed marginal reduction of these pathogens at ≤500 ppm concentration of cinnamaldehyde and sporran (data not shown). Treatment with thyme oil, oregano oil, or carvacrol at 0.025% reduced up to 1.6 log *Salmonella* in biofilms after a 1-h exposure (Soni *et al.*, 2013). They observed complete removal of *Salmonella* in biofilms at 0.05–0.1% concentrations of these antimicrobials. In our study, cinnamaldehyde and sporran at 3000 ppm concentration reduced *Salmonella* in biofilms to undetectable level (<6 log reduction). Cinnamaldehyde and sporran at ≤1000 ppm has significantly reduced *Salmonella* on fresh produce (Yossa *et al.*, 2012, 2013). Four *Salmonella* Typhimurium strains were undetectable at lower concentrations (2000 ppm) of cinnamaldehyde suggesting differences in sensitivity of these isolates to cinnamaldehyde. In this study, the need for higher concentrations of these antimicrobials for complete removal of *Salmonella* in biofilms could be attributed to environmental fitness of *Salmonella* strains. Chlorine is used at different concentrations ranging from 50 to 200 ppm in commercial processing facilities as a chemical sanitizer to reduce microbial load on fresh produce (Cherry, 1999; Taormina and Beuchat, 1999). In our study, chlorine significantly reduced *Salmonella* in biofilm at 25 and 50 ppm concentrations.

Conclusions

Our study confirms that environmental isolates of *Salmonella* form biofilm under unfavorable conditions, which could facilitate their persistence and dissemination in the farm environment and food chain. Natural antimicrobials such as cinnamaldehyde and sporran are effective in reducing *Salmonella* in biofilms. These natural antimicrobials at higher concentrations can serve as an alternative to chemical sanitizers toward meeting consumers' preference for natural interventions in the food system. Furthermore, natural antimicrobials could overcome the potential concern of chlorine-tolerant bacteria in water. This study provides evidence for the role of natural antimicrobials in removing biofilms and potential concerns to the overall cost–benefit analysis when compared with chlorine.

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Disclosure Statement

The mention of trade names or commercial products does not imply recommendation or endorsement to the exclusion of other products by the U.S. Department of Agriculture.

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