Eggs constitute a vital part of the American diet with an annual per capita consumption of approximately 250 eggs (USDA, 2012). Because of the universal acceptance of eggs as an economical and nutritious food source, and considering the public health significance, the microbiological safety of this product is critical (Howard et al., 2012). The primary source of bacterial contamination in eggs is Salmonella enterica serovar Enteritidis, which is the most common serotype of Salmonella (Braden, 2006), transmitted to humans largely due to the consumption of infected eggs (Mead et al., 1999). It is also the most frequently isolated Salmonella from chickens, especially layer flocks (Baird-Parker, 1990; Gast et al., 2005). The primary colonization site of Salmonella Enteritidis in chickens is the ceca (Allen-Vercoe and Woodward, 1999; Stern, 2008), with cecal carriage of the pathogen leading to transmission of the organism either via contaminated eggs from infected ovaries, or contaminated eggshell with feces (Keller et al., 1995; Gantois et al., 2009). In the former case, contamination of egg contents (yolk, albumen, and eggshell membranes) by Salmonella Enteritidis occurs before oviposition (Miyamoto et al., 1997; Okamura et al., 2001), where Salmonella originating from infected ovaries invades and multiplies in the preovulatory follicles of the reproductive tract (Thiagarajan et al., 1994, 1996). In the latter, Salmonella Enteritidis contamination could result from penetration of the bacteria through the eggshell from contact with

**ABSTRACT** Salmonella Enteritidis is a common food-borne pathogen transmitted to humans largely by consumption of contaminated eggs. The external surface of eggs becomes contaminated with Salmonella Enteritidis from various sources on farms, the main sources being hens’ droppings and contaminated litter. Therefore, effective egg surface disinfection is critical to reduce pathogens on eggs and potentially control egg-borne disease outbreaks. This study investigated the efficacy of GRAS (generally recognized as safe) status, plant-derived antimicrobials (PDA), namely trans-cinnamaldehyde (TC), carvacrol (CR), and eugenol (EUG), as an antimicrobial wash for rapidly killing Salmonella Enteritidis on shell eggs in the presence or absence of chicken droppings. White-shelled eggs inoculated with a 5-strain mixture of nalidixic acid (NA) resistant Salmonella Enteritidis (8.0 log cfu/mL) were washed in sterile deionized water containing each PDA (0.0, 0.25, 0.5, or 0.75%) or chlorine (200 mg/kg) at 32 or 42°C for 30 s, 3 min, or 5 min. Approximately 6.0 log cfu/mL of Salmonella Enteritidis was recovered from inoculated and unwashed eggs. The wash water control and chlorine control decreased Salmonella Enteritidis on eggs by only 2.0 log cfu/mL even after washing for 5 min. The PDA were highly effective in killing Salmonella Enteritidis on eggs compared with controls (P < 0.05). All treatments containing CR and EUG reduced Salmonella Enteritidis to undetectable levels as rapidly as within 30 s of washing, whereas TC (0.75%) completely inactivated Salmonella Enteritidis on eggs washed at 42°C for 30 s (P < 0.05). No Salmonella Enteritidis was detected in any PDA or chlorine wash solution; however, substantial pathogen populations (~4.0 log cfu/mL) survived in the antibacterial-free control wash water (P < 0.05). The CR and EUG were also able to eliminate Salmonella Enteritidis on eggs to undetectable levels in the presence of 3% chicken droppings at 32°C (P < 0.05). This study demonstrates that PDA could effectively be used as a wash treatment to reduce Salmonella Enteritidis on shell eggs. Sensory and quality studies of PDA-washed eggs need to be conducted before recommending their use.

**Key words:** Salmonella Enteritidis, plant-derived antimicrobial, egg wash
fees infected with the pathogen during or after oviposition (Messens et al., 2006). Trans-shell contamination of eggs with *Salmonella* Enteritidis may occur through environmental sources such as farmers, pets, rodents, contaminated feed, litter, and water (Jones et al., 1995; Latimer et al., 2002). Following oviposition, *Salmonella* Enteritidis survival on the outer shell surface of eggs is supported by the presence of chicken manure and other moist organic materials (Gantois et al., 2009). Once the egg is subjected to processing, eggshell contamination can occur at the processing facilities from transfer belts and packaging materials as well (Mayes and Takeballi, 1983). In view of multiple sources of egg contamination, the cleanliness and disinfection of the eggshell is pivotal in controlling *Salmonella* Enteritidis contamination on eggs (Kuo et al., 1997; Park et al., 2005). Therefore, reducing or eliminating *Salmonella* Enteritidis population on shell eggs could potentially result in microbiologically safer egg products.

For reducing the microbiological load on shell eggs, including *Salmonella*, a variety of disinfectants for egg washing have been investigated with varying degrees of success. The commonly employed antimicrobials include chlorine and iodine-based sanitizers (Knape et al., 1999), hydrogen peroxide (Padron, 1995), ozone (Koidis et al., 2000), quaternary ammonium compounds (Wang and Slavik, 1998), and electrolyzed oxidizing water (Russell, 2003). However, many of the aforementioned chemicals have been shown to possess a limited antimicrobial effect, especially in the presence of organic matter, and do not render eggs pathogen-free (Moats, 1998; Wang and Slavik, 1998).

The use of natural antimicrobial molecules for inactivating pathogenic microorganisms has received renewed attention due to toxicity concerns of synthetic chemicals (Salamci et al., 2007; Isman 2000). Historically, plants have served as sources of novel drugs, contributing to human health and well-being. Plants are capable of synthesizing a large number of molecules, many of which are phenolic compounds or their derivatives (Geissman, 1963). *Trans*-cinnamaldehyde (TC) is an aldehyde present as a major component of bark extract of cinnamon (*Cinnamomum zeylandicum*). Carvacrol (CR) is an antimicrobial ingredient in oregano oil obtained from *Origanum glandulosum*. Eugenol (EUG) is an active ingredient in the oil obtained from cloves (*Eugenia caryophillata*). The aforementioned molecules are classified by the US Food and Drug Administration as GRAS (generally regarded as safe; Adams et al., 2004, 2005; Knowles et al., 2005). Previous research conducted in our laboratory has shown that various plant-derived antimicrobials (PDA), including TC, CR, and EUG, were effective in inactivating *Salmonella* Enteritidis and *Campylobacter jejuni* in chicken cecal contents in vitro (Kollanoor Johny et al., 2010b). We also previously reported that the PDA increased the sensitivity of *Salmonella* Typhimurium DT104 to several antibiotics (Kollanoor Johny et al., 2010a), and 2 of the PDA, namely TC and EUG, significantly reduced *Salmonella* Enteritidis populations in the cecum of young and market-age broiler chickens (Kollanoor Johny et al., 2012a,b). The objective of the present study was to investigate the efficacy of TC, CR, and EUG as wash treatments for reducing *Salmonella* Enteritidis on the eggshell surface.

**MATERIALS AND METHODS**

**Bacterial Strains and Culture Conditions**

Five isolates of *Salmonella* Enteritidis preinduced for resistance to 50 µg/mL of nalidixic acid (NA; catalog no. N4382, Sigma-Aldrich, St. Louis, MO) were used for the study. The strains included *Salmonella* Enteritidis 12 (chicken liver, phage type 14b), *Salmonella* Enteritidis 22 (chicken intestine, phage type 8), *Salmonella* Enteritidis 28 (chicken ovary, phage type 13a), *Salmonella* Enteritidis 31 (chicken gut, phage type 13a), and *Salmonella* Enteritidis 90 (human, phage type 8). Each strain was cultured separately in 10 mL of tryptic soy broth (Difco, Becton Dickinson, Sparks, MD) containing 50 µg/mL of NA, and incubated at 37°C for 24 h. After 3 passages, equal volumes of the cultures were combined and sedimented by centrifugation (3,600 × g for 15 min at 4°C). The pellet was washed twice, resuspended in PBS (pH 7.0), and used as the inoculum. The bacterial count in the individual cultures and the 5-strain mixture was confirmed by plating 0.1-mL portions of appropriate dilutions on tryptic soy agar (TSA+NA; Difco) containing 50 µg/mL of NA and xylose lysine desoxycholate agar (XLD+NA; Difco) containing 50 µg/mL of NA, and incubating the plates at 37°C for 24 h (Kollanoor Johny et al. 2010b).

**Preparation and Inoculation of Eggs**

Freshly laid eggs from Single-Comb White Leghorn layer chickens obtained from the University of Connecticut poultry farm were washed in sterile deionized water at room temperature (23°C) to remove visible dirt, if any, and kept for drying under a laminar flow hood for 15 min. A batch of 8 eggs was dipped completely in 500 mL of sterile PBS inoculated with ~8.0 log cfu/mL of a 5-strain mixture containing 50 µg/mL of NA and xylolysis desoxycholate agar (XLD+NA; Difco) containing 50 µg/mL of NA, and incubating the plates at 37°C for 24 h (Kollanoor Johny et al. 2010b).

**PDA Treatment and Microbiological Analysis of Eggs**

Each egg was placed in a separate Whirlpak bag with 100 mL of sterile deionized water containing 0.0, 0.25, 0.5, or 0.75% of TC, CR, or EUG, and washed in a shaker water bath at 32 or 42°C for 1, 3, or 5 min. The sterile deionized water used was previous-
ly tempered for 5 min in the shaker before applying treatments and was monitored using a thermocouple throughout the experiment. Water with 200 mg/kg of chlorine was included as chlorine control. After treatment, each egg was transferred to a sterile stomacher bag containing 30 mL of neutralizing broth (Fisher) and was gently rubbed by hand for 1 min (Park et al., 2005). The surviving *Salmonella* Enteritidis on eggs was determined by both enumeration and enrichment. *Salmonella* Enteritidis was enumerated by plating the neutralizing broth directly or after 10-fold serial dilutions on XLD+NA and TSA+NA plates. The plates were incubated at 37°C for 48 h before counting the colonies. Ten milliliter aliquots of the neutralizing broth were added to 100 mL of selenite cysteine broth (Difco) and enriched at 37°C for 48 h. The culture was streaked on Brilliant Green Agar (Oxoid) plates and incubated at 37°C for 3 days. Representative bacterial colonies from the Brilliant Green Agar plates were confirmed as *Salmonella* Enteritidis using the *Salmonella* rapid detection kit (Microgen Bioproducts Ltd., Camberley, UK). In addition, we also tested the egg wash solution (de-ionized water with/without PDA) after each washing period for the presence of *Salmonella* Enteritidis.

**Statistical Analysis**

Five eggs per treatment at every sampling point for each temperature were included in all 3 replicated experiments. Each experiment was a completely randomized design with a 3 × 6 × 3 × 2 factorial treatment structure. The factors included 3 compounds (CR, EUG, TC), 6 treatments (baseline, chlorine, 0%, 0.25%, 0.5%, and 0.75% of respective compounds), 3 sampling times (30 s, 3 min, and 5 min), and 2 temperatures (32 and 42°C). The experiment was repeated in the presence of 3% organic matter utilizing a completely randomized design with 3 × 4 × 3 factorial treatment structure. The factors included 3 compounds, 4 treatments (baseline, chlorine, 0% and 0.5% of respective compounds), and 3 sampling times (30 s, 3 min, and 5 min). All experiments were replicated 3 times. Data were analyzed using PROC MIXED procedure of the Statistical Analysis Software (SAS Institute Inc., Cary, NC). Differences among means were detected at \( P < 0.05 \) using the Fisher’s least significant difference test with appropriate corrections for multiple comparisons.

**RESULTS AND DISCUSSION**

Cleaning and sanitation of shell eggs by washing is a common practice mandatory for retail shell eggs from plants operating under voluntary USDA grade standards (USDA, 2000) and also required by individual state laws in the United States. Zeidler (2002) reported that washing eggs under optimum conditions could potentially reduce the total bacterial load by 2 to 3 log cfu/mL. An ideal egg wash antimicrobial should be effective in reducing large populations of the target pathogen in a rapid time frame, even in the presence of organic matter. Further, it should be safe to workers and the environment, cost effective (Scott and Sweetnam, 1993), and be easily incorporated into a hazard analysis and critical control points plan.

Chlorine and chlorine-containing compounds are the most commonly used antimicrobial agents for egg washing (Cao et al., 2009). However, chlorine is minimally effective in reducing pathogen loads on the egg surface, and does not render the egg pathogen-free (Wang et al., 2010). For example, Park et al. (2005) observed that washing of eggs in water containing 200 mg/kg of chlorine or electrolyzed oxidizing water for 5 min decreased *Salmonella* Enteritidis only by a maximum of ~3 log cfu/mL. Similarly, Knape et al., (1999) reported that washing of eggs in chlorinated water reduced total bacterial population by ~2 to 3 log cfu/mL. In yet another study, washing of eggs in water containing ozone at 3 mg/kg for 30, 60, and 90 s decreased *Salmonella* Enteritidis counts by 2.1 to 2.34 and 2.47 log cfu/mL, respectively (Koidis et al., 2000). Moreover, chlorine can combine with organic matter releasing trihalomethanes and other organochlorine compounds, which are potentially carcinogenic. Therefore, it is critical to develop safe and effective antimicrobial interventions to wash eggs to reduce or eliminate *Salmonella* Enteritidis on the eggshell surface.

In the current study, we investigated the efficacy of 3 PDA, namely, CR, TC, and EUG, added to wash solutions for reducing *Salmonella* Enteritidis on shell eggs in the presence and absence of organic matter. Testing of eggs for any inherent *Salmonella*, including NA-resistant bacteria, revealed that the eggs were devoid of the pathogen. Because selective media can inhibit the recovery of bacteria stressed by exposure to antimicrobials, we used a nonselective medium (TSA) for enumerating *Salmonella* Enteritidis from treated eggs, although a selective medium (XLD) was also used. However, we did not find any significant difference \( (P < 0.05) \) between the *Salmonella* Enteritidis counts recovered on the selective and nonselective media (data not shown). Therefore, *Salmonella* counts from the XLD plates were used for statistical analysis and discussion.

Because the USDA (2000) recommends 32°C as the minimum temperature for the water used for egg washing, the efficacy of PDA as antimicrobial wash for killing *Salmonella* Enteritidis on shell eggs was investigated at this temperature. A higher temperature of 42°C was also chosen to determine if an increase in the wash water temperature could enhance the efficacy of PDA. The effect of various egg washing treatments at 32°C on *Salmonella* Enteritidis counts in the absence of organic matter is depicted in Figures 1A-C. The average *Salmonella* Enteritidis count recovered from unwashed eggs after inoculation (baseline) was ~6.4 log cfu/mL. Washing of eggs in water or water containing chlorine (200 mg/kg) decreased *Salmonella* Enteritidis counts by ~2.0 log cfu/mL \( (P < 0.05) \). However, washing of eggs in water containing CR (0.5 and 0.75%) decreased
Figure 1. Effects of A) carvacrol (CR), B) eugenol (EUG), and C) trans-cinnamaldehyde (TC) at 0.25, 0.5, and 0.75% on Salmonella Enteridis inoculated on shelled eggs at 32°C. Control (0%) and chlorine (200 mg/kg) under similar experimental conditions were also tested. Five eggs per treatment per sampling point (30 s, 3 min, and 5 min) were included, and the experiment was repeated 3 times. The differences between the means were compared at a significance level of 5%. Error bars represent SEM (n = 15). Means that do not show a common letter (a–d) differ from one another within a given time point.
Figure 2. Effects of A) carvacrol (CR), B) eugenol (EUG), and C) trans-cinnamaldehyde (TC) at 0.25, 0.5, and 0.75% on *Salmonella* Enteritidis inoculated on shelled eggs at 42°C. Control (0%) and chlorine (200 mg/kg) under similar experimental conditions were also tested. Five eggs per treatment per sampling point (30 s, 3 min, and 5 min) were included, and the experiment was repeated 3 times. The differences between the means were compared at a significance level of 5%. Error bars represent SEM (n = 15). Means that do not show a common letter (a–e) differ from one another within a given time point.
Salmonella Enteritidis counts to undetectable levels by 30 s, and the eggs consistently tested negative for the pathogen (by plating and enrichment) throughout the subsequent sampling points (3 and 5 min, \( P < 0.05 \); Figure 1A). Although 0.25% CR reduced Salmonella Enteritidis to undetectable levels by plating at all time points, the eggs tested positive for the pathogen on enrichment at 30 s. Similarly at 32°C, EUG (0.5 and 0.75%) completely inactivated Salmonella Enteritidis (negative by enrichment) at all sampling time points, whereas 0.25% EUG reduced Salmonella Enteritidis populations by ~5.0 log cfu/mL at 5 min of washing (\( P < 0.05 \); Figure 1B). On the other hand, TC at its highest tested concentration of 0.75% decreased Salmonella Enteritidis counts on eggs by ~5.0 cfu/mL at the end of 5 min (\( P < 0.05 \); Figure 1C).

At 42°C, all concentrations of CR and EUG reduced Salmonella Enteritidis counts on eggs to undetectable levels as quickly as 30 s of washing (\( P < 0.05 \); Figure 2A and 2B). In the case of TC, the highest concentration (0.75%) completely inactivated Salmonella Enteritidis on eggs at 30 s (negative on enrichment), whereas 0.25 and 0.5% TC brought about the same magnitude of reduction in Salmonella Enteritidis populations at 5 min of washing (\( P < 0.05 \); Figure 2C).

The presence of organic matter could potentially reduce the efficacy of antimicrobials used in egg wash. For example, Knape et al. (2002) observed that although distilled deionized water and chlorine (200 mg/kg) decreased Salmonella Enteritidis populations on eggs compared with dry egg controls, the efficacy of egg sanitizers was affected by the level of total dissolved compounds in the wash water. Therefore, we investigated the efficacy of the aforementioned PDA in the presence of poultry droppings, which is one of the common contaminants on eggshell surfaces. We examined the efficacy of 0.5% of each PDA on Salmonella Enteritidis at 32°C in the presence of chicken droppings. We observed that CR decreased Salmonella Enteritidis to undetectable levels as rapidly as 30 s (enrichment negative), whereas EUG completely inactivated the pathogen at 3 min (\( P < 0.05 \); Figures 2A and 3). However, TC could reduce Salmonella Enteritidis counts on eggs by 5.0 log cfu/mL only after 5 min (\( P < 0.05 \)).

It was found that ~4.0 log cfu/mL of Salmonella Enteritidis survived in the antimicrobial-free deionized wash water after treating the eggs, whereas no bacteria were recovered from the water containing chlorine or the PDA. The recovery of viable Salmonella Enteritidis in the water after washing eggs is of concern due to potential cross-contamination or recontamination of sequential batches of eggs, if the same solution is used for washing. In addition, the disposal of wash water needs to be addressed to prevent potential environmental contamination.

We found significant difference between the 2 temperatures (i.e., 32 and 42°C), especially the efficacy of EUG and TC in reducing Salmonella Enteritidis on...
eggs ($P < 0.05$). For example, EUG at 0.25% decreased *Salmonella* Enteritidis counts by $>5.0$ log cfu/mL only after 5 min at 32°C, whereas same concentration of the compound completely inactivated the pathogen after 30 s of washing at 42°C (Figure 1B and 2B). Similarly, none of the tested concentrations of TC reduced *Salmonella* Enteritidis counts by more than 5.0 log cfu/mL at 32°C (Figure 1C), whereas all 3 TC concentrations decreased the pathogen to undetectable levels after 5 min of washing at 42°C (Figure 2C). The antimicrobial activity of lipid-soluble PDA is attributed to their hydrophobicity and deleterious effects on bacterial cell membrane (Sikkema et al., 1994; Cox et al., 2006). The heat-induced damage of bacterial plasma membrane potentiates the effect of PDA, thereby resulting in an enhanced bactericidal effect with increase in temperature. A similar finding was reported by Shibasaki and Kato (1978), who observed that heating makes the bacterial plasma membrane more fluid, thereby increasing the antimicrobial activity of lipid-soluble small molecules.

To conclude, the results of this study indicate that the PDA, especially CR and EUG, were effective ($P < 0.05$) in rapidly reducing *Salmonella* Enteritidis on shell eggs compared with washing in untreated or chlorine-treated water. Although washing of eggs by immersion is not permitted by the USDA-Food Safety and Inspection Service, we used this method as a first step to investigate the potential ability of PDA for rapidly reducing *Salmonella* Enteritidis on eggs. Based on the results of this study, our future research will ascertain the PDA efficacy for sanitizing eggs in a continuous flow system to mimic commercial application. Moreover, although washing of eggs with the PDA revealed no visible difference in shell color or consistency compared with control eggs, sensory and quality analyses of PDA-treated eggs are required before recommending their use.

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