Trans-cinnamaldehyde nanoemulsion wash inactivates *Salmonella* Enteritidis on shelled eggs without affecting egg color

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ABSTRACT *Salmonella* Enteritidis is a major foodborne pathogen that causes enteric illnesses in humans, primarily through the consumption of contaminated poultry meat and eggs. Despite implementation of traditional disinfection approaches to reduce *S.* Enteritidis contamination, egg-borne outbreaks continue to occur, raising public health concerns and adversely affecting the popularity and profitability for the poultry industry. Generally Recognized as Safe (**GRAS**) status phytochemicals such as *Trans*-cinnamaldehyde (**TC**) have previously shown to exhibit anti-*Salmonella* efficacy, however, the low solubility of TC is a major hurdle in its adoption as an egg wash treatment. Therefore, the present study investigated the efficacy of *Trans*-cinnamaldehyde nanoemulsions (**TCNE**) prepared with emulsifiers Tween 80 (**Tw.80**) or Gum Arabic and lecithin (**GAL**) as dip treatments, at 34°C, for reducing *S.* Enteritidis on shelled eggs in presence or absence of 5% chicken litter. In addition, the efficacy of TCNE dip treatments in reducing trans-shell migration of *S.* Enteritidis across shell barrier was investigated. The effect of wash treatments on shell color were evaluated on d 0, 1, 7, and 14 of refrigerated storage. TCNE-Tw.80 or GAL treatments (0.06, 0.12, 0.24, 0.48%) were effective in inactivating *S.* Enteritidis by at least 2 to 2.5 log cfu/egg as early as 1 min of washing time ($P < 0.05$). In presence of organic matter, nanoemulsions (0.48%) reduced *S.* Enteritidis counts by ∼ 2 to 2.5 log cfu/egg as early as 1 min, ($P < 0.05$). Nanoemulsion wash also inhibited trans-shell migration of *S.* Enteritidis, as compared to control ($P < 0.05$). The nanoemulsion wash treatments did not affect shell color ($P > 0.05$). Results suggest that TCNE could potentially be used as an antimicrobial wash to reduce *S.* Enteritidis on shelled eggs, although further studies investigating the effect of TCNE wash treatments on organoleptic properties of eggs are necessary.

Key words: *trans*-cinnamaldehyde, *salmonella* enteritidis, nanoemulsion, food-grade emulsifier, egg

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INTRODUCTION

Shelled eggs constitute an important part of the American diet. The current per capita consumption of eggs in the United States is ∼286 per person (USDA, 2021). By end of 2022, the per capita consumption is projected to increase to 288.1 (WASDE, 2022). Despite numerous health benefits of eggs and heightened consumption, eggs can serve as a primary vehicle in transmitting foodborne pathogens to humans. Among the foodborne pathogens transmitted through poultry products, *Salmonella* Enteritidis (**SE**) is the leading pathogen responsible for egg contamination (FDA, 2009; De Knegt et al., 2015; Gast et al., 2019). During 1985−1998, a total of 360 outbreaks of *S.* Enteritidis infection were investigated, with egg consumption associated with 279 or 82% of those outbreaks, suggesting *S.* Enteritidis as the leading cause of human salmonellosis associated with the consumption of raw, undercooked, or contaminated table eggs (CDC, 1992; CDC, 2000; Kimura et al., 2004; Lublin et al., 2015). Annually, ∼79,000 cases of egg-borne illness and 30 deaths are caused by consumption of eggs contaminated with *S.* Enteritidis (FDA, 2020). The latest *S.* Enteritidis egg-borne outbreak in 2018 affected 11 states, resulting in 44

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cases and 12 hospitalizations (CDC, 2018). Shelled eggs can become contaminated with *S.* Enteritidis within the hen's reproductive tract by vertical transmission or transovarian infection of the developing ovum prior to shell deposition, which leads to infection of egg albumen, yolk, or vitelline membrane (Messens et al., 2006; Gantois et al., 2009; Lublin et al., 2015). Moreover, egg contamination can occur by bacterial surface attachment or penetration of the eggshell during or following oviposition through contact with contaminated chicken litter, feedstuff, feces, or equipment (Messens et al., 2006; Gantois et al., 2009). This route of transmission is known as on-shell or trans-shell contamination with bacterial penetration of the eggshell increasing with length of time eggs encounter the contaminated material (FDA, 2009). Considering the multiple sources of egg contamination, the cleanliness and disinfection of the eggshell is critical for reducing *S.* Enteritidis contamination of eggs.

The operation for controlling transmission of *Salmonella* from table eggs to humans, involves incorporating specific safety regulations regarding sanitation, storage, and transportation of eggs. Currently, the Egg Safety Final rule issued by FDA requires egg producers with 3,000 or more laying hens to implement measures to prevent *S.* Enteritidis egg contamination during production, storage, and transportation (FDA, 2009, 2020). In the United States, shelled eggs are washed collectively in plastic-coated wire baskets, fiberboard flats, or on moving conveyor belts using water containing a cleaning solution. The wash solution should be able to blanket large surface areas, displaying antimicrobial activity against microbes (ATTRA, 2009). The use of synthetic chemicals to reduce pathogen load have been explored, with varying degrees of success. The current intervention strategies in reducing *S.* Enteritidis on eggs include chlorine and iodine-based sanitizers, hydrogen peroxide, ozone, quaternary ammonium compounds, and electrolyzed oxidizing water (EPA, 1999; Upadhyaya et al., 2013; Al-Ajeeli et al., 2016). However, the aforementioned strategies are challenged with concerns over residues, discoloration, and limited antimicrobial efficacy, especially in the presence of organic matter (Stringfellow et al., 2009; Upadhyaya et al., 2013; Wagle et al., 2019). Furthermore, use of chlorine-based sanitizers has been linked with production of carcinogenic byproducts such as chloramines, trihalomethanes, and other organochlorine compounds (Zhang and Farber, 1996; Richardson, 1998; Donato and Zani, 2010). The potential health risks associated with the use of synthetic chemicals as well as consumer preference toward natural antimicrobials has led to an increase in studies investigating the potential of Generally Recognized as Safe (**GRAS**) status plant-derived compounds (**PDAs**) for egg decontamination.

Obtained from various plant sources, PDAs are secondary metabolites that exhibit significant ability to inhibit the growth of bacteria, yeasts, and molds (Chen et al., 2012). The secondary metabolites are produced by plants in response to microbial infection or animal predation (Reichling, 2010; Upadhyaya et al., 2015). The PDAs selected for egg washing must be safe and effective for use on table eggs. *Trans*-cinnamaldehyde (**TC**) is a PDA obtained from bark extract of cinnamon (*Cinnamomum zeylandicum*) and has been classified by FDA as GRAS (approval TC-21CFR182.60). *Trans*-cinnamaldehyde has been shown to be effective against Gram-positive and Gram-negative microorganisms (Kollanoor-Johny et al., 2008; Jo et al., 2015; Upadhyaya et al., 2015; Upadhyay and Venkitanarayanan, 2016). While TC has been investigated as an antimicrobial to counteract foodborne pathogens at the pre- and postharvest level (Upadhyaya et al., 2013, 2015), the poor water solubility is a significant challenge for widespread application in food safety (Doyle and Stephens, 2019; FAO, 2023; PubChem, 2023). Therefore, in this study, TC nanoemulsions (**TCNE**) were prepared for increasing its dispersion in the aqueous medium followed by investigating the efficacy of TCNE dip treatments in reducing *S.* Enteritidis survival on eggs (in the presence or absence of organic matter). Furthermore, the efficacy of TCNE dip treatments in reducing trans-shell migration of *S.* Enteritidis from surface to interior of egg and effect on egg color was investigated.

MATERIALS AND METHODS

Preparation and Characterization of Trans-*cinnamaldehyde Nanoemulsion*

An oil-in-water nanoemulsion of TC was prepared using sonication, a high energy method. *Trans*-cinnamaldehyde (99%, catalogue no. AC110350010, Fisher Scientific, Waltham, MA) was combined with either Tw.80 (catalog no. 28329, Fisher Scientific), a synthetic non-ionic surfactant or a combination of gum Arabic (catalogue no. G9752, Sigma-Aldrich, St. Louis, MO) and lecithin (catalogue no. O3376250, Fisher Scientific). The preparation of TCNE with Tw.80 replicates a previously published protocol of Bhargava et al. (2015), where hydrophobic oil and surfactant were mixed at a 2:1 mass ratio for 30 min at constant speed (200−400 rpm). Under continuous stirring, deionized water (**DI** water) was added dropwise and stirred for 30 min at 23° C. The mixture (5% stock solution) was subjected to continuous sonication for 20 min using an ultrasonicator (QSonica 700, QSonica L.L.C, Newtown, CT) at 75 W at 23°C. The preparation of TCNE with gum Arabic and lecithin (**GAL**) was performed as per published protocol (Hu et al., 2016). Briefly, the stock solutions of gum Arabic and lecithin were separately prepared by dissolving in water at 4% (w/v). The stock solutions were mixed at equal volume in DI water to prepare aqueous phase. Different quantity of oil (1.7, 1.25, 0.8, or 0.4%) was first pre-mixed with ethanol, used as a co-surfactant. The mixture was added dropwise to aqueous phase containing DI water and GAL, then mixed for 30 min at room temperature. After mixing oil and aqueous phase, the mixture (1.25% stock solution) was

subjected to sonication for 10 min at 75 W, with 10 s pulse On and 5 s pulse Off cycle.

The freshly prepared nanoemulsions were diluted 1:2 with DI water and then subjected to physicochemical characterizations such as particle size, polydispersity index (**PDI**) and zeta potential (ζ), determined by dynamic light scattering (**DLS**) at 25°C using a Nano Zeta-sizer ZS (Malvern Instruments Ltd, Malvern, WR, UK). The measurements were recorded from 3 replicates of stock solutions. The stability of nanoemulsion was determined after storage at both room temperature (23°C) and refrigeration temperature (4°C) for 8 wk. At each time point, samples were characterized for particulate attributes as described above.

Preparation of Bacterial Cultures

A 4-strain cocktail of *S.* Enteritidis with SE12 (chicken liver isolate, phage type 14b), SE28 (chicken ovary isolate, phage 13a), SE31 (chicken gut isolate, phage type 13a), and SE90 (human isolate, phage type 8) were used in the study. Each strain was cultured individually in 10-mL sterile tryptic soy broth (**TSB**; BD Bacto) and incubated at 37°C for 24 h. After 3 successful passages, equal volumes of cultures were combined and sedimented by centrifugation (7,000 rpm for 10 min at 25°C). The pellet was washed thrice, re-suspended in phosphate buffer saline (pH 7.0), and used as the inoculum (~8.0 log cfu/mL). The bacterial population of the individual cultures and 4-strain cocktail was estimated by plating 0.1-mL portions of appropriate dilutions on xylose lysine deoxycholate agar (**XLD**; BD Difco, Sparks, MD) and tryptic soy agar (**TSA**; BD Difco), and then incubated at 37°C for 24 h (Upadhyaya et al., 2013).

Antimicrobial Activity of Trans-cinnamaldehyde Nanoemulsion During Room and Refrigerated Temperature Storage

Tw.80 and GAL prepared nanoemulsions were stored at 23 and 4°C for 8 wk. At each time point (0, 2, 4, 8 wk), the Minimum Bactericidal Concentration (**MBC**) was estimated to determine the impact of temperature and storage on nanoemulsion antimicrobial activity as described below.

Four strains of *S.* Enteritidis, SE12, SE28, SE31, and SE90 were used in the study. Each strain was cultured separately in 10 mL sterile TSB and incubated at 37°C for 24 h. The antimicrobial activity of TCNE was determined as previously described (CLSI, 2012). The MBC was determined by broth dilution assay. MBC was defined as the compound concentration that induced a 99.9% decrease in viable cells upon exposure to the compound for 24 h. The 2 types of nanoemulsion were serially diluted in TSB in a sterile 24-well plate (Fisher, Corning Costar) to attain nanoemulsion concentrations ranging from 0.015, 0.03, 0.06 to 0.24%. Ten mL sterile TSB was inoculated with 50 μL culture containing ~6.0 log cfu/mL of individual *S.* Enteritidis strain. TSB containing *S.* Enteritidis was added to control and treatment wells followed by gentle mixing and incubation at 37°C for 24 h. To determine MBC, 0.1-mL aliquots from wells were diluted and plated on TSA. TSA plates were incubated at 37°C for 24 h to observe *S.* Enteritidis growth. TSB only and TSB broth containing ethanol or TC oil with culture served as controls.

Transmission Electron Microscopy

Transmission electron microscopy (**TEM**) was conducted to examine the morphology and size of nanoemulsions. Freshly prepared nanoemulsions (3 μL) was transferred to a freshly glow discharged TEM copper coated 400 mesh grid and stained with a drop of 0.5% uranyl acetate solution, followed by drying for 2 min at 23°C (Yildirim et al., 2017). Once the sample was completely dried, the grid was loaded into the sample chamber of Tecnai T12 and images were obtained by a CCD camera (AMT 2K XR40).

Efficacy of TCNE Wash Treatments in Inactivating S. Enteritidis on Eggs

Freshly laid eggs from Single-Comb White Leghorn layer chickens were obtained from the University of Connecticut poultry farm. The eggs (maintained at 23°C) were spot inoculated with 200 μL (~7.0 log cfu/egg) of a 4-strain cocktail of *S.* Enteritidis followed by drying to facilitate bacterial attachment under a laminar flow hood for 1 h at 23°C (Upadhyaya et al., 2013).

S. Enteritidis inoculated eggs were treated in separate Whirl-Pak bags (catalog no. 01812120, Fisher Scientific) containing 75 mL sterile DI water with or without 0.01, 0.02, 0.03, 0.06, 0.12, 0.24, or 0.48% TCNE prepared with Tw.80 or GAL emulsifiers, as well as pure oil TC at the aforementioned concentrations. Eggs were washed in dip treatments at 34°C for 1, 3, or 5 min. Sterile DI water containing 0.02% or 200 ppm chlorine was included as an industry control. After treatment, each egg was transferred aseptically to a sterile stomacher bag containing 100 mL Dey-Engley Neutralizing broth (Fisher) and was rubbed by hand for 1 min to detach bacteria from eggshell. Using light force, eggs were cracked gently to release internal contents and stomached for 1 min at 75 rpm. The number of surviving *S.* Enteritidis on combined eggshells and egg contents was determined by both enumeration and enrichment. *S.* Enteritidis was enumerated by plating the D/E neutralizing broth directly or after 10-fold serial dilution on XLD agar plates. The plates were incubated at 37°C for 48 h for the enumeration of *Salmonella* colonies. For enrichment, 10 mL aliquots of D/E neutralizing broth were added to 100 mL selenite cysteine broth (Difco) and enriched at 37°C for 48 h. The samples were streaked on XLD agar plates and incubated at 37°C for

48 h for the presence of bacterial colonies. In addition, egg wash solution (DI water with/without phytochemical) was enumerated after each washing period. The detection limit for *S.* Enteritidis by plating was 3 log cfu/egg while the detection limit by enrichment was 2 log cfu/egg.

Efficacy of TCNE Treatments in Inactivating S. Enteritidis on Eggs in Presence of Organic Matter

The efficacy of select TCNE treatments (0.24, 0.48%) in inactivating *S.* Enteritidis on eggs was investigated in presence of 5% organic load (pathogen free chicken litter). The litter material collected for this experiment consisted of pine shavings used for rearing one flock of broiler chickens. The methodology for the experiment was the same as described above.

Efficacy of TCNE Treatments in Inactivating S. Enteritidis on Eggs Washed in Batches

Since eggs are washed in batches in the industry, we wanted to validate the anti-*Salmonella* efficacy of TCNE treatments on eggs when washed collectively. Pathogen inoculation was performed as described above. *S.* Enteritidis inoculated eggs (10 eggs per treatment) were treated in a sterile aluminum tray containing 1,500 mL sterile DI water containing 0.02% or 200 ppm chlorine, TCNE-Tw.80, GAL, or TC 0.48% at 34°C for 5 min in presence or absence of 5% pathogen free chicken litter. Surviving *S.* Enteritidis population on eggshells was enumerated on XLD agar as described above.

Efficacy of TCNE Treatments in Reducing Trans-shell Migration of S. Enteritidis

Freshly laid unwashed eggs from Single-Comb White Leghorn layer chickens were procured from the University of Connecticut poultry farm. The eggs were inoculated with *S.* Enteritidis as described previously (De Reu et al., 2006; Lublin et al., 2015). Briefly, the eggs were spot inoculated on narrow end with 200 μL (~7.0 log cfu/egg) of a 4-strain cocktail of *S.* Enteritidis, followed by attachment/drying time of ~2 h at 23°C. To differentiate inoculated versus uninoculated side of egg, a vertical line was drawn on shell center to determine the beginning of inoculation from vertical line toward the narrow end. This technique allowed for separation of SE inoculated narrow end from uninoculated broad end, reducing contamination. The inoculated eggs were washed with dip treatments containing TCNE-Tw.80, GAL or TC (0.24, 0.48%) at 34°C for 1, 3, or 5 min. Chlorine 0.02% or 200 ppm was included as an industry control. Eggs were kept for drying for 1 h at 23°C. After drying, the blunt end of eggs was disinfected with 70% ethanol and an egg cracker opener device was used to remove shell. Egg internal contents were aseptically transferred to 50 mL selenite cysteine broth, massaged by hand for 30 s, then incubated at 37°C for 48 h, followed by streaking on XLD agar. XLD plates were incubated at 37°C for 48 h for the presence or absence of *Salmonella.*

Effect of TCNE Wash Treatments on Color of Shelled Eggs

Un-inoculated shelled eggs were washed using the highest concentration (0.48%) and longest duration (5 min) of TCNE treatments. Egg shell color was measured using a chroma meter (MiniScan XE Plus, Hunter Associates Laboratory, Inc., Reston, VA) during 2 wk of refrigerated storage following the guidelines from International Commission on Illumination (**CIE**) for L*, a*, b* values. L* measures lightness, a* measures green/red and b* measures blue/yellow of samples. The chroma meter was calibrated against a white ceramic surface before measurements were recorded on d 0, 1, 7, and 14. Egg samples were stored for 2 wk at 4°C. Three readings were taken on the lateral side of each sample for each timepoint.

Statistical Analysis

The experiment was conducted using a completely randomized design with 3 eggs per treatment at every time point and replicated at least 3 times. Collective egg wash experiments were a completely randomized design, where the receptable for egg washing was considered the experimental unit (n = 1) containing 10 eggs/treatment and replicated at least 3 times. Trans-shell inactivation experiments involved 5 eggs per treatment at every time point, with a trial as the experimental unit. The experiment was a completely randomized design and replicated 6 times, with results expressed in percent positivity (%) of *S.* Enteritidis. Color analysis experiments were a completely randomized design with repeated measures. Two trials were conducted with 3 replicates per treatment.

For all experiments, data were analyzed using one-way analysis of variance (**ANOVA**) of R software (version 4.0.2). Differences among means were detected at $P < 0.05$ using the Tukey's test with appropriate corrections for multiple comparisons.

RESULTS

Mean Particle Size, PDI, Zeta Potential, and MBC Values of Nanoemulsion

The particle size, PDI and zeta potential of freshly prepared TCNE-Tw.80 nanoemulsion were ~135 ± 1.46 nm, 0.131 ± 0.02, -9.35 ± 1.62 mV whereas the particle size, PDI, and zeta potential of TCNE-GAL nanoemulsion were 80 ± 0.57 nm, 0.249 ± 0.01, -29.3

Table 1. The nanoemulsions were stored at 23 or 4°C for 8 wk. Dynamic light scattering (DLS) was used to measure mean particle size, PDI (polydispersity index), and zeta potential (ZP) values at wk 0, 2, 4, and 8.[1]

Temperature (°C)	Storage time (weeks)	Nanoemulsion type	Size (nm)	PDI	ZP (mV)
23	0	Tween 80	135 ± 1.46^{a}	0.131 ± 0.02^{b}	−9.35 ± 1.62^{a}
	2		129 ± 0.55^{b}	0.239 ± 0.001^{a}	−5.36 ± 0.32^{ab}
	4		136 ± 0.95^{a}	0.274 ± 0.003^{a}	−1.45 ± 0.22^{b}
	8		127 ± 0.99^{b}	0.238 ± 0.006^{a}	−5.36 ± 0.86^{ab}
	0	Gum Arabic Lecithin	80 ± 0.57^{c}	0.249 ± 0.01^{a}	−29.3 ± 1.98^{a}
	2		91 ± 1.33^{b}	0.227 ± 0.01^{a}	−25.8 ± 0.32^{a}
	4		89 ± 4.08^{bc}	0.156 ± 0.09^{a}	−28.2 ± 1.99^{a}
	8		155 ± 0.95^{a}	0.162 ± 0.005^{a}	−32.1 ± 1.20^{a}
4	0	Tween 80	135 ± 1.46^{a}	0.131 ± 0.02^{d}	−9.35 ± 1.62^{a}
	2		106 ± 0.34^{c}	0.352 ± 0.003^{a}	−0.69 ± 0.08^{c}
	4		113 ± 0.47^{b}	0.302 ± 0.0006^{b}	−3.11 ± 0.57^{bc}
	8		117 ± 0.83^{b}	0.225 ± 0.006^{c}	−6.24 ± 0.96^{ab}
	0	Gum Arabic Lecithin	80 ± 0.57^{b}	0.249 ± 0.01^{a}	−29.3 ± 1.98^{a}
	2		93 ± 1.36^{a}	0.258 ± 0.01^{a}	−25.3 ± 0.26^{ab}
	4		84 ± 0.77^{b}	0.204 ± 0.004^{a}	−22.1 ± 0.43^{ab}
	8		96 ± 1.61^{a}	0.219 ± 0.02^{a}	−26.9 ± 0.64^{b}

[1]Values presented as mean ± standard error of mean.
[a,b,c,d]Means with different superscripts within a column, within a temperature and nanoemulsion type treatment differ significantly ($P < 0.05$).

± 1.98 mV, respectively. The distribution of particle size was unimodal, and solutions were homogenous. The stability of the nanoemulsion was determined after storage at 23 and 4°C for 8 wk (Table 1). In accordance with physicochemical parameters, TCNE-GAL stored at 4°C maintained its particle size (∼80−90 nm), and PDI (∼0.219 ± 0.02) and zeta potential (−26.9 ± 0.64 mV) after 8 wk, respectively ($P > 0.05$). However, a decrease in zeta potential was observed after 8 wk of storage when TCNE-GAL was stored at 4°C as compared to wk 0 ($P < 0.05$). When stored at 23°C, an increase in particle size was observed at 8 wk as compared to wk 0 ($P < 0.05$). No significant change in the PDI or zeta potential was observed after 8 wk of storage at 23°C ($P > 0.05$). For TCNE-Tw.80, slight variation in the particle size, PDI, and zeta potential were observed at both 23 and 4°C storage ($P < 0.05$). However, all values were within the acceptable range of good quality nanoemulsions. The MBC of TCNE or TC oil was 0.06% for all 4 individual strains of *S.* Enteritidis (data not shown). There were no shifts in MBC observed throughout storage at 23 and 4°C for 8 wk ($P > 0.05$).

TEM Imaging of Nanoemulsion

TEM analysis was conducted to confirm the particle shape and size of 2 kinds of TCNE as indicated in Figures 1A and 1B. Results revealed that nanoemulsions were spherical in shape with a size of ∼80 to 100 nm, which were consistent with the results of DLS.

Efficacy of TCNE Wash Treatment in Inactivating S. Enteritidis on Shelled Eggs in Presence or Absence of 5% Organic Matter

The effect of TCNE wash treatments at 34°C on survival of *S.* Enteritidis (**SE**) is depicted in Table 2. For baseline (SE inoculated eggs, not washed), ∼6.28 log cfu SE/egg were recovered (data not shown). After washing eggs with water for 5 min (control) ∼4.44 log cfu SE/egg were recovered. The pathogen was also recovered from wash water at ∼3.95 log cfu SE/mL indicating that washing with water reduces pathogen load by ∼1.8 log cfu/mL as compared to baseline ($P < 0.05$) but is not sufficient to completely eliminate SE on eggs. Moreover, the pathogen survives in treatment solution. Washing eggs with water containing 200 ppm chlorine reduced SE counts by ∼2.41 log cfu/egg as early as 1 min, as compared to control ($P < 0.05$). Increasing the treatment time from 1 min to 3 or 5, did not increase the antimicrobial efficacy of chlorine ($P > 0.05$).

When washed with TC, only highest concentrations (0.12, 0.24, and 0.48%) were effective in reducing SE by ∼1.77, 2.84, 2.53 log cfu/egg, respectively, as compared to control by 1 min ($P < 0.05$). In contrast, 4 TCNE-Tw.80 treatments (0.06, 0.12, 0.24, 0.48%) were effective in killing SE by ∼2 to 2.5 log cfu/egg as early as 1 min ($P < 0.05$). At 0.03% concentration and 5 min treatment time, TCNE-Tw.80 was more effective in reducing SE counts on eggs as compared to TC ($P < 0.05$). At 0.06% concentration (wash time 1 or 3 min), both TCNE-Tw.80 and TCNE-GAL were more effective in reducing SE counts on eggs as compared to TC ($P < 0.05$). At 0.48% concentration (wash time 5 min), both TCNE-Tw.80 and TCNE-GAL reduced SE counts on eggs to below detection limit ($P < 0.05$). There were no significant differences between nanoemulsions, except that at 0.03%-5 min treatment, TCNE-Tw.80 was more effective in reducing SE counts on eggs as compared to TCNE-GAL ($P < 0.05$).

S. Enteritidis was detected up to 4 log cfu/mL in wash water of control groups. Highest concentrations of TCNE-Tw.80, TCNE-GAL, TC treatments (0.24, 0.48%) and chlorine 200 ppm effectively reduced SE population in wash water to undetectable levels at all treatment times ($P < 0.05$), while 0.06, 0.12% TC treatments had *Salmonella* present in wash water at

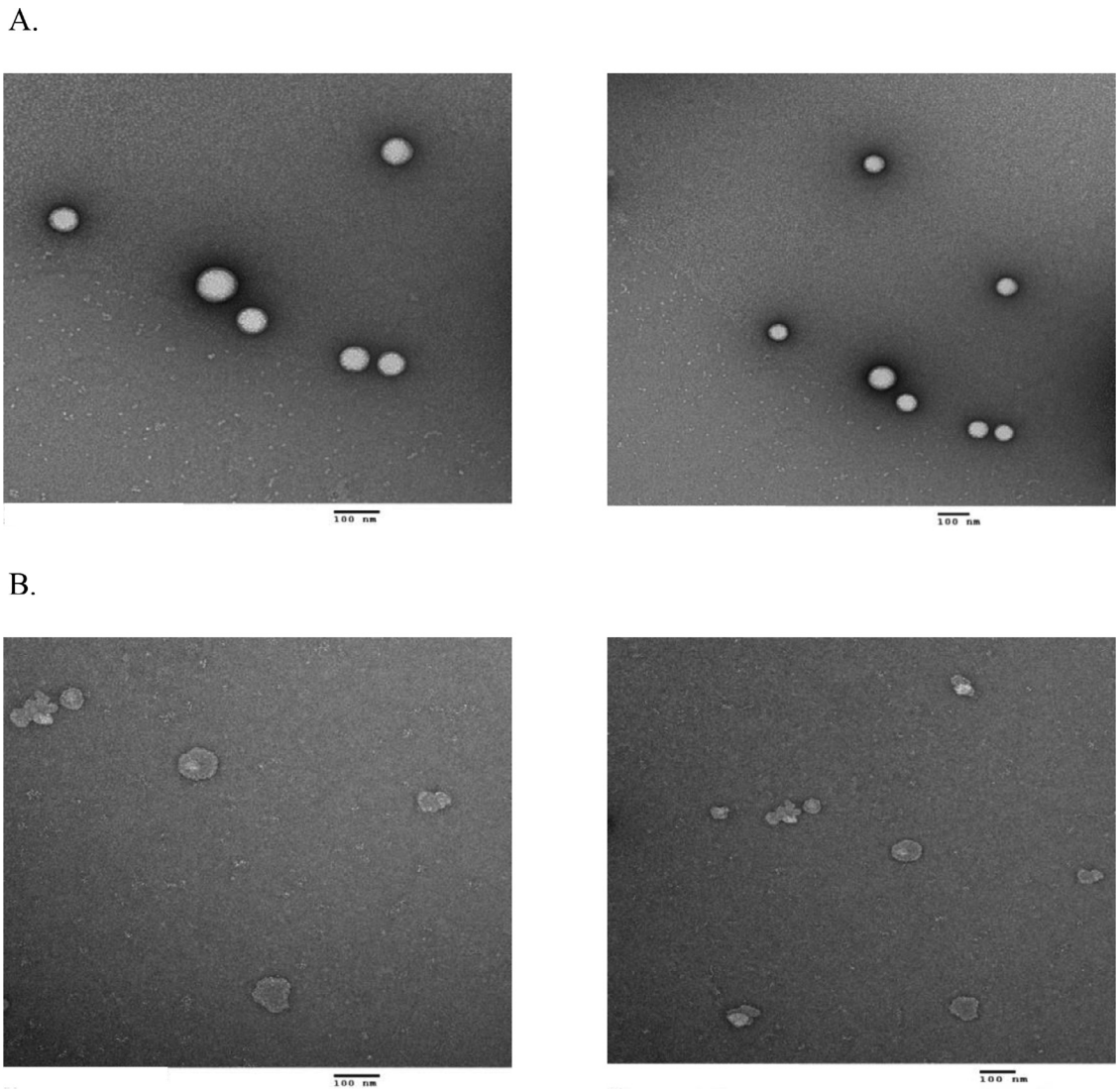

Figure 1. Transmission electron microscopy (TEM) images depicting morphology of nanoparticles of Tween 80 prepared *Trans*-cinnamaldehyde nanoemulsion (A) or Gum Arabic and lecithin *Trans*-cinnamaldehyde nanoemulsion (B).

~0.25 to 3.52 log cfu/mL (data not shown). Enrichment results showed that water containing chlorine 200 ppm, TCNE-Tw.80, TCNE-GAL, or TC 0.06, 0.12, 0.24, 0.48% tested positive for SE after 5 min treatment (data not shown).

The presence of organic matter could potentially reduce the effectiveness of antimicrobials used in the wash water. Therefore, we investigated the anti-*Salmonella* efficacy of higher dose of TCNE (0.24, 0.48%) in the presence of 5% chicken litter at 34°C. The results are presented in Table 3. In the absence of organic matter, after 5 min of wash time, chlorine 200 ppm was effective in reducing SE counts by ~2 log cfu/egg ($P < 0.05$). However, in the presence of organic matter, chlorine 200 ppm was not effective in reducing SE counts on eggs even after 5 min of treatment as compared to control and a pathogen load of ~4.86 log cfu/egg was observed on the eggs ($P > 0.05$). We observed similar results for TC 0.24 and 0.48%, where the phytochemical treatments were not effective in reducing SE load on eggs in the presence of organic matter, as compared to control ($P > 0.05$). However, TCNE-Tw.80 and TCNE-GAL treatments, at 0.48% concentration, reduced SE counts by ~2 to 2.5 log cfu/egg, as early as 1 min, in the presence of organic matter ($P < 0.05$). By 5 min of treatment time, both 0.24 and 0.48% dose of TCNE treatments were effective in reducing pathogen load by at least 2 log cfu/egg, both in the presence or absence of organic matter ($P < 0.05$).

The effect of TCNE in inactivating *S.* Enteritidis on shelled eggs, washed in batches, in presence or absence of 5% organic matter at 34°C is presented in Table 4. The highest concentration (0.48%) and longest duration (5 min) of treatment was selected from the individual egg wash experiment. For baseline ~6.58 log cfu SE/egg were recovered. In the absence of organic matter, ~4.67 log cfu SE/egg were recovered after washing with DI water for 5 min (control). Washing eggs with water containing TCNE-Tw.80, TCNE-GAL, or TC 0.48%, reduced SE counts below detection limit ($P < 0.05$). Chlorine was also effective in reducing SE population after 5 min of washing and a reduction of ~2 log cfu/egg was observed ($P < 0.05$). However, in presence of 5% chicken litter, the

Table 2. Individual egg wash treatments included Control, Chlorine (0.02%), TCNE-Tw.80, GAL, or TC (0.01, 0.02, 0.03, 0.06, 0.12, 0.24, 0.48%) at time points 1, 3, or 5 min. Each experiment consisted of 3 replicates per treatment per time point per experiment and each experiment was repeated at least three times (n = 9). Pathogen detection limit by plating was 3 log cfu/egg whereas detection limit by enrichment was 2 log cfu/egg.[1]

Treatments		1 min	3 min	5 min
Controls	DI water	4.96 ± 0.09^{a}	4.69 ± 0.12^{a}	4.44 ± 0.15^{a}
	0.02% chlorine	2.55 ± 0.30^{bc}	2.48 ± 0.28^{cd}	2.20 ± 0.20^{bc}
TCNE-Tw.80	0.01%	5.52 ± 0.10^{a}	4.86 ± 0.23^{a}	5.07 ± 0.21^{a}
	0.02%	4.99 ± 0.22^{a}	5.14 ± 0.10^{a}	4.95 ± 0.21^{a}
	0.03%	4.94 ± 0.12^{a}	4.32 ± 0.09^{a}	3.07 ± 0.28^{b}
	0.06%	2.33 ± 0.33^{bc}	2.51 ± 0.34^{cd}	2.91 ± 0.32^{bc}
	0.12%	3.02 ± 0.37^{bc}	2.36 ± 0.24^{cd}	2.11 ± 0.11^{bc}
	0.24%	2.54 ± 0.37^{bc}	2.13 ± 0.13^{d}	2.31 ± 0.23^{bc}
	0.48%	2.55 ± 0.24^{bc}	2.37 ± 0.19^{cd}	2.00 ± 0.00^{c}
TCNE-GAL	0.01%	5.32 ± 0.12^{a}	5.09 ± 0.11^{a}	4.99 ± 0.17^{a}
	0.02%	5.32 ± 0.10^{a}	5.25 ± 0.11^{a}	5.10 ± 0.10^{a}
	0.03%	4.91 ± 0.06^{a}	4.85 ± 0.11^{a}	4.51 ± 0.10^{a}
	0.06%	3.35 ± 0.45^{b}	2.40 ± 0.27^{cd}	2.78 ± 0.41^{bc}
	0.12%	2.85 ± 0.39^{bc}	2.32 ± 0.21^{cd}	2.14 ± 0.14^{bc}
	0.24%	2.66 ± 0.34^{bc}	2.00 ± 0.00^{d}	2.28 ± 0.15^{bc}
	0.48%	2.00 ± 0.00^{c}	2.00 ± 0.00^{d}	2.00 ± 0.00^{c}
TC oil	0.01%	5.21 ± 0.20^{a}	4.66 ± 0.09^{a}	4.89 ± 0.15^{a}
	0.02%	5.17 ± 0.26^{a}	5.11 ± 0.20^{a}	5.05 ± 0.23^{a}
	0.03%	4.88 ± 0.11^{a}	4.70 ± 0.13^{a}	4.55 ± 0.23^{a}
	0.06%	5.11 ± 0.05^{a}	4.09 ± 0.09^{ab}	2.22 ± 0.15^{bc}
	0.12%	3.19 ± 0.44^{bc}	3.31 ± 0.41^{bc}	2.67 ± 0.34^{bc}
	0.24%	2.12 ± 0.12^{bc}	2.00 ± 0.00^{d}	2.00 ± 0.00^{c}
	0.48%	2.43 ± 0.29^{bc}	2.25 ± 0.25^{d}	2.51 ± 0.30^{bc}

[1]Values ($\log_{10}$ cfu/egg) presented as mean ± standard error of mean.
[a,b,c,d]*S.* Enteritidis counts within column with different superscript differ significantly ($P < 0.05$).

efficacy of chlorine and TC oil was reduced and SE counts on the eggs were similar to control ($P > 0.05$). In contrast, TCNE-Tw.80 or TCNE-GAL 0.48% treatments were effective in reducing SE population by at least 2.56 log cfu/egg.

Efficacy of TCNE Wash Treatment in Reducing Trans-shell Migration of S. *Enteritidis at 34°C*

On-shell or trans-shell contamination is a major source of egg contamination as *Salmonella* penetrates the eggshell and contaminates the egg internal contents such as yolk or albumen (De Reu et al., 2006). Therefore, we investigated the efficacy of TCNE dip treatments in reducing trans-shell migration of *S.* Enteritidis from surface to interior of eggs using selected treatments from the egg wash experiment (*Efficacy of TCNE wash treatments in inactivating S. Enteritidis on eggs)* described above. Results are presented in Figure 2. In the case of baseline (inoculated but unwashed eggs), ~51.11% egg contents were positive for SE. When SE inoculated eggs were washed with water (control) for 1 or 3 min, ~32.5, and 17.5% egg contents were found to be SE positive, respectively. We observed no SE positives in eggs

Table 3. Individual egg wash treatments included Control, Chlorine (0.02%), TCNE-Tw.80, GAL ,or TC (0.24, 0.48%) at time points 1, 3, or 5 min. Each experiment consisted of 3 replicates per treatment per time point per experiment and each experiment was repeated at least three times (n = 9). Pathogen detection limit by plating was 3 log cfu/egg whereas detection limit by enrichment was 2 log cfu/egg.[1]

		Absence of organic matter		
Treatments		1 min	3 min	5 min
Controls	DI water	4.93 ± 0.30^{ab}	4.72 ± 0.32^{a}	4.89 ± 0.13^{a}
	0.02% chlorine	3.00 ± 0.37^{cde}	2.83 ± 0.39^{cde}	2.78 ± 0.33^{bc}
TCNE-Tw.80	0.24%	3.05 ± 0.37^{cde}	2.59 ± 0.30^{de}	2.16 ± 0.16^{c}
	0.48%	2.11 ± 0.11^{e}	2.16 ± 0.16^{e}	2.42 ± 0.28^{c}
TCNE-GAL	0.24%	3.05 ± 0.38^{cde}	2.63 ± 0.28^{de}	2.42 ± 0.28^{c}
	0.48%	2.11 ± 0.11^{e}	2.11 ± 0.11^{e}	2.14 ± 0.14^{c}
TC oil	0.24%	2.22 ± 0.15^{e}	2.20 ± 0.20^{e}	2.00 ± 0.00^{c}
	0.48%	2.33 ± 0.23^{e}	2.00 ± 0.00^{e}	2.30 ± 0.30^{c}
Presence of organic matter				
Controls	DI water	5.31 ± 0.08^{a}	5.28 ± 0.08^{a}	5.20 ± 0.13^{a}
	0.02% chlorine	5.24 ± 0.09^{a}	4.86 ± 0.10^{a}	4.86 ± 0.20^{a}
TCNE-Tw.80	0.24%	4.33 ± 0.51^{abcd}	4.15 ± 0.43^{abc}	3.28 ± 0.32^{bc}
	0.48%	3.36 ± 0.41^{bcde}	3.21 ± 0.41^{bcde}	2.63 ± 0.32^{c}
TCNE-GAL	0.24%	4.94 ± 0.33^{ab}	4.13 ± 0.42^{abc}	3.39 ± 0.41^{bc}
	0.48%	2.83 ± 0.43^{de}	3.88 ± 0.31^{abcd}	2.44 ± 0.33^{c}
TC oil	0.24%	4.24 ± 0.48^{abcd}	4.54 ± 0.43^{ab}	4.09 ± 0.41^{ab}
	0.48%	4.44 ± 0.23^{abc}	4.35 ± 0.26^{ab}	4.07 ± 0.44^{ab}

[1]Values ($\log_{10}$ cfu/egg) presented as mean ± standard error of mean.
[a,b,c,d,e]*S.* Enteritidis counts within column with different superscript differ significantly ($P < 0.05$).

Table 4. Collective/batch egg wash treatments included Control, Chlorine (0.02%), TCNE-Tw.80, GAL, or TC (0.48%) at 5 min time point. Each experiment consisted of 10 shelled eggs per treatment, where the wash receptable was considered the experimental unit (n = 1). Each experiment was replicated at least three times. Pathogen detection limit by plating was 3 log cfu/egg whereas detection limit by enrichment was 2 log cfu/egg.[1]

Treatments	Absence of organic matter		Presence of organic matter
Controls	DI water	4.67 ± 0.24[ab]	4.56 ± 0.26[b]
	0.02% chlorine	2.03 ± 0.11[c]	4.51 ± 0.23[b]
TCNE-Tw.80	0.48%	2.00 ± 0.00[c]	2.00 ± 0.00[c]
TCNE-GAL	0.48%	2.00 ± 0.00[c]	2.04 ± 0.14[c]
TC oil	0.48%	2.00 ± 0.00[c]	4.90 ± 0.28[a]

[1]Values ($\log_{10}$ cfu/egg) presented as mean ± standard error of mean.
[a,b,c]*S.* Enteritidis counts within column with different superscript differ significantly ($P < 0.05$).

washed with water for 5 min ($P < 0.05$). Eggs washed in treatments including chlorine 200 ppm, TCNE-Tw.80, GAL or TC 0.24, 0.48%, for 1, 3, or 5 min, resulted in no SE positive egg contents as compared to baseline ($P < 0.05$).

Effect of TCNE Wash Treatment on Eggshell Color

The effect of TCNE wash (34°C for 5 min) on eggshell color during refrigerated storage for 2 wk is presented in Table 5. The lightness value L* represents the darkest black L* = 0, and the brightest white at L* = 100. The −a∗ to +a∗ and −b∗ to +b∗ represent green-red and blue-yellow, respectively (Pathare et al., 2013; Faitarone et al., 2016). Results revealed that washing with TCNE, chlorine or TC treatments did not significantly change the L* value of eggshell as compared to unwashed eggs and eggs washed with water, during the storage period of 14 d ($P > 0.05$). However, changes were observed in the a* and b* values.

DISCUSSION

In the current study, we investigated the efficacy of *Trans*-cinnamaldehyde nanoemulsion (**TCNE**) prepared with 2 types of emulsifiers, Tween 80 (Tw.80) or gum Arabic and lecithin combination (GAL) as dip treatments in reducing *S.* Enteritidis on shelled eggs, in the presence or absence of organic matter. The rationale for selecting Tween 80 as an emulsifier was for its prominent use in cosmetics, pharmaceuticals, and food products, as well as approval from FDA for use (up to 1% concentration) in foods (Chassaing et al., 2015; Mehmood and Ahmed, 2020). Nanoemulsions incorporating Tween 80 are economically feasible containing a single, hydrophilic, nonionic surfactant (Chassaing et al., 2015; Mehmood and Ahmed, 2020). Gum Arabic and lecithin were selected as natural, food grade emulsifiers, and their combination provides good emulsification properties and increases the stability of nanoemulsion

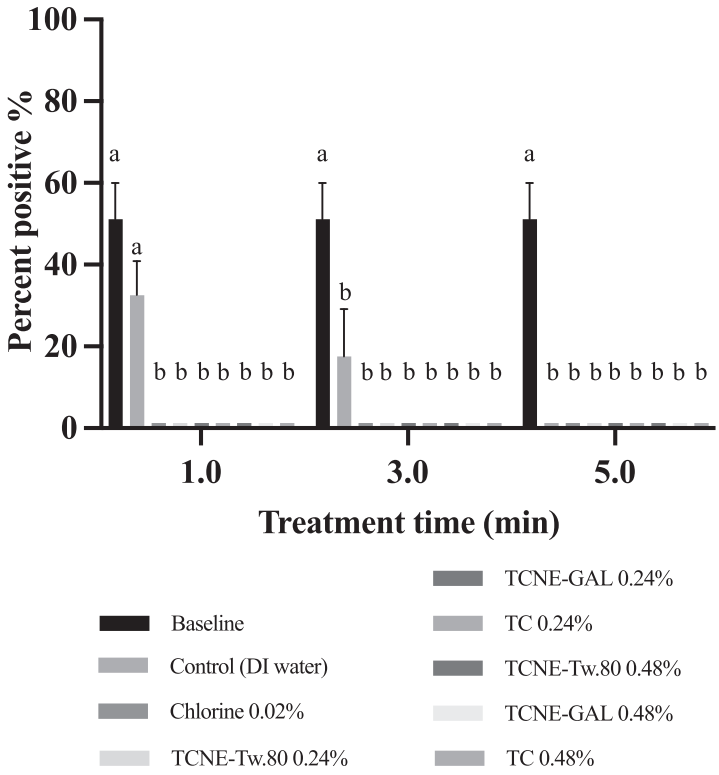

Figure 2. Effect of *Trans*-cinnamaldehyde nanoemulsion (TCNE) wash on trans-shell migration of *S.* Enteritidis. The treatments included Baseline (unwashed egg), Control (DI water), Chlorine (0.02% or 200 ppm), TCNE-Tw.80, GAL, or TC (0.24, 0.48%) at 34°C for 1, 3, or 5 min time point. Each experiment consisted of 5 sampling units (eggs) per treatment and was replicated 6 times (n = 6). Values expressed in percent positivity (%) of *S.* Enteritidis. Error bars indicate standard error of mean. Means with different superscript differ significantly ($P < 0.05$).

Table 5. The treatments included Unwashed, Chlorine (0.02%), TCNE-Tw.80, GAL or TC (0.48%) at 5 min time point. Each experiment consisted of 3 replicates per treatment repeatedly measured on d 0, 1, 7, and 14 of refrigerated storage. L* measures lightness, a* measures green/red and b* measures blue/yellow of samples.[1]

Storage time (days)	Treatments		L	a	b
	Unwashed		74.56 ± 1.40[a]	0.91 ± 0.13[a]	3.45 ± 0.36[ab]
	Controls	DI water	75.24 ± 2.72[a]	1.03 ± 0.17[a]	3.92 ± 0.33[ab]
0		0.02% chlorine	79.02 ± 2.02[a]	1.16 ± 0.16[a]	4.79 ± 0.36[ab]
	TCNE-Tw.80	0.48%	77.32 ± 1.89[a]	1.01 ± 0.21[a]	3.90 ± 0.52[ab]
	TCNE-GAL	0.48%	78.50 ± 1.56[a]	1.18 ± 0.07[a]	5.11 ± 0.33[a]
	TC oil	0.48%	80.13 ± 1.37[a]	0.80 ± 0.05[a]	3.17 ± 0.12[b]
	Unwashed		73.73 ± 2.53[a]	0.87 ± 0.13[a]	3.49 ± 0.43[ab]
	Controls	DI water	75.74 ± 1.67[a]	1.13 ± 0.18[a]	4.32 ± 0.43[ab]
1		0.02% chlorine	77.12 ± 1.77[a]	1.29 ± 0.13[a]	5.09 ± 0.36[ab]
	TCNE-Tw.80	0.48%	78.84 ± 1.80[a]	1.03 ± 0.21[a]	4.02 ± 0.53[ab]
	TCNE-GAL	0.48%	75.74 ± 0.68[a]	1.25 ± 0.05[a]	5.32 ± 0.28[a]
	TC oil	0.48%	76.52 ± 1.64[a]	0.80 ± 0.08[a]	3.26 ± 0.21[b]
	Unwashed		76.05 ± 1.15[a]	0.71 ± 0.04[b]	3.36 ± 0.11[b]
	Controls	DI water	77.32 ± 2.41[a]	1.00 ± 0.21[ab]	4.25 ± 0.48[ab]
7		0.02% chlorine	78.48 ± 1.51[a]	1.54 ± 0.18[a]	6.09 ± 0.63[a]
	TCNE-Tw.80	0.48%	78.71 ± 0.72[a]	1.11 ± 0.21[ab]	4.63 ± 0.56[ab]
	TCNE-GAL	0.48%	79.00 ± 2.58[a]	1.30 ± 0.12[ab]	6.16 ± 0.37[a]
	TC oil	0.48%	80.59 ± 2.01[a]	0.78 ± 0.04[ab]	3.64 ± 0.13[b]
	Unwashed		76.89 ± 1.87[a]	0.88 ± 0.04[a]	3.71 ± 0.39[a]
	Controls	DI water	79.97 ± 0.98[a]	1.13 ± 0.20[a]	4.85 ± 0.48[a]
14		0.02% chlorine	79.34 ± 1.07[a]	1.49 ± 0.20[a]	5.76 ± 0.67[a]
	TCNE-Tw.80	0.48%	80.24 ± 1.30[a]	1.12 ± 0.26[a]	4.56 ± 0.80[a]
	TCNE-GAL	0.48%	78.34 ± 1.63[a]	1.22 ± 0.07[a]	5.68 ± 0.47[a]
	TC oil	0.48%	78.85 ± 1.43[a]	0.91 ± 0.12[a]	4.02 ± 0.37[a]

[1]Values presented as mean ± standard error of mean.
[a,b]L*, a*, b* values within column with different superscript differ significantly ($P < 0.05$).

(Hu et al., 2016; Dammak et al., 2020). The as-prepared TCNE-Tw.80 and TCNE-GAL values for particle size, PDI, and zeta potential were within acceptable parameters of being classified a nanoemulsion of high quality (Table 1). TEM imaging (Figures 1A and 1B) also confirmed spherical shaped nanoparticles within nanometric range. The stability of nanoemulsion was determined after storage at 23 and 4°C for 8 wk. TCNE prepared with GAL was considered stable at both temperatures up to 8 wk as determined by physicochemical properties (Table 1). Hu et al. (2016) reported a similar finding where eugenol nanoemulsion prepared with food grade emulsifiers maintained its physicochemical properties up to 14 d under room and refrigeration temperatures. In contrast, TCNE prepared with Tw.80 decreased in zeta potential by the second to fourth week of storage (under room and refrigeration temperatures), suggesting as early as 2 wk of storage, the surface of charged particles in TCNE-Tw.80 had minimal repulsion (Table 1). The difference in stability between nanoemulsions could be attributed to the selection of emulsifier. Previously published work has shown that zeta potential was attributed to the surfactant lecithin with abundant phosphate groups that were negatively charged (Hu et al., 2016). Lecithin in comparison to gum Arabic has a higher hydrophobic characteristic, which allows the emulsifier to stay closely at the oil/water interface, surrounded by hydrophilic gum Arabic (Hu et al., 2016). Tween 80, however, is a nonionic surfactant, carrying no charge, and therefore this might justify the inability for TCNE-Tw.80 to achieve a zeta potential of ± 30 mV. An increase in negative charge would aid in maintaining its stability throughout storage. Moreover, storage of nanoemulsions at 23 and 4°C for 8 wk did not result in MBC fluctuation; therefore, the antimicrobial activity of TCNE was sustained during storage.

As per experimentation, nanoemulsions were supplemented in wash water as a treatment to imitate the egg washing step in a shell egg cleaning operation. The USDA (2020) recommends wash water temperature of eggs should be at least 90°F (~32.2°C) and should be at least 20°F warmer than the eggs. Considering the above parameters, and the temperature of the laboratory environment, we selected to wash the eggs at 34°C in our experiments. This temperature met the USDA recommendations for egg washing.

The results of this study revealed that both types of TC nanoemulsions (0.06−0.48%) were effective in reducing *S.* Enteritidis on eggshell as early as 1 min (Table 2). In addition, the antimicrobial efficacy of select TCNE treatments improved when treatment time was increased to 5 min. Similar findings were reported by Upadhyaya et al. (2013) who determined washing white shelled eggs with TC oil in the presence and absence of organic matter was effective in inactivating *S.* Enteritidis. However, the concentration of TC used in the wash treatments were higher (0.25, 0.5, 0.75%) as compared to our treatments. Thus, our findings indicate that, when used in nanoemulsion form, a lower concentration of TC could achieve similar pathogen reduction on eggshell as observed by Upadhyaya et al. (2013).

Comparable to TC oil's efficacy, chlorine was equally effective in reducing *Salmonella* on eggshells. However, in the presence of organic matter, chlorine, and phytochemical treatment was reduced in efficacy. Interestingly, TC nanoemulsions were effective on shelled eggs

at higher concentrations when organic matter was present (Table 3). In addition, the TC nanoemulsion treatments were effective when applied to batches of eggs as well (Table 4). This could be due to uniform distribution of TCNE droplets in the solution that facilitated sustained antimicrobial efficacy in the presence of organic load.

On-shell or trans-shell contamination is a major source of egg contamination as *S.* Enteritidis penetrates the eggshell and contaminates the egg internal contents such as yolk or albumen (De Reu et al., 2006; Messens et al., 2006, 2007; Gantois et al., 2009). Moreover, eggshell characteristics such as thickness, pore area, and number do not significantly influence *S.* Enteritidis capacity of trans-shell contamination (De Reu et al., 2006). Our result indicated that *S.* Enteritidis inoculated unwashed eggs had the most egg contents positive for the pathogen indicating penetration of *Salmonella* through the eggshell, meaning the consumer is more at risk for salmonellosis when eggs are unwashed. Although egg washing practices are implemented, as per the result, eggs washed with water for less than 5 min contained *S.* Enteritidis positive egg contents. In this study, eggs washed with TCNE-Tw.80 or TCNE-GAL resulted in no *S.* Enteritidis positives in egg contents at 1, 3, or 5 min of washing, suggesting TCNE has the ability to inhibit trans-shell migration. Similarly, eggs washed with water at 3 and 5 min were effective in inhibiting trans-shell migration, as well as chlorine and TC at 1, 3 or 5 min of washing. To our knowledge, this is the first study reporting the effect of washing of eggs with phytochemical nanoemulsions on the trans-shell contamination capability of *S.* Enteritidis.

The color of eggshell is important in determining consumer decision to purchase the product. The lightness value L* represents the darkest black L* = 0, and the brightest white at L* = 100. The −a∗ to +a∗ and −b∗ to +b∗ represent green-red and blue-yellow, respectively (Pathare et al., 2013; Faitarone et al., 2016). Color analysis results revealed both kinds of nanoemulsion did not alter shell color when stored for 14 d suggesting that the TCNE treatments will not potentially affect color-based consumer perception of the eggs. However, changes were observed in the a* and b* values.

In conclusion, TCNE prepared with Tw.80 or GAL as an antimicrobial wash treatment resulted in significant reductions of *S.* Enteritidis population on shelled eggs. In several treatments, TCNE was more effective than TC oil resulting in ∼2 to 2.5 log reduction of *Salmonella* on eggs. TC significantly reduced in efficacy in the presence of organic matter, however, nanoemulsion maintained its efficacy. In addition, TCNE did not alter eggshell color. Thus, TCNE could potentially be used as a novel alternative to conventional chemicals to reduce *S.* Enteritidis on shelled eggs. However, future investigations testing the effects of TCNE in an industrial setting and effects on organoleptic properties of eggs are warranted prior to implementing treatments in the poultry industry.

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DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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