



Applications of Edible Coatings Formulated with Antimicrobials Inhibit *Listeria monocytogenes* Growth on Queso Fresco

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Despite efforts to control *Listeria monocytogenes* in dairy processing environments, contamination and subsequent outbreaks of listeriosis continue to occur. The ability of *L. monocytogenes* to grow during refrigerated storage necessitates strategies to prevent contamination, reduce pathogen numbers, and limit growth during storage. The objective of this study was to determine the efficacy of edible antimicrobial coatings to control *L. monocytogenes* on Queso Fresco (QF) when applied before [precoated (PC)] or after [preinoculated (PI)] surface contamination. Coating solutions were formulated to contain 2% chitosan and either 5% hydrogen peroxide (HP), 5% lauric arginate (LAE), 25% acidified calcium sulfate with lactic acid (ACSL), or combinations of 10% sodium caprylate (SC) with either LAE or ACSL. Fresh QF samples (25 g) were inoculated with *L. monocytogenes* at ~4 log CFU/g prior to, or following, antimicrobial coating application. Cheeses were then vacuum packaged and stored at 7°C for 35 days with weekly enumeration of *L. monocytogenes*. Aside from ACSL and LAE + SC, there was no effect of coating application timing (PC vs. PI) on the change in *L. monocytogenes* counts over time. Chitosan coatings without additional antimicrobials were more effective than controls but did not inhibit *L. monocytogenes* growth beyond 7 days. Coatings containing HP at 5% were equally effective when applied before or after *L. monocytogenes* inoculation, significantly reducing *L. monocytogenes* counts by more than 3 log CFU/g and inhibiting growth through 35 days of storage. Coatings formulated with ACSL at 25% were more effective when applied to PI cheeses but neither application produced significant reductions in *L. monocytogenes* counts or inhibited growth. Although LAE coatings were more effective than ACSL, neither were more effective than chitosan coatings alone. The addition of SC to ACSL and LAE coatings enhanced their antimicrobial activity as ACSL + SC and LAE + SC coatings reduced *L. monocytogenes* counts by >1 log CFU/g after 24 h and were listericidal through 28 and 35 days, respectively. The identification of listericidal and listeristatic edible antimicrobial coating applications that are effective when applied before or after contamination events identifies a new approach for the control of *L. monocytogenes* on fresh cheese.

Keywords: *Listeria monocytogenes*, chitosan, cheese, antimicrobials, queso fresco, coating

INTRODUCTION

According to the Centers for Disease Control and Prevention's Foodborne Outbreak Online Database, there were 19 reported outbreaks of listeriosis linked to dairy products between 2003 and 2016 resulting in 144 illnesses, 108 hospitalizations, and 21 deaths. Eleven of these outbreaks were linked to cheese known to be produced from pasteurized milk, six of which were Mexican or Hispanic-style soft cheeses including Queso Fresco (QF) (Centers for Disease Control and Prevention, 2017). Microbial risk assessments of *Listeria monocytogenes* in ready-to-eat foods suggest that preventing contamination altogether, followed by preventing the occurrence of high levels of contamination at consumption, would have the greatest impact on reducing illness rates [Chen et al., 2003; Food and Agriculture Organization and World Health Organization (FAO/WHO, 2004); U.S. Food and Drug Administration et al., 2003]. The application of antimicrobial coatings can inactivate, extend the lag-phase, and reduce the growth rate and maximum populations of microorganisms (Quintavalla and Vicini, 2002; Coma et al., 2003). Coatings can also provide an extra physical barrier to potentially limit initial contamination (Duan et al., 2007; Chen et al., 2012).

Chitosan is a natural, nontoxic, biodegradable, cationic polysaccharide obtained by alkaline deacetylation of the nitrogenous polysaccharide, chitin. Chitosan films and coatings have demonstrated antimicrobial activity against both bacteria and fungi both intrinsically and as a carrier of various antimicrobials (Kong et al., 2010). Chitosan coatings and films have been used to inhibit the growth of *L. monocytogenes* in a variety of foods and the incorporation of antimicrobials (e.g., essential oils, enzymes, bioactive compounds, etc.), can further enhance their efficacy (Pranoto et al., 2005; Zivanovic et al., 2005; Duan et al., 2007; Beverly et al., 2008; Jiang et al., 2011; Petrou et al., 2012; Guo et al., 2014; Shekarforoush et al., 2015; Paparella et al., 2016). Previous research has identified effective antimicrobial treatments for the control of *L. monocytogenes* in broth and whole milk including acidified calcium sulfate with lactic acid (ACSL), hydrogen peroxide (HP), lauric arginate (LAE), and sodium caprylate (SC) (Kozak et al., 2017, 2018a). However, application of these antimicrobials to the surface of QF as aqueous dips were comparably less effective in inactivating *L. monocytogenes* and inhibiting growth during storage (Kozak et al., 2018b). Incorporation of these antimicrobials into edible chitosan films may enhance their efficacy, present a protective barrier, and allow for the progressive release of antimicrobials during storage. Maintaining high concentrations at the food surface may help extend the lag phase or inhibit growth following initial inactivation (Oussalah et al., 2004; Fajardo et al., 2010).

Although the use of chitosan coatings to extend shelf life has been tested in various cheeses (Coma et al., 2003; Altieri et al., 2005; Gammariello et al., 2008; Cerqueira et al., 2010), research on the use of chitosan coatings alone or formulated with antimicrobial compounds to control pathogens on cheese is limited. Generalizing from previous research is also difficult because the antimicrobial activity of chitosan and other antimicrobials is influenced by pH, bacterial strain, incubation temperature, and food matrix (Devlieghere et al., 2004; Fernandez-Saiz et al., 2010).

Therefore, the objective of this study was to determine the efficacy of antimicrobial chitosan coatings, applied before or after surface contamination, to control *L. monocytogenes* on QF during storage at 7°C.

MATERIALS AND METHODS

Bacterial Strains, Growth Conditions, and Inoculum Preparation

A cocktail was prepared as previously described (Kozak et al., 2017) using eight *L. monocytogenes* strains associated with outbreaks linked to soft cheeses or isolated from cheese processing environments (Table 1). The cocktail was serially diluted in Butterfield's phosphate buffer (BPB), pelleted through centrifugation (30 min, 4,000 g at 4°C) (Thermo Scientific Sorvall Legend X1R, ThermoFisher Scientific, Waltham, MA, USA), and resuspended in BPB to attain ~7 log CFU/mL.

Cheese Manufacture and Analysis

Queso Fresco was manufactured in the University of Connecticut Creamery according to a standard protocol as previously described (Kozak et al., 2018b). Compositional targets including ~52% moisture, ~22% fat, ~2.0% salt, ~67% moisture in the nonfat substance (MNFS), ~45% fat in dry matter (FDM), ~4% salt in moisture (SM), and pH of ~6.4, were based on previous work (Kozak et al., 2018b). Cheese blocks were vacuum packaged (Ultravac 250, UltraSource LLC, Kansas City, MO, USA) and stored at 4°C prior to cutting into experimental units.

Physicochemical analysis was conducted on each independent batch of cheese, in duplicate, at the time of cutting including: pH (Potentiometric Method) (Hooi et al., 2004) (Accumet AB150 with microtip electrode, Fisher Scientific International Inc., Hampton, NH, USA), dry matter (DM) (Forced Draft Oven Method) (Hooi et al., 2004), fat (Babcock Method) (Hooi et al., 2004), and salt (Quantab Chloride, Hach, Loveland, CO, USA). SM, MNFS, and FDM were determined using the following formulae:

$$\% \text{ SM} = (\% \text{ salt} / (100 - \% \text{ DM})) \times 100$$

$$\% \text{ MNFS} = (100 - \% \text{ DM} / (100 - \% \text{ fat})) \times 100$$

$$\% \text{ FDM} = (\% \text{ fat} / \% \text{ DM}) \times 100.$$

TABLE 1 | Name, source, ribotype, and serotype information for *Listeria monocytogenes* strains used in cocktail.

Strain name	Source information	Ribotype	Serotype
F5069/ATCC 51414	Milk-related outbreak	DUP-1044B	4b
CWD 675-3	Hispanic-style cheese-related outbreak	DUP-1053A	1a
CWD 1567	Hispanic-style cheese-related outbreak	DUP-1038B	4b
Scott A	Milk-related outbreak	DUP-1042B	4b
2012L-5323	Ricotta salata cheese-related outbreak	NA	1/2a
2014L-6025	Hispanic-style cheese-related outbreak	NA	1/2b
DJD 1	Washed-rind cheese-related outbreak	NA	NA
CWD 193-10 U5-2	Dairy plant food contact surface	DUP-1030B	NA

NA, not available.

Cheese Sample Preparation and Inoculation

For each trial, cheeses were cut into 25 ± 2 g samples using sterile knives within 48 h of manufacture and randomly assigned to one of two groups: preinoculated (PI) or precoated (PC). For inoculation of cheese in both groups, 100 μ L of the *L. monocytogenes* suspension in BPB was spread over a single surface of the cheese sample with a sterile spreader in order to attain a target contamination level of ~ 4 log CFU/g of cheese. Inoculated cheese samples with or without antimicrobial coating were allowed a 30-min drying period in a biosafety cabinet to enable bacterial attachment.

Coating Preparation and Application

Antimicrobial treatments for incorporation in chitosan coatings were selected based on previous work (Kozak et al., 2017, 2018a,b) and included: 25% ACSL, 5% HP, 5% LAE, 25% ACSL + 10% SC, and 5% LAE + 10% SC. Preparation and application of chitosan coating solutions were adapted from previously published methods (No et al., 2002; Chen et al., 2012). Briefly, low-molecular-weight (LMW) chitosan (50–190 kDa; Sigma Aldrich, St. Louis, MO, USA) was added at 2% wt/v to sterile deionized water (SDW) acidified with acetic acid (AA) ($\geq 99.5\%$, Sigma-Aldrich, St. Louis, MO, USA) at 1% (v/v). For coating solutions containing ACSL (Mionix Corporation, Scottsdale, AZ, USA), chitosan was added to 1% AA solution after the addition of ACSL (25% v/v). SC (10%, wt/v) (Sigma Aldrich) and chitosan were added to 1% AA solution at the same time for LAE + SC and prior to the addition of ACSL for ACSL + SC coatings. Antimicrobials in liquid form including HP (30%, Acros Organic, Pittsburgh, PA, USA), and LAE (Cytoguard LA2X, A + B Ingredients, Fairfield, NJ, USA) were added directly to mixed chitosan solutions on a v/v basis. Coating solutions were adjusted to pH 4.5 ± 0.05 with AA or 10 N NaOH and mixed overnight in an orbital shaker at room temperature before use.

Antimicrobial coatings were applied before or after inoculation with *L. monocytogenes* for PC and PI groups, respectively. Cheese samples (uninoculated for the PC group or inoculated for the PI group) were submerged in SDW (control), 1% AA solution (AA control), 2% chitosan coating solution (chitosan), or the respective antimicrobial coating solution for 1 min at room temperature. Samples were then removed using sterile tongs, placed onto a sterile drying rack, and the coating was evenly distributed across the exposed cheese surfaces with a sterile spreader. Samples were allowed to dry in a biosafety cabinet for up to 30 min to ensure a dry coating surface (Duan et al., 2007). After drying, samples in the PC group were inoculated with the *L. monocytogenes* cocktail as previously described. Inoculated and coated samples were placed in pouches (3 mil, oxygen transmission rate: 50–70 cm^3/m^2 24 h) (UltraSource LLC), vacuum-sealed (Ultravac 250, UltraSource LLC), and stored at 7°C to mimic retail storage and mild temperature abuse along the food supply chain.

Sampling and Enumeration

Duplicate samples from each treatment and control were removed from storage on days 1, 7, 14, 21, 28, and 35 postinoculation. Samples were homogenized in 100 mL of Dey-Engley broth (DE)

(BD Difco, Becton, Dickinson and Company, Sparks, MD, USA) in a Smasher stomacher (Biomérieux, Marcy-l'Étoile, France) for 1 min at 560 strokes/min to neutralize antimicrobials. Following serial dilutions in BPB, homogenates were plated onto modified Oxford agar (MOX) (BD Difco), incubated at 37°C for 48 h, and enumerated. Two inoculated, uncoated samples were processed after the bacterial attachment period to verify initial inoculation levels. When applicable, 1 mL of the homogenate was plated over four MOX plates (250 μ L per plate) to achieve a limit of detection (LOD) of ≥ 5 CFU/g. Two uninoculated negative controls were also processed to verify the absence of *L. monocytogenes* in uninoculated QF based on this enumeration procedure.

Data Analysis

A completely randomized design with 15×6 factorial treatment structure (15 treatments at 6 time points) was followed. Experiments were performed in triplicate using three independently produced batches of cheese from three different days. Fresh working antimicrobial stock solutions were prepared for each biological replicate. Two cheese samples (technical replicates) were included for each treatment/time point/trial. Counts below the LOD of ≥ 5 CFU/g were recorded as 2.5 CFU/g (LOD/2) and counts from technical replicates were averaged for each trial ($n = 3$). One trial of HP (PI- and PC-HP) was removed from analysis after the stock solution used in coatings was found to be expired. PC-AA treatment also consists of only two biological replicates. Mean bacterial counts were log transformed and pooled data were analyzed using the PROC GLM procedure of SAS (ver. 9.4, SAS Institute, Cary, NC, USA). The model included treatment and time as main effects as well as treatment \times time interactions. Pairwise comparisons were performed using LSMEANS with the Tukey or Tukey–Kramer method. Significance was defined as $P < 0.05$. Inhibitory and bactericidal activity were defined as < 1 log CFU/g increase and ≥ 3 log CFU/g reduction in *L. monocytogenes* counts at a given time point compared to the initial inoculum level, respectively [National Advisory Committee on Microbiological Criteria for Foods (NACMCF, 2010); US Food and Drug Administration (FDA, 2017)].

RESULTS AND DISCUSSION

The physicochemical properties of the experimental QF batches were within target ranges (Table 2). Previous studies have estimated the shelf life of QF of similar composition packaged

TABLE 2 | Composition of Queso Fresco.

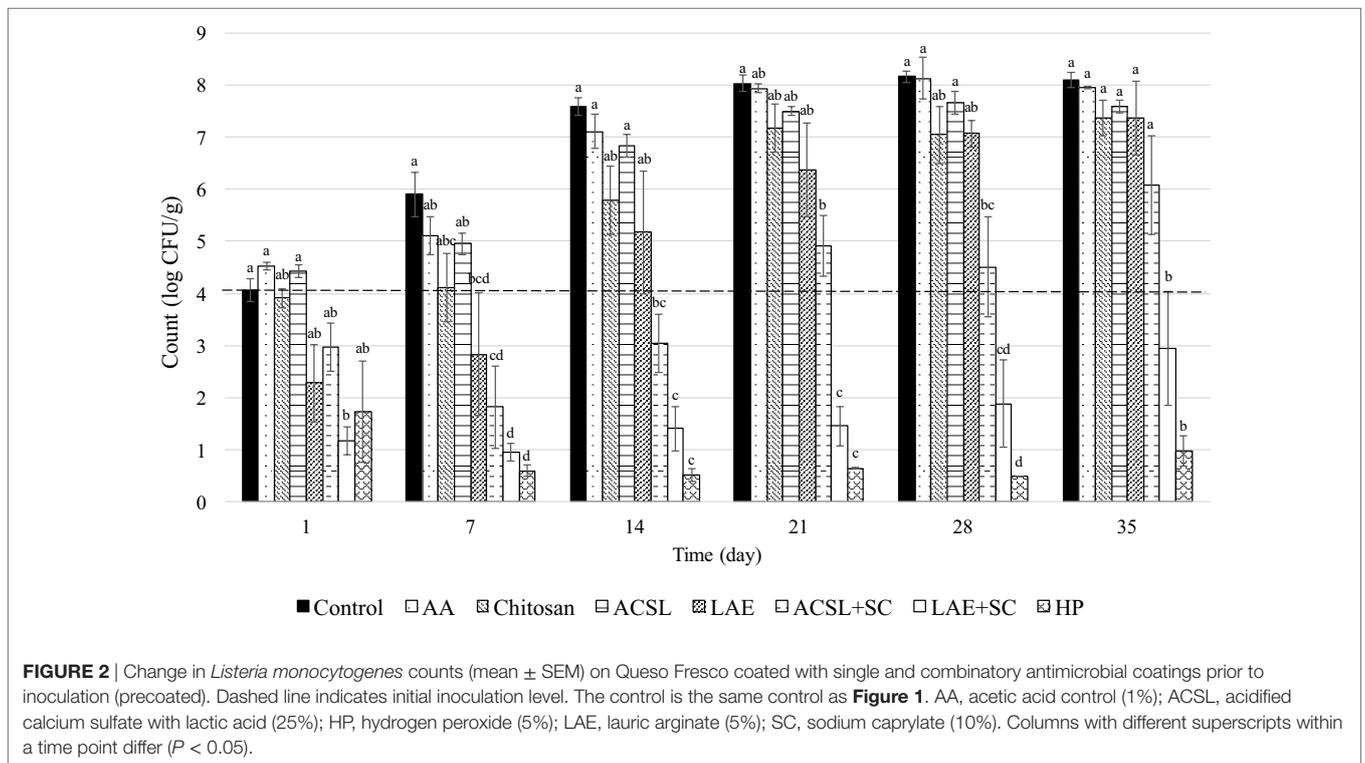
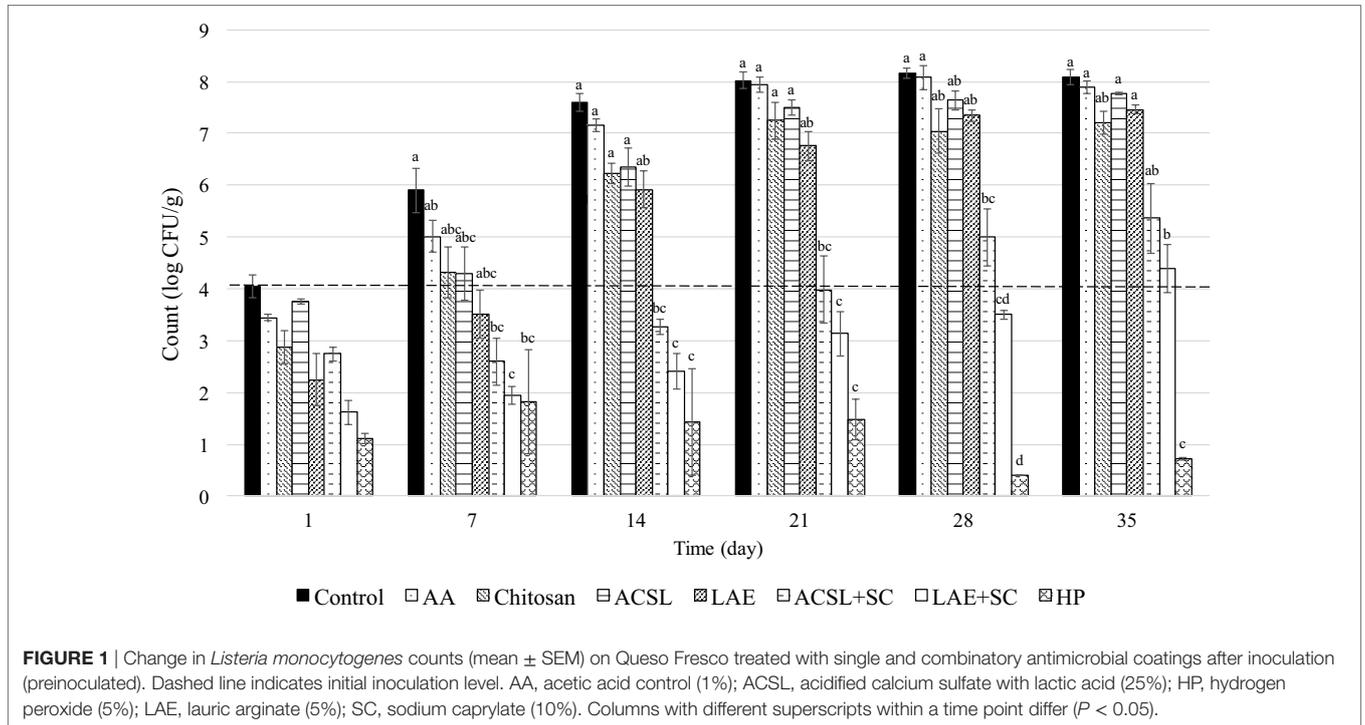
	Mean \pm SEM
Dry matter	44.4 \pm 1.3%
Moisture	55.6 \pm 1.3%
Fat	18.6 \pm 0.2%
Salt	2.0 \pm 0.0%
MNFS	68.3 \pm 1.5%
SM	3.7 \pm 0.1%
FDM	41.9 \pm 0.8%
pH	6.44 \pm 0.01

MNFS, moisture in the nonfat substance; SM, salt in moisture; FDM, fat in dry matter.

under vacuum to be between 14 and 21 days (Brown et al. under review)¹. Enumeration of untreated controls on day 0 verified an initial inoculation level of 4.1 ± 0.2 CFU/g. No listeriae were

detected in uninoculated samples from each of the three batches of cheese. After a 24 h lag, *L. monocytogenes* grew rapidly on the surface of QF without antimicrobial application reaching counts >8 log CFU/g at day 21 with no additional change through 35 days (Figures 1 and 2), which is in agreement with previous studies (Soni et al., 2010; Kozak et al., 2018b). Treatment and time

¹Brown, S. R. B., Forauer, E. C., and D'Amico, D. J. (2018). Effect of modified atmosphere packaging on the growth of spoilage microorganisms and *Listeria monocytogenes* on fresh cheese. Under review.



effects as well as treatment \times time interaction were observed for both PI and PC applications when treatments and controls were compared altogether ($P < 0.001$). Overall, the surface application of 1% AA to PI cheese (PI-AA control) had a significant effect on *L. monocytogenes* counts when compared to SDW control ($P = 0.004$) (Figure 1). The same effect was not observed for PC-AA control cheeses ($P = 0.22$) (Figure 2), which suggests that the antimicrobial effect of AA may rely on more immediate contact with the acid or that acidity decreases following application to cheese and is less effective at the time of subsequent contamination.

Antimicrobial Activity of Chitosan Coating

Chitosan is typically applied to foods as either coatings or films. Coatings may be more suitable for the application to cheese to ensure direct contact in the presence of irregular surfaces (Guo et al., 2014). Chitosan is also thought to be more available as an antimicrobial in a coating solution compared to when it is used to form films (Zivanovic et al., 2005; Vascenez et al., 2009). The application of chitosan coatings to PI cheese in the present study (PI-chitosan) resulted in a 1.2 log CFU/g reduction in mean *L. monocytogenes* counts at day 1 of sampling (Figure 1). This reduction was twice that of the PI-AA control (0.6 log CFU/g) suggesting that the initial antimicrobial effect of chitosan coatings is not solely attributed to the presence of AA in the formulation. Counts on PI-chitosan treated cheeses returned to inoculation levels at day 7 and reached final population levels of ~ 7 log CFU/g on day 21 (Figure 1). Similar reductions (~ 1 log CFU/g) in *L. monocytogenes* counts on Mozzarella cheese were also reported 24 h after application of chitosan coatings. Because untreated Mozzarella did not support the growth of *L. monocytogenes* during 14-day refrigerated storage, no subsequent growth was observed on chitosan-coated cheeses (Duan et al., 2007). Coma et al. (2003) reported substantial reductions in *L. innocua* after 3 days of storage following application of chitosan to Emmental cheese, but samples were stored at 37°C as opposed to lower temperatures more representative of typical refrigerated storage conditions. This is important considering lower temperatures may reduce chitosan efficacy due to reductions in the number of surface binding sites or electronegativity (Tsai and Su, 1999).

In contrast to coating PI cheese, the precoating of QF with chitosan (PC-chitosan) produced minimal reductions at day 1 with counts (3.9 log CFU/g) similar to initial inoculation levels (4.1 log CFU/g) and the PC-AA control (4 log CFU/g) (Figure 2). This may be explained in the same way as AA controls where initial antimicrobial activity relies on initial contact with the AA or chitosan in the coating or both. In the absence of diffusion, only organisms in direct contact with the active sites of chitosan are inhibited (Coma et al., 2003). It is possible that preapplication of chitosan coatings limits interaction between microorganisms with active sites compared to direct application of a liquid coating. Longer exposure to the components of the food matrix may also have inhibited the antimicrobial activity of chitosan. While the influence of fat may be negligible, protein and NaCl may inhibit the antimicrobial activity of chitosan as a result of competition for the positive charges on chitosan and the negative charges on the cell surfaces of the bacteria (Devlieghere et al., 2004). However,

aside from differences in initial reductions after 24 h, counts were similar between treatments (PI vs. PC) from day 7 onward and no treatment effects were observed between PC and PI-chitosan treatments. Significant treatment effects were observed for both PI- and PC-chitosan treatments when compared to SDW control ($P < 0.001$) and their corresponding PI- and PC-AA controls ($P < 0.001$ and $P = 0.005$, respectively) (Figures 1 and 2).

Antimicrobial Activity of Chitosan Coating with HP

According to the Standards of Identity for cheese, HP is approved for use in certain varieties of cheese in the United States (i.e., cheddar, colby, washed or soaked curd, granular or stirred curd, swiss, and emmentaler) when added to milk at levels up to 500 ppm followed by neutralization with catalase (21 CFR 133) [U.S. Food and Drug Administration (FDA), 2016]. HP has been shown to reduce *L. monocytogenes* counts and inhibit subsequent growth during storage when added to whole milk or applied as an aqueous dip to QF (Kozak et al., 2018a,b). In agreement with these reports, chitosan coatings formulated with HP at 5% were effective listericidal and listeristatic treatments (Figures 1 and 2). Aside from a single treatment (PC-LAE + SC), there were significant treatment \times time interactions when either PI- or PC-HP treatments were compared to all other treatments and controls. Application of HP coating to inoculated cheeses (PI-HP) reduced mean *L. monocytogenes* counts by ≥ 3 log CFU/g at day 1 and counts were < 1 log CFU/g at days 28 and 35 (Figure 1). Mean counts on cheeses PC with HP coatings (PC-HP) were < 1 log CFU/g at day 7 where they remained through 35 days of storage. Despite the lower counts observed with the PC-HP treatment, there was no effect of coating application timing (PI-HP vs. PC-HP) on *L. monocytogenes* counts.

Antimicrobial Activity of Chitosan Coating with ACSL

Acidified calcium sulfate with lactic acid is generally recognized as safe (GRAS) and suitable for use in several animal food products [U.S. Department of Agriculture, Food Safety and Inspection Service (USDA, 2009)]. Application of ACSL as an aqueous dip (25%) on QF reduced *L. monocytogenes* counts after 24 h, but growth was not inhibited throughout the remaining storage period (Kozak et al., 2018b). Because coatings have been shown to maintain high concentrations of antimicrobials at the food surface through their progressive release during storage (Oussalah et al., 2004; Fajardo et al., 2010), the incorporation ACSL into chitosan coatings could potentially limit growth following initial reductions. However, the application of chitosan coatings formulated with ACSL at 25% yielded minimal changes in *L. monocytogenes* counts by day 1 in both the PI- and PC-ACSL treatments and both treatments were only inhibitory through day 7 (4.3 and 4.4 log CFU/g for PI- and PC-ACSL, respectively) (Figures 1 and 2). Although there was a significant effect of treatment type (PI vs. PC) with lower counts on PI-ACSL cheeses compared to PC-ACSL ($P = 0.046$), counts on cheeses for both treatments were lower than SDW control ($P < 0.001$) and their corresponding AA controls ($P < 0.001$ and $P = 0.022$ for PI and PC, respectively). In

contrast, both treatments resulted in counts on cheeses that were higher than chitosan coating alone ($P = 0.04$ and $P = 0.011$ for PI-ACSL and PC-ACSL, respectively), suggesting that the addition of ACSL at 25% does not enhance the antimicrobial activity of chitosan coatings.

Overall, the efficacy of ACSL incorporated into chitosan coatings appears to be less than that observed when applied to QF as an aqueous dip, which produced initial reductions of 1.4 log CFU/g after 24 h (Kozak et al., 2018b). Mean counts reported for ACSL dip applications were more than one log lower than those on ACSL-coated cheeses in the present study through 14 days (5.2 log CFU/g) (Kozak et al., 2018b). Mean counts on both the PI- and PC-ACSL treated cheeses also exceeded 7 log CFU/g at day 21, which was one week earlier than reported for dip application (Kozak et al., 2018b). Because the mechanism of action for ACSL is attributed to the dissociation and reprotonation of lactic acid (Kemp et al., 2003), buffering the pH of the coating (pH 4.5) in the present study could have contributed to a loss of antimicrobial activity.

Antimicrobial Activity of Chitosan Coating with LAE

Similar to ACSL, LAE has been shown to produce initial inactivation of *L. monocytogenes* on the surface of QF when applied as an aqueous dip followed by subsequent growth (Kozak et al., 2018b). The application of coating solutions formulated with LAE at 5% reduced mean *L. monocytogenes* counts on QF by 1.7 and 1.8 log CFU/g when applied before (PC-LAE) or after inoculation (PI-LAE), respectively (Figures 1 and 2). Despite a difference in counts of 1.2 log CFU/g at day 7, there was no effect of coating application timing (PI-LAE vs. PC-LAE) on *L. monocytogenes* counts. Significant treatment effects were observed when both PI- and PC-LAE cheeses were compared to SDW control ($P < 0.001$) and their corresponding AA controls ($P < 0.001$ and $P = 0.003$ for PI and PC, respectively). PI-LAE and PC-LAE were also more effective in reducing *L. monocytogenes* counts when compared to cheeses coated with ACSL ($P < 0.001$ and $P = 0.002$, respectively), which is in contrast with results reported for aqueous dip application of these antimicrobials (Kozak et al., 2018b). Similar to ACSL coatings, *L. monocytogenes* counts on both the PI- and PC-LAE cheeses did not differ from PI or PC-chitosan treatments suggesting that the addition of LAE at 5% does not enhance the antimicrobial activity of chitosan coatings. *L. monocytogenes* counts following LAE application as an aqueous dip at 5% to surface contaminated QF (Kozak et al., 2018b) were also similar to those observed in the present study, suggesting that formulating LAE in a chitosan-based antimicrobial coating may not enhance its antimicrobial effects.

Guo et al. (2014) demonstrated that antimicrobial coating treatments with 1.94 mg/cm² chitosan (~2% LMW chitosan) and 0.388 mg/cm² LAE reduced *L. innocua* counts by ~4.6 log CFU/cm² on turkey deli meat after 24 h at 10°C (Guo et al., 2014). Although this is a significant reduction compared to those observed in the present study, it is not known whether *L. innocua* counts increased during subsequent storage. However, chitosan coating treatments formulated with LAE (~4% LAE) reduced initial counts and inhibited the growth of *L. monocytogenes* on roast

beef throughout the 30-day storage period at 4°C when inoculated at 2.8 and 5.3 log CFU/cm² (Wang et al., 2015). Chitosan coatings containing 1% LAE also reduced *Salmonella* populations on egg shells by 5.6 log units at day 1 and then to undetectable levels on day 3 during storage at 7°C (Jin et al., 2013). In addition to differences in pH, bacterial species, incubation temperature, and food matrices (Devlieghere et al., 2004; Fernandez-Saiz et al., 2010), the differences in antimicrobial activity between studies could be attributed to type of acid used to produce coating solutions. Previous studies have demonstrated that the combination of lactic, acetic, and levulinic acids used in the formulation of these coating solutions has antimicrobial activity against *Listeria* and *Salmonella* in various foods (Chen et al., 2012; Jin and Gurtler, 2012).

It is possible that higher concentrations of LAE would be more effective in the control of *L. monocytogenes* on QF. However, LAE is only considered GRAS for use as an antimicrobial agent in cheeses (e.g., curd and whey cheeses, cream, natural, grating, processed, spread, and dip cheeses) at levels up to 200 ppm [U.S. Food and Drug Administration (FDA), 2005]. The impact of higher concentrations of LAE on the sensory properties of cheese is also not known. LAE can bind to anionic biopolymers naturally present within the mouth leading to a perceived bitterness or astringency, but the impact of complexation interactions (i.e., LAE and chitosan) on the sensory attributes of LAE (i.e., ability to cause astringency) are also not known (Bonnaud et al., 2010). As an alternative, the use of combinations of antimicrobials that work additively or synergistically to inhibit or inactivate *L. monocytogenes* can potentially enhance antimicrobial efficacy without increasing individual usage concentrations (Kozak et al., 2017).

Antimicrobial Activity of Chitosan Coating with SC in Combination with ACSL or LAE

Submerging QF in 10% solutions of SC inhibited *L. monocytogenes* growth to <1 log CFU/g through 21 days of storage (Kozak et al., 2018b). Addition of SC to LAE dip treatments also inhibited *L. monocytogenes* growth on QF through 21 days and worked synergistically with ACSL to inhibit *L. monocytogenes* counts on QF through 28 days of storage (Kozak et al., 2018b). Based on these findings, coatings were formulated to include ACSL and LAE in combination with SC to limit growth during storage. Caprylic acid, but not SC, is currently considered GRAS at levels not to exceed current good manufacturing practices, which result in maximum levels, as served, of 400 ppm for cheeses and 50 ppm for frozen dairy desserts [U.S. Food and Drug Administration (FDA), 2017a]. Application of ACSL + SC coating to inoculated cheese (PI-ACSL + SC) reduced mean *L. monocytogenes* counts by 1.3 and 1.5 log CFU/g at days 1 and 7, respectively (Figure 1). Counts eventually returned to initial inoculation levels at day 21 but the treatment was listeristatic through day 28 with counts reaching 5 log CFU/g. Significant time × treatment interactions were observed when counts on PI-ACSL + SC coated cheeses were compared to SDW control ($P = 0.009$), PI-AA control ($P = 0.002$), PI-chitosan ($P = 0.018$), and PI-LAE ($P = 0.005$). Counts were also lower than PI-ACSL coating alone ($P = 0.045$) (Figure 1). The efficacy of PC-ASCL + SC was similar to that of

PI-ACSL + SC. Mean *L. monocytogenes* counts were reduced by 1.1 log CFU/g by the day 1 sampling point and reached a low of 1.8 log CFU/g on day 7. Counts increased thereafter but the treatment was listeristic through 28 days (Figure 2). Treatment \times time interactions were observed when counts on PC-ACSL + SC treated cheeses were compared to SDW control ($P = 0.037$) and treatment effects were seen when compared to PC-AA control ($P < 0.001$), PC-chitosan ($P < 0.001$), PC-LAE ($P = 0.011$), and PC-ACSL ($P < 0.001$) (Figure 2). As with ACSL coatings, the application of ACSL + SC coatings were less effective against *L. monocytogenes* on QF when compared with aqueous dip applications (Kozak et al., 2018b).

Aside from PI-HP, PI-LAE + SC was more effective than all other treatments and controls with treatment \times time interactions observed when compared to SDW control ($P < 0.001$), PI-AA control ($P < 0.001$), PI-chitosan ($P = 0.003$), PI-ACSL ($P = 0.003$), and PI-LAE ($P < 0.001$) (Figure 1). Though no significant interaction was observed, counts were lower than PI-ACSL + SC ($P < 0.001$) as well. At 24 h, mean counts were reduced to 1.6 log CFU/g and the treatment was listeristic through 35 days with a final count of 4.4 log CFU/g on day 35 of storage (Figure 1).

Precoating cheese with LAE + SC reduced mean *L. monocytogenes* counts by 2.9 log CFU/g at 24 h and counts gradually increased during storage to 3 log CFU/g at 35 days (Figure 2). There was a significant treatment effect for LAE + SC applications whereby this combinatory antimicrobial coating was more effective in reducing *L. monocytogenes* counts on QF when applied before (PC-LAE + SC) than after inoculation (PI-LAE + SC) ($P < 0.001$). Treatment \times time interactions were observed when the change in *L. monocytogenes* counts on PC-LAE + SC treated cheeses were compared to SDW control ($P = 0.007$), PC-ACSL ($P = 0.038$), and PC-LAE ($P < 0.001$). No significant interaction was observed but counts were lower than PC-AA control ($P < 0.001$), PC-chitosan ($P < 0.001$), PC-LAE ($P < 0.001$), and PC-ACSL + SC ($P < 0.001$) (Figure 2). In contrast to ACSL + SC coatings, counts at 35 days for both PI- and PC-LAE + SC were lower than those reported for LAE + SC application as an aqueous dip, which only inhibited growth through 28 days reaching 4.7 log CFU/g at day 35 (Kozak et al., 2018b).

The addition of SC in the formulation of ACSL + SC solutions increased the pH to 4.5, which was similar to that of chitosan alone. When SC was added to the LAE coating solution the pH increased to ~ 7.25 . Because higher pH levels impede the solubility of chitosan and for consistency between treatments, the pH of LAE + SC coating solutions was reduced to 4.5 using glacial AA. Synergistic activity between organic acids and chitosan (Rhoades and Roller, 2000) in addition to any antimicrobial effects of SC alone may help explain the enhanced efficacy of this treatment compared to LAE alone. Because the antimicrobial efficacy of chitosan is enhanced with increased acidity (Wang, 1992; No et al., 2002; Duan et al., 2007; Kong et al., 2010), future studies are needed to determine the efficacy of coatings formulated at a lower pH.

Although a formal sensory evaluation was outside the scope of the present study, chitosan coatings can bind water and lipids and may therefore serve as a protective barrier against moisture loss and delay lipid oxidation (Sathivel, 2005; Vázquez et al.,

2009). Previous studies on chitosan application to chicken breast (Petrou et al., 2012), cold-smoked salmon (Jiang et al., 2011), and turkey meat (Vardaka et al., 2016) suggest that chitosan may not negatively influence the sensory characteristics of these products. Studies on the effects of antimicrobials alone or formulated in chitosan coatings or films on the sensory properties of foods are limited. Sensory panels evaluating medium-well roast beef samples coated with a chitosan solution formulated with LAE were able to identify the difference between treated and control samples immediately after surface coating treatments but not on day 15 (Wang et al., 2015). Similarly, Otero et al. (2014) reported that polypropylene and polyethylene terephthalate films coated with LAE did not significantly affect the organoleptic properties of Zamorano cheese slices. Therefore, chitosan solutions formulated with LAE may not negatively influence the organoleptic properties of cheese, but future studies are warranted.

In addition to those studies previously discussed, antimicrobial coatings and films formulated with compounds other than chitosan have also been investigated for the control of *Listeria* on cheeses with some similar results. For example, application of a nisin-incorporated sodium caseinate film resulted in a ~ 1.1 log CFU/g reduction in *L. innocua* counts in surface-inoculated processed cheese samples after 1 week of storage at 4°C as compared to control samples (Cao-Hoang et al., 2010). Similarly, chitosan-grafted lactic acid packaging reduced *L. monocytogenes* populations on fresh cheese by ~ 1 log CFU/g by day 7 at 4°C (Sandoval et al., 2016). Zein (protein from maize) and zein-carnauba wax composite films formulated with lysozyme were effective in suppressing the growth of *L. monocytogenes* in fresh cheeses (Kashar) during cold-storage with significant reductions in *L. monocytogenes* counts occurring when films were formulated with sustained lysozyme-release (Ünalán et al., 2013).

The focus of this study was to determine the efficacy of chitosan-based antimicrobial coatings applied to the surface of QF before or after surface contamination to control *L. monocytogenes* growth during cold storage. Efficacy of treatments was based on current FDA guidance whereby a listeristic formulation that combines one or more antimicrobial substances is generally considered to be effective as process controls if growth studies show an increase of < 1 log cycle over two or more time intervals during product shelf life [U.S. Food and Drug Administration (FDA), 2017b]. The present study identifies effective listericidal and listeristic controls. Coatings containing HP at 5% were equally effective when applied before or after *L. monocytogenes* inoculation, significantly reducing *L. monocytogenes* counts by more than 3 log CFU/g and inhibiting growth through 35 days of storage. Coatings formulated with ACSL at 25% were more effective when applied to PI cheeses but neither application produced significant reductions in *L. monocytogenes* counts or inhibited growth. The application of coating solutions formulated with LAE at 5% reduced mean *L. monocytogenes* counts on the QF by 1.7–1.8 log CFU/g whether applied before or after inoculation. Although LAE coatings were more effective than those containing ACSL, neither were more effective than chitosan coatings alone, suggesting that the addition of LAE at 5% or ACSL at 25% does not enhance the antimicrobial activity of chitosan coatings applied before or after *L. monocytogenes*

contamination. The addition of SC to ACSL and LAE coatings enhanced their effectiveness. ACSL + SC coatings reduced *L. monocytogenes* counts by 1.1–1.3 log CFU/g after 24 h and were listeristatic through 28 days. LAE + SC treatments were more effective in reducing *L. monocytogenes* counts on QF when applied before inoculation than after, but both treatments produced > 1 log CFU/g reductions at 24 h and were listeristatic through 35 days.

This study identifies a novel approach to control *L. monocytogenes*. Applications of edible antimicrobial coatings containing HP or SC in combination with ACSL or LAE were effective in controlling postlethality *L. monocytogenes* contamination of QF. Effective coating formulations not only inactivate and inhibit *L. monocytogenes* already present on the surface of cheese, but provide a protective barrier to proliferation when applied before contamination events. Future studies are needed to evaluate potential changes to the sensory properties of QF and similar fresh and soft cheeses containing antimicrobials at effective concentrations to ensure consumer acceptance.

DATA AVAILABILITY

Datasets analyzed for this study supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

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AUTHOR CONTRIBUTIONS

DD conceived and designed the experiments. SK and SB performed the experiments. SB performed the statistical analysis. SB and DD wrote sections of the manuscript. DD revised the manuscript. All authors read and approved the submitted version.

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The reviewer LG and handling editor declared their shared affiliation.

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