Control of *Listeria monocytogenes* in whole milk using antimicrobials applied individually and in combination

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ABSTRACT

Dairy product recalls and dairy-related illnesses are often the result of contamination with *Listeria monocytogenes*, which can occur throughout the dairy production and supply chains. The use of antimicrobial compounds is one practical approach for controlling pathogen survival and growth in foods. The goal of this study was to use fluid milk as a model system to identify listeristatic or listericidal treatments that show promise for application in fluid milk and for further evaluation in other dairy products (e.g., cheese). Caprylic acid (CA), ϵ -polylysine (EPL), hydrogen peroxide, lauric arginate (LAE), and sodium caprylate (SC) were added individually or in combination to whole milk inoculated with *L. monocytogenes* at $\sim 4 \log_{10}$ cfu/mL. Samples were stored at 7°C for 21 d, and *L. monocytogenes* counts were determined weekly. Inhibitory concentrations of LAE (800 mg/L) and EPL (100–400 mg/L), as well as SC and CA (3,200 mg/L each), were identified. The addition of EPL at 800 mg/L reduced *L. monocytogenes* counts by $>3 \log_{10}$ cfu/mL from initial inoculation levels after 21 d. Addition of hydrogen peroxide to milk reduced counts by $>3 \log_{10}$ cfu/mL from initial inoculation within 24 h (400 and 800 mg/L) or by d 7 (200 mg/L). Although the combinatory treatments of EPL + CA, EPL + LAE, and LAE + SC were characterized as indifferent, EPL + SC worked synergistically to reduce *L. monocytogenes* populations in milk over 21 d. Overall, these data identify potential antimicrobial treatments to control *L. monocytogenes* in milk and serve as a foundation for the continued development of antimicrobial controls for *L. monocytogenes* in dairy products.

Key words: *Listeria monocytogenes*, milk, antimicrobial, synergy

INTRODUCTION

The food-pathogen combination of dairy and *Listeria monocytogenes* was recently ranked fifth in terms of annual disease burden in the United States when considering the loss of quality adjusted life years (a measure of health-related quality of life) and cost of illness (Batz et al., 2012). Contamination of dairy products with *L. monocytogenes* can occur throughout the food production and supply chains (Almeida et al., 2013) and outbreaks have been linked to both pasteurized and unpasteurized milk and milk products (CDC, 2017). According to the Centers for Disease Control and Prevention's Foodborne Outbreak Online Database (CDC, 2017), 18 reported outbreaks of listeriosis were linked to dairy products between 2003 and 2015, resulting in 166 illnesses, 130 hospitalizations, and 22 deaths. Thirteen of these outbreaks were linked to cheese and ice cream milkshakes produced from pasteurized milk as the result of postpasteurization cross-contamination (CDC, 2017). Because contaminated milk can be consumed directly or used in the manufacture of dairy products, there is a continuous need to identify methods to inhibit and inactivate *L. monocytogenes*. Milk also serves as a model food system to assess antimicrobial activity in the presence of interfering compounds including proteins and lipids (Chen and Zhong, 2017).

The use of antimicrobial compounds is a practical approach for controlling pathogen survival and growth in foods. According to US Food and Drug Administration (FDA) guidance on the control of *L. monocytogenes* in ready-to-eat foods, a listeristatic formulation that combines one or more antimicrobial substances is generally considered to be effective as a process control if growth studies that include samples at various points during product shelf life show an increase of $<1 \log_{10}$ cycle over 2 or more time intervals (e.g., days, weeks, and so on, based on sampling plan) in the number of *L. monocytogenes* during replicate trials with the food of interest (FDA, 2017b). Several antimicrobials generally recognized as safe (GRAS) by the FDA including caprylic acid (CA; FDA, 2015), hydrogen peroxide (HP;

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FDA, 2015), lauric arginate (**LAE**; FDA, 2005a,b), and ϵ -polylysine (**EPL**; FDA, 2011) have demonstrated efficacy in controlling *L. monocytogenes* under varying experimental conditions (Luchansky et al., 2005; Amrouche et al., 2010; Kozak et al., 2017), including applications in milk (Romanova et al., 2002; Geornaras et al., 2007; Ma et al., 2013) and cheese (Soni et al., 2010; Gadotti et al., 2014). Limited data are available on the efficacy of sodium caprylate (**SC**) against *L. monocytogenes* (Kozak et al., 2017), but several fatty acid derivatives including SC have demonstrated antimicrobial effects against gram-positive bacteria (Kabara et al., 1972; Nair et al., 2004).

The application of antimicrobials in foods can be affected by cost, regulatory limits, and potential changes to sensory properties (Nuñez de Gonzalez et al., 2006; Gadotti et al., 2014; Smith et al., 2015). The use of combinations of antimicrobials that work additively or synergistically to inhibit or inactivate *L. monocytogenes* can potentially enhance antimicrobial efficacy while allowing for reductions in individual usage concentrations (Kozak et al., 2017). Despite evidence of antimicrobial efficacy when compounds are used alone, the effect of potential synergistic combinations of antimicrobials in milk and other complex food matrices remains under investigated. To date, data on the efficacy of antimicrobials and their combinations in milk are scant and do not typically consider the potential shelf life of milk (Gaysinsky et al., 2007; Shah et al., 2013), which can extend beyond 21 d with little to no sensory defects (Ma et al., 2000). Determining the effectiveness of antimicrobial treatments in milk during cold storage also informs their potential use in other dairy products such as ice cream and fresh cheese. Therefore, the goal of this study was to use fluid milk as a model system to identify listeristatic or listericidal treatments that show promise for application in fluid milk and for further evaluation in other dairy products (e.g., cheese). More specifically, the objectives of this study were to (1) determine the efficacy of antimicrobial compounds used alone and in combination to control the growth of *L. monocytogenes* in whole milk throughout 21 d of storage at 7°C and (2) to characterize combinations as antagonistic, indifferent, additive, or synergistic.

MATERIALS AND METHODS

Bacterial Strains, Growth Conditions, and Inoculum Preparation

A cocktail of 8 *L. monocytogenes* strains representing a diversity of subtypes and sources including those associated with outbreaks linked to dairy products was prepared as previously described (Kozak et al., 2017).

The cocktail was serially diluted in Butterfield's phosphate buffer (**BPB**), pelleted through centrifugation (15 min, $4,000 \times g$ at 4°C; Thermo Scientific Sorvall Legend X1R, Thermo Fisher Scientific, Waltham, MA), and re-suspended in UHT pasteurized whole milk (Parmalat, Parma, Italy). Working volumes of 25 mL of UHT milk in 50-mL conical tubes (Fisher Scientific International Inc., Hampton, NH) were then inoculated with the *L. monocytogenes* suspension to the target count of $\sim 4 \log_{10}$ cfu/mL for use in the assays below. Viable numbers of *L. monocytogenes* in suspension were verified by enumeration after serial dilution in BPB, spread-plating on brain heart infusion agar (BBL, Becton Dickinson, Franklin Lakes, NJ), and incubating plates at $37 \pm 1^\circ\text{C}$ for 24 to 48 h.

Preparation of Antimicrobial Solutions

Working antimicrobial stock solutions of CA (Sigma Aldrich, St. Louis, MO), EPL (San Keeper #381; San-Ei Gen FFI Inc., New York, NY), HP (30%, Acros Organic, Pittsburgh, PA), LAE (Cytoguard LA2X, A+B Ingredients, Fairfield, NJ), and SC (Sigma Aldrich) were prepared in sterile deionized water. Caprylic acid, SC, and LAE stocks were stored at room temperature, whereas EPL and HP were stored in the dark at 4°C. Stock solutions were vortexed vigorously before use to ensure homogeneity and added to milk to achieve target concentrations (mg/L) of the active compound in milk, accounting for the concentrations in the original antimicrobial product.

Decomposition of Hydrogen Peroxide

The decomposition of HP at the treatment concentrations (100 to 800 mg/L) in whole milk with and without *L. monocytogenes* ($4 \log_{10}$ cfu/mL) during storage at 7°C was determined by measuring HP concentrations daily throughout storage using commercially available colorimetric peroxide test strips (1–3–10–30–100 mg/L; EMD Chemicals Inc., Gibbstown, NJ). When the color was between break points, the break point values were averaged (e.g., between 10 and 30 = 20 mg/L) and the mean value was recorded.

Time-Kill Assays

The efficacy of antimicrobial compounds used alone and in combination against *L. monocytogenes* in milk was determined using a standard broth dilution assay (Andrews, 2001). Experiments were conducted in UHT whole milk to eliminate background microbiota as a variable for this screening and to account for the

potential effect of milkfat, which has been shown to reduce the efficacy of antimicrobials such as LAE (Soni et al., 2010). Antimicrobial stock solutions were serially diluted in sterile deionized water and 100- μ L aliquots of each dilution were added to 25 mL of UHT milk to achieve the desired antimicrobial concentrations. Experimental treatments as listed in Table 1 were selected based on previous reports in the literature where available, while considering the potential effect of the food matrix and temperature (Lingbeck et al., 2014). Combination treatments were selected based on reported additive and synergistic interactions (Kozak et al., 2017) and were formulated to contain antimicrobials at 0.5 $\times$ their inhibitory concentration in milk to observe the effect of combinations (Ma et al., 2013; Shi et al., 2017). Inoculated UHT milk samples without antimicrobials were included as positive controls, uninoculated UHT milk containing each of the antimicrobial treatments individually served as antimicrobial controls, and uninoculated UHT milk containing no antimicrobial served as a negative control. The pH of each treatment was measured following the addition of antimicrobials (Accumet AB150, Fisher Scientific International Inc.). Samples were stored for 21 d at 7°C to mimic retail storage and mild temperature abuse along the food supply chain (Kennedy et al., 2005). Samples were serially diluted in BPB and spread onto brain heart infusion agar before the addition of antimicrobial compounds (initial inoculation) and on d 1, 7, 14, and 21 following antimicrobial addition. Counts of *L. monocytogenes* were determined following incubation at 37°C for 24 to 48 h.

Data Analysis

All assays were performed independently in triplicate using independently propagated cultures on 3 different days, with 2 technical replicates per treatment per sampling time point. Fresh working antimicrobial stock solutions were prepared for each biological replicate. Counts below the detection limit (≥ 1 cfu/mL) were recorded as 0 cfu/mL. Counts from technical replicates were averaged for each trial ($n = 3$). Mean bacterial counts were $\log_{10}$ transformed for statistical analysis. Data were analyzed using the PROC MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC) to determine the effects of treatment, time, and treatment $\times$ time interactions on *L. monocytogenes* counts. Pairwise comparisons including pH values were performed using LSMEANS with the Tukey-Kramer method. The effects of the presence/absence of *L. monocytogenes*, time, and the presence/absence of *L. monocytogenes* over time on hydrogen peroxide concentrations in milk were determined using the GLM procedure for repeated

measures (SPSS for Windows, version 24, SPSS Inc., Chicago, IL). Significance was defined at $P < 0.05$.

Inhibitory and bactericidal activity were defined as $< 1 \log_{10}$ cfu/mL of growth and $\geq 3 \log_{10}$ cfu/mL reduction in *L. monocytogenes* counts at a given time point compared with the initial inoculum level, respectively (NACMCF, 2010; FDA, 2017b). Antimicrobial combinations were characterized based on common established criteria (NCCLS, 1999; Steenbergen et al., 2009; Shi et al., 2017) modified for longer-term experiments in food. Briefly, combinations resulting in counts $\geq 2 \log_{10}$ cfu/mL lower than the most active constituent alone after 21 d were defined as synergistic. Combinations were considered additive if counts after 21 d were < 2 but $> 1 \log_{10}$ cfu/mL lower than those obtained using the most active constituent alone. Combinations with counts $< 2 \log_{10}$ and $\geq 2 \log_{10}$ cfu/mL higher than the least active constituent alone were defined as indifferent and antagonistic, respectively.

RESULTS AND DISCUSSION

Efficacy of Individual Antimicrobial Treatments

Antimicrobial Activity of CA and SC. Caprylic acid is currently GRAS at levels not to exceed current

Table 1. Antimicrobial treatments, concentrations, and resulting pH of sterile whole milk

Treatment ¹	Concentration (mg/L)	pH $\pm$ SD
Control		6.67 $\pm$ 0.02
CA	400	6.61 $\pm$ 0.09
	800	6.64 $\pm$ 0.02
	1,600	6.36 $\pm$ 0.04***
	3,200	5.44 $\pm$ 0.06***
EPL	100	6.67 $\pm$ 0.02
	200	6.71 $\pm$ 0.01
	400	6.83 $\pm$ 0.01***
	800	6.78 $\pm$ 0.04**
HP	100	6.66 $\pm$ 0.01
	200	6.68 $\pm$ 0.00
	400	6.68 $\pm$ 0.01
	800	6.68 $\pm$ 0.00
LAE	100	6.57 $\pm$ 0.02*
	200	6.53 $\pm$ 0.02***
	400	6.52 $\pm$ 0.02***
	800	6.53 $\pm$ 0.02***
SC	400	6.72 $\pm$ 0.01
	800	6.75 $\pm$ 0.01
	1,600	6.74 $\pm$ 0.03
	3,200	6.73 $\pm$ 0.12
EPL + SC	50 + 1,600	6.70 $\pm$ 0.02
EPL + CA	50 + 1,600	6.25 $\pm$ 0.06***
LAE + SC	400 + 1,600	6.64 $\pm$ 0.05
EPL + LAE	50 + 400	6.65 $\pm$ 0.01

¹Treatments: CA = caprylic acid; EPL = ϵ -polylysine; HP = hydrogen peroxide; LAE = lauric arginate; SC = sodium caprylate.

*pH of treatment differs from control ($P < 0.05$); **pH of treatment differs from control ($P < 0.01$); ***pH of treatment differs from control ($P < 0.001$).

good manufacturing practices, which result in maximum levels, as served, of 400 mg/kg for cheeses and 50 mg/kg for frozen dairy desserts (FDA, 2017a). Addition to milk has yet to be added as an intended use. Neither compound is an approved ingredient according to the standards of identity for milk and cream (21 CFR 131), cheese and related cheese products (21 CFR 133), and frozen dairy desserts (21 CFR 135). However, addition of CA to nonstandardized products like soft cheeses would be allowed if used within the GRAS limits of 400 mg/kg or less. All concentrations of CA or SC in the present study inhibited *L. monocytogenes* growth in milk to $\leq 1 \log_{10}$ cfu/mL through 7 d of storage. However, only SC at 3,200 mg/L and CA at 1,600 and 3,200 mg/L were inhibitory through the entire 21-d storage period (Figures 1 and 2). The inhibitory concentration of CA in milk at 7°C (3,200 mg/L) is similar to that reported in broth incubated at 37°C (3,250 mg/L; Kozak et al., 2017) despite differences in the assay medium, temperature, and pH. In contrast, the MIC for SC in milk (3,200 mg/L) was lower than that reported in broth (Kozak et al., 2017), suggesting that the milk matrix or lower temperature or both may enhance antimicrobial activity. We observed no significant difference between counts of *L. monocytogenes* on d 21 in milk containing CA or SC at 3,200 mg/L ($P = 1$), suggesting these compounds have similar efficacy against *L. monocytogenes* in milk. Inhibitory concentrations of CA

significantly reduced milk pH, whereas all concentrations of SC increased milk pH (Table 1), which could affect potential future applications in milk and dairy foods where such changes in pH are not acceptable. Because both CA and SC have shown increased effectiveness in acidic environments (Kozak et al., 2017), they may be promising surface treatments to inhibit *L. monocytogenes* growth in acidified dairy products such as fresh cheese (e.g., Ricotta). Higher concentrations may be necessary to produce bactericidal effects. For example, Nair et al. (2004) reported significant reductions in *L. monocytogenes* counts ($\sim 2.7 \log_{10}$ cfu/mL) after 48 h at 8°C when CA was added to sterile whole milk at 50 mM ($\sim 7,200$ mg/L; pH 5.4), whereas 25 mM ($\sim 3,600$ mg/L; pH 5.6) produced little change. However, addition of CA at these concentrations reduced milk pH and increases the risk of negative changes to consumer acceptability. For example, the addition of CA to fresh cheese curds during manufacture at 1.09 g/kg (1,090 mg/kg) resulted in cheeses with significantly reduced consumer acceptability scores when compared with untreated and commercial controls (Lourenço et al., 2017).

Antimicrobial Activity of LAE. Lauric arginate is currently GRAS in the United States for use in certain animal-based foods as well as cheeses (e.g., curd and whey cheeses, cream, natural, grating, processed, spread, and dip cheeses) at levels up to 200

Figure 1. Change in *Listeria monocytogenes* counts (mean $\log_{10}$ cfu/mL of 3 replicates $\pm$ SEM) in sterile whole milk with caprylic acid when held at 7°C. Columns with different letters within a time point differ ($P < 0.05$).

mg/kg (FDA, 2005a,b). Similar to CA, LAE is not an approved ingredient in standardized cheeses or other standardized dairy products but could be used in non-standardized products like soft cheese. Though LAE is known to bind to anionic biopolymers naturally present within the mouth, which may lead to a perceived bitterness or astringency (Bonnaud et al., 2010), the use of LAE is not expected to negatively affect the aroma, flavor, texture, or overall acceptability of products like fresh soft cheese (Soni et al., 2010). Applications have been shown to inhibit a broad spectrum of foodborne pathogens over a wide pH range (3–7). Binding of LAE to the plasma membrane lipid bilayer can result in structural changes and interrupt metabolic processes thereby inhibiting cell proliferation (Rodriguez et al., 2004; EFSA, 2007). The addition of LAE to milk at 100, 200, and 400 mg/L allowed for growth similar to that of the control throughout storage and we observed no significant differences in bacterial counts between these treatments at any time point (Figure 3). Treatments at 800 mg/L steadily reduced *L. monocytogenes* levels throughout storage with final counts reaching 1.68 log₁₀ cfu/mL lower than the starting inoculum of 4.5 log₁₀ cfu/mL and ~5 log₁₀ cfu/mL less than the control on d 21 (Figure 3). All LAE treatments produced significant reductions in milk pH from 6.67 to 6.57–6.53. Soni et al. (2010) reported inhibition of *L. monocytogenes* growth with LAE at concentrations as low as 400 mg/L in skim

milk. The authors also observed that 800 mg/L of LAE reduced *L. monocytogenes* counts to undetectable levels in both skim and whole milk after 24 h, which persisted throughout 15 d of storage at 4°C. Similarly, Ma et al. (2013) found that 750 mg/L of LAE in 2% reduced fat skim milk at 32°C reduced *L. monocytogenes* Scott A by 6.20 log₁₀ cfu/mL after 24 h. However, reductions of ~2 and 3 log₁₀ cfu/mL were reported after 24 and 48 h, respectively, when the treatment temperature was reduced to 21°C, suggesting that antimicrobial efficacy decreases with decreasing temperatures. Other studies have also demonstrated reductions in the antimicrobial activity of LAE at lower incubation temperatures (Lingbeck et al., 2014). Therefore, differences in results between studies may be attributed to differences in the commercial LAE products used, fat contents of milk, *L. monocytogenes* strains used, as well as time and temperature of the experiments. The effect of temperature, fat content, and other food components on the efficacy of LAE is also supported by a more than 100-fold increase in the inhibitory concentrations observed in refrigerated whole milk (~800 mg/L) compared with that reported for the same strains of *L. monocytogenes* in broth at 37°C (6.25 mg/L; Kozak et al., 2017).

Antimicrobial Activity of EPL. ε-Polylysine is a polypeptide comprised of repeating lysine subunits that electrostatically adsorb onto the surface of, and interfere with, microbial cell membranes. As an anti-

Figure 2. Change in *Listeria monocytogenes* counts (mean log₁₀ cfu/mL of 3 replicates ± SEM) in sterile whole milk with sodium caprylate when held at 7°C. Columns with different letters within a time point differ ($P < 0.05$).

Figure 3. Change in *Listeria monocytogenes* counts (mean log₁₀ cfu/mL of 3 replicates $\pm$ SEM) in sterile whole milk with lauric arginate when held at 7°C. Columns with different letters within a time point differ ($P < 0.05$).

microbial, EPL is effective against both gram-positive and gram-negative bacteria, as well as yeasts and mold (Shima et al., 1984; Yoshida and Nagasawa, 2003). The use of EPL as an antimicrobial in milk and dairy products is desirable owing to its solubility in water, efficacy at neutral pH and lower temperatures (Geornaras and Sofos, 2005), and limited organoleptic changes when applied to foods (Hiraki et al., 2003). In the present study, the addition of EPL at concentrations ≥ 400 mg/L slightly, but significantly ($P < 0.001$ and $P = 0.003$ for 400 and 800 mg/L, respectively), increased milk pH (Table 1), consistent with previous reports (Geornaras et al., 2007). Counts resulting from 25 mg/L EPL treatments did not differ from control at any time point. Counts in the 50 mg/L treatments were lower than control on d 7 ($P = 0.037$) and 14 ($P = 0.018$), but similar by the final day of sampling. Concentrations of 100 and 200 mg/L of EPL inhibited the growth of *L. monocytogenes* through 21 d with counts in the 200 mg/L treatments lower than the starting inoculum of 4.78 log₁₀ cfu/mL ($P < 0.001$) on d 21 (Figure 4). Treatments at 400 and 800 mg/L resulted in a gradual decrease in pathogen counts over 21 d with 800 mg/L defined as bactericidal based the 3.33 log₁₀ cfu/mL reduction from the initial inoculation. These results are similar to those reported by Geornaras et al. (2007) where *L. monocytogenes* counts were reduced to undetectable levels in diluted fat-free and whole milk with 200 mg/L of EPL after 6 d of storage. Bactericidal activity at the lower concentration of 200 mg/L may

be attributed to dilution of potentially inhibitory milk components (i.e., fat and protein) in preparation of the milk extracts. The inhibitory concentration of EPL was ~ 10 -fold higher in milk than that reported for broth at pH 7.4 (20 mg/L; Kozak et al., 2017) and is most likely attributed to the interaction of EPL with food components in milk. Potential interactions between LAE and EPL with negatively charged proteins (i.e., casein micelles) could reduce their ability to interact with target bacterial cells. Regardless, the results presented here identify EPL as a promising antimicrobial for use in milk and other dairy products. Currently, EPL has GRAS status for use in cream and various cheeses at a level up to 250 mg/kg in the United States (FDA, 2011) but is not an approved ingredient in standardized dairy products.

Antimicrobial Activity of HP and Its Decomposition in Milk. The use of HP is desirable owing to its decomposition into nontoxic byproducts and its long history of use as an antimicrobial in the dairy industry to preserve raw milk in the absence of refrigeration (Roundy, 1958). Direct addition of HP to raw milk at high concentrations (i.e., 900 mg/L) has been shown to reduce total bacteria counts when held at 6°C or ambient temperature for 48 h (Martin et al., 2014). Hydrogen peroxide has also been shown to increase the lag phase of *L. monocytogenes* in skim milk held at 20°C (Siragusa and Johnson, 1989) and inhibit growth in raw milk held at 4 and 8°C (Gaya et al., 1991) when added at 0.25 to 0.3 mM as part of the lactoperoxidase-thio-

cyanate-HP system. Hydrogen peroxide has been shown to rapidly inactivate *L. monocytogenes* in broth over a range of concentrations at 37°C (Liu and Ream, 2008) and when added to sterilized milk at 495 mg/L and stored at 4°C (Dominguez et al., 1987). A concentration of 100 mg/L inhibited growth of *L. monocytogenes* over 21 d at 7°C in the present study with an overall $2.5 \log_{10}$ cfu/mL reduction from initial inoculation by the end of storage reaching $1.49 \pm 0.93 \log_{10}$ cfu/mL. Concentrations of 400 and 800 mg/L were bactericidal within 24 h, and 200 mg/L reduced counts by more than $3 \log_{10}$ cfu/mL from initial inoculation ($4.01 \log_{10}$ cfu/mL) by d 7 of storage (Figure 5). Counts remained below the limit of detection (≥ 1 cfu/mL) from d 7 through d 21 for treatments of 200, 400, and 800 mg/mL.

Decomposition assays demonstrate the gradual degradation of HP during storage over 21 d at 7°C. Table 2 shows the change in HP concentration over time for the initial concentrations (d 0) and odd sampling days (i.e., 1, 3, 5, and so on). Only data for samples containing *L. monocytogenes* are shown because the presence of *L. monocytogenes* was only found to significantly affect the decomposition of HP over time when added at 800 mg/L ($P = 0.036$). Hydrogen peroxide concentrations in the 100 mg/L treatments were determined to be 30 mg/L within 48 h and remained at 1 ± 1 mg/L from d 8 through 21 (Table 2). Measurements from treatment concentrations of 200, 400, and 800 mg/L remained at ≥ 100 mg/L through d 2, 3, and 4, respectively.

Concentrations gradually declined in these treatments and remained in the 10 to 30 mg/L range through the remaining storage period. The decomposition in UHT whole milk reported here differs from that observed in raw milk by Martin et al. (2014), where the predicted probability of detecting HP in raw milk after 48 h of storage at 6 or 21°C using peroxide test strips was quite low at most concentrations. The authors concluded that this, when combined with the absence of continued decrease in bacterial numbers, suggests that the HP had decomposed. Based on the time-kill results in the present study, it is likely that the higher concentrations fully inactivated *L. monocytogenes* populations during these initial time points with residual peroxide preventing any potential recovery and growth as observed in the 100 mg/L treatment.

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 at levels up to 500 mg/L followed by neutralization with catalase (21 CFR 133; FDA, 2016). Treatments within this limit were effective and did not alter milk pH (Table 1). Although these treatments were effective at reducing levels of *L. monocytogenes* in fluid milk, evidence indicates that HP applied as low as 10 mg/L may inhibit acid production by starter cultures even after residual HP is neutralized using catalase (Subramanian and Olson, 1968),

Figure 4. Change in *Listeria monocytogenes* counts (mean $\log_{10}$ cfu/mL of 3 replicates $\pm$ SEM) in sterile whole milk with ϵ -polylysine when held at 7°C. Columns with different letters within a time point differ ($P < 0.05$).

Figure 5. Change in *Listeria monocytogenes* counts (mean $\log_{10}$ cfu/mL of 3 replicates $\pm$ SEM) in sterile whole milk with hydrogen peroxide when held at 7°C. Columns with different letters within a time point differ ($P < 0.05$). *Mean counts below the limit of detection of ≥ 1 cfu/mL for all 3 biological replicates. Treatments at 200, 400, and 800 mg/L were not included in statistical analyses.

thereby potentially limiting its use in the production of cultured dairy products. Oxidative rancidity has also been known to occur when adding HP to foods with high fat content (Wambura and Yang, 2011) due to free radical production. Therefore, additional studies are needed to investigate the rate of pathogen inactivation within the first 24 h of treatment, the effect of catalase neutralization on the antimicrobial activity against *L. monocytogenes* populations, and potential lipid oxidation in milk and dairy products.

Efficacy of Antimicrobials in Combination

Combinations of antimicrobials added at 0.5 $\times$ their individual inhibitory concentrations in whole milk were evaluated to identify antagonistic, indifferent, additive, and synergistic effects (Ma et al., 2013). Effective combinatorial treatments allow for reductions in use concentrations for individual antimicrobials to help alleviate potential limitations posed by cost and effects on organoleptic properties of foods. Data on the combination of antimicrobials in milk are generally limited to bacteriocins and plant-derived antimicrobials (Singh et al., 2001; Cava et al., 2007; López-Expósito et al., 2008; Ma et al., 2013). For example, Ma et al. (2013) reported synergistic antilisterial activity when combining thymol (3,000 mg/L) with LAE (375 mg/L) in 2% reduced fat

milk at 21°C. Previous research in nutrient broth has demonstrated synergistic inactivation of *L. monocytogenes* at pH 7.4 using various combinations of EPL and LAE at comparably low concentrations, suggesting this combination as a potential candidate for use in milk and other low acid foods (Kozak et al., 2017). The combination of EPL and LAE in milk in the present study was inhibitory through 7 d, after which counts increased to $7.4 \pm 0.28 \log_{10}$ cfu/mL by 21 d (Figure 6). These counts were not different ($P = 1$) than the most effective constituent alone (LAE at 400 mg/L; Table 3), suggesting the combination was indifferent. Similarly, EPL + CA combination treatments in milk at 7°C were inhibitory through 7 d (Figure 6) after which counts increased to levels that did not differ ($P = 1$) from CA alone at 1,600 mg/L at d 21 (Table 3), suggesting indifference as well. This is in contrast to observations in broth where the combination of EPL + CA worked synergistically to inactivate *L. monocytogenes* at pH 5.5 with additive interactions for pathogen inhibition and inactivation at pH 7.4 (Kozak et al., 2017). The combination of LAE + SC limited *L. monocytogenes* growth to $1.21 \log_{10}$ cfu/mL higher than initial inoculum and 2.29 cfu/mL lower than control at 21 d (Figure 6). Counts resulting from the combination treatment in milk at 21 d (5.84 ± 0.01 cfu/mL) were significantly lower than LAE alone at 400 mg/L ($P = 0.025$; 8.21

Table 2. Change in concentrations (measured as mg/L) of hydrogen peroxide (mean of 3 independent replicates $\pm$ SEM) in sterile whole milk with *Listeria monocytogenes* over time

Treatment ¹	Time (d)										
	0	1	3	5	7	9	11	13	15	17	21
Control	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0.75 $\pm$ 0.25	0 $\pm$ 0	0.25 $\pm$ 0.25	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
100 mg/L HP	$\geq 100^2$	65 $\pm$ 0	30 $\pm$ 0	4.75 $\pm$ 1.75	2.5 $\pm$ 0.5	1 $\pm$ 0	1 $\pm$ 0	1 $\pm$ 0	0.25 $\pm$ 0.25	0.5 $\pm$ 0	0 $\pm$ 0
200 mg/L HP	≥ 100	≥ 100	65 $\pm$ 0	20 $\pm$ 0	20 $\pm$ 0	25 $\pm$ 5	25 $\pm$ 5	20 $\pm$ 0	15 $\pm$ 5	20 $\pm$ 0	15 $\pm$ 5
400 mg/L HP	≥ 100	≥ 100	≥ 100	30 $\pm$ 0	30 $\pm$ 0	47.5 $\pm$ 17.5	30 $\pm$ 0	30 $\pm$ 0	30 $\pm$ 0	30 $\pm$ 0	25 $\pm$ 5
800 mg/L HP	≥ 100	≥ 100	≥ 100	65 $\pm$ 0	65 $\pm$ 0	30 $\pm$ 0	30 $\pm$ 0	30 $\pm$ 0	25 $\pm$ 5	30 $\pm$ 0	20 $\pm$ 0

¹Treatment: control = sterile milk with *L. monocytogenes*; HP = hydrogen peroxide in whole milk with *Listeria monocytogenes* (4 log cfu/mL).²Results reported as ≥ 100 indicate that all 3 replicates were at or above the limit of detection of 100 mg/L.

log₁₀ cfu/mL; Table 3) but not SC alone at 1600 mg/L ($P = 0.9$; 6.59 log₁₀ cfu/mL; Table 3). Because the difference in counts between the most active constituent (SC at 1,600 mg/L; Table 3) and the combination was <1 log₁₀ cfu/mL, the combination was characterized as indifferent. The combination of EPL + SC, which has demonstrated bactericidal synergy in broth at pH 5.5 and 7.4 (Kozak et al., 2017), was the most effective combination tested, reducing *L. monocytogenes* populations in milk by >1 log₁₀ cfu/mL to a final count of 3.56 ± 0.59 log₁₀ cfu/mL (Figure 6) at 21 d. With a >3 log₁₀ cfu/mL difference in counts at 21 d between the combination treatment and the most active constituent alone ($P < 0.001$; SC at 6.59 ± 0.21 log₁₀ cfu/mL; Table 3) the combination was defined as synergistic. The combination also inhibited growth to counts similar to SC or EPL alone at their inhibitory concentrations (3,200 and 100 mg/L, respectively; Figures 2 and 4, respectively) through 21 d, highlighting the ability of synergistic combinations to reduce individual use concentrations without diminishing overall antimicrobial efficacy.

CONCLUSIONS

The focus of this study was to determine the efficacy of antimicrobials to control *L. monocytogenes* in whole milk. Overall, concentrations of LAE (800 mg/L) and EPL (100–400 mg/L), as well as SC and CA (3200 mg/L each) capable of inhibiting *L. monocytogenes* growth in whole milk over 21 d at 7°C were identified. The addition of EPL at 800 mg/L reduced *L. monocytogenes* counts by more than 3 log₁₀ cfu/mL from initial inoculation levels after 21 d. Addition of HP to milk at 400 and 800 mg/L was bactericidal within 24 h, whereas 200 mg/L reduced counts by >3 log₁₀ cfu/mL from initial inoculation levels by d 7. Although combinatorial treatments containing EPL + CA, EPL + LAE, and LAE + SC were characterized as indifferent, EPL + SC was characterized as synergistic, allowing for reductions in individual use concentrations while achieving similar pathogen inhibition. Considering the initial pathogen levels in the present study are higher than would be expected, these results indicate even greater potential for effective *L. monocytogenes* control. Overall, these data identify effective antilisterial treatments in milk and serve as a foundation for the continued development of antimicrobial treatments to control *L. monocytogenes* in dairy products. Surface applications are not expected to affect native microbiota, but further studies are necessary if antimicrobials are to be used in the manufacture of cultured products or in raw milk to determine the effect on desirable microbiota. Considering concentrations of antimicrobials to effectively control pathogens in dairy products will vary based on

Table 3. Difference in mean counts (log₁₀ cfu/mL) between combinatory antimicrobial treatments and their individual constituents at d 21¹

Treatment	Difference in mean counts (combination – constituent) at d 21			
	EPL	SC	CA	LAE
EPL + SC	–4.82***	–3.03***		
CA + EPL	–1.23		0.22	
LAE + SC		–0.75		–2.37*
EPL + LAE	–0.98			–0.81

¹CA = caprylic acid (1,600 mg/L); EPL = ε-polylysine (50 mg/L); LAE = lauric arginate (400 mg/L); SC = sodium caprylate (1,600 mg/L). Counts for combinatory antimicrobial treatments and their individual constituents at d 21 are also depicted in Figures 1 to 6.

*Counts between combination and single antimicrobial treatment differ at *P* < 0.05.

***Counts between combination and single antimicrobial treatment differ at *P* < 0.001.

product composition, the presence of additional inhibitors, and so on, and that the perception of off flavors and odors will differ between products, future studies will be needed to evaluate potential changes to the sensory properties of products containing antimicrobials at effective concentrations to ensure consumer acceptance. Although many antimicrobials are GRAS for use in milk and other dairy products including cheese, products formulated with these and other antimicrobials discussed herein may not meet the current standards of identity. The continued identification and development of process controls for foodborne pathogens suggests revisions to the Standards of Identity to include anti-

microbial applications with demonstrated efficacy and safety are warranted.

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Figure 6. Change in *Listeria monocytogenes* counts (mean log₁₀ cfu/mL of 3 replicates ± SEM) in sterile whole milk with antimicrobials added in combination when held at 7°C. CA = caprylic acid (1,600 mg/L); EPL = ε-polylysine (50 mg/L); LAE = lauric arginate (400 mg/L); SC = sodium caprylate (1,600 mg/L). Columns with different letters within a time point differ (*P* < 0.05).

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