Lactose oxidase: An enzymatic approach to inhibit *Listeria monocytogenes* in milk

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ABSTRACT

Listeria monocytogenes is a ubiquitous pathogen that can cause morbidity and mortality in immunocompromised individuals. Growth of *L. monocytogenes* is possible at refrigeration temperatures due to its psychrotrophic nature. The use of antimicrobials in dairy products is a potential way to control *L. monocytogenes* growth in processes with no thermal kill step, thereby enhancing the safety of such products. Microbial-based enzymes offer a clean-label approach for control of *L. monocytogenes* outgrowth. Lactose oxidase (LO) is a microbial-derived enzyme with antimicrobial properties. It oxidizes lactose into lactobionic acid and reduces oxygen, generating H_2O_2 . This study investigated the effects of LO in UHT skim milk using different *L. monocytogenes* contamination scenarios. These LO treatments were then applied to raw milk with various modifications; higher levels of LO as well as supplementation with thiocyanate were added to activate the lactoperoxidase system, a natural antimicrobial system present in milk. In UHT skim milk, concentrations of 0.0060, 0.012, and 0.12 g/L LO each reduced *L. monocytogenes* counts to below the limit of detection between 14 and 21 d of refrigerated storage, dependent on the concentration of LO. In the 48-h trials in UHT skim milk, LO treatments were effective in a concentration-dependent fashion. The highest concentration of LO in the 21-d trials, 0.12 g/L, did not show great inhibition over 48 h, so concentrations were increased for these experiments. In the lower inoculum, after 48 h, a 12 g/L LO treatment reached levels of 1.7 log cfu/mL, a reduction of 1.3 log cfu/mL from the initial inoculum, whereas the control grew out to approximately 4 log cfu/mL, an increase of 1 log cfu/mL from the inoculum on d 0. When a higher challenge inoculum of 5 log cfu/mL was used, the 0.12 g/L and 1.2 g/L treatments reduced the levels by 0.2 to 0.3 log cfu/mL

below the initial inoculum and the 12 g/L treatment by >1 log cfu/mL below the initial inoculum by hour 48 of storage at refrigeration temperatures. After the efficacy of LO was determined in UHT skim milk, LO treatments were applied to raw milk. Concentrations of LO were increased, and the addition of thiocyanate was investigated to supplement the effect of the lactoperoxidase system against *L. monocytogenes*. When raw milk was inoculated with 2 log cfu/mL, 1.2 g/L LO alone and combined with sodium thiocyanate reduced ~0.8 log cfu/mL from the initial inoculum on d 7 of storage, whereas the control grew out to >1 log cfu/mL from the initial inoculum. Furthermore, in the higher inoculum, 1.2 g/L LO combined with sodium thiocyanate reduced *L. monocytogenes* counts from the initial inoculum by >1 log cfu/mL, whereas the control grew out 2 log cfu/mL from the initial inoculum. Results from this study suggest that LO is inhibitory against *L. monocytogenes* in UHT skim milk and in raw milk. Therefore, LO may be an effective treatment to prevent *L. monocytogenes* outgrowth, increase the safety of raw milk, and be used as an effective agent to prevent *L. monocytogenes* proliferation in fresh cheese and other dairy products. This enzymatic approach is a novel application to control the foodborne pathogen *L. monocytogenes* in dairy products.

Key words: *Listeria*, lactose oxidase, milk, enzyme, lactoperoxidase

INTRODUCTION

Listeria monocytogenes is a ubiquitous, gram-positive, facultative anaerobe that grows at refrigeration temperatures (Berger, 2017; FDA, 2019b). The pathogen is found throughout the environment, specifically in soil and water. *Listeria monocytogenes* may survive in food that has a relatively high acid and salt content and can tolerate high and low temperatures (FDA, 2018). Due to its ubiquitous nature and the ready-to-eat nature of dairy products, *L. monocytogenes* is a pathogen of concern for dairy processors (Boor et al., 2017). It can contaminate bulk tank milk samples from nonhygienic sampling of the cow udder or from

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the cow-milking equipment (Fedio and Jackson, 1992). *Listeria monocytogenes* may contaminate raw milk products as observed from past outbreaks, but it can also contaminate products postpasteurization from the dairy environment where it can survive for years, making it a danger in both raw and pasteurized dairy products (Boor et al., 2017).

Ingestion of *L. monocytogenes* has the potential to cause the disease listeriosis among children, immunocompromised individuals, pregnant women, and the elderly. Epidemiological studies in the United States have shown that *L. monocytogenes* has some of the highest hospitalization and case-fatality rates of all the foodborne pathogens (Mead et al., 1999), making it a danger to society if improper protocols are followed in food processing facilities. It was estimated by de Noordhout et al. (2014) that globally the burden of listeriosis was 23,150 illnesses and 5,463 deaths in 2010. In the United States, dairy products, such as fresh soft cheeses, hard cheeses, and soft-ripened cheeses were most commonly associated with *L. monocytogenes* contamination in the cheese category between 1986 and 2008 and have resulted in numerous deaths (FDA, 2015). Furthermore, there have been 7 outbreaks in the United States associated with *L. monocytogenes* since 2011 that caused illness and death from dairy product consumption (Centers for Disease Control and Prevention, 2020). Globally, contamination of soft and soft-ripened cheeses with *L. monocytogenes* continues to occur, as these cheese types have low salt content, near-neutral pH, and high water activity (Barria et al., 2020). Therefore, if contaminated postpasteurization, *L. monocytogenes* can grow to dangerous levels (Van Tassell et al., 2015; Lawton et al., 2020).

In addition to fresh cheeses, *L. monocytogenes* may pose a threat to cheeses made with raw milk. In the United States, unpasteurized milk cheeses must be aged at a temperature of at least 1.7°C and held for 60 d before distribution (21 CFR 133.182(a); FDA, 2020a). In a raw milk, soft-ripened cheese risk assessment, the US Food and Drug Administration (FDA) predicted that 4.7% of cheeses had *L. monocytogenes* contamination. According to this risk assessment, reducing contamination in the manufacture of raw milk cheese by a factor of 3 log₁₀, 4 log₁₀, or 5 log₁₀ would reduce the average risk of an immunocompromised individual or pregnant woman from becoming sick from cheese contaminated with *L. monocytogenes*. The addition of an antimicrobial on the cheese surface that could reduce *L. monocytogenes* contamination by 2 log₁₀ cfu would provide a lower risk to immunocompromised individuals than consuming cheese with no antimicrobial treatment (FDA, 2015).

The ubiquitous nature of *L. monocytogenes* and its potential to contaminate dairy products has led to the evaluation of different methods to inhibit the pathogen in milk and cheese products (Boor et al., 2017). High pressure processing as a control strategy has the advantage of inactivating *L. monocytogenes* with minimal disruption of cheese quality (Tomasula et al., 2014). Addition of antimicrobials including bacteriocins and other generally recognized as safe (GRAS) substances such as organic acids have been evaluated to be applied alone or in combination to reduce the amount needed for protection (Van Tassell et al., 2015; Kozak et al., 2018). Protective cultures, commonly lactic acid bacteria, can inhibit pathogen growth through production of acids or bacteriocins or competition for nutrients. This strategy is dependent on the specific cheese matrix and how cultures thrive in its environment due to factors such as water activity and pH (Coelho et al., 2014; Lawton et al., 2020). *Listeria monocytogenes* outgrowth over time has been shown in different experiments that tested different methodology at inhibiting the pathogen (Van Tassell et al., 2015; Kozak et al., 2018). D'Amico et al. (2008) found that *L. monocytogenes* still had the potential to grow even after the 60-d storage period required by the 21 CFR in both raw and pasteurized milk cheeses, meaning an effective method to combat this outgrowth is necessary.

Listeria monocytogenes outgrowth and consumption in raw milk or contaminated cheeses can pose a serious health risk, especially as consumers move toward a trend of eating unprocessed or minimally processed food (Ricchi et al., 2019). Some consumers view common production methods and ingredients as “unnatural” or “artificial,” causing difficulties in the addition of antimicrobial treatments. There is currently no definition of a clean label by a regulatory authority. Consumers often view foods produced with an artificial ingredient as not falling under the clean-label category (Asioli et al., 2017).

With consumers moving toward a trend of desiring “clean labels,” clean-label antimicrobial treatments that are effective against *L. monocytogenes* are needed in dairy products. Lactose oxidase (LO) is an enzyme that many consumers would categorize in the clean-label category because it is commercially produced by a strain of mold, *Microdochium nivale*. Enzymes made from plant or microbial products are often used as effective coagulants for cheese, so consumers may be primed to view LO as naturally occurring in dairy products (Ben Amira et al., 2017).

Lactose oxidase oxidizes lactose into lactobionic acid (LBA; Ahmad et al., 2004) as it concurrently reduces O₂ into H₂O₂ (Nordkvist et al., 2007). H₂O₂ is GRAS

Table 1. Strains of *Listeria monocytogenes* used to produce a cocktail used for inoculation of milk samples

Identification	Outbreak	Source type	Source site	Isolate date	Serotype
FSL-X1-0001	Laboratory strain 10403S				1/2a
FSL-R9-5621	2012 Ricotta cheese	Food	Cheese	6/19/2012	1/2a
FSL-R9-5623	2013 Semi fresh style cheese	Human	Placenta	5/29/2013	4b
FSL-R9-5625	2014 Soft cheese	Human	Blood	7/6/2014	4b
FSL-R9-5624	2013 Queso fresco	Human	Blood	8/14/2013	1/2b

and can be used as an antimicrobial agent in different production processes within the United States (FDA, 2019a). In the European Union, H₂O₂ is only allowed as a processing aid for certain foodstuffs, such as the whitening of fish (Himonides et al., 1999); however, the use of glucose oxidase, an enzyme which also produces hydrogen peroxide (Duke et al., 1969) is allowed as an additive in certain foods (EFSA CEP Panel et al., 2019). H₂O₂ has been shown as an effective agent in inhibiting *L. monocytogenes* in milk, and its combination with thiocyanate (TCN) has been deemed to be an effective activator of the lactoperoxidase system (LPDS), a natural antimicrobial system in milk (Kussendrager and van Hooijdonk, 2000; FAO, 2006). The LO inhibited *L. monocytogenes* both alone and in combination with TCN using an inhibition assay in a previous study (Lara-Aguilar and Alcaine, 2019b). Its usefulness in fluid milk and cheese products as an inhibitor of *L. monocytogenes* has not yet been explored.

The purpose of this study was to evaluate LO as an antimicrobial in fluid milk inoculated with *L. monocytogenes*. The first part of this study explored the efficacy of LO on the growth of *L. monocytogenes* in UHT skim milk inoculated at 2 log cfu/mL and 4 log cfu/mL over a 21-d storage period. These results were then used to determine if increasing concentrations of LO showed improved efficacy against *L. monocytogenes* inoculated at 3 log cfu/mL and 5 log cfu/mL in UHT skim milk over 48 h. Last, it was investigated whether LO inhibited *L. monocytogenes* growth in raw milk when stored at refrigeration temperatures. Our results showed that LO has potential as a prevention strategy for *L. monocytogenes* outgrowth in fluid dairy products.

MATERIALS AND METHODS

Listeria monocytogenes Cocktail Preparation

Five isolates of *Listeria monocytogenes* (Table 1), 4 isolated from fresh cheese outbreaks and 1 local laboratory strain, were obtained from the Food Safety Laboratory at Cornell University (Ithaca, NY). Each isolate was streaked onto Brain Heart Infusion (BHI) agar and incubated at 37°C for 24 h. After incubation, a sterile inoculation loop was used to transfer an

individual colony of each strain into 5 mL of BHI broth. Broth cultures were grown at 37°C for 18 h to obtain cultures of optical density = 1.00 (9 log cfu/mL). A *Listeria* cocktail was prepared by transferring 1,000 µL of each broth into a sterile tube and vortexed to combine. The cocktail was serially diluted in PBS and plated onto BHI agar to confirm target inoculum levels.

Application of LO in UHT Skim Milk

We used UHT-processed skim milk (Parmalat USA Corp.) to minimize background microflora. Twenty-five milliliters of milk was aseptically transferred to individual 50-mL tubes (VWR International) and treated with various levels of LO to produce final concentrations of 0.006, 0.012, and 0.12 g/L (LactoYield, Chr. Hansen). One hundred microliters of the *L. monocytogenes* cocktail described above were added into each treatment to obtain an approximate microbial load of 2 log cfu/mL or 4 log cfu/mL. Inoculated milk with no LO was used as a positive control. Milk with no LO that was not inoculated with *L. monocytogenes* was used as a negative control to confirm absence of *L. monocytogenes* in the commercial product. Samples were stored at 6°C for 21 d with samples for microbiological load taken on d 0, 2, 4, 7, 14, and 21. At each time point, each treatment was sampled from the same tube in duplicate for all experiments. Before plating, serial dilutions of each sample were prepared in PBS. *Listeria* counts were enumerated at all sampling points by spread plating in duplicate on Modified Oxford Agar (MOX), and plates were incubated at 30°C (Curtis et al., 1989; Henderson et al., 2019) for 48 h. The pH of each sample was taken in duplicate at all sampling points using established methodology (Wehr and Frank, 2004). For all pH measurements, an InLab Smart Pro-ISM pH probe (Mettler Toledo) connected to an iCinac (AMS Alliance) was used. The experiment was performed in triplicate.

High Concentration of LO in UHT Skim Milk Over 48 h

Twenty-five milliliters of UHT skim milk (Parmalat USA Corp.) was aseptically transferred to individual tubes and treated with various levels of LO to produce

final concentrations of 0.12, 1.2, and 12 g/L (LactoYield, Chr. Hansen). One hundred microliters of the *Listeria* cocktail were inoculated into each treatment to obtain an approximate microbial load of 3 log cfu/mL or 5 log cfu/mL. Inoculated milk with no LO was used as the positive control. Milk with no LO that was not inoculated with *L. monocytogenes* was used as the negative control to confirm the absence of the pathogen in the commercial product. Samples were stored at 6°C for 48 h of storage with samples for microbiological load taken at h 0, 2, 4, 6, 8, 12, 24, 36, and 48. Before plating, serial dilutions of each sample were prepared in PBS. *Listeria* counts were enumerated at all sampling points by spread plating in duplicate on MOX, and plates were incubated at 30°C for 48 h. The pH of each sample was taken in duplicate at all sampling points. The experiment was repeated in triplicate.

Application of LO and TCN in Raw Milk

Raw whole milk was collected from the Cornell University Dairy Plant 3 h after milking and stored at refrigeration temperatures for 1 d (24 h) before performing the experiment. Raw milk was shaken before sampling to ensure homogeneity. Twenty-five milliliters of milk was aseptically transferred to tubes and treated with various levels of LO and TCN (VWR International). We added LO to milk to produce final concentrations of 0.12, 0.6, and 1.2 g/L. Following the addition of LO, TCN was added to obtain a final concentration of 14 mg/L. One hundred microliters of the *Listeria* cocktail was inoculated into each treatment to obtain an approximate microbial load of 2 log cfu/mL or 4 log cfu/mL. One treatment of each LO concentration did not include the addition of TCN, and inoculated milk with no LO was used as the positive control. Milk without LO that was not inoculated with *L. monocytogenes* was used as the negative control to confirm the absence of the pathogen in the raw milk produced from Cornell. Samples were stored at 6°C for 7 d of storage with samples for microbiological load taken on d 0, 2, 4, and 7. Before plating, serial dilutions of each sample were prepared in PBS. *Listeria* counts were enumerated by spread plating in duplicate on MOX, and plates were incubated at 30°C for 48 h. The total aerobic count of the negative control was determined by plating the serial dilutions on Standard Plate Count (SPC) agar and incubating plates at 32°C for 48 h. The SPC counts were not taken in the UHT trials because the high heat and time of UHT pasteurization produces a microbially shelf-stable product. The pH of each sample was taken in duplicate at all sampling points. The experiment was repeated in triplicate.

Statistical Analysis

All statistical analyses were performed using R software (version 3.5.2, <https://www.r-project.org/>). We performed ANOVA and Tukey's honest significant difference tests at each time point to determine log differences in *Listeria* counts between all treatments and the positive control. The same tests were performed at each time point to determine differences in pH values between all treatments and the negative control.

RESULTS AND DISCUSSION

Application of LO in UHT Skim Milk Results in a Bacteriostatic Effect Against *L. monocytogenes*

In previous work, it was shown that background microbiota could have an effect on LO efficacy (Lara-Aguilar and Alcaine, 2019a) on the target bacteria. To understand the interaction between LO and *L. monocytogenes*, we initially used UHT skim milk to have a dairy environment that was free from background microbiota. Multiple concentrations of LO were added to UHT milk, and their efficacy to control *L. monocytogenes* outgrowth was monitored. Four of the isolates used for the *L. monocytogenes* challenge cocktail were from fresh cheese outbreaks, and one was a reference laboratory strain. The treatments were challenged with both a high, 4 log cfu/mL, and a low, 2 log cfu/mL, inoculum to represent variable contamination scenarios.

When *L. monocytogenes* was inoculated at a target inoculum of 2 log cfu/mL, all LO treatments resulted in a reduction in the number of *L. monocytogenes* when compared with the control starting at d 2 of storage (Figure 1). Within 2 d of storage, a 0.78 log cfu/mL outgrowth of the control was observed, whereas each treatment group inhibited outgrowth of *L. monocytogenes*. This trend continued throughout the rest of the 21-d trial (Table 2). Significant differences ($P < 0.05$) between each treatment and the control were shown starting on d 2 of storage and continued throughout the trial. By d 21, the control outgrowth reached 8 log cfu/mL, whereas each treatment group fell below the limit of detection (LOD).

When *L. monocytogenes* was inoculated at a higher level of 4 log cfu/mL (Figure 2), the control differed significantly ($P < 0.05$) from groups treated with LO within 48 h of storage. By d 4 of storage, the *L. monocytogenes* outgrowth was observed in the control, whereas each treatment group remained below the initial inoculation level. By d 7, outgrowth in the control reached 7.2 log cfu/mL, whereas each treatment group fell below that in a concentration-dependent fashion (0.006

Figure 1. *Listeria monocytogenes* counts inoculated at 2 log cfu/mL in UHT skim milk treated with lactose oxidase (LO) during storage at 6°C. Numbers on the treatment label indicate the concentration of LO solution (g/L). Bars with different letters (a–d) indicate significant differences ($P < 0.05$) between treatments on the same day. For counts lower than the limit of detection, a value of 0.5 log₁₀ cfu/mL was used. A horizontal line was drawn at the limit of detection to represent $y = 1.0$ log cfu/mL. The 0.006 and 0.012 g/L treatments were undetectable in 21 d, and the 0.12 g/L treatment was undetectable in 14 d. Error bars represent the SD.

g/L = 3.6, 0.012 g/L = 3.4, 0.12 = 2.8 log cfu/mL). On d 14, the control reached levels of above 8 log cfu/mL, and reduction of *L. monocytogenes* was shown for each treatment group. Even at the lowest concentration of LO (0.006 g/L), the treatment fell to 2.0 log cfu/mL compared with the initial inoculation of approximately 4 log cfu/mL. At a concentration of 0.012 g/L, the count of *L. monocytogenes* dropped to 1.6 log cfu/mL. At the highest concentration of LO, the number of *L. monocytogenes* was reduced below the LOD. By d 21 of storage, all treatment groups fell below the LOD.

These results suggested the production of H₂O₂ from the presence of LO in milk was sufficient to cause *L.*

monocytogenes to fall below the LOD with no regrowth after long-term storage. A previous study (Lara-Aguilar and Alcaine, 2019b) screened LO against a set of dairy-relevant microorganisms, including *L. monocytogenes* using a well-diffusion assay. A zone of clearing was observed around the LO wells. However, when catalase was spotted next to the well, *L. monocytogenes* growth was observed, suggesting that the production of H₂O₂ was the driving cause of bacterial inhibition of LO.

In both our contamination scenarios, *L. monocytogenes* outgrowth was inhibited by LO in comparison to the control starting at d 2 of storage and lasted throughout the rest of the trial. According to the FDA

Table 2. pH ($\pm$ SD) of UHT skim milk treated with lactose oxidase (LO) stored at 6°C

Treatment ¹	Time (d)					
	0	2	4	7	14	21
Control	6.62 $\pm$ 0.02 ^a	6.67 $\pm$ 0.05 ^a	6.72 $\pm$ 0.06 ^a	6.70 $\pm$ 0.01 ^a	6.70 $\pm$ 0.05 ^a	6.71 $\pm$ 0.01 ^a
LO						
0.006 g/L	6.62 $\pm$ 0.01 ^a	6.67 $\pm$ 0.06 ^a	6.66 $\pm$ 0.03 ^a	6.59 $\pm$ 0.02 ^b	6.55 $\pm$ 0.08 ^b	6.47 $\pm$ 0.07 ^b
0.012 g/L	6.61 $\pm$ 0.01 ^a	6.65 $\pm$ 0.03 ^a	6.66 $\pm$ 0.03 ^a	6.57 $\pm$ 0.04 ^b	6.51 $\pm$ 0.01 ^b	6.42 $\pm$ 0.08 ^b
0.12 g/L	6.61 $\pm$ 0.02 ^a	6.65 $\pm$ 0.07 ^a	6.57 $\pm$ 0.02 ^b	6.47 $\pm$ 0.09 ^c	6.37 $\pm$ 0.14 ^c	6.17 $\pm$ 0.10 ^c

^{a–c}Means within a column with different superscript letters are significantly different ($P < 0.05$) between treatments.

Figure 2. *Listeria monocytogenes* counts inoculated at 4 log cfu/mL in UHT skim milk treated with lactose oxidase (LO) during storage at 6°C. Numbers on the treatment label indicate the concentration of LO solution (g/L). Bars with different letters (a–d) indicate significant differences ($P < 0.05$) between treatments on the same day. For counts lower than the limit of detection, a value of 0.5 log₁₀ cfu/mL was used. A horizontal line was drawn at the limit of detection to represent $y = 1.0$ log cfu/mL. The 0.006 and 0.012 g/L treatments were undetectable in 21 d, and the 0.12 g/L treatment was undetectable in 14 d. Error bars represent the SD.

risk assessment, a listeristatic process control measure shows an average increase of less than 1 log cycle over 2 or more time intervals in the number of *L. monocytogenes* in replicate trials with the food of interest. A listericidal process control is one that provides a reduction of 5 orders of magnitude (FDA, 2015). According to these results, even at the lowest concentration of LO, listeristatic inhibition was observed.

The relative safety of H₂O₂ means it has many uses in the food industry (Linley et al., 2012), such as a sanitizer on food contact surfaces to provide an antimicrobial effect (Govaert et al., 2019), and as a food additive to reduce microbial contamination. According to the 21 Code of Federal Regulations, H₂O₂ may be added to milk and other food products at a maximum concentration of 0.05% to milk intended for cheesemaking [21 CFR 184(a); FDA, 2020b]. Main advantages of using H₂O₂ in the food industry are that it has broad-spectrum activity and lack of environmental toxicity following its complete degradation (Linley et al., 2012). H₂O₂ has a long history of use worldwide for milk preservation (Martin et al., 2014). It has also been shown to be an effective antimicrobial in different food products. It was used as an effective antimicrobial to reduce

counts of *L. monocytogenes* on mung bean sprouts after combination with a hot water treatment. These counts were reduced by approximately 2 log cfu/g on mung beans after storage (Trzaskowska et al., 2018). Kozak et al. (2018) found that a concentration of H₂O₂ of 100 mg/L inhibited growth of *L. monocytogenes* when stored at 7°C in whole milk. Overall, there was a 2.5 log cfu/mL reduction in the number of *L. monocytogenes* when H₂O₂ was added at this concentration. Concentrations of 400 mg/L were bactericidal within 24 h of storage. Our results are consistent with the reduction of *L. monocytogenes* found in previous studies due to H₂O₂ production.

There is little literature regarding the exact mechanism behind bacterial death via H₂O₂. It is an oxidative biocide, meaning it removes electrons from susceptible chemical groups, oxidizing them and then becoming reduced. Oxidative biocides may severely damage microbial structures, causing the release of intracellular compounds, which are then oxidized (Finnegan et al., 2010). H₂O₂ may then act via the formation of hydroxy radicals that oxidize thiol groups in enzymes and proteins (Russell, 2003). It is currently believed that the Fenton reaction leading to the production of free hy-

droxy radicals is the basis of the reaction, and evidence exists for the reaction of this leading to the oxidation of DNA, proteins, and lipids in vivo (Linley et al., 2012).

Lactase oxidase also produces LBA at the same time as generating H_2O_2 , and a significant difference in pH ($P < 0.001$) was observed between the control sample and UHT skim milk treated with the highest LO concentration (0.12 g/L) at d 4 of storage. By d 14 of storage, these significant differences remained. By d 7, all treatments were significantly lower ($P < 0.05$) in pH than the control. By the end of the trial, d 21, the control remained at approximately 6.7, whereas the lower LO treatment groups (0.006 and 0.012 g/L) were above 6.4, and the highest concentration of LO (0.12 g/L) was 6.2. The presence of lactic acid bacteria was likely not the driving cause of this pH reduction, as UHT skim milk was used, and the pH of the control increased throughout the trial. Therefore, in the treatment groups, the production of LBA by the LO reaction in solution likely resulted in the pH reduction.

Lactobionic acid is obtained from the oxidation of lactose, which has metal-chelating and humectant properties. It has many potential applications in foods and pharmaceutical products (Gutiérrez et al., 2012). Lactobionic acid has shown inhibition of *L. monocytogenes* when combined with other antimicrobial compounds at 21°C in 2% milk, but has not resulted in inhibition when added to milk alone (Chen and Zhong, 2017). Therefore, the combination of LBA with other antimicrobials such as H_2O_2 may show further inhibition in milk. Results from the same study showed that the addition of LBA to 2% milk significantly reduced the pH of the milk after addition by 0.44 units at a concentration of 10 mg/L (Chen and Zhong, 2017), consistent with the results obtained from this study. The exact mechanism of action of LBA is currently not well known; however, it is thought that it is due to its chelating and oxidation properties. Furthermore, in our study, measuring a reduction in pH was an indirect measurement of LBA concentration in the milk. It is not well known exactly how much LBA is produced by this reaction; therefore, future studies should determine the titratable acidity of the milk to quantify this value.

High Concentration With LO Inhibits *L. Monocytogenes* Outgrowth in UHT Skim Milk Over 48 h

In the previously established LO treatment levels, *L. monocytogenes* counts did not increase between the start of the trial and d 2 (Figures 1 and 2). We were interested if higher concentrations of LO would reduce *L. monocytogenes* levels during those 48 h compared with the control. We challenged the LO with a moder-

ate, 3 log cfu/mL, and high, 5 log cfu/mL, inoculum of *L. monocytogenes*. At the moderate inoculum, the control grew approximately 1 log cfu/mL in 48 h, as shown in Figure 3. The lowest concentration of LO, 0.12 g/L, was significantly different from the control ($P < 0.0001$) at h 8 of storage. At h 48, this concentration was 1.2 log cfu/mL below the control. At h 24 of storage, the control reached 3.5 log cfu/mL, and the 1.2 g/L LO treatment group was reduced to 2.7 log cfu/mL. This inhibition continued to h 48 of storage when the control grew out to 4.0 log cfu/mL and the treatment was further reduced to 2.3 log cfu/mL. The highest concentration, 12 g/L, showed significant differences from the control group throughout the entire period of storage. At h 12, this treatment fell to 2.4 log cfu/mL, whereas the control reached a level of 3.5 log cfu/mL. On h 48 of storage, the control reached levels of 4 log cfu/mL and the treatment group was further reduced to 1.6 log cfu/mL. Each of these treatments showed a bacteriostatic effect throughout the entire trial.

In the high inoculum challenge with 5 log cfu/mL, all treatment groups were significantly lower than the ($P < 0.05$) from the control from h 2 and remained significantly lower throughout the trial (Figure 4). By hour 48 of storage, the 0.12 g/L concentration of LO was 1.1 log cfu/mL less than the control. The second highest LO treatment, 1.2 g/L, was not statistically significant from the 0.12 g/L treatment throughout the entire trial. By h 48 of storage, this treatment demonstrated a 1.2 log cfu/mL reduction from the control. The highest concentration of LO, 12 g/L, showed the most efficacy toward reduction of *L. monocytogenes* in UHT skim milk when inoculated at 5 log cfu/mL. By h 24, this treatment was significantly different from both the control ($P < 0.05$) and the 0.12 and 1.2 g/L treatments ($P < 0.05$). At h 36, this treatment was approximately 0.8 log cfu/mL below the control. By h 48, the control grew out to almost 6 log cfu/mL, whereas the 12 g/L treatment was reduced to 4 log cfu/mL.

However, the higher levels of LO showed significant differences in the pH from the control over the 48-h trial (Table 3). By h 12, the pH of the highest concentration of LO, 12 g/L, dropped to 6.55 and was significantly different ($P < 0.05$) from the pH of the control. Significant differences in pH were not observed between the control and the lowest LO level of 0.12 g/L throughout the trial. By h 48 of storage, the 1.2 g/L LO concentration was significantly different ($P < 0.05$) from the control. The 12 g/L LO concentration reached a pH of 6.44 by the end of the 48-h storage period, a 0.23-unit drop below the control.

The higher concentrations of LO used in the 48-h trials showed greater inhibition of *L. monocytogenes*. As noted earlier, these observations were likely due to

Figure 3. *Listeria monocytogenes* counts inoculated at 3 log cfu/mL in UHT skim milk treated with lactose oxidase (LO) during storage at 6°C. Numbers on the treatment label indicate the concentration of LO solution (g/L). Bars with different letters (a–d) indicate significant differences ($P < 0.05$) between treatments on the same day. Error bars represent the SD.

greater production of H_2O_2 that LO produces. Kozak et al. (2018) found that a concentration of 100 mg/L of H_2O_2 inhibited *L. monocytogenes* growth in UHT whole milk over a period of 21 d, whereas concentrations of 400 mg/L and 800 mg/L were bactericidal within 24 h of storage. Furthermore, Martin et al. (2014) concluded that the higher concentration of H_2O_2 in raw milk reduced total bacterial concentrations in raw milk when stored at 6°C. Our results that showed that higher concentrations of LO produced a greater antimicrobial effect were consistent with these studies. However, the large drop in pH, particularly at the highest LO concentration used, means that there is a potential effect on the milk in a way that may not be sensorially acceptable. Furthermore, LO may produce oxidative flavors that consumers may not find acceptable, which should be investigated in future studies. Future research is needed to investigate whether other methods for LO use, such as immobilization, might allow for the use of higher levels of the enzyme to produce high level of H_2O_2 to achieve the bactericidal effects observed in the Kozak et al. (2018) study, and to also minimize the effect on pH by controlling exposure time of the milk to LO. If milk is to be made into cheese using starter cultures, topical application of the treatment to prevent environmental contamination of *L. monocytogenes* may

be a way to minimize the antimicrobial effect of LO on catalase-negative starter cultures.

Effect of LO on Raw Milk pH

The LO treatments significantly reduced the pH of the raw milk comparison to the control by d 2 of storage (Table 4). By d 7 of storage, the 0.6 g/L LO and 0.6 g/L LO-TCN treatments had a pH approximately 0.3 units lower than the control. Furthermore, the 1.2 g/L LO and 1.2 g/L LO-TCN treatments were more than 0.3 pH units below the control on d 7 of storage. Supplementation with TCN appeared to have no effect on the pH in comparison to the LO treatment. As noted earlier, the observed pH drop was due to LBA produced by LO. The implications of the pH drop on sensory acceptance of the raw milk or of cheese made from LO-treated raw milk need further investigation.

Application of LO and TCN Provide a Bacteriostatic Effect on *L. monocytogenes* in Raw Milk

Supplementation with TCN assists activation of the LPDS, a natural antimicrobial system present in raw milk. The LPDS is comprised of the following 3 components: lactoperoxidase, TCN, and H_2O_2 (Kussendrager

Figure 4. *Listeria monocytogenes* counts inoculated at 5 log cfu/mL in UHT skim milk treated with lactose oxidase (LO) during storage at 6°C. Numbers on the treatment label indicate the concentration of LO solution (g/L). Bars with different letters (a–d) indicate significant differences ($P < 0.05$) between treatments on the same day. Error bars represent the SD.

and van Hooijdonk, 2000). Ultra-high temperature pasteurization completely inactivates the lactoperoxidase enzyme (Barrett et al., 1999), leading to no possible activation of the LPDS in UHT skim milk. Also, Lara-Aguilar and Alcaide (2019a) determined that supplementation of raw milk with TCN alone had no antimicrobial efficacy on *Pseudomonas fragi* counts, which reinforced the theory of TCN supplementation having a synergistic effect with LO. For these reasons, we did not test TCN supplementation in UHT skim milk or TCN supplementation alone in raw milk and its efficacy against *L. monocytogenes*. Furthermore, in real-world situations, *L. monocytogenes* contamination is more of a concern in milk that will not see a pasteurization step, and thus it is important to understand how LO works in such products.

Similar to the UHT skim milk experiments, LO-treated raw milk was challenged with 2 inoculation scenarios with a low and high load of 2 log cfu/mL and 4 log cfu/mL, respectively. In both scenarios, *L. monocytogenes* exhibited a slower outgrowth in raw milk than in UHT skim milk (Figures 5 and 6), with low inoculum reaching 5.3 log cfu/mL (Figure 1) in UHT skim milk in 7 d, whereas it only reached 3.1 log cfu/mL in the raw milk (Figure 5). Similarly, outgrowth of the positive control

with the high inoculum reached >7 log cfu/mL after 7 d (Figure 2) in the UHT milk, whereas it only reached a level of 5.8 log cfu/mL (Figure 6) in the raw milk.

Multiple reasons can explain the discrepancy between the growth of *L. monocytogenes* in raw milk versus UHT skim milk, with the background microbiota being the primary cause. Raw milk contains a variety of microorganisms, including a large lactic acid bacteria population. In descending order, this lactic acid bacteria population typically includes *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Leuconostoc*, and *Enterococcus* spp. (Quigley et al., 2013). Psychrotrophic bacteria, including *Pseudomonas*, *Acinetobacter*, and *Aeromonas* spp. are present in raw milk and thrive at cold temperatures (Raats et al., 2011). The background microbiota present in raw milk may make it difficult for *L. monocytogenes* to proliferate. A study by Jia et al. (2020) concluded that *L. monocytogenes* growth was affected by background microbiota in commercially-produced salmon when compared with a sterile salmon sample. Furthermore, Gonzales-Barron et al. (2020) found that *L. monocytogenes* could grow slightly in pasteurized milk and raw milk cheeses; however, the pasteurized milk cheese showed greater outgrowth of *L. monocytogenes* than raw milk cheese, likely due to a

Table 3. pH ($\pm$ SD) of UHT skim milk treated with lactose oxidase (LO) during storage at 6°C (n = 9)

Treatment ¹	Time (h)								
	0	2	4	6	8	12	24	36	48
Control	6.62 ± 0.06 ^a	6.62 ± 0.06 ^a	6.62 ± 0.06 ^a	6.63 ± 0.05 ^a	6.63 ± 0.04 ^a	6.64 ± 0.04 ^a	6.65 ± 0.05 ^a	6.66 ± 0.02 ^a	6.67 ± 0.02 ^a
LO	6.69 ± 0.05 ^a	6.66 ± 0.03 ^a	6.65 ± 0.03 ^a	6.65 ± 0.03 ^a	6.65 ± 0.03 ^a	6.65 ± 0.03 ^a	6.65 ± 0.03 ^a	6.65 ± 0.04 ^a	6.65 ± 0.03 ^{ab}
0.12 g/L	6.62 ± 0.06 ^a	6.62 ± 0.01 ^a	6.62 ± 0.02 ^a	6.61 ± 0.02 ^a	6.61 ± 0.02 ^a	6.61 ± 0.02 ^{ab}	6.60 ± 0.02 ^a	6.59 ± 0.03 ^a	6.58 ± 0.03 ^b
1.2 g/L	6.63 ± 0.03 ^a	6.61 ± 0.01 ^a	6.59 ± 0.00 ^a	6.57 ± 0.01 ^a	6.56 ± 0.01 ^a	6.55 ± 0.02 ^b	6.52 ± 0.02 ^b	6.48 ± 0.08 ^b	6.44 ± 0.10 ^c

^{a,b}Means within a column with different superscript letters are significantly different ($P < 0.05$) between treatments.

less complex background microbiota. Our results were consistent with those of other studies that show that *L. monocytogenes* better proliferates in environments with fewer other microorganisms present and grows to higher levels in environments with fewer microorganisms present.

Figure 7 shows the SPC of the microbial population present in raw milk control samples with no *L. monocytogenes* inoculation over a 7-d storage period. The high and low *L. monocytogenes* inocula are included in the graphs for reference. These figures show that the initial concentration of microorganisms present in the raw milk was approximately 3.4 ± 0.5 log cfu/mL on d 0 of storage. Within 2 d of storage at refrigeration temperatures, this concentration increased to 6.0 ± 1.5 log cfu/mL. After 7 d of storage, these counts reached 8.3 ± 0.16 log cfu/mL, indicating that a complex background microbiota was present in the raw milk at this time point. The growth of microorganisms in the raw milk by d 2 of storage can help explain why *L. monocytogenes* exhibited a slow outgrowth within the raw milk samples. On d 2 of storage with both the 2 log cfu/mL and 4 log cfu/mL inoculation trials, *L. monocytogenes* exhibited only slight outgrowth, suggesting it competes poorly in the presence of other microorganisms.

At low concentrations, LO efficacy was reduced in raw milk, but the supplementation with TCN restored some efficacy. For example, the effect of an LO treatment of 0.12 g/L in raw milk on both low and high inocula of *L. monocytogenes* did not inhibit outgrowth in comparison to the control (Figures 5 and 6), though that concentration was able to inhibit growth in UHT milk (Figures 1 and 2). This reduction of efficacy may be due to the fact that in raw milk, there are other factors that affect H₂O₂ such as the presence of background microbiota that in essence divert the activity of H₂O₂ away from *L. monocytogenes*. There may also be species in the raw milk that produce a sufficient level of catalase to degrade the H₂O₂ produced by this low level of LO, as well as the lactoperoxidase present in the milk. However, with the supplementation of TCN at 14 mg/mL, the 0.12 g/L LO was sufficient to inhibit *L. monocytogenes* outgrowth (Figures 5 and 6).

This was also observed with *Pseudomonas fragi* inhibition in raw milk (Lara-Aguilar and Alcaine, 2019a). H₂O₂ activates the LPDS, which uses H₂O₂ to oxidize TCN, and in turn leads to the formation of antimicrobial compounds (Seifu et al., 2005). It is possible that the level of TCN in raw milk is insufficient to produce enough antimicrobial compounds to inhibit *L. monocytogenes*, and the excess H₂O₂ produced by LO is lost. With supplementation of TCN, there is more substrate for lactoperoxidase to produce antimicrobial compounds and thus inhibit *L. monocytogenes*. This

Table 4. pH ($\pm$ SD) of raw milk treated with thiocyanate (TCN) and lactose oxidase (LO) during storage at 6°C (n = 4)

Treatment ¹	Time (d)			
	0	2	4	7
Control	6.78 $\pm$ 0.01 ^a	6.78 $\pm$ 0.01 ^a	6.78 $\pm$ 0.03 ^a	6.68 $\pm$ 0.05 ^a
LO				
0.12 g/L	6.77 $\pm$ 0.02 ^a	6.74 $\pm$ 0.01 ^a	6.69 $\pm$ 0.00 ^a	6.63 $\pm$ 0.06 ^a
0.6 g/L	6.75 $\pm$ 0.02 ^a	6.67 $\pm$ 0.03 ^{ab}	6.60 $\pm$ 0.09 ^b	6.40 $\pm$ 0.12 ^b
1.2 g/L	6.73 $\pm$ 0.03 ^a	6.62 $\pm$ 0.05 ^b	6.51 $\pm$ 0.07 ^b	6.36 $\pm$ 0.15 ^b
TCN with LO				
0.12 g/L	6.77 $\pm$ 0.03 ^a	6.73 $\pm$ 0.01 ^a	6.69 $\pm$ 0.00 ^a	6.62 $\pm$ 0.05 ^a
0.6 g/L	6.72 $\pm$ 0.02 ^a	6.67 $\pm$ 0.04 ^b	6.55 $\pm$ 0.04 ^b	6.41 $\pm$ 0.15 ^b
1.2 g/L	6.73 $\pm$ 0.02 ^a	6.61 $\pm$ 0.07 ^b	6.52 $\pm$ 0.08 ^b	6.27 $\pm$ 0.19 ^b

^{a,b}Means within a column with different superscript letters are significantly different ($P < 0.05$) between treatments.

also suggests the mechanism of *L. monocytogenes* inhibition by LO in UHT milk versus LO in raw milk is different, and further research is needed to understand these differences.

Treatments with higher levels of LO were effective at inhibiting *L. monocytogenes* with and without TCN (Figures 5 and 6). When the 2 log cfu/mL inoculum was used, concentrations of 0.6 g/L LO alone and combined with TCN and 1.2 g/L alone and combined with

TCN differed from the control as well as the 0.12 g/L LO treatment by d 4 of the trial. By d 7 of treatment, the 0.6 g/L LO combined with TCN and the 1.2 g/L LO and 1.2 g/L combined with TCN reduced the level of *L. monocytogenes* to below the LOD. Similarly, when *L. monocytogenes* was inoculated into the raw milk at a concentration of 4 log cfu/mL, each of these treatments were significantly different from the control by d 4 of storage. The 0.6 LO-TCN, 1.2 g/L LO, and 1.2 g/L

Figure 5. *Listeria monocytogenes* counts inoculated at 2 log cfu/mL in raw milk treated with lactose oxidase (LO) and thiocyanate (TCN) during storage at 6°C. Numbers on the treatment label indicate the concentration of LO solution (g/L). Bars with different letters (a–c) indicate significant differences ($P < 0.05$) between treatments on the same day. For counts lower than the limit of detection, a value of 0.5 log₁₀ cfu/mL was used. A horizontal line was drawn at the limit of detection to represent $y = 1.0$ log cfu/mL. The LO treatments were undetectable in 21 d. Error bars represent the SD.

Figure 6. *Listeria monocytogenes* counts inoculated at 4 log cfu/mL in raw milk treated with lactose oxidase (LO) and thiocyanate (TCN) during storage at 6°C. Numbers on the treatment label indicate the concentration of LO solution (g/L). Bars with different letters (a–d) indicate significant differences ($P < 0.05$) between treatments on the same day. Error bars represent the SD.

LO-TCN treatments all reduced levels of *L. monocytogenes* to below 3 log cfu/mL; a bacteriostatic effect was present using these levels. Higher concentrations of LO had more of an effect against *L. monocytogenes* in raw milk.

CONCLUSIONS

The goal of this study was to explore the possible antimicrobial effect of LO on *L. monocytogenes* in various milk products as a possible safety enhancement to prevent the pathogen's outgrowth. We first explored the antimicrobial effects of LO to reduce *L. monocytogenes* counts in UHT skim milk and then used these results for investigation of further application in other milk products. Our results showed that very low concentrations (0.006, 0.012, and 0.12 g/L LO) were effective at inhibiting *L. monocytogenes* inoculated at both 2 log cfu/mL and 4 log cfu/mL concentrations in UHT skim milk. Each of these treatments had minimal effect on the pH until d 4 of storage; by d 7 of storage all treatments differed significantly in pH from the control due to the production of LBA. We observed that the highest concentration of LO that proved effective in the

UHT trials did not have the same effect on *L. monocytogenes* in raw milk, likely due to the presence of a diverse microbiota as well as the presence of the LPDS in raw milk. Therefore, we increased concentrations of LO and supplemented with sodium TCN. These concentrations proved inhibitory to outgrowth of *L. monocytogenes* during a 1-wk storage period, demonstrating that LO and LO supplemented with TCN are effective and could be leveraged to improve the food safety of dairy products. Future studies will evaluate the efficacy of LO as an inhibitor of *L. monocytogenes* and other pathogenic organisms in other dairy products, such as cheese and yogurt.

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Figure 7. (A) Total bacterial growth curve in raw milk. The 2 log cfu/mL inoculum of *Listeria monocytogenes* in raw milk was plotted for comparison. Error bars represent the SD. (B) Total bacterial growth curve in raw milk. The 4 log cfu/mL inoculum of *L. monocytogenes* was plotted for comparison. Error bars represent the SD.

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