IMPROVING THE SAFETY OF DAIRY PRODUCTS BY EVALUATION OF LACTOSE OXIDASE AS AN ENZYMATIC CONTROL METHOD FOR *LISTERIA MONOCYTOGENES* GROWTH AND QUALITATIVE ANALYSIS OF ARTISAN DAIRY FOOD SAFETY WORKSHOPS

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ABSTRACT

Listeria monocytogenes is a pathogen of concern in the dairy industry due to its ubiquitous nature and ability to thrive and proliferate at refrigeration temperatures. Certain dairy products are ready-to-eat (RTE) and provide an environment for *L. monocytogenes* to thrive. Consumption of the pathogen by consumers can cause morbidity and mortality in immunocompromised individuals and the fetuses of pregnant women. Consumers are currently moving toward a trend of desiring clean-label products, so a clean-label antimicrobial that is effective at controlling *L. monocytogenes* growth in dairy products would be beneficial to the dairy industry. Lactose oxidase (LO) would fall into this clean label category because it is produced by a strain of mold. LO oxidizes lactose into lactobionic acid (LBA) with the concurrent reduction of oxygen into hydrogen peroxide (H_2O_2). H_2O_2 has long been used in the dairy industry as an antimicrobial and has shown promising results on inhibition of *L. monocytogenes* in dairy products in previous studies. Incorporation of LO into dairy products may provide an effective control method for *L. monocytogenes* growth. H_2O_2 could also activate the lactoperoxidase system (LPS), an antimicrobial system that is present in raw milk to provide further control of the pathogen. We first evaluated the effect of different concentrations of LO on *L. monocytogenes* growth in UHT skim milk in both the long-term and the short-term and then evaluated different concentrations of LO combined with sodium thiocyanate (TCN), a component of the LPS, in fluid raw milk. We found that LO and LO-TCN combinations yielded promising results on inhibition of *L. monocytogenes* in fluid milk, and then moved to

evaluate the effect of LO and LO-TCN combinations against *L. monocytogenes* in a laboratory-scale fresh cheese model. Various contamination scenarios and applications of LO and LO-TCN combinations were tested in this fresh cheese model to observe the antimicrobial effect of the combinations at various points in the production process. LO alone was discovered to inhibit *L. monocytogenes* at a concentration of 0.12 and 0.6 g/L at low inoculum levels on the surface of the cheese and at both higher and lower inoculum levels when the enzyme was added into fluid milk, indicating that the production of H_2O_2 was sufficient to reduce pathogenic growth.

We also analyzed qualitative data from artisan dairy food safety workshops to determine the effect of formal education on artisan dairy producers in terms of their food safety knowledge, attitudes to food safety practices, and levels of food safety implementation in their facilities. It was found that these workshop enhanced participants' knowledge of food safety, and post-workshop, they had a more optimistic perspective of various components of their food safety plans. These results will be used to determine if formal education changed the food safety knowledge, attitudes to food safety practices, and levels of food safety implementation of artisan producers, post-workshop. In the future, these results will be used to evaluate and improve artisan dairy food safety workshop courses that Cornell holds throughout various geographic locations in the United States.

BIOGRAPHICAL SKETCH

Brenna is from Fresno, California. She graduated from California State University, Fresno with a bachelor's degree in Food Science and Nutrition, food science option. During her studies, she worked part-time as a Statistics tutor and Supplemental Instructor for Biology and Physics. As Brenna thought about what she wanted to do in the future during her undergraduate studies, she determined she wanted to delve deeper into Food Microbiology and Food Safety.

In 2019, Brenna joined the Alcaine Research Group as a master's student. Her research focused on improving the safety of dairy products by exploring a novel application to reduce *Listeria monocytogenes* growth. Specifically, her work involved application of lactose oxidase as an enzymatic control method to inhibit *L. monocytogenes* in milk and cheese products. She also worked on data analysis for artisan dairy food safety workshops. Brenna was an active participant in the Food Science Department. She served as the MS representative for the Food Science Graduate Students Organization and worked on an extension project regarding hemp processing and production in Geneva.

Upon graduation, Brenna aspires to keep serving the food industry in a role related to food safety and regulations. She hopes to use the knowledge gained in graduate school to educate food processors and producers on how to prevent pathogenic outbreaks and improve the safety of products destined for customer consumption.

To my parents for their ongoing love and support and to the best kitty who's ever lived, Pebbles, for keeping me company (and keeping me sane) in graduate school.

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CHAPTER 1

LACTOSE OXIDASE: AN ENZYMATIC APPROACH TO INHIBIT *LISTERIA MONOCYTOGENES* IN MILK

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 (TCN) were added to utilize 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 14-21 days 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-day 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 hours, 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, while the control grew out to approximately 4 log CFU/mL, an increase of 1 log CFU/mL from the inoculum on d0.

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 *L. monocytogenes* levels by 0.2-.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 TCN 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 fell ~0.8 log CFU/mL below the initial inoculum on d7 of storage, while 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 while 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 and 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, *Listeria monocytogenes*, in dairy products.

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

INTRODUCTION

Listeria monocytogenes is a ubiquitous, Gram-positive, facultative anaerobe that grows at refrigeration temperatures (Berger, 2017, U.S. Food and Drug Administration, 2019b). The pathogen is found throughout the environment, specifically in soil and water. *L. monocytogenes* may survive in food that has a relatively high acid and salt content and can tolerate high and low temperatures (U.S. Food and Drug Administration, 2018). Due to its ubiquitous nature and the RTE 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 non-hygienic sampling of the cow udder or from the cow milking equipment (Fedio and Jackson, 1992). *L. monocytogenes* may contaminate raw milk products as observed from past outbreaks, but it can also contaminate products post-pasteurization 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 one of the highest hospitalization and case fatality rates of all the foodborne pathogens (Paul 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., that globally the burden of listeriosis was 23,150 illnesses and 5,463 deaths in 2010 (de Noordhout et

al., 2014). 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 (U.S. Food and Drug Administration, 2015). Furthermore, since 2011, there have been seven outbreaks in the United States associated with *L. monocytogenes* 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 (Barría et al., 2020). Therefore, if contaminated post-pasteurization, *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 days before distribution (21 CFR 133.182(a)). In a raw milk, soft-ripened cheese risk assessment, the 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 $3\log_{10}$, $4\log_{10}$, or $5\log_{10}$ 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 $\log_{10}$ CFU would provide a lower risk to

immunocompromised individuals than consuming cheese with no antimicrobial treatment (U.S. Food and Drug Administration, 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 in order 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). *L. 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-day storage period required by the 21 Code of Federal Regulations (**CFR**) in both raw and pasteurized milk cheeses, meaning an effective method to combat this outgrowth is necessary.

L. monocytogenes outgrowth and consumption in raw milk or contaminated cheeses can pose a serious health risk, especially as consumers are moving 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 (Amira et al., 2017).

LO oxidizes lactose into lactobionic acid (**LBA**) (Ahmad et al., 2004) while it concurrently reduces O_2 into H_2O_2 (Nordkvist et al., 2007). H_2O_2 is GRAS and can be used as an antimicrobial agent in different production processes within the United States (U.S. Food and Drug Administration, 2019a). In the European Union, hydrogen peroxide 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). H_2O_2 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 (**LPS**), a natural antimicrobial system in milk (Kussendrager and van Hooijdonk, 2000, FAO, 2006). LO was shown to inhibit *L. monocytogenes* in the presence of both LO and 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 explores 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-day 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 hours. Lastly, it was investigated whether LO inhibited *L. monocytogenes* growth in raw milk when stored at refrigeration temperatures. Our results show 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), four isolated from fresh cheese outbreaks and one 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 hours. After incubation, a sterile inoculation loop was used to transfer an individual colony of each strain into five mL of BHI broth. Broth cultures were grown at 37 °C for 18 hours to obtain cultures of OD = 1.00 (9 log CFU/mL). A *Listeria* cocktail was prepared by transferring 1000 μL of each broth into a sterile tube and vortexed to combine. The cocktail was serially diluted in phosphate buffer saline (PBS) and plated onto BHI agar to confirm target inoculum levels.

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

ID	Outbreak	Source Type	Source Site	Isolate Date	Serotype
FSL-X1-0001	Lab 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

Application of LO in UHT Skim Milk

Ultra-high temperature (UHT) processed skim milk (Parmalat, USA, Corp., Grand Rapids, MI) was used to minimize background microflora. Twenty-five mL of milk was aseptically transferred to individual 50 mL tubes (VWR International, Solon, OH) and treated with various levels of LO to produce final concentrations of 0.006, 0.012, and 0.12 g/L (LactoYield, Chr. Hansen, Milkwukee, WI). One hundred µL 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 days with samples for microbiological load taken on days 0, 2, 4, 7, 14, and 21. At each time point, each treatment was sampled from the same tube in duplicate for all experiments. Prior to 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 hours. The pH of each sample was taken in duplicate at all sampling points using established methodology (Standard methods for the examination of dairy products). For all pH measurements, an InLab Smart Pro-ISM pH probe (Mettler Toledo; Columbus, OH, USA) connected to an iCinac was used. The experiment was performed in triplicate.

High concentration application of LO in UHT skim milk over 48 hours

Twenty-five mL 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, Milwaukee, WI). One hundred μL 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 hours of storage with samples for microbiological load taken at hours 0, 2, 4, 6, 8, 12, 24, 36, and 48. Prior to 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 hours. 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 hours after milking and stored at refrigeration temperatures for one day (24 h) before performing the experiment. Raw milk was shaken before sampling to ensure homogeneity. Twenty-five mL of milk was aseptically transferred to tubes and treated with various levels of LO and thiocyanate (TCN; VWR International, Solon, OH). LO was added 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 μL 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 days of storage with samples for microbiological load taken on days 0, 2, 4, and 7. Prior to 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 hours. 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 hours. SPC counts were not taken in the UHT trials because UHT pasteurization produces a product free from bacterial growth. 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, R Development Core Team, Vienna, Austria). Analysis of Variance and Tukey's Honest Significant Difference tests were performed 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 impact on LO efficacy (Lara Agulair and Alcaine, 2019a) on the target bacteria. To retain consistency with previous protocols, UHT skim milk was used to investigate the impact of LO on the *L. monocytogenes* strains in an environment free from background microbiota to determine its initial effect on the pathogen in this environment. Multiple concentrations of LO were added to UHT skim 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 to the control starting at day 2 of storage (Figure 1). Within 2 days of storage, a 0.78 log CFU/mL outgrowth of the control was observed while each treatment group inhibited outgrowth of *L. monocytogenes*. This trend continued throughout the rest of the 21-day trial. Significant differences ($p < 0.05$) between each treatment and the control were shown starting on day 2 of storage and continued throughout the trial. By

day 21, the control outgrowth reached 8 log CFU/mL while each treatment group fell below the LOD.

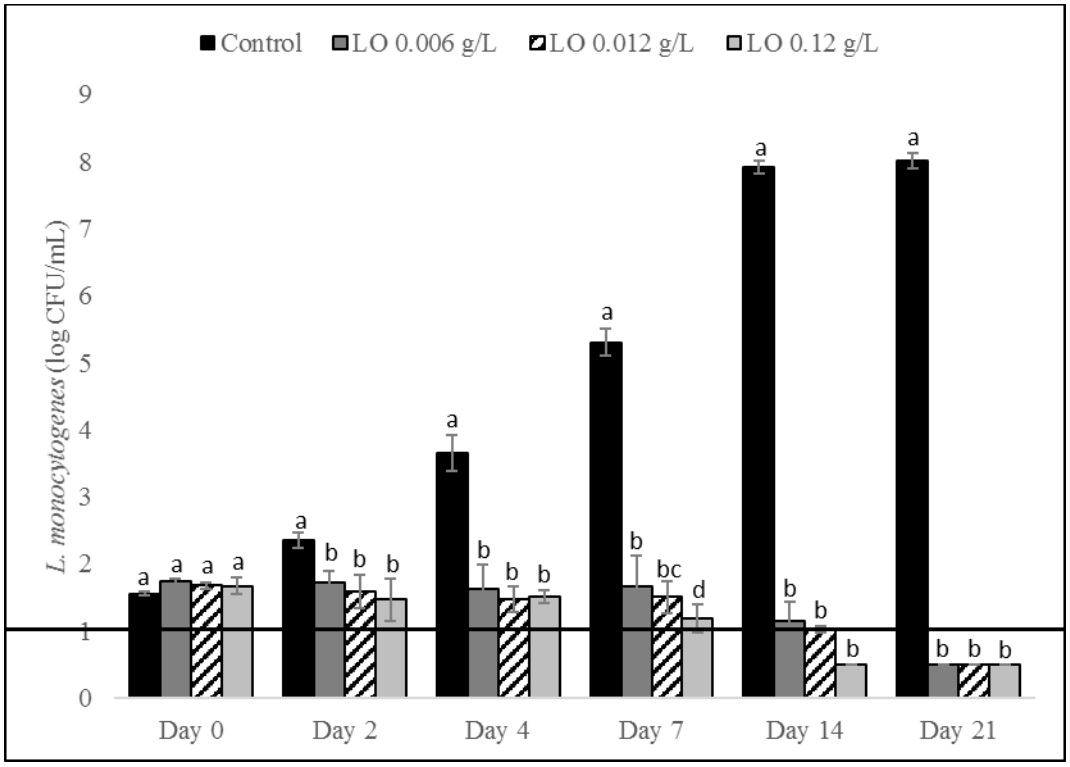

Figure 1. *L. monocytogenes* counts inoculated at 2 $\log_{10}$ CFU/mL in UHT skim milk treated with lactose oxidase (LO) during storage at 6 °C. Numbers on the treatment label indicate the concentration of LO solution (g/L). Bars with different letters indicate significant differences ($p < 0.05$) between treatments on the same day. For counts lower than the limit of detection a value of 0.5 $\log_{10}$ CFU/mL was used. A horizontal line was drawn at the limit of detection to represent y = 1.0 $\log_{10}$ CFU/mL. The 0.006 and 0.012 g/L treatments were undetectable in 21 days and the 0.12 g/L treatment was undetectable in 14 days. Error bars represent the SD.

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 hours of storage. By day 4 of storage, the *L. monocytogenes* outgrowth was observed in the control while each treatment group remained below the initial inoculation level. By day 7 outgrowth in the control reached 7.2 log CFU/mL while each treatment group fell below that in a concentration-dependent fashion (0.006 g/L= 3.6, 0.012 g/L= 3.4, 0.12= 2.8 log CFU/mL). On day 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 to 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 limit of detection (LOD). By day 21 of storage, all treatment groups fell below the LOD.

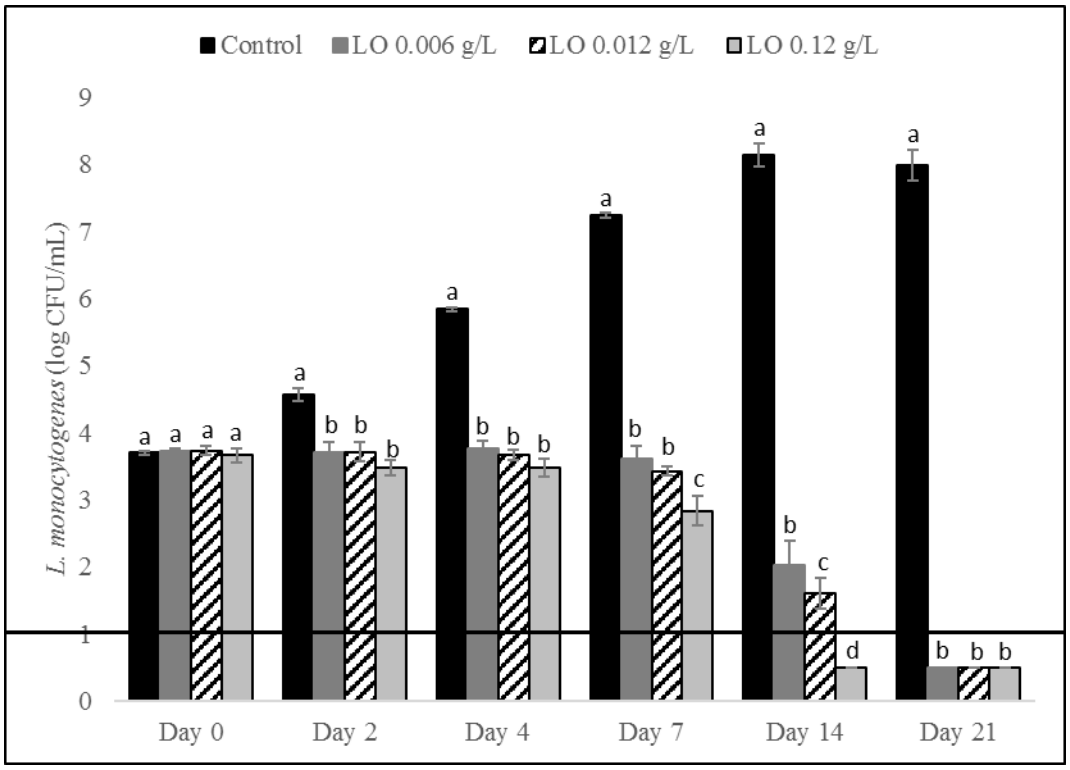

Figure 2. *L. monocytogenes* counts inoculated at 4 $\log_{10}$ CFU/mL in UHT skim milk treated with lactose oxidase (LO) during storage at 6 °C. Numbers on the treatment label indicate the concentration of LO solution (g/L). Bars with different letters indicate significant differences ($p < 0.05$) between treatments on the same day. For counts lower than the limit of detection a value of 0.5 $\log_{10}$ 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 days and the 0.12 g/L treatment was undetectable in 14 days. Error bars represent the SD.

These results suggest the production of H_2O_2 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, 2019) showed

inhibition of *L. monocytogenes* treated LO in an overlay-inhibition assay. To determine the cause of microbial inhibition was the production of H_2O_2, catalase was spotted next to the well where the compound would be formed, and growth of *L. monocytogenes* occurred, suggesting production of H_2O_2 is the driving cause of bacterial inhibition when LO is applied to dairy products.

In both contamination scenarios, *L. monocytogenes* outgrowth was inhibited by LO in comparison to the control starting at day 2 of storage and lasted throughout the rest of the trial. According to the FDA risk assessment, a listeriostatic process control measure shows an average increase of less than 1 log cycle over two 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 five orders of magnitude (U.S. Food and Drug Administration, 2015). According to these results, even at the lowest concentration of lactose oxidase, listeriostatic inhibition was observed.

The relative safety of H_2O_2 means it has many uses in the food industry (Ezra 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 CFR, H_2O_2 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)). Main advantages of using H_2O_2 in the food industry are that it has broad-spectrum activity and lack of environmental toxicity following its complete

degradation (Ezra et al., 2012). H_2O_2 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 after storage (Trzaskowska et al., 2018). Kozak et al. (2018) found that a concentration of H_2O_2 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 H_2O_2 was added at this concentration. Concentrations of 400 mg/L were bactericidal within 24 hours of storage. Our results are consistent with the reduction of *L. monocytogenes* found in previous studies due to H_2O_2 production.

There is little literature regarding the exact mechanism behind bacterial death via H_2O_2. 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). H_2O_2 may then act via the formation of hydroxy radicals which oxidize thiol groups in enzymes and proteins (Russell, 2003). It is currently believed that the Fenton reaction leading to the production of free hydroxy 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* (Ezra et al., 2012).

LO also produces LBA while generating H_2O_2, and a significant difference in pH ($p < 0.05$) was observed between the control sample and UHT skim milk treated with the highest LO concentration (0.12 g/L) at day 4 of storage. By day 14 of storage, these significant differences remained. By day 7 all treatments were significantly lower in pH than the control. By the end of the trial, day 21, the control remained at approximately 6.7 while the lower 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 is 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 lactose oxidase reaction in solution likely resulted in the pH reduction.

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

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

[a,b,c] Means within columns with different lowercase letters (a-c) are significantly different ($p<0.05$) between treatments

LBA is an acid obtained from the oxidation of lactose that has metal chelating and humectant properties. It has many potential applications in foods and pharmaceutical products (Gutiérrez et al., 2012). LBA has shown inhibition of *L. monocytogenes* when combined with other antimicrobial compounds at 21° C in 2% milk but did not result 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, however, future studies should determine the titratable acidity of the milk to quantify this value.

High concentration treatment with LO inhibits *L. monocytogenes* outgrowth in UHT skim milk over 48 hours.

In the previously established LO treatment levels, *L. monocytogenes* counts did not increase between the start of the trial and day 2 (Figure 1 and 2). We were interested if higher concentrations of LO would reduce *L. monocytogenes* levels during those 48 hours compared to the control, and we challenged the LO with a moderate, 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 hours, as shown in Figure 3. The lowest concentration of LO, 0.12 g/L, was significantly different from the control ($p < 0.05$) at hour 8 of storage. At hour 48 this concentration was 1.2 log CFU/mL below the control. At hour 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 hour 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 hour 12, this treatment fell to 2.4 log CFU/mL while the control reached a level of 3.5 log CFU/mL. On hour 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.

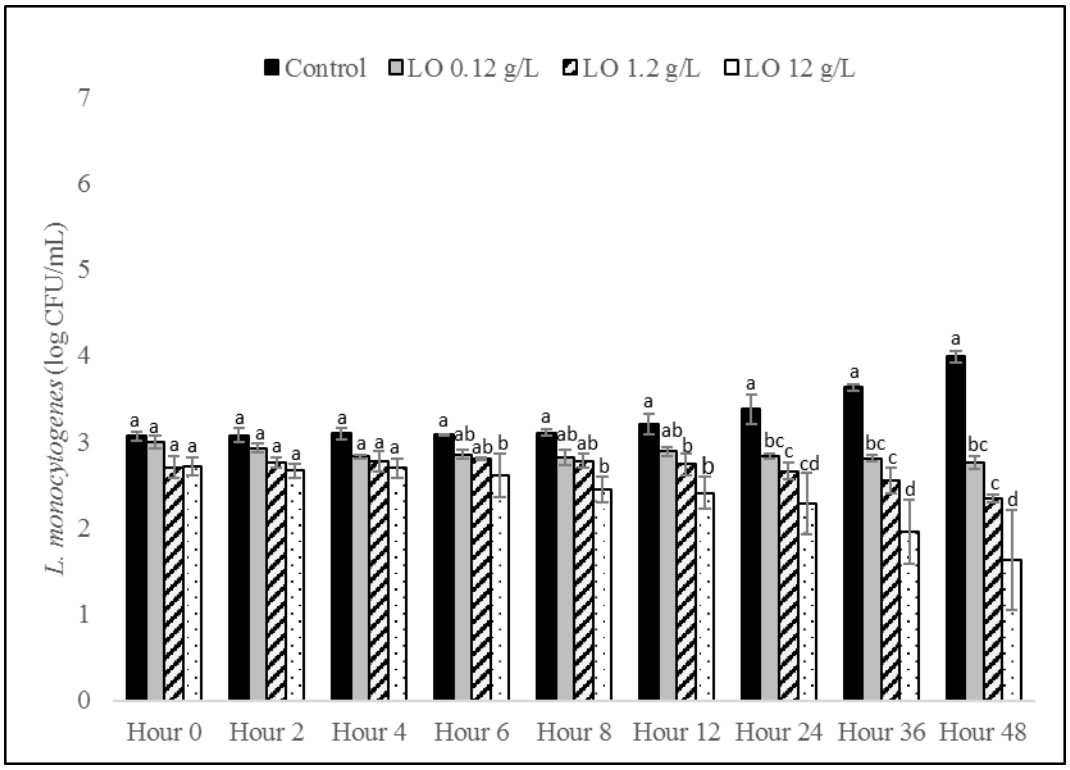

Figure 3. *L. monocytogenes* counts inoculated at 3 $\log_{10}$ CFU/mL in UHT skim milk treated with lactose oxidase (LO) during storage at 6 °C. Numbers on the treatment label indicate the concentration of LO solution (g/L). Bars with different letters indicate significant differences ($p < 0.05$) between treatments on the same day. Error bars represent the SD.

In the high inoculum challenge with 5 log CFU/mL all treatment groups were significantly lower ($p < 0.05$) from the control starting at hour 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 hour 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 hour 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 hour 36, this treatment was approximately 0.8 log CFU/mL below the control. By hour 48, the control grew out to almost 6 log CFU/mL while the 12 g/L treatment was reduced to 4 log CFU/mL.

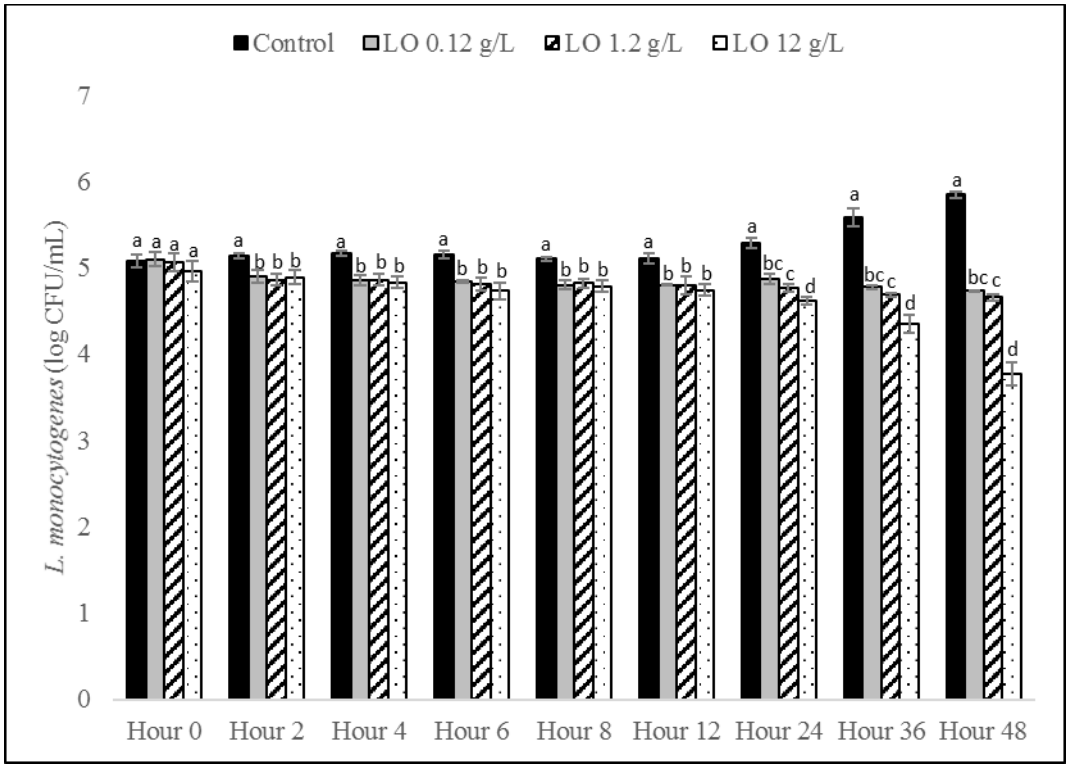

Figure 4. *L. monocytogenes* counts inoculated at 5 $\log_{10}$ CFU/mL in UHT skim milk treated with lactose oxidase (LO) during storage at 6 °C. Numbers on the treatment label indicate the concentration of LO solution (g/L). Bars with different letters

indicate significant differences ($p < 0.05$) between treatments on the same day. Error bars represent the SD.

The higher levels of LO, however, did show significant differences in the pH from the control over the 48-hour trial (Table 3). By hour 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 hour 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-hour storage period, a 0.23 unit drop below the control.

Table 3. pH (± SD) of UHT skim milk treated with lactose oxidase during storage at 6 °C

	Time (h)								
Treatment[1]	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									
0.12 g/L	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]
1.2 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]
12 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 lowercase letters are significantly different ($p < 0.05$) between treatments.

[1] LO = lactose oxidase; n = 9

The higher concentrations of LO utilized in the 48-hour trials showed greater inhibition of *L. monocytogenes*. As noted earlier, these observations are likely due to 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 days while concentrations of 400 mg/L and 800 mg/L were bactericidal within 24 hours 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 show that higher concentrations of LO produce a greater antimicrobial effect is consistent with these studies. However, the large drop in pH, particularly at the highest LO concentration used, means that there is potential impact to the milk in a way that may not be sensorial 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 utilization, like immobilization, might allow for the use of higher levels of the enzyme to thus produce high levels of H_2O_2 to achieve the bactericidal effects observed in the Kozak et. al (2018) study, while minimizing the impact 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.

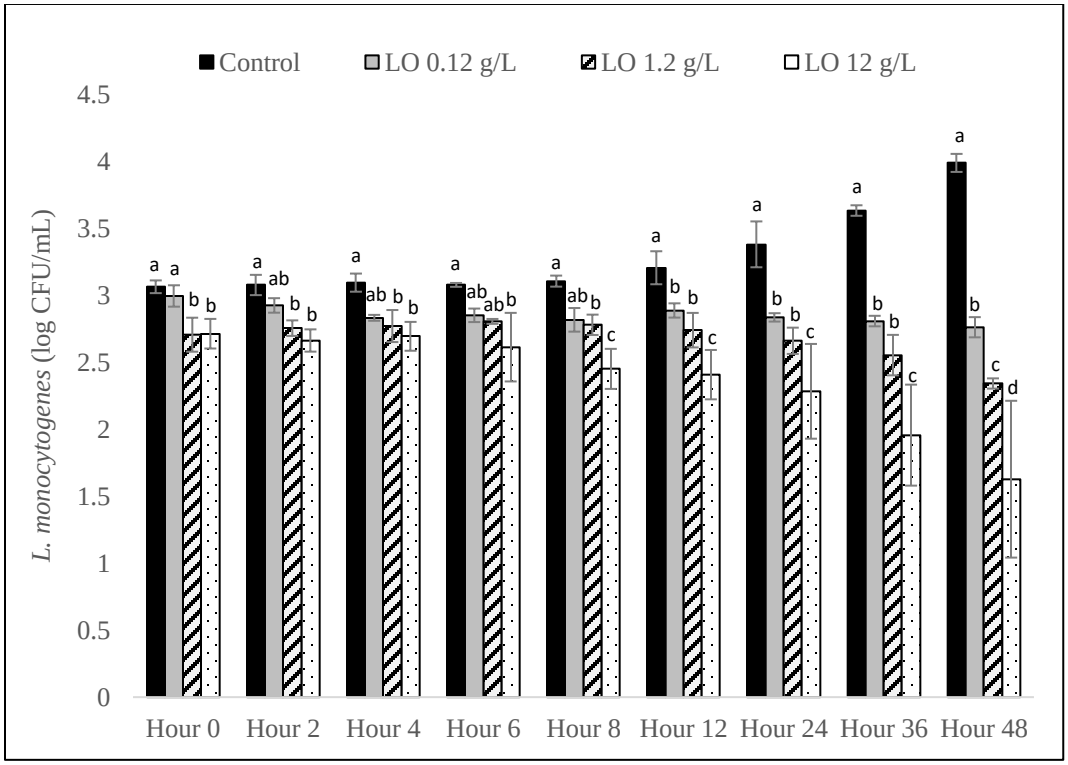

Figure 3. *L. monocytogenes* counts inoculated at 3 $\log_{10}$ CFU/mL in UHT skim milk treated with lactose oxidase (LO) during storage at 6 °C. Numbers on the treatment label indicate the concentration of LO solution (g/L). Bars with different letters indicate significant differences ($p < 0.05$) between treatments on the same day. Error bars represent the SD.

In the high inoculum challenge with 5 log CFU/mL all treatment groups were significantly lower ($p < 0.05$) from the control from hour 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 hour 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 hour 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 hour 36, this treatment was approximately 0.8 log CFU/mL below the control. By hour 48, the control grew out to almost 6 log CFU/mL while the 12 g/L treatment remained below 4 log CFU/mL.

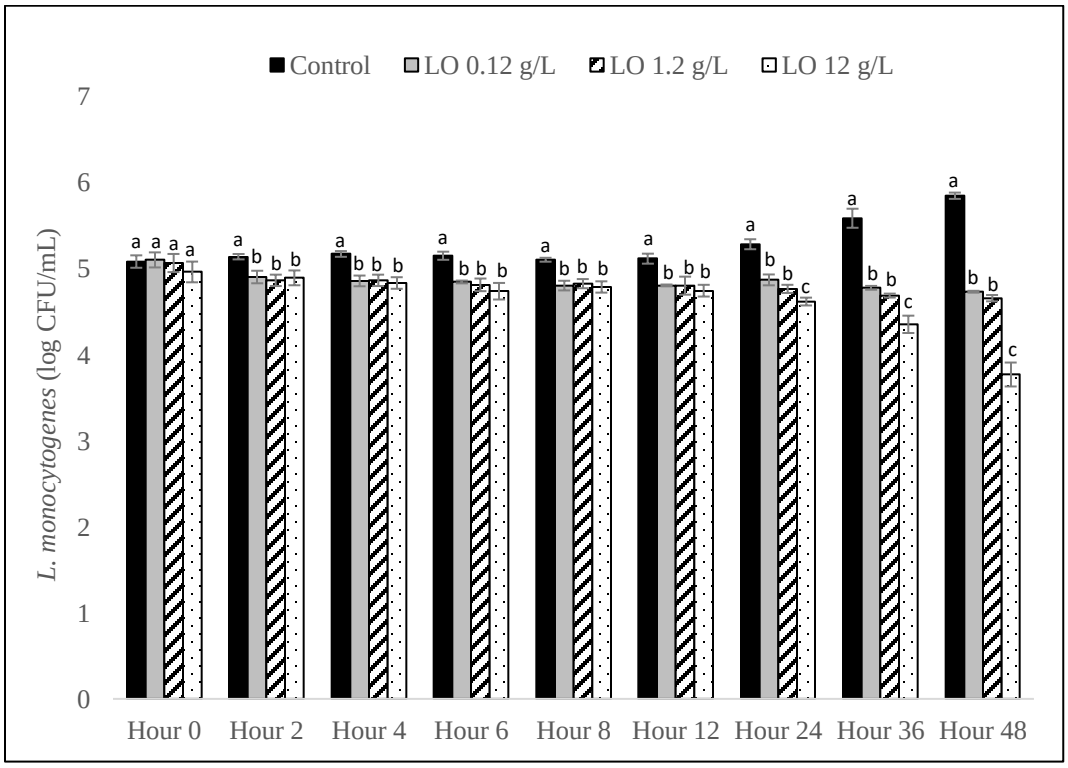

Figure 4. *L. monocytogenes* counts inoculated at 5 $\log_{10}$ CFU/mL in UHT skim milk treated with lactose oxidase (LO) during storage at 6 °C. Numbers on the treatment label indicate the concentration of LO solution (g/L). Bars with different letters indicate significant differences ($p < 0.05$) between treatments on the same day. Error bars represent the SD.

The higher levels of LO, however, did show significant differences in the pH from the control over the 48-hour trial (Table 3). By hour 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 hour 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-hour storage period, a 0.23 unit drop below the control.

Table 3. pH (± SD) of UHT skim milk treated with lactose oxidase during storage at 6 °C.

	Time (h)								
Treatment[1]	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^{ab}	6.64 ± 0.04^{a}	6.65 ± 0.05^{a}	6.66 ± 0.02^{a}	6.67 ± 0.02^{a}
LO 0.12 g/L	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}
1.2 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^{ab}	6.61 ± 0.02^{ab}	6.60 ± 0.02^{a}	6.59 ± 0.03^{a}	6.58 ± 0.03^{b}
12 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^{b}	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 lowercase letters are significantly different ($p < 0.05$) between treatments.

[1] LO = lactose oxidase; n = 9

The higher concentrations of LO utilized in the short-term trials showed greater inhibition of *L. monocytogenes*. As noted earlier, these observations are likely due to 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 days while concentrations of 400 mg/L and 800 mg/L were bactericidal within 24 hours 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 show that higher

concentrations of LO produce a greater antimicrobial effect is consistent with these studies. However, the large drop in pH, particularly at highest LO concentration used, means that there is potential impact to the milk in a way that may not be sensorially acceptable. Future research is needed to investigate whether other methods for LO utilization, like immobilization, might allow for the use of higher levels of the enzyme to thus produce high levels of H_2O_2 to achieve the bactericidal effects observed in the Kozak et. al (2018) study, while minimizing the impact on pH by controlling exposure time of the milk to LO.

Impact of LO on raw milk pH

LO treatments significantly reduced the pH of the raw milk comparison to the control by day 2 of storage (Table 4). By day 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 day 7 of storage. Supplementation with TCN appears to have no effect on the pH in comparison to the LO treatment. As noted earlier, the observed pH drop is 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.

Table 4. pH (± SD) of raw milk treated with thiocyanate and lactose oxidase during storage at 6 °C

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

[a.b,c] Means within a column with different letters are significantly different ($p < 0.05$) between treatments

[1] LO = lactose oxidase; TCN = thiocyanate; n = 4.

Application of LO and TCN provide a bacteriostatic effect on *L. monocytogenes* in raw milk

Supplementation with TCN assists activation of the lactoperoxidase system (LPS), a natural antimicrobial system present in raw milk. The LPS is comprised of three components: lactoperoxidase, thiocyanate, and H_2O_2 (Kussendrager and van Hooijdonk, 2000). UHT pasteurization completely inactivates the lactoperoxidase enzyme (Barrett et al., 1999), leading to no possible activation of the LPS in UHT skim milk. Also, Lara-Aguilar-Alcaine (2019) determined that supplementation of raw milk with TCN alone had no antimicrobial efficacy on *P. 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, so 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 two 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 (Figure 5 and 6), with low inoculum reaching 5.3 log CFU/mL (Figure 1) in UHT skim milk in 7 days, 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 days (Figure 2) in the UHT milk whereas it only reached a level of 5.8 log CFU/mL (Figure 6) in the raw milk.

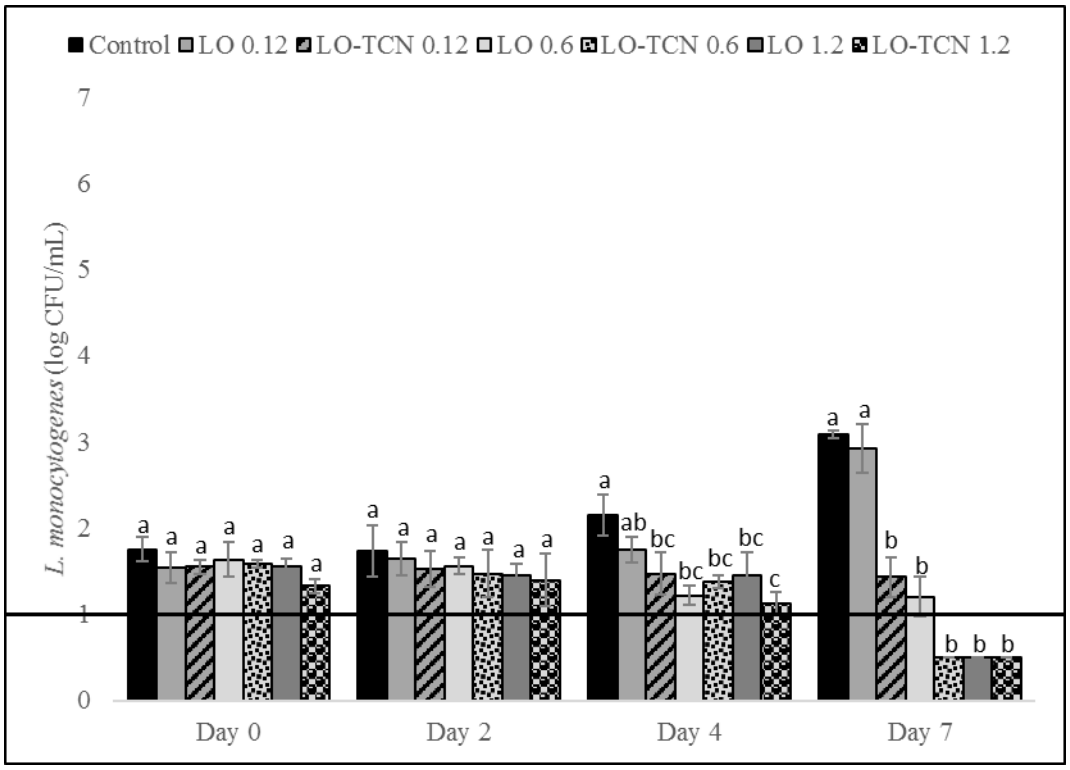

Figure 5. *L. monocytogenes* counts inoculated at 2 $\log_{10}$ CFU/mL in raw milk treated with lactose oxidase (LO) and thiocyanate (TCN) during storage at 6 °C. Numbers on the treatment label indicate the concentration of LO solution (g/L). Bars with different letters indicate significant differences ($p < 0.05$) between treatments on the same day. For counts lower than the limit of detection a value of 0.5 $\log_{10}$ CFU/mL was used. A horizontal line was drawn at the limit of detection to represent y = 1.0 $\log_{10}$ CFU/mL. The LO treatments were undetectable in 21 days. Error bars represent the SD.

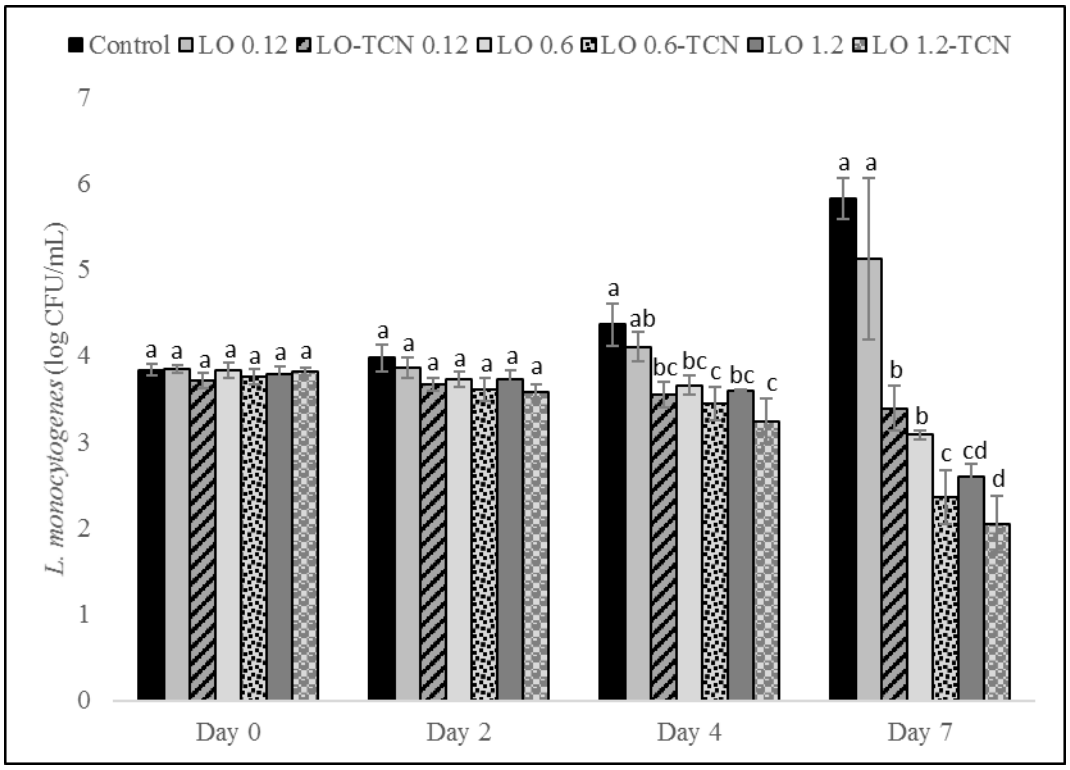

Figure 6. *L. monocytogenes* counts inoculated at 4 $\log_{10}$ CFU/mL in raw milk treated with lactose oxidase (LO) and thiocyanate (TCN) during storage at 6 °C. Numbers on the treatment label indicate the concentration of LO solution (g/L). Bars with different letters indicate significant differences ($p < 0.05$) between treatments on the same day. Error bars represent the SD.

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 (LAB) population. In descending order this LAB population typically includes *Lactococcus, Streptococcus, Lactobacillus, Leuconostoc,* and *Enterococcus spp.* (Quigley et al., 2013). Psychrotrophic bacteria, including *Psuedomonas,*

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

Figures 7a and 7b show the standard plate count of the microbial population present in raw milk control samples with no *L. monocytogenes* inoculation over a 7-day 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 day 0 of storage. Within 2 days of storage at refrigeration temperatures this concentration increased to 6.0 ± 1.5 log CFU/mL. After 7 days 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 timepoint. The growth of microorganisms in the raw milk by day 2 of storage can help explain why *L. monocytogenes* exhibited a slow outgrowth within the raw milk

samples. On day 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.

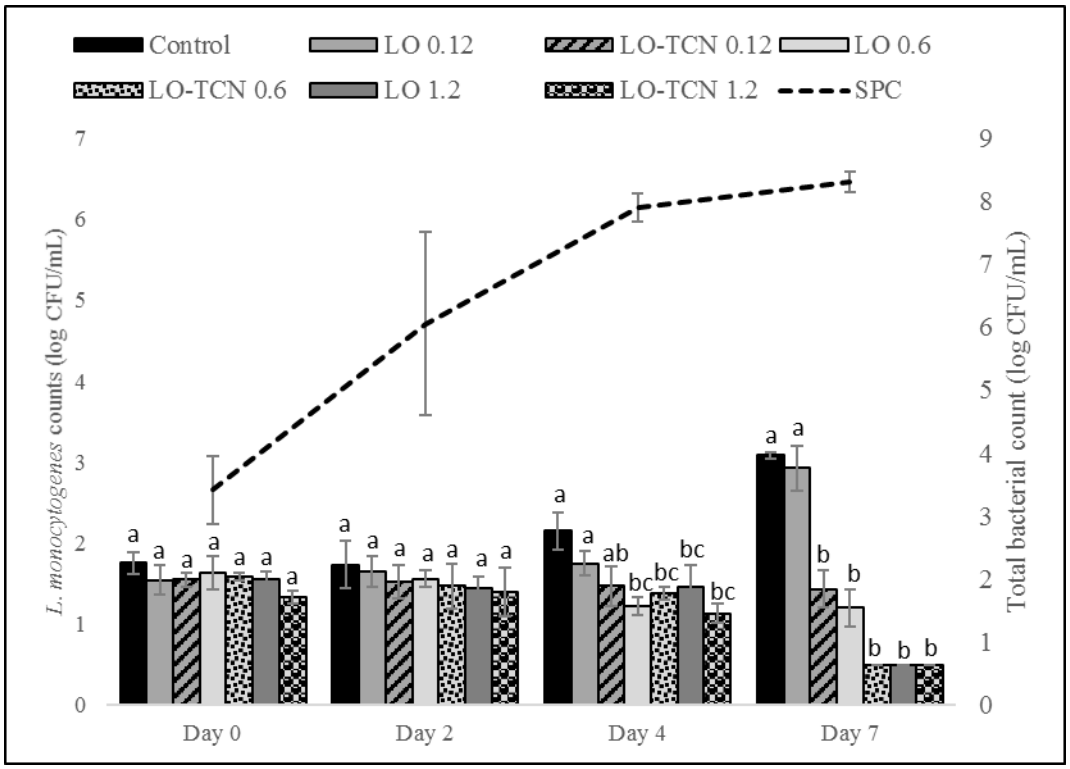

Figure 7a. Total bacterial growth curve in raw milk. The 2 $\log_{10}$ CFU/mL inoculum of *L. monocytogenes* in raw milk was plotted for comparison. Error bars represent the SD.

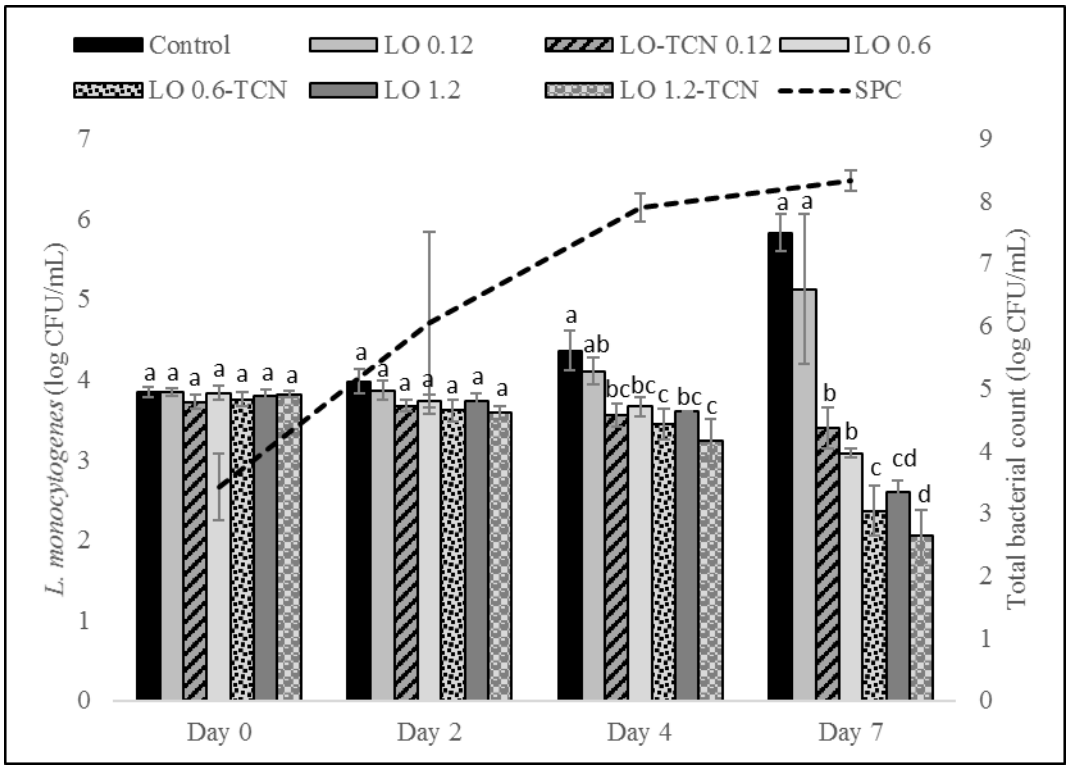

Figure 7b. Total bacterial growth curve in raw milk. The 4 $\log_{10}$ CFU/mL inoculum of *L. monocytogenes* was plotted for comparison. Error bars represent the SD.

At low concentrations, LO efficacy was reduced in raw milk, but the supplementation with TCN restored some efficacy. For example, the impact 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 impact H_2O_2 such as the presence of background microbiota that in essence divert the activity of H_2O_2 away from *L. monocytogenes*. There may also be species in the raw milk that produce a sufficient level of catalase to degrade the H_2O_2 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/L, the 0.12 g/L of 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, 2019). H_2O_2 activates the lactoperoxidases system (LPS) which uses H_2O_2 to oxidize TCN, which 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 H_2O_2 produced by LO is being lost. With supplementation TCN there is more substrate for lactoperoxidase to produce antimicrobial compounds and thus inhibit *L. monocytogenes.* This also suggests the mechanism of *L. monocytogenes* inhibition by LO in UHT milk vs 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 day 4 of the trial. By day 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 day 4 of storage. The 0.6 LO-TCN, 1.2 g/L LO, and 1.2 g/L 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.

CONCLUSION

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 as a model system for further application in other milk products. Our results show that very low concentrations, 0.006, 0.012, and 0.12 g/L of LO were effective at inhibiting *L. monocytogenes* inoculated at both 2 log CFU/mL and 4 log CFU/mL in UHT skim milk. Each of these treatments had minimal effect on the pH until day 4 of storage; by day 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 lactoperoxidase system in raw milk. Therefore, we increased concentrations of LO and supplemented with sodium thiocyanate. These concentrations proved inhibitory to outgrowth of *L. monocytogenes* during a 1-week 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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CHAPTER 2: EVALUATION OF LACTOSE OXIDASE AS AN ENZYME-BASED ANTIMICROBIAL FOR CONTROL OF *LISTERIA MONOCYTOGENES* IN FRESH CHEESE

ABSTRACT

Listeria monocytogenes is a ubiquitous pathogen that can cause morbidity and mortality in the elderly, immune compromised, and the fetuses of pregnant women. The intrinsic properties of fresh cheese—high aW, low salt content, and near-neutral pH—make it susceptible to *L. monocytogenes* contamination and growth at various points in the production process. The aim of this study was to investigate the effect of lactose oxidase (LO), a naturally derived enzyme that is considered a clean label product, on inhibition of *L. monocytogenes* growth in fresh cheese during various points of the production process. Lab scale queso fresco was produced and inoculated with *L. monocytogenes* at final concentrations of 1 log CFU/mL and 1 CFU/100 mL through inoculation of the milk during the cheese make. Lactose oxidase and lactose oxidase sodium thiocyanate combinations were incorporated into the milk at final concentrations of 0.12 and 0.6 g/L LO and 0.12 and 0.6 g/L LO combined with 14 mg/L sodium thiocyanate (TCN). Surface application was also tested with inoculation of *L. monocytogenes* at concentrations of 200 CFU/g and 2 CFU/g onto the surface of the cheese. Lactose oxidase and lactose oxidase combined with sodium thiocyanate were applied to the surface of the cheese at the same concentrations of the previous experiments utilizing the treatments in the milk. A positive control inoculated with *L.*

monocytogenes and no lactose oxidase treatment and an uninoculated negative control were included for all experiments. When *L. monocytogenes* was inoculated into the milk used for the cheese make, by Day 28, the positive control grew to above 7 log CFU/g while the 0.6 g/L treatment (LO and LO + TCN) fell below the limit of detection (LOD) of 1.3 log CFU/g. In the lower inoculum, the positive control grew to above 7 log CFU/g and the treatment groups fell below the LOD by Day 21 and continued through Day 28 of storage. For surface application, outgrowth occurred with the treatments in the higher inoculum, but some inhibition was observed. In the lower inoculum, the higher LO and LO-TCN concentrations (0.6 g/L) reduced *L. monocytogenes* counts to below the LOD, while the control grew out to above 7 log CFU/g, a > 5 log difference between the control and the treatment. These results suggest that LO could be leveraged as an effective control for *L. monocytogenes* in a fresh cheese.

Key Words: *Listeria*, lactose oxidase, enzymes, queso fresco

INTRODUCTION

Listeria monocytogenes, a ubiquitous, intracellular pathogen (Farber and Peterkin, 1991), has the ability to proliferate at refrigeration temperature and can often contaminate products post-pasteurization (Boor et al., 2017), after which there is no further kill step to prevent ingestion of the pathogen by consumers. *L. monocytogenes* is of particular concern for certain dairy processors due to its ubiquitous presence in the environment and the physical properties of some ready-to-eat (RTE) dairy products. Consumption of products contaminated with *L. monocytogenes* may cause listeriosis, which primarily affects the immune compromised, neonates, and the elderly (Swaminathan and Gerner-Smidt, 2007). *Listeria* infection in pregnant women may lead to sudden abortion, death, or premature delivery of the fetus (Voetsch et al., 2007). In the immune compromised and the elderly, listeriosis can result in gastroenteritis, meningitis, and septicemia. Nearly all cases of listeriosis are thought to be foodborne (Mead et al., 1999) and it is estimated that *L. monocytogenes* is among the top five pathogens that cause loss of quality adjusted life years (9,000). Foodborne listeriosis cases cost the U.S. approximately 2.6 billion dollars annually (Hoffman et al., 2012). The incidence of listeriosis varies between 0.1 and 11.3/1,000,000 in various countries and although this incidence is low, the average case-fatality rate of listeriosis, even with adequate antimicrobial treatment, is 20-30% (Swaminathan and Gerner-Smidt, 2007).

The consumption of Hispanic-style cheeses is increasing in popularity in the United States (Hnosko et al., 2009). This upsurge in popularity is likely due to the growth of the Hispanic population in the U.S., which reached 18.4% of the total population in 2019 (United States Census Bureau, 2019). Queso fresco, the most popular Hispanic-style fresh cheese (Van Hekken et al., 2017), is a rennet coagulated cheese with intrinsic properties such as high moisture content, low salt content, and near-neutral pH (Hnosko et al., 2009), that make it susceptible it to *L. monocytogenes* outgrowth when contaminated. According to the Centers for Disease Control and Prevention (CDC), there were eight foodborne outbreaks associated with *L. monocytogenes* contamination in cheese products between 2014 and 2018. The outbreaks included products such as quesarito (fresh cheese) curd (Centers for Disease Control and Prevention, 2014), soft raw milk cheese (Centers for Disease Control and Prevention, 2017), and most recently, in queso fresco (Centers for Disease Control and Prevention, 2021). According to the National Outbreak Reporting System (NORS), there have been 5 outbreaks, 46 illnesses, 34 hospitalizations, and 2 deaths associated with *L. monocytogenes* contamination in queso fresco in the United States between 1971 and 2018 (Centers for Disease Control and Prevention, 2018), which demonstrates that an effective control strategy is necessary.

There have been studies dedicated to controlling *L. monocytogenes* in queso fresco using a range of processing and antimicrobial treatments. High-hydrostatic-pressure-processing (HPP) was found to immediately reduce *L. monocytogenes* contamination when queso fresco was treated at 600 MPa and held at a temperature of 20 °C for 3

minutes. However, this method is currently not cost-effective, and significant wheying-off was observed and the queso fresco was found to have different textural and rheological properties when compared to a control at 20 °C (Tomasula et al., 2014). The efficacy of antimicrobial combinations of nisin, lauric arginate, and ε-polylysine (Martínez-Ramos et al., 2020), protective cultures (Lawton et al., 2020), and phage endolysin PlyP100 (Ibarra-Sánchez et al., 2018) have all been explored to inhibit *L. monocytogenes* in queso fresco, demonstrating that finding an effective control method for this pathogen is a priority for the dairy industry. Consumers are currently moving toward a trend of desiring products with "clean labels" (Asioli et al., 2017). Therefore, finding effective antimicrobial agents that fall into the clean label category and are effective at preventing or reducing *L. monocytogenes* contamination in queso fresco would be beneficial to both consumers and producers by preventing further foodborne outbreaks and their associated human and financial costs.

Lactose oxidase (**LO**) is a naturally-derived enzyme produced by a strain of mold called *Microdochium nivale* (Nordkvist et al., 2007) that consumers would likely categorize under the clean-label category. Some cheeses are coagulated enzymatically with rennet (United States. Agricultural Research Service. Dairy Products et al., 1969), therefore, consumers may be primed to view enzymes as a normal occurrence in dairy products. LO oxidizes lactose into lactobionic acid (**LBA**) (Ahmad et al., 2004) with the concurrent reduction of oxygen into hydrogen peroxide (**H_2O_2**) (Nordkvist et al., 2007). The structure of LBA consists of a polyhydroxy gluconic acid that is bonded to a glucose sugar ($C_{12}H_{22}O_{12}$) (Cardoso et al., 2019). It has been shown

to inhibit counts of *L. monocytogenes* in milk when combined with other antimicrobial agents such as nisin and thymol (Chen and Zhong, 2017). Its effect on *L. monocytogenes* in a complex food matrix, such as cheese, when combined with hydrogen peroxide may yield promising results. H_2O_2 has been used in the dairy industry to preserve raw milk and breaks down into nontoxic compounds in solution (Roundy, 1958). It is approved for use at a concentration of 0.05% in solution for the milk used to produce several varieties of cheese, such as Colby, Cheddar, Swiss, and Emmentaler (21 CFR 184.1366). Treatment of fresh cheese with H_2O_2 was found to reduce *L. monocytogenes* counts by 3 log CFU/g when the cheese was exposed to a 10% solution for 5 s (Robinson and D'Amico, 2021) and concentrations of 400 and 800 mg/L were bactericidal on *L. monocytogenes* within 24 h in a previous study (Kozak et al., 2018).

LBA and H_2O_2 have shown to be effective inhibitors of *L. monocytogenes* in dairy products, demonstrating that the production of these antimicrobial agents by LO itself may be an effective strategy to control *L. monocytogenes* in cheese. In a previous inhibition assay study, LO combined with sodium thiocyanate (**TCN**) was shown to inhibit growth of *L. monocytogenes* (Lara-Aguilar and Alcaine, 2019b). Sodium thiocyanate combined with hydrogen peroxide has been shown to be an effective activator of the lactoperoxidase system, a natural antimicrobial system present in raw milk (Kussendrager and van Hooijdonk, 2000, FAO, 2006). In our previous study, LO and LO-TCN combinations were shown to inhibit *L. monocytogenes* in UHT skim milk and raw milk (Flynn, B. et al., 2021 in review).

The efficacy of LO at inhibiting *L. monocytogenes* in a complex food matrix such as fresh cheese has not yet been investigated. The purpose of this study was to test multiple methods that LO could be utilized, and the use of a laboratory-scale cheese model allowed various *L. monocytogenes* contamination scenarios and applications of LO and LO-TCN combinations to be explored. The first part of this study looks at the scenario in which the milk was contaminated with *L. monocytogenes* and looked at the efficacy of adding either LO or LO-TCN to the milk prior to the cheese make on the subsequent outgrowth of *L. monocytogenes* in the final cheese. The second part of this study investigates scenarios in which the surface of the queso fresco is contaminated by *L. monocytogenes* from the environment, and the subsequent effect of LO and LO-TCN combinations application to the surface to prevent outgrowth. Queso fresco was inoculated onto the surface of the cheese at concentrations of either 200 CFU/g or 2 CFU/g and LO and LO-TCN combinations were then topically applied to the cheese surface. The addition of LO into the milk used for cheesemaking or topical application both represent potential control strategies that could be implemented by cheese makers to reduce the risk of *L. monocytogenes* outbreaks attributed to queso fresco and similar cheeses.

MATERIALS AND METHODS

Preparation of *Listeria monocytogenes* inoculum

A cocktail of *L. monocytogenes* was prepared using five isolates of *L. monocytogenes* (Table 5). The strains, four from fresh cheese outbreaks and one laboratory strain,

were obtained from Dr. Martin Wiedmann's Food Safety Laboratory at Cornell University (Ithaca, NY). Each strain was streaked onto Brain Heart Infusion (BHI) agar (Beckton, Dickinson and Co.) and incubated at 37 °C for 24 hours. An individual colony of each strain from each streak plate was used to separately inoculate 5 mL of BHI broth. Broth cultures were incubated at 37 °C to obtain a concentration of OD = 1.00 (9 log CFU/mL). One mL of each culture was combined to produce a cocktail for inoculation of milk or cheese samples. The *L. monocytogenes* cocktail was serially diluted in phosphate buffer saline (PBS) solution to the appropriate concentration for each experiment. The appropriate serial dilutions of the cocktail were enumerated on BHI agar for each experiment to confirm target inoculum concentrations.

Table 5. Strains of *Listeria monocytogenes* used to produce a cocktail used for inoculation of milk samples.

ID	Outbreak	Source Type	Source Site	Isolate Date	Serotype
FSL-X1-0001	Lab 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

Cheese make

Laboratory-scale queso fresco was produced following previous methods with modifications dependent on the goal of each experiment that will be discussed as follows with modifications from a previously established method (Lawton et al., 2020). Pasteurized, homogenized milk was collected from the Cornell Dairy. Milk (600 mL) was aseptically added to 1 L sterile bottles and brought to 35 °C over the course of one hour. The following were added to each bottle: 1875 µL of a 32% calcium chloride ($CaCl_2$) solution (Dairy Connection Inc., Madison, WI) and 78 µL of double-strength rennet (Chy-Max Extra; Chr. Hansen, Milwaukee, WI). Each bottle was swirled to mix and poured into individual plastic cheese vats. The vats were incubated in a water bath at 35 °C for 65 minutes to promote curd formation. After 65 minutes, the curd was cut and allowed to heal for 10 minutes at 35 °C. The temperature of the water bath was then slowly brought to 43 °C over the course of 30 minutes and the vats were incubated for 30 minutes at this temperature. Then 60 mL of whey were removed from each cheese vat and replaced with 60 mL of a 0.16 g/mL NaCl solution. Each vat was returned to the water bath and incubated at a temperature set at 43 °C for 20 minutes. The whey was then drained using sterile cheesecloth for 1 hour. After drainage, 6 g (±0.2 g) of cheese curd was aseptically transferred into 12-well plates (Corning, Inc., Corning, NY). The curd was then pressed for 16 hours overnight using cheese weights provided from the Cornell Dairy to produce a final curd weight of approximately 5 (±0.2 g) grams.

Application of LO and TCN for inhibition of *L. monocytogenes* in the milk

Cheese was made as described in the earlier section with the following modifications. During the first step, the prepared *L. monocytogenes* cocktail was inoculated into the milk at final concentrations of approximately either 1 log CFU/mL or 1 CFU/100 mL. The bottles were inverted to ensure distribution of the inoculum throughout the milk. Following the one-hour incubation step to heat the milk, either lactose oxidase on its own or a combination of lactose oxidase and sodium thiocyanate (TCN) were added to each inoculated treatment. Separate experiments were performed using the LO treatments on its own or the LO-TCN combined treatments. Lactose oxidase was added into the milk to reach a final concentration of either 0.12 or 0.6 g/L in the milk. Sodium thiocyanate was added into the milk to reach a final concentration of 14 mg/L. A positive control with no added LO was used for each inoculum, and a negative control with no added LO or inoculum was used. The samples were stored at 6 °C for the entirety of the trial. For each of these LO and LO-TCN experiments, batches of cheese were made in triplicate to measure the pH and aW in duplicate throughout the storage conditions of the treatment following the same sampling period as microbiological analysis.

Enumeration of samples for microbiological analysis

For each trial, cheese samples were enumerated for microbiological analysis using the same method. Samples were enumerated on d 0, 1, 2, 4, 7, 14, 21, and 28 for *L. monocytogenes* counts. Each sample was aseptically added to a Whirl-Pak filter bag (Nasco, Fort Atkinson, WI) and a 1:10 (w/v) dilution using PBS was performed. The samples were digested on normal speed for 60 s using a stomacher. The digested

samples were diluted in 9 mL PBS blanks to the appropriate dilution and vortexed. Dilutions were then spread-plated on MOX in duplicate and incubated for 48 h at 37 °C. *L. monocytogenes* counts were taken after 48 h of incubation. Each experiment was performed at least in triplicate.

Preparation of LO and TCN solutions for surface application

The LO and TCN solutions for surface application were prepared as follows. A 16% lactose solution was combined with 5 mL of a 0.24 g/L LO solution and filter sterilized. A 1.2 g/L LO solution was combined with a 16% lactose solution and filter sterilized. Final concentrations of 0.12 g/L LO 8% lactose and 0.6 g/L LO 8% lactose in solution were produced when combined. A 14 mg/L solution of TCN was made and filter sterilized. Each treatment was stored at 6 °C for the entirety of the trial. For each of these LO and LO-TCN surface experiments, batches of cheese were made to measure the pH and aW in duplicate throughout the storage conditions of the treatment following the same sampling period as microbiological analysis.

Surface application of LO and TCN for inhibition of *L. monocytogenes* on cheese surface

Laboratory-scale cheese was produced using the method described in the cheese make section. Following the overnight press, 100 µL of *L. monocytogenes* cocktail was inoculated on the surface of the cheese to obtain final concentrations of approximately 200 and 2 CFU/g. Cheese with no inoculum was included as a negative control. The inoculum was allowed to attach for approximately 45 minutes.

For each experiment, the following treatments were then added to the cheese. For the LO treatments, 100 μL of either the 0.12 g/L LO or 0.6 g/L LO 8% lactose solution was dispensed onto the surface of the cheese. For the TCN treatment, the respective LO solution was dispensed onto the surface of the cheese with 100 μL of TCN solution. For the negative and positive control, 100 uL of sterile MilliQ water was dispensed onto the cheese. Cheese samples were enumerated as described in the previous section.

Statistical Analysis

Each experiment was repeated in triplicate. All statistical analyses were performed using R software (Version 3.5.2, R Development Core Team, Vienna, Austria). Analysis of Variance and Tukey's Honest Significant Difference tests were performed at each time point to determine log differences in *L. monocytogenes* counts between all treatments and the positive control. The same tests were performed at each time point to determine differences in pH and aW values between all treatments and the negative control.

RESULTS AND DISCUSSION

LO application in the milk during the cheese make controls L. monocytogenes outgrowth

Final concentrations of 0.12 and 0.6 g/L LO in solution were added to the milk during the cheese make process to determine their efficacy against *L. monocytogenes* in laboratory-scale queso fresco. *L. monocytogenes* inoculum was added into the milk to

achieve concentrations of 1 log CFU/mL and 1 CFU/100 mL, representing variable contamination scenarios. *L. monocytogenes* can contaminate bulk tank milk samples from non-aseptic sampling of the cow udder or milking equipment (Fedio and Jackson, 1992) where it may then proliferate during the cheese make. This experiment aimed to determine the antimicrobial effect of LO on *L. monocytogenes* contamination that could occur with the incoming milk product used for a cheese make.

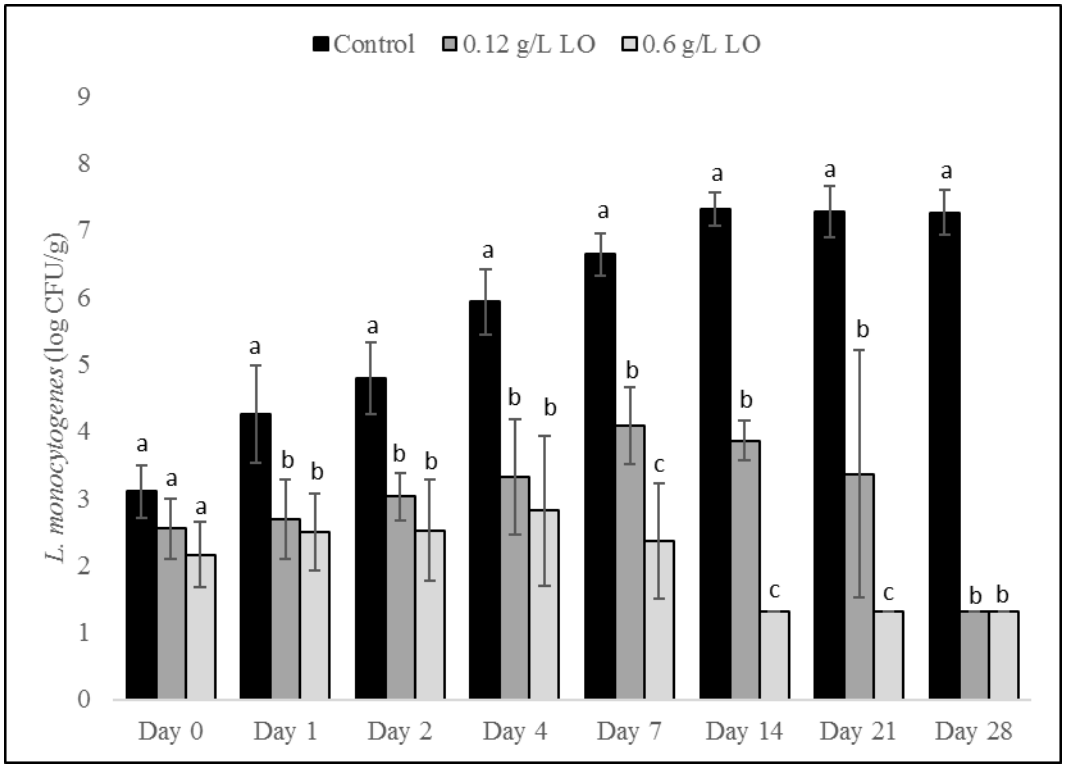

Figure 8. *L. monocytogenes* counts presented as $\log_{10}$ CFU/g in pasteurized queso fresco that was inoculated at 1 $\log_{10}$ CFU/mL in the milk used for the cheese make and treated with lactose oxidase (LO) during storage at 6 °C. Numbers on the treatment label indicate the concentration of LO solution (g/L). Bars with different letters indicate significant differences ($p < 0.05$) between treatments on the same day. For

counts lower than the limit of detection a value of 1.3 $\log_{10}$ CFU/g was used. Error bars represent the SD.

At *L. monocytogenes* inoculation levels of 1 log CFU/mL into the milk, both the 0.12 g/L LO and 0.6 g/L LO treatments showed less outgrowth of *L. monocytogenes* than the control (Figure 8). Inoculation of the milk at a concentration of 1 log CFU/mL resulted in a final concentration of approximately 3 CFU/g in the final cheese control on d 0 of storage. On d 0, there was no significant difference in *L. monocytogenes* counts between the treatments and the control. However, significant differences ($p < 0.05$) between the treatment groups and the control were observed starting at d 1 of storage and continued throughout the entire trial. Both treatments grew out slightly from the initial inoculum, with the 0.12 g/L LO treatment reaching a maximum of 4 log CFU/g on d 7 of storage and the 0.6 g/L LO treatment reaching a maximum of 2.8 log CFU/g on d 4 of storage. After d 7 of storage, the *L. monocytogenes* counts dropped in the treatment groups while the control presented outgrowth. On d 14, 21, and 28 the control grew to above 7 log CFU/g, while the 0.6 g/L LO treatment fell below the limit of detection (LOD) of 1.3 log CFU/g. The *L. monocytogenes* counts for the 0.12 g/L treatment dropped starting on d 14 and fell below the LOD on d 28 of storage. On d 14, 21, and 28 a listericidal effect was observed using the 0.6 g/L LO treatment and on d 28 the same effect was present with the 0.12 g/L treatment because the treatment groups were reduced to levels below the initial inoculum.

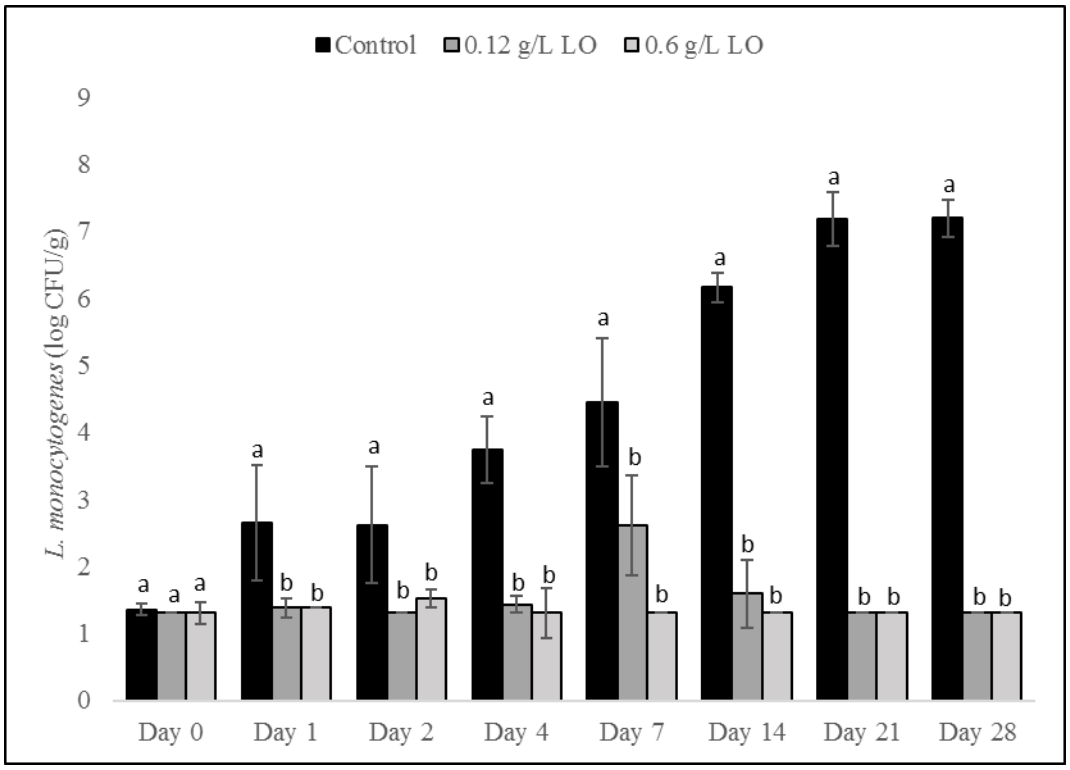

Figure 9. *L. monocytogenes* counts presented as $\log_{10}$ CFU/g in pasteurized queso fresco that was inoculated at 1 CFU/100 mL in the milk used for the cheese make and treated with lactose oxidase (LO) during storage at 6 °C. Numbers on the treatment label indicate the concentration of LO solution (g/L). Bars with different letters indicate significant differences ($p < 0.05$) between treatments on the same day. For counts lower than the limit of detection a value of 1.3 $\log_{10}$ CFU/g was used. Error bars represent the SD.

When *L. monocytogenes* was inoculated into the milk at a concentration of 1 CFU/100 mL, counts for both treatments and the control did not reach above the LOD (1.3 log CFU/g) until d 1 of storage (Figure 9). Both treatments inhibited *L. monocytogenes* outgrowth throughout the entirety of the trial. The 0.12 g/L treatment reached a

maximum concentration of approximately 2.5 CFU/g on d 7 of storage, while the control reached a level above 4 log CFU/g on that day. Except for d 7, both treatment groups averaged below 2 CFU/g throughout the trial. On d 21 and 28, the control reached levels above 7 log CFU/g while both treatment groups fell below the LOD.

These results suggest that the production of H_2O_2 by LO is sufficient to inhibit *L. monocytogenes* counts at different concentrations in queso fresco. A previous study (Lara-Aguilar and Alcaine, 2019b) tested the effect of LO as an antimicrobial against *L. monocytogenes* using an overlay inhibition assay and microbial inhibition was observed. Catalase was added to the treatments, and *L. monocytogenes* growth then occurred, suggesting that a primary cause of bacterial inhibition was production of H_2O_2 by the LO reaction in solution. In both contamination scenarios, LO inhibited or reduced *L. monocytogenes* outgrowth throughout the entire trial. The application of hydrogen peroxide to reduce *L. monocytogenes* counts has been shown to be effective in a variety of products, such as mung bean sprouts (Trzaskowska et al., 2018), organic fresh lettuce (Back et al., 2014), milk (Kozak et al., 2018), and high moisture soft cheese (Robinson and D'Amico, 2021). Robinson and D'Amico (2021) found that treatment of queso fresco with a 10% H_2O_2 solution significantly reduced *L. monocytogenes* counts by 2.27 log CFU/g in the first 30 minutes of treatment and by approximately 0.5 log CFU/g after the first 24 h of storage, with no regrowth after storage. Kozak (et. al 2018) found that in milk, a 400 mg/L H_2O_2 solution was bactericidal against *L. monocytogenes*. Our results utilizing H_2O_2, a product of LO in

dairy applications, as an antimicrobial for *L. monocytogenes* growth are consistent with these previous studies.

Impact of LO addition to milk used to produce queso fresco on pH

Significant differences were observed starting at d 1 of storage between the LO treatment groups and the control and these differences continued until day 28 of the experiment (Table 6). By d 2 of the experiment the control, 0.12 g/L LO, and 0.6 g/L LO treatment groups were all statistically significant from one another. This trend lasted throughout the entirety of the trial. By the end of the trial the pH of the treatment groups were 0.57 and 1.02 pH units lower than the control for the 0.12 and 0.6 g/L treatments, respectively.

Table 6. pH (±SD) of pasteurized queso fresco treated with lactose oxidase during storage at 6°C.

	Time (d)							
Treatment[1]	0	1	2	4	7	14	21	28
Control	6.36 ± 0.08[a]	6.32 ± 0.03[a]	6.29 ± 0.04[a]	6.31 ± 0.01[a]	6.32 ± 0.02[a]	6.33 ± 0.02[a]	6.30 ± 0.00[a]	6.40 ± 0.10[a]
LO 0.12 g/L	6.29 ± 0.05[a]	6.21 ± 0.04[b]	6.18 ± 0.01[b]	6.15 ± 0.04[b]	6.08 ± 0.04[b]	5.94 ± 0.02[b]	5.93 ± 0.10[b]	5.83 ± 0.07[b]
0.6 g/L	6.25 ± 0.03[a]	6.14 ± 0.04[b]	6.02 ± 0.03[c]	5.94 ± 0.07[c]	5.83 ± 0.06[c]	5.69 ± 0.05[c]	5.59 ± 0.07[c]	5.38 ± 0.15[c]

[a,b,c] Means within a column with different letters are significantly different ($p < 0.05$) between treatments

[1] LO = lactose oxidase; n = 8

The drop in pH observed in this study is likely due to the oxidation of lactose into LBA from the addition of LO into the queso fresco product. In this cheese make no starter cultures were added, and while there may be some background lactic acid bacteria in the pasteurized milk, the steady pH of the control cheese (Table 6) suggests that bacterial acidification is not the driver of the pH drop in the samples. LBA is comprised of one galactose molecule that is attached to one molecule of gluconic acid via an ether-like linkage. The use of LBA has been investigated in the dairy industry as a key ingredient in novel dairy technologies (Alonso et al., 2013). A study showed that LBA exhibited antimicrobial properties against both Gram-negative and Gram-positive bacteria in pasteurized whole milk (Kang et al., 2020). Our previous study (Flynn, 2021) showed that a pH drop was exhibited in milk at low concentrations from the addition of LO. Our current results are consistent with this data. Further sensory analysis should be explored to determine the consumer perception of the pH drop in queso fresco. Sensory analysis was performed with the addition of LBA to whole milk (Kang et al., 2020) and it delayed the deterioration of sensory qualities in the milk. Therefore, although a pH drop is present in our study, there could be a potential to use LO as a biopreservation method in further studies to reduce spoilage in cheese, as well as its application to reduce pathogenic growth. Furthermore, the pH reduction over time may also explain why *L. monocytogenes* counts fell below the LOD in the microbiological study by d 14 of storage for the higher concentration treatment (0.6 g/L LO) and by d 28 of storage of the lower concentration treatment (0.12 g/L LO).

A previous study (Engstrom et al., 2020) evaluated the effect of different acids on the outgrowth of *L. monocytogenes* in queso fresco. Dependent on the acid type, moisture content, and salt content of the cheese, the addition of certain acids inhibited *L. monocytogenes* outgrowth over an 8-week period. When acetic acid and propionic acid were added to the cheese to produce final pH values of 5.25-5.75, at all moisture content and salt content percentages, there was no weekly growth of *L. monocytogenes* in the cheese. When lactic acid was added, outgrowth was observed at pH levels above 5.25, with moisture content of 50-56%, and an NaCl concentration of 1.25%. In our study, the pH of the 0.6 g/L LO treatment dropped to 5.69-5.38 (d 14 and d 28) and *L. monocytogenes* counts fell below the LOD in both challenge studies. When the pH reached 5.83 on d 28 of storage, the 0.12 g/L LO treatment also fell below the LOD. It is thus possible that the drop in pH due to LBA production, particularly in the 0.6 g/L treatment, also played a role in controlling *L. monocytogenes* outgrowth.

Addition of LO into the milk during the cheese make does not affect water activity

The water activity of the control and treatment groups remained consistent throughout the entirety of the experiment (Table 7). The aW remained at 0.97 for all treatments throughout the whole trial, therefore, there was no significant difference between the control and the treatment groups. The standard aW value for queso fresco is 0.98 (Trmčić et al., 2017), our results are consistent with this standard. Therefore, from the results provided by this study, the addition of LO to milk does not influence the water activity of queso fresco.

Table 7. aW (±SD) of pasteurized queso fresco treated with lactose oxidase during storage at 6°C.

	Time (d)							
Treatment[1]	0	1	2	4	7	14	21	28
Control	0.97 ± 0.00[a]	0.97± 0.01[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]
LO 0.12 g/L	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97± 0.00[a]	0.97 ± 0.00[a]	0.97± 0.00[a]
0.6 g/L	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]

[a] Means with columns with the same letter (a) are not significantly different ($p > 0.05$) between treatments.

[1] LO = lactose oxidase; n = 8

Addition of LO-TCN into the milk during the cheese make controls L. monocytogenes outgrowth

LO addition into the milk alone showed antimicrobial properties on *L. monocytogenes* in queso fresco over time. Since LO alone was shown to have a bacteriostatic effect, we then explored the addition of sodium thiocyanate (TCN) combined with LO to investigate if this combination had further antimicrobial properties. In our previous study (Flynn et al., 2021), LO-TCN combinations inhibited *L. monocytogenes* in raw milk more effectively than LO alone. Therefore, these concentrations were utilized to investigate the effect against *L. monocytogenes* in lab scale queso fresco.

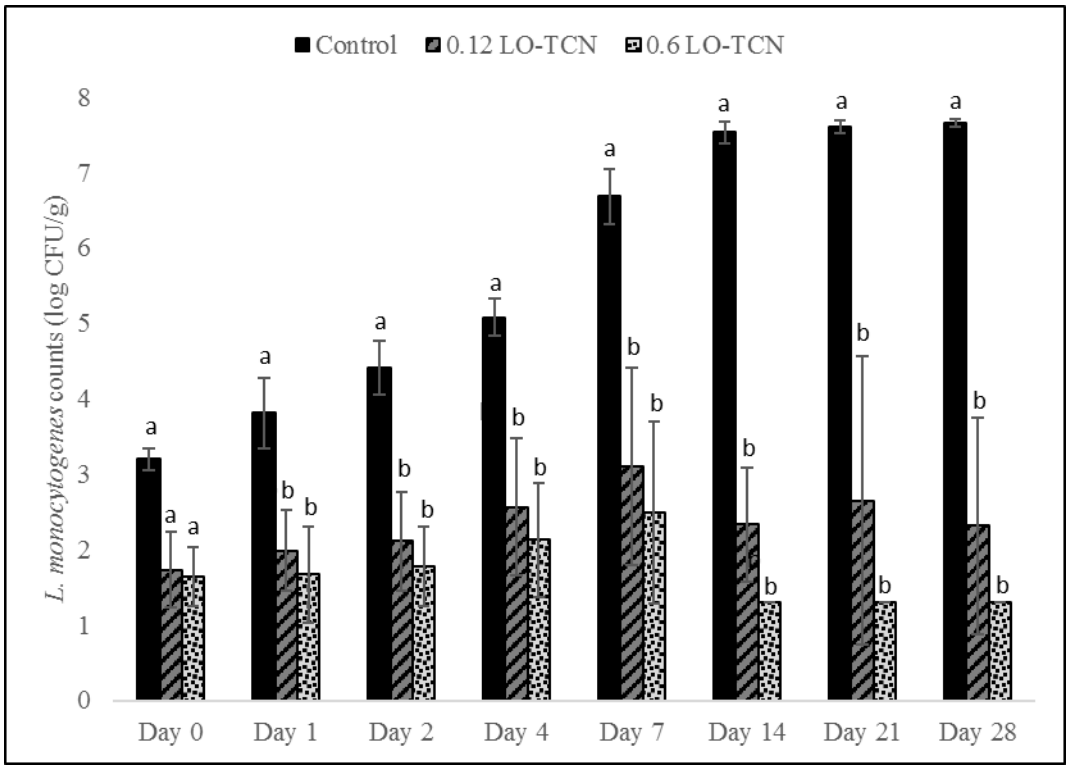

Figure 10. *L. monocytogenes* counts presented as $\log_{10}$ CFU/g in pasteurized queso fresco that was inoculated at 1 $\log_{10}$ CFU/mL in the milk used for the cheese make and treated with lactose oxidase (LO) and 14 mg/L sodium thiocyanate (TCN) during storage at 6 °C. Numbers on the treatment label indicate the concentration of LO solution (g/L). Bars with different letters indicate significant differences ($p < 0.05$) between treatments on the same day. For counts lower than the limit of detection a value of 1.3 $\log_{10}$ CFU/g was used. Error bars represent the SD.

LO-TCN treatments displayed a similar level of growth as in our LO experiments when the milk during the cheese make was inoculated with an *L. monocytogenes* cocktail at a final concentration of 1 log CFU/mL (Figure 10). Except for d 0, during the entirety of the trial, both the low (0.12) and high (0.6) g/L LO treatments displayed

significant differences ($p < 0.05$) in *L. monocytogenes* outgrowth in comparison to the control. The 0.12 and 0.6 g/L LO treatments reached the highest level of outgrowth on d 7, where they reached levels of approximately 3.1 and 2.5 log CFU/g, respectively. These levels were reduced throughout the rest of the trial. By d 14 of storage and continuing to d 28, the *L. monocytogenes* counts for the 0.6 g/L treatment dropped below the LOD, while the control displayed outgrowth to above 7 log CFU/g.

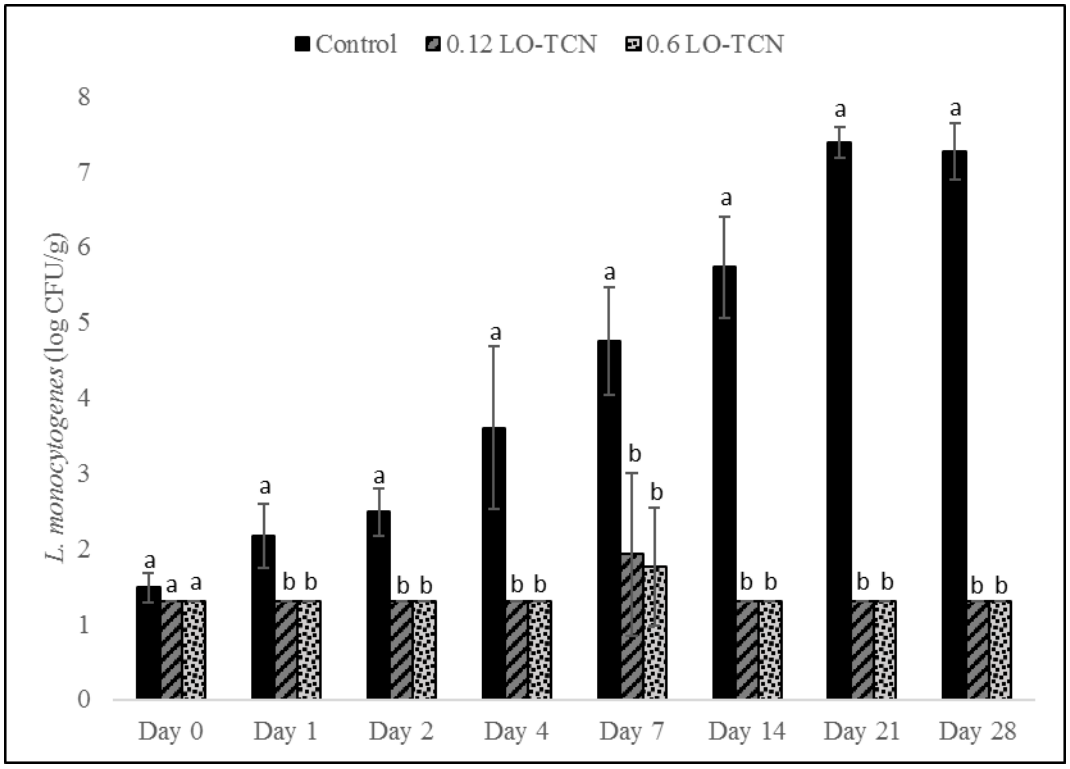

Figure 11. *L. monocytogenes* counts presented as $\log_{10}$ CFU/g in pasteurized queso fresco that was inoculated at 1 CFU/100 mL in the milk used for the cheese make and treated with lactose oxidase (LO) and 14 mg/L sodium thiocyanate (TCN) during storage at 6 °C. Numbers on the treatment label indicate the concentration of LO solution (g/L). Bars with different letters indicate significant differences ($p < 0.05$)

between treatments on the same day. For counts lower than the limit of detection a value of 1.3 $\log_{10}$ CFU/g was used. Error bars represent the SD.

At the lower challenge level of 1 CFU/100 mL, supplementation with TCN resulted in lower *L. monocytogenes* outgrowth (Figure 11) in comparison to treatment with LO alone. The treatment groups (0.12 and 0.6 g/L LO-TCN) fell below the LOD on all days except for d 7. By d 21 and 28 of the experiment, the control grew out to levels above 7 log CFU/g, while the treatment groups only reached the LOD of 1.3 log CFU/g.

The addition of TCN into raw milk activates the lactoperoxidase system (LPS), a natural antimicrobial system that is present in raw milk. The LPS is comprised of 3 components: hydrogen peroxide, thiocyanate, and lactoperoxidase. Lactoperoxidase catalyzes the oxidation of thiocyanate by hydrogen peroxide, which generates compounds like hypothiocyanite ions, which act as antimicrobials. Efficacy of the LPS varies and relies on the concentration of thiocyanate and hydrogen peroxide. Thiocyanate in raw milk is present in close to optimal concentrations, but hydrogen peroxide must be added by other means, such as the addition of LO, to optimize the effect of the LPS in dairy products (Kussendrager and van Hooijdonk, 2000).

The concentration of lactoperoxidase in bovine raw milk is 1.2-16.2 ppm. In pasteurized milk, lactoperoxidase retains approximately 70% of its residual activity when pasteurized at 72 °C for 15 s and complete deactivation of the enzyme occurs

when milk is pasteurized at 80 °C for 15 s (Barrett et al., 1999). The results for TCN supplementation at both the high (1 log CFU/mL) and low (1 CFU/100 mL) inoculum were similar to that of the LO trials. Therefore, either the LPS produced a slight antimicrobial effect against *L. monocytogenes* in these trials, especially in the lower inoculum, or the inhibition was due to H_2O_2 production by LO.

Impact of LO-TCN addition to milk during the cheese make on pH.

Table 8. pH (±SD) of pasteurized queso fresco treated with lactose oxidase and sodium thiocyanate during storage at 6°C.

	Time (d)							
Treatment[1]	0	1	2	4	7	14	21	28
Control	6.32 ± 0.08[a]	6.34 ± 0.04[a]	6.36 ± 0.01[a]	6.42 ± 0.07[a]	6.41 ± 0.10[a]	6.25 ± 0.10[a]	6.34 ± 0.04[a]	6.33 ± 0.04[a]
LO-TCN 0.12 g/L	6.30 ± 0.03[a]	6.29 ± 0.02[a]	6.24 ± 0.02[a]	6.13 ± 0.15[b]	5.95 ± 0.11[b]	5.88 ± 0.05[b]	5.91 ± 0.18[b]	5.79 ± 0.03[b]
0.6 g/L	6.29 ± 0.04[a]	6.20 ± 0.05[a]	6.11 ± 0.01	5.96 ± 0.09[b]	5.77 ± 0.09[c]	5.86 ± 0.21[b]	5.54 ± 0.07[c]	5.45 ± 0.14[c]

[a,b,c] Means within a column with different letters are significantly different ($p < 0.05$) between treatments

[1]LO-TCN = lactose oxidase; TCN = sodium thiocyanate; n = 8

Significant differences in pH began on d 2 of the trial and the pH was reduced throughout the entirety of the experiments (Table 8). By d 28, the pH of the 0.12 g/L treatment dropped to 5.78 while the 0.6 g/L treatment group dropped to 5.45 and the pH of the control remained at 6.33. The production of lactobionic acid, as discussed

previously, is likely the reason for this pH reduction. The addition of sodium thiocyanate did not prevent a pH reduction throughout the trial.

Addition of LO-TCN to the milk during the cheese make does not produce a change in aW

The data in Table 9 demonstrates that no significant difference ($p > 0.05$) was displayed throughout the entirety of the trial using 0.12 and 0.6 g/L LO-TCN combinations. The aW of the control and treatments fell between 0.97 and 0.98 units during the entire trial, close to the 0.98 industry standard. Therefore, the addition of LO-TCN combinations does not influence water activity, which is promising for further sensory analysis.

Table 9. aW (±SD) of pasteurized queso fresco treated with lactose oxidase and sodium thiocyanate during storage at 6°C.

	Time (d)							
Treatment[1]	0	1	2	4	7	14	21	28
Control	0.97 ± 0.00[a]	0.98± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]
LO-TCN 0.12 g/L	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.98 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97± 0.00[a]	0.97 ± 0.00[a]	0.97± 0.00[a]
0.6 g/L	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.98 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]

[a] Means with columns with the same letter (a) are not significantly different (P > 0.05) between treatments.

[1] LO-TCN = lactose oxidase; TCN = sodium thiocyanate; n = 8

Addition of LO and LO-TCN combinations on the surface of the cheese produce variable effects

L. monocytogenes may contaminate dairy products by contaminating the raw materials (i.e. contaminated or improperly pasteurized milk) used to make products or through the post-pasteurization contamination from the processing environment (Boor et al., 2017). The second part of this study aimed to explore surface contamination with *L. monocytogenes* of queso fresco from the processing environment. Cheese was made in the laboratory scale fashion and *L. monocytogenes* was inoculated onto the surface of the cheese at concentrations of 200 CFU/g or 2 CFU/g and solutions of lactose oxidase or lactose oxidase with sodium thiocyanate were topically applied to examine their effect on surface *L. monocytogenes* contamination.

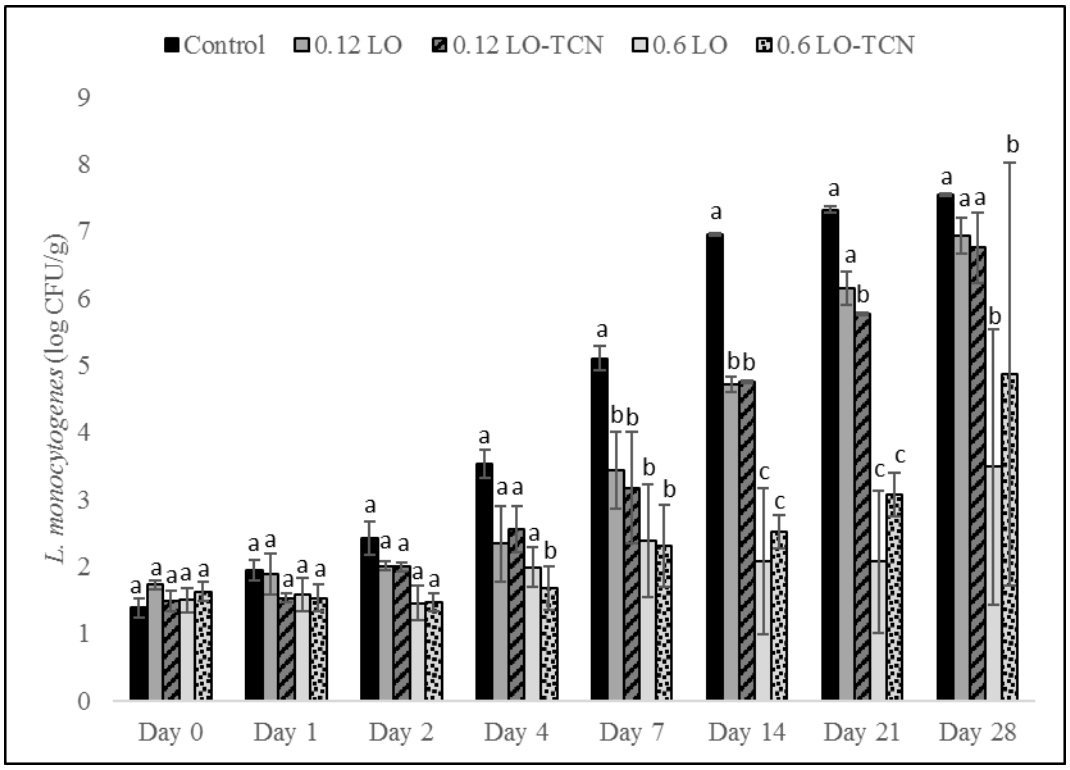

Figure 12**.** *L. monocytogenes* counts presented as $\log_{10}$ CFU/g in pasteurized queso fresco that was inoculated at 200 CFU/g on the surface of the cheese and topically treated with lactose oxidase (LO) and 14 mg/L sodium thiocyanate (TCN) during storage at 6 °C. Numbers on the treatment label indicate the concentration of LO solution (g/L). Bars with different letters indicate significant differences ($p < 0.05$) between treatments on the same day. For counts lower than the limit of detection a value of 1.3 $\log_{10}$ CFU/g was used. Error bars represent the SD.

When samples were inoculated at an *L. monocytogenes* concentration of 200 CFU/g, significant differences between the treatment groups and the control were not observed until d 7 of storage (Figure 12). These differences remained significant throughout the rest of the trial. The greatest antimicrobial inhibition was observed on d 14 of storage,

when the 0.12 g/L treatments fell to below 5 log CFU/g and the 0.6 g/L treatment groups fell below 4 log CFU/g, while the control grew out to above 6.5 log CFU/g. Average levels of outgrowth did not reach that of the control throughout the trial, however, *L. monocytogenes* counts did increase in the treatments throughout storage.

High standard deviations occurred for both 0.6 g/L treatments on d 28 of storage. This was due to variation between trials for both treatment groups. The 0.6 g/L treatment had levels of growth that were below the LOD for one trial and reached 3.8 and 5.4 log CFU/g for the other two trials. The 0.6 g/L LO-TCN treatment had levels of growth that reached 6 to 7 log CFU/g for two of the trials but fell below the LOD for one trial. These large discrepancies could be due to variability in the way LO and TCN treatments were topically applied to each small cheese enumerated per day of storage. When applying LO treatments in the food industry, a more methodical approach to surface application, such as spraying, could be used to ensure the entire surface of the cheese is covered with the treatment. Furthermore, there was no significant difference between topical application of LO alone and LO in combination with TCN in these trials. The LO-TCN treatments exhibited slightly more outgrowth than the LO treatments alone on d 14, 21, and 28.

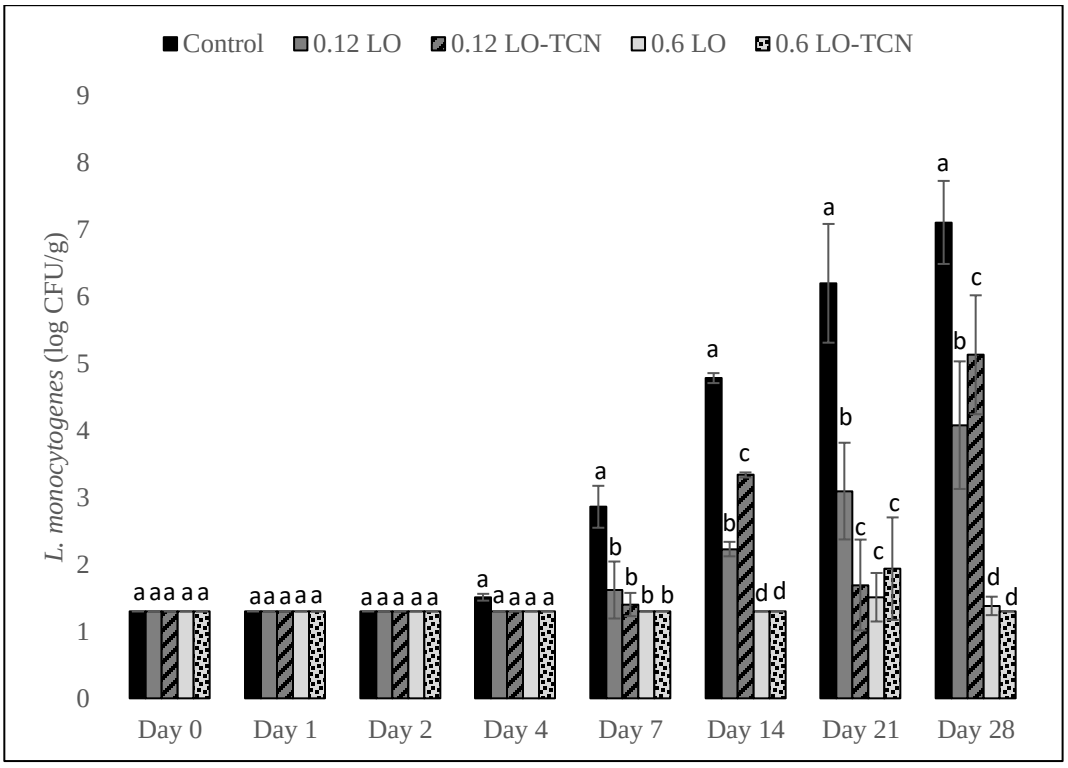

Figure 13. *L. monocytogenes* counts presented as $\log_{10}$ CFU/g in pasteurized queso fresco that was inoculated at 2 CFU/g on the surface of the cheese and topically treated with lactose oxidase (LO) and 14 mg/L sodium thiocyanate (TCN) during storage at 6 °C. Numbers on the treatment label indicate the concentration of LO solution (g/L). Bars with different letters indicate significant differences ($p < 0.05$) between treatments on the same day. For counts lower than the limit of detection a value of 1.3 $\log_{10}$ CFU/g was used. Error bars represent the SD.

No *L. monocytogenes* growth occurred until d 4 of storage in the low inoculum challenge (Figure 13). After d 4, the control exhibited outgrowth while the treatments inhibited growth of *L. monocytogenes*. The 0.12 g/L LO and 0.12 g/L LO-TCN treatments grew out from the original inoculum, however, they still exhibited

significant differences ($p < 0.05$) from the control throughout the storage period and fell approximately 3 log CFU/g below the control on d 21 of storage, before growing out on d 28. The 0.6 g/L LO and 0.6 g/L LO-TCN treatments fell below the LOD on all days except for d 21 and 28 of storage, and on d 21 and 28 only slight outgrowth occurred. By d 28 of storage, these treatments fell more than 5 log CFU/g below the control.

The purpose of this experiment was to study if the outgrowth due to surface contamination by *L. monocytogenes* could be controlled with the topical application of LO and LO-TCN combinations in queso fresco. At higher inoculum levels, outgrowth of *L. monocytogenes* occurred in both the control and the treatment groups, with some inhibition by LO treatment. While we were applying concentrations of 0.12 and 0.6 g/L LO and 14 mg/L TCN concentrations onto the surface of the cheese, the final concentration of components in the cheese is much lower, and thus much lower than the LO and TCN concentrations we used in the earlier experiments where the components were directly added to the milk. The total amount of LO and TCN used in the cheese was calculated as follows. Since each cheese sample was pressed in its own well in a 12-well plate, the use of these wells as cheese molds produced uniform cheeses that had a surface area of 3.8 cm^2. When solutions of 0.6 and 0.12 g/L LO were topically applied, the final concentration of LO solutions was 1.58×10^{-5} and $3.16\ 10^{-6}$ g LO per cm^2 of cheese, respectively. The TCN was added at a concentration of 3.68×10^{-4} mg TCN/cm^2 of cheese. In the final cheese product, LO concentrations were incorporated at 1.20×10^{-5} (0.6 g/L LO solution addition) and $2.40\ 10^{-6}$ (0.12 g/L

LO solution addition) g LO per g of cheese. The TCN solution was incorporated at 2.80 10^{-4} mg TCN per g of cheese when a 14 mg/L solution was topically applied.

While surface application means there is more oxygen available for LO, because of its lower concentration in the cheese, this would have resulted in lower total hydrogen peroxide production by LO in comparison to the treatments where LO was added directly to the milk. Still, at the lower challenge inoculum (2 CFU/g) outgrowth was inhibited by the 0.6 g/L LO and LO-TCN combinations on the surface of the cheese.

Additional supplementation with lactoperoxidase enzyme in pasteurized milk may increase the antimicrobial effect of the LPS, as displayed by a previous study that used LO and the LPS to inhibit spoilage in milk (Lara-Aguilar and Alcaine, 2019a). Future studies should explore the optimization of LO, TCN, and LPS levels to produce the greatest antimicrobial inhibition of *L. monocytogenes* in a laboratory scale queso fresco. Furthermore, future studies should also explore these same concentrations topically applied at concentrations of 0.12 and 0.6 g/L LO total in the cheese.

Impact of LO and LO-TCN combinations on cheese pH

The 0.12 g/L LO and 0.12 g/L LO-TCN combination did not display significant differences ($p > 0.05$) in pH from the control, suggesting that production of LBA was minimal for these treatments when compared to the experiments that added LO into the milk (Table 10). The pH of the 0.6 g/L LO and 0.6 g/L LO-TCN combination displayed significant differences from the control by d 7 of storage and this drop

continued until d 28. By the end of the trial, both treatments were more than 0.20 pH units below the control. The reduced pH of the 0.6 g/L LO and 0.6 g/L LO-TCN treatments may have caused greater antimicrobial reduction when compared to the 0.12 g/L LO and 0.12 g/L LO-TCN combinations.

Table 10. pH ±SD of queso fresco treated with thiocyanate and lactose oxidase during storage at 6 °C.

	Time (d)							
Treatment[1]	0	1	2	4	7	14	21	28
Control	6.36 ± 0.01[a]	6.35 ± 0.03[a]	6.38 ± 0.01[a]	6.33 ± 0.04[a]	6.34 ± 0.02[a]	6.38 ± 0.12[a]	6.31 ± 0.03[a]	6.31 ± 0.03[a]
LO 0.12 g/L	6.8 ± 0.02[a]	6.37 ± 0.02[a]	6.34 ± 0.01[a]	6.34 ± 0.01[a]	6.35 ± 0.03[a]	6.29 ± 0.05[ab]	6.24 ± 0.05[a]	6.23± 0.02[a]
0.6 g/L	6.38 ± 0.03[a]	6.45 ± 0.09[a]	6.35 ± 0.02[a]	6.35 ± 0.02[a]	6.30 ± 0.08[b]	6.19 ± 0.05[b]	6.10 ± 0.05[bc]	6.04 ± 0.01[b]
LO-TCN 0.12 g/L	6.39 ± 0.02[a]	6.46 ± 0.07[a]	6.42 ± 0.01[a]	6.47 ± 0.06[b]	6.36 ± 0.06[a]	6.30 ± 0.06[ab]	6.25 ± 0.03[ab]	6.22 ± 0.02[a]
0.6 g/L	6.41 ± 0.02[a]	6.42 ± 0.05[a]	6.40 ± 0.10[a]	6.34 ± 0.01[a]	6.35 ± 0.10[a]	6.16 ± 0.05[b]	6.09 ± 0.05[c]	6.02 ± 0.03[b]

[a.b,c] Means within a column with different letters are significantly different ($P<0.05$) between treatments

[1] LO = lactose oxidase; TCN = thiocyanate; n = 8.

When compared to the treatments where LO and TCN were added into the milk, there was not as large of a pH reduction when the treatments were applied topically. When the LO treatments were added into the milk used for the cheese make, the pH was reduced to 5.38 and 5.83 for the 0.6 and 0.12 g/L treatments, respectively. When treatments were topically applied, the pH remained above 6.0 throughout the entire trial for all treatments. When various organic acids were added to queso fresco to produce a pH of 6.0 in a previous study, *L. monocytogenes* was able to grow at all moisture and NaCl levels (Engstrom et al., 2020).

Outgrowth was inhibited when the lower inoculum was applied to the surface of the cheese and the 0.6 g/L LO and LO-TCN combinations were topically applied, although the pH remained above 6.0 (Figure 6). Outgrowth was also slowed with the 0.12 g/L LO and LO-TCN treatments. This suggests that either of the antimicrobial products, H_2O_2 and LBA, of LO were sufficient to inhibit *L. monocytogenes* growth at low levels of incidental contamination on a cheese surface.

Addition of LO and LO-TCN combinations does not change the aW of queso fresco

The aW of the treatments when compared to the control did not change throughout the entire trial (Table 11). The aW remained between 0.97-0.98 for both the control and the treatments. Therefore, the LO and LO-TCN combinations do not change the water activity of the cheese over time when topically applied.

Table 11. aW ±SD of queso fresco treated with thiocyanate and lactose oxidase during storage at 6 °C.

	Time (d)							
Treatment[1]	0	1	2	4	7	14	21	28
Control	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.98 ± 0.01[a]	0.97 ± 0.01[a]	0.97 ± 0.01[a]	0.98 ± 0.01[a]
LO 0.12 g/L	0.98 ± 0.01[a]	0.97 ± 0.01[a]	0.97 ± 0.01[a]	0.96 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[ab]	0.97 ± 0.00[a]	0.97± 0.00[a]
0.6 g/L	0.98 ± 0.01[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97± 0.00[a]
LO-TCN 0.12 g/L	0.98 ± 0.00[a]	0.97 ± 0.01[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.01[a]	0.98 ± 0.01[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]
0.6 g/L	0.97 ± 0.00[a]	0.97 ± 0.01[a]	0.97 ± 0.00[a]	0.97 ± 0.00[a]	0.98± 0.00[a]	0.97 ± 0.00[a]	0.97 ± 0.01[a]	0.98 ± 0.01[a]

[a] Means within a column with the same letters are not significantly different ($p > 0.05$) between treatments

[1] LO = lactose oxidase; TCN = thiocyanate; n = 8.

CONCLUSION

In this study, we explored the effect of lactose oxidase on its own and in combination with sodium thiocyanate as method to control *L. monocytogenes* outgrowth in a laboratory scale fresh cheese model. We first explored the inoculation of *L. monocytogenes* and addition of LO and LO-TCN combinations into the incoming raw milk. In these experiments, we determined that LO and LO-TCN combinations inhibit

growth of *L. monocytogenes* in a concentration-dependent fashion. The 0.12 and 0.6 g/L treatments both inhibited *L. monocytogenes* outgrowth, however the higher enzyme concentrations, were able to reduce *L. monocytogenes* to fall below the LOD 21 days faster than the lower concentration. Furthermore, we determined that LO is effective as a listeriostatic control method with both a high (1 log CFU/mL) and a low (1 CFU/100 mL) inoculum in the milk used during the cheese make. These treatments did cause a significant change in the pH of the cheese, which may affect sensory analysis and should be explored further in future studies.

We then explored the efficacy of LO and LO-TCN combinations as a topical application for surface contamination of queso fresco. We made the laboratory-scale fresh cheese in the same manner as in the previous experiment, but inoculated *L. monocytogenes* onto the surface of the cheese and topically applied the treatments. With a higher inoculum, there was outgrowth by d 28 for all of the treatments, with some inhibition throughout the trial. At a lower challenge inoculum (2 CFU/g) there was a reduction in outgrowth and the 0.6 g/L LO and 0.6 g/L LO-TCN combinations remained below the LOD on most days of treatment. The level of initial surface contamination by *L. monocytogenes* in the real world is difficult to know definitely, and obviously varies by the conditions of the event. Our results suggests that surface application of LO, with or without TCN supplementation, could be useful in controlling incidental, low level *L. monocytogenes* from the environment on to the surface of the cheese.

Overall, the aim of this study was to explore a novel method to control *L. monocytogenes* outgrowth in a laboratory-scale queso fresco to improve the safety of high-risk cheeses. In conjunction with good hygienic practices, LO represent a novel tool that cheesemakers could use to improve the safety of their cheeses. Further research is needed to optimize the use of LO and understand the potential synergies with other antimicrobials that can be used to control *L. monocytogenes*.

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CHAPTER 3: IMPROVING THE SAFETY OF ARTISAN DAIRY PRODUCTS BY QUALITATIVE DATA ANALYSIS

INTRODUCTION

The demand for artisanal food products is increasing (Martinez, 2010) and within the artisan category falls artisan dairy products. In 2016, the American Cheese Society estimated that there were over 900 artisinal cheese producers in the United States, and that this number would grow exponentially future years. Many of these producers are small, with limited funds for their business. If underfunded, these artisan cheesemakers may lack the assets necessary to invest in the technical expertise required for food safety training and if they lack proper food safety training and implementation, artisan dairy producers may produce products that are at a higher risk of pathogenic contamination.

Many artisan cheese makers utilize milk produced from their own farm for their products or raw milk, which poses a higher danger to the production of products due to outbreaks associated with *Listeria monocytogenes*, *Camplyobacter jejuni*, *Escherichia coli* O157:H7, *Salmonella* spp., and *Staphylococcus aureus*. Further, some producers may rely on the 60-day aging requirement to inhibit pathogens in raw milk cheeses, which has questionable efficacy at preventing pathogenic outbreaks (D'Amico et al., 2008, U.S. Food and Drug Administration, 2018).

There were multiple outbreaks associated with artisan cheese produced between 2010-2017. These included *E. coli* O157:H7 contamination in raw milk gouda and cheddar produced in 2010 (McCollum et al., 2012), *E. coli* O157:H7 contamination mold-ripened soft cheese produced by Sally Jackson Creamery where *L. monocytogenes* was also isolated in the cheese and the facility in 2010 (Flynn, 2011), in 2013 a widespread outbreak of listeriosis was traced back to Crave Brothers Farmstead of Waterloo, WI (Choi Mary et al., 2014), and in the early Spring of 2017, a listeriosis outbreak was traced back to a raw milk outbreak by Vulto Creamery in Walton, NY (FDA, 2017). These outbreaks demonstrated that knowledge gaps exist among artisan dairy producers that can be filled by formal food safety education.

There is little literature and knowledge regarding the level of food safety awareness and programs in place that implemented by artisan cheese makers. However, a qualitative study found that many artisan producers found food safety programs, such as HACCP, burdensome to implement due to the amount of effort required to build such programs. Also, many of the producers that participated in the study did not have the formal training due to lack of funds and resources to implement these programs (Le et al., 2014), meaning there is a need for formal food safety education among these individuals.

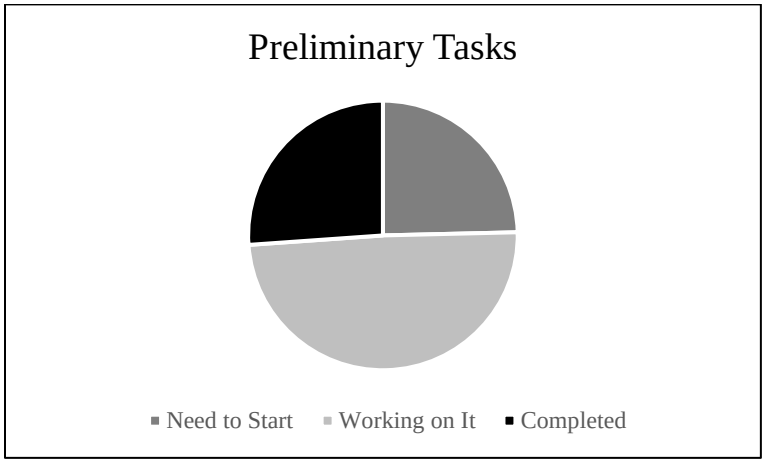
The ultimate goal of this project was to develop a training program to educate artisanal dairy producers on food safety and how to implement food safety programs (ie a Food Safety Plan). To do this, three goals were established. These goals were to execute

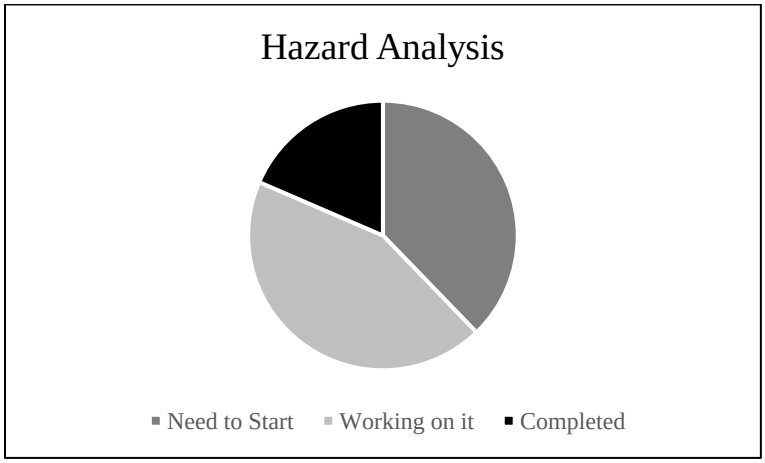
yearly food safety workshops across multiple geographical locations in the United States, determine the impact of food safety training on participant knowledge, behavior change, and food safety plan implementation, and to generate a white paper detailing the current challenges to and opportunities for food safety plan implementation. The results highlighted in this chapter describe the results gathered from the second objective of the project: determination of the impact of this food safety training on participant knowledge, behavior change, and food safety plan implementation.

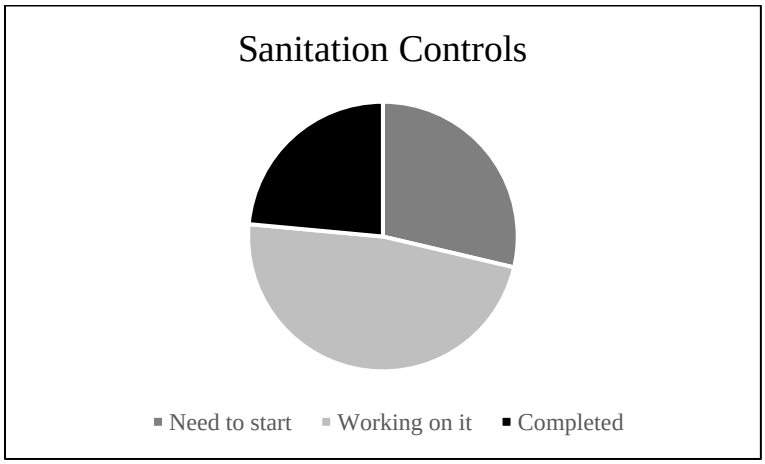
As part of preliminary preparation for the artisan dairy food safety course, participants were asked to fill out a survey. This survey was designed to evaluate participants' pre-workshop food safety knowledge, attitudes to food safety practices, and gauge the current level of food safety implementation at their facility. At the end of the workshop, before participants left, they were asked to fill out a similar survey as the pre-workshop survey to identify changes in food safety knowledge, attitude, and expected changes in food safety plan implementation. The results in the following pages highlight the participants' pre-workshop and post-workshop food safety knowledge, attitudes to food safety practices, and current and expected changes in food safety plan implementation.

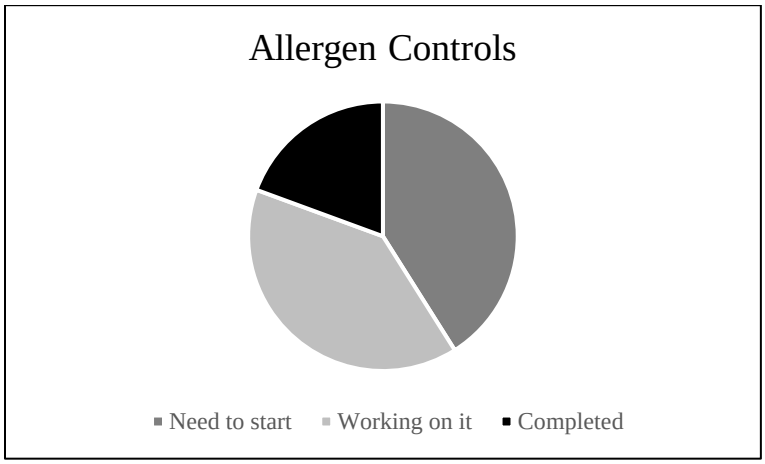
SURVEY DATA ANALYSIS RESULTS

PIE CHARTS





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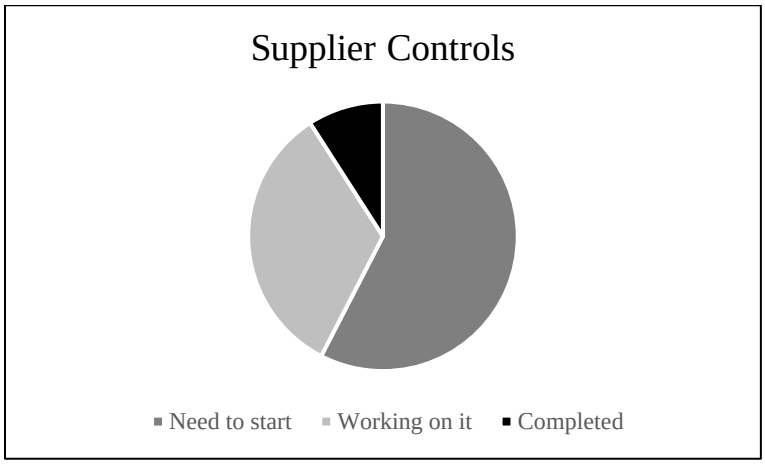
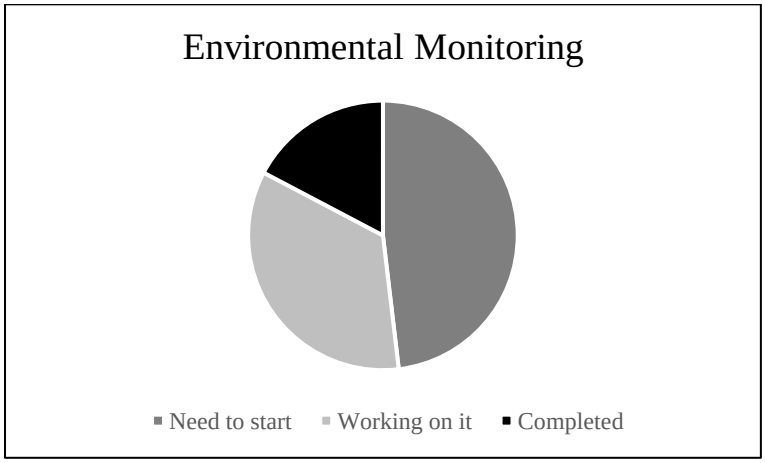
Need to start
Working on it
Completed


Supplier Controls
Need to start
Working on it
Completed


Environmental Monitoring
Need to start
Working on it
Completed

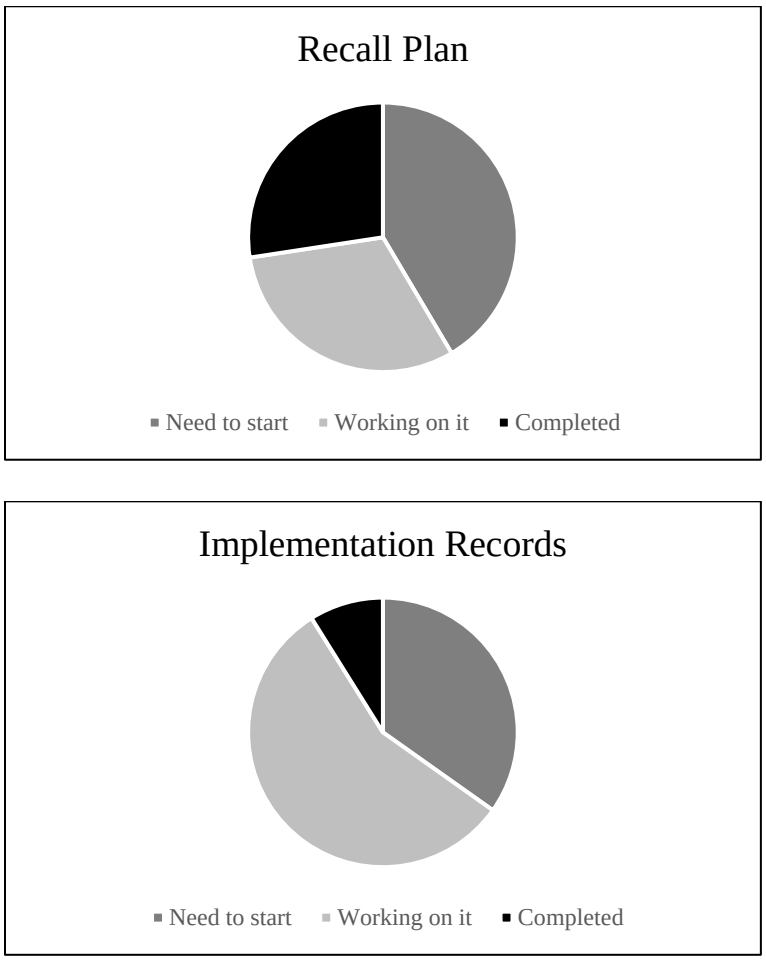


Figure 14. The extent of development that artisan dairy food safety workshop participants had established the different components of their food safety plan, pre-workshop. These components included preliminary tasks, hazard analysis, sanitation controls, allergen controls, supplier controls, environmental monitoring, a recall plan, and implementation records.

TABLES

Behavioral Questions

Table 12. Artisan dairy food safety workshop plan participants' identification of dairy products produced in their facilities, pre-workshop. Participants selected all products that applied to their facilities.

Dairy products produced	*(n = 139)*
Pasteurized milk cheese	82
Raw milk cheese	42
Ice cream	41
Yogurt	28
Other	38
Pasteurized fluid milk	14
Buttermilk	4
Butter	4
Eggnog	2
Heat treated cheese	2
Pudding	1
Flavored milk	1
Kefir	1
Fudge	1
Whey protein	1
Gelato	1

Table 13. Artisan dairy food safety workshop plan participants' identification of food-safety related courses and workshops that they had previously taken, pre-workshop. Participants selected all courses that they had previously taken.

Courses previously taken	*(n = 139)*
Basic food safety	73
Hazard analysis and critical control points (HACCP)	33
Preventive Controls for Human Food (PCQI)	32
The "Food Safety Basics for Artisan Cheesemakers" online course from NC University	23
Good Manufacturing Practices (GMPs)	21
Environmental Monitoring	8
Other	31
ServSafe	8
Milk Quality	3
Cornell Vat Pasteurizer	1
GAP	1

Allergen Awareness	1
NYC Food Handler	1

Table 14. Artisan dairy food safety workshop plan participants' identification of components of their food safety plan they hoped to specifically work on during the workshop, pre-workshop. Participants selected all components they hoped to work on.

Food safety plan components	*(n = 139)*
Hazard analysis	102
Implementation records	95
Sanitation controls	87
Supply chain controls	87
Process controls	84
Environmental monitoring	78
Allergen controls	76
Recall plan	75
Preliminary tasks	63
Other	12
Review of all the above	5
Flowcharts	1
Sense of scale of operations	1
Labeling	1
Food safety instructions to distributors	1
Where to start developing a food safety plan	1

Table 15. Artisan dairy food safety workshop plan participants' identification of biological, chemical, and physical hazards that they were concerned about in their products, pre-workshop. All hazards that participants identified are listed. If participants did not respond or were not concerned they were listed under the "no response/none" category.

Hazards	*(n = 139)*
Biological Hazards	
Pathogens	53
Listeria monocytogenes	41
Salmonella	15
E. coli	14
Staphylococcus aureus	5
Clostridium botulinum	1
Coliforms	1
No response/none	25
Chemical Hazards	
Allergens	30
Cleaners/sanitizers	26
Antibiotics/drug residues	10
Toxins	2
No response/none	73
Physical Hazards	
Metal	19
Glass	8
Plastic	8
Hair	6
Equipment pieces	6
Pests	5
Shells	1
No response/none	92

Table 16. Artisan dairy food safety workshop plan participants' identification of concerns regarding building and implementing their food safety plan. The data in the table displays all concerns participants had. If participants did not respond they were listed under the "no response" category.

Concerns	*(n = 139)*
Correctness of the plan	38
Time to complete the plan	22
Completing the plan	18
Overwhelmed by the scope of the plan	12
Documentation	9
Ensuring employees follow the plan	8
HACCP	3
Environmental monitoring	2

No response	33

Knowledge Questions

Table 17. Artisan dairy food safety workshop plan participants' identification of the required components of a food safety plan, pre-workshop. Participants were told to select all answers that applied.

Required components of a food safety plan	*(n = 139)*
Facility overview and Food Safety Team	89
Product description	89
Hazard analysis	124
Process description	96
Flow diagram	91
Preventive controls	127
Recall plan	117
Procedure for monitoring, corrective actions, and verification	111
No response	12

Table 18. Artisan dairy food safety workshop plan participants' identification of the required components of a food safety plan, post-workshop. Participants were told to select all answers that applied.

Required components of a food safety plan	*(n = 158)*
Facility overview and Food Safety Team	103
Product description	103
Hazard analysis	152
Process description	111
Flow diagram	98
Preventive controls	152
Recall plan	147
Procedure for monitoring, corrective actions, and verification	142
No response	2

Table 19. Artisan dairy food safety workshop plan participants' identification of the three categories of hazards, pre-workshop. All participant responses were recorded. If participants did not answer they were listed under the "don't know/no response" category.

Hazards	*(n = 139)*
Biological	89
Chemical	96
Physical	94
Allergen	7
Environmental	7
People	2
Contamination	2
Ingredients	2
Sanitation	2
Temperature	2
Lack of training	1
Listeria	1
Don't know/no response	33

Table 20. Artisan dairy food safety workshop plan participants' identification of the three categories of hazards, post-workshop. All participant responses were recorded. If participants did not answer they were listed under the "don't know/no response" category.

Hazards	*(n = 158)*
Biological	133
Chemical	132
Physical	134
Allergen	3
People	3
Environmental	11
Bacterial	3
Safety	1
Process	1
Supply chain	1
Sanitation	1
Toxins	1
Don't know/no response	14

Table 21. Artisan dairy food safety workshop plan participants' identification of potential sources of hazards in their facility, pre-workshop. If participants did not answer they were listed under the "don't know/no response" category.

Hazards	*(n = 139)*
People	128
Ingredients	126
Environment	127
Don't know/no response	6

Table 22. Artisan dairy food safety workshop plan participants' identification of potential sources of hazards in their facility, post-workshop. If participants did not answer they were listed under the "don't know/no response" category.

Hazards	*(n = 158)*
People	154
Ingredients	153
Environment	153
Don't know/no response	4

Table 23. Artisan dairy food safety workshop plan participants' identification of bacterial pathogens that represent a significant risk for dairy producers, pre-workshop. If participants did not answer they were listed under the "don't know/no response" category.

Pathogen	*(n = 139)*
Listeria monocytogenes	117
Salmonella	15
E. coli	17
Campylobacter jejuni	2
Coliforms	2
Staphylococcus aureus	1
Mold	1
Don't know/no response	16

Table 24. Artisan dairy food safety workshop plan participants' identification of bacterial pathogens that represent a significant risk for dairy producers, post-workshop. All participant responses were recorded. If participants did not answer they were listed under the "don't know/no response" category.

Pathogen	*(n = 158)*
Listeria monocytogenes	124
Salmonella	19
E. coli	6
Staphylococcus aureus	1
Clostridium botulinum	1

Don’t know/no response	10

Table 25. Artisan dairy food safety workshop plan participants’ identification of an example of a process control, pre-workshop. All participant responses were recorded. If participants did not answer they were listed under the “don’t know/no response” category.

Process control	*(n = 139)*
Pasteurization	58
Temperature monitoring	15
Sanitation	10
Heat treatment	5
Allergen testing	4
Cleaning	2
Cooking	3
Handwashing	1
PPE	1
Environmental monitoring	2
GMPs	1
pH testing	1
Approved supplier program	1
Don’t know/no response	38

Table 26. Artisan dairy food safety workshop plan participants’ identification of an example of a process control, post-workshop. All participant responses were recorded. If participants did not answer they were listed under the “don’t know/no response” category.

Process control	*(n = 158)*
Pasteurization	82
Temperature monitoring	21
Allergen controls	7
Sanitation	6
Labeling	6
Cleaning	4
Monitoring procedures	5
Cooking	3
Metal detection	2
Record keeping	2
Heat treatment	1
Ingredient processing	1
Acidification	1
Boiling	1
Don’t know/no response	31

Table 27. Artisan dairy food safety workshop plan participants' identification of when an environmental monitoring plan is required, pre-workshop. If participants did not answer they were listed under the "don't know/no response" category.

Answer	*(n = 139)*
Always	76
Only if the food product has a kill step	1
If the potential to contaminate the product exists and there is no subsequent kill step to control the hazard	40
If economically feasible	0
Never	5
Don't know/no response	17

Table 28. Artisan dairy food safety workshop plan participants' identification of when an environmental monitoring plan is required, post-workshop. If participants did not answer they were listed under the "don't know/no response" category.

Answer	*(n = 158)*
Always	68
Only if the food product has a kill step	0
If the potential to contaminate the product exists and there is no subsequent kill step to control the hazard	63
If economically feasible	4
Never	9
Don't know/no response	15

Table 29. Artisan dairy food safety workshop plan participants' identification of when a preventive control value needs to be recorded, pre-workshop. If participants did not answer they were listed under the "don't know/no response" category.

Answer	*(n = 139)*
At the time observed	113
Within the shift	0
By the end of the day	2
Don't know/no response	24

Table 30. Artisan dairy food safety workshop plan participants' identification of when a preventive control value needs to be recorded, post-workshop. If participants did not answer they were listed under the "don't know/no response" category.

Answer	*(n = 158)*
At the time observed	136
Within the shift	12
By the end of the day	4
Don't know/no response	6

Table 31. Artisan dairy food safety workshop plan participants' identification of when monitoring records must be reviewed, pre-workshop. If participants did not answer they were listed under the "don't know/no response" category.

Answer	*(n = 139)*
24 hours	34
7 days	48
2 years	5
There is no requirement	29
Don't know/no response	23

Table 32. Artisan dairy food safety workshop plan participants' identification of when monitoring records must be reviewed, post-workshop. If participants did not answer they were listed under the "don't know/no response" category.

Answer	*(n = 158)*
24 hours	24
7 days	114
2 years	3
There is no requirement	0
Don't know/no response	17

Table 33. Artisan dairy food safety workshop plan participants' identification of if calibration records for monitoring equipment should be kept, pre-workshop. If participants did not answer they were listed under the "don't know/no response" category.

Answer	*(n = 139)*
Should be kept, but are not required	15
Are required records for food safety plan	111
Are not needed	0
Don't know/no response	13

Table 34. Artisan dairy food safety workshop plan participant's identification of if calibration records for monitoring equipment should be kept, post-workshop. If participants did not answer they were listed under the "don't know/no response" category.

Answer	*(n = 158)*
Should be kept, but are not required	17
Are required records for food safety plan	130
Are not needed	0
Don't know/no response	11

Table 35. Artisan dairy food safety workshop plan participants' identification of sources useful to support the validation of their preventive controls, pre-workshop. Participants were told to select all answers that applied. If participants did not answer they were listed under the "don't know/no response" category.

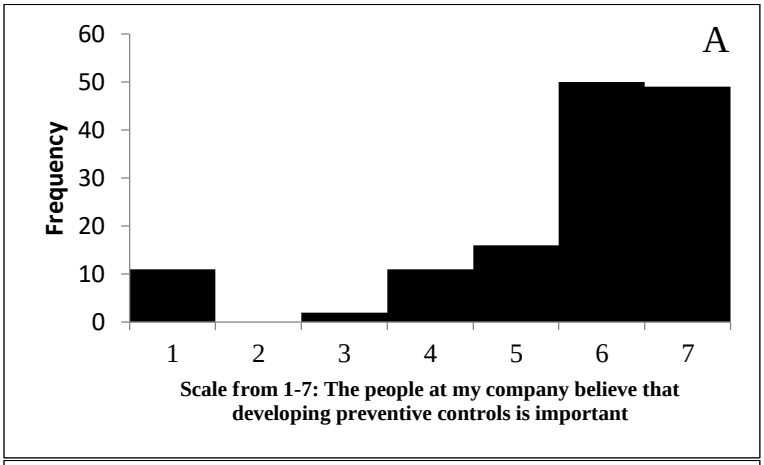
Sources	*(n = 138)*
Peer reviewed scientific literature	90
FDA Hazards Guide	119
Twitter/Facebook	1
Popular magazines	0
Trade association guidance documents (ACS, NDC, etc.)	72
Blogs	1
University cooperative extension	96
websites	14
Don't know/no response	

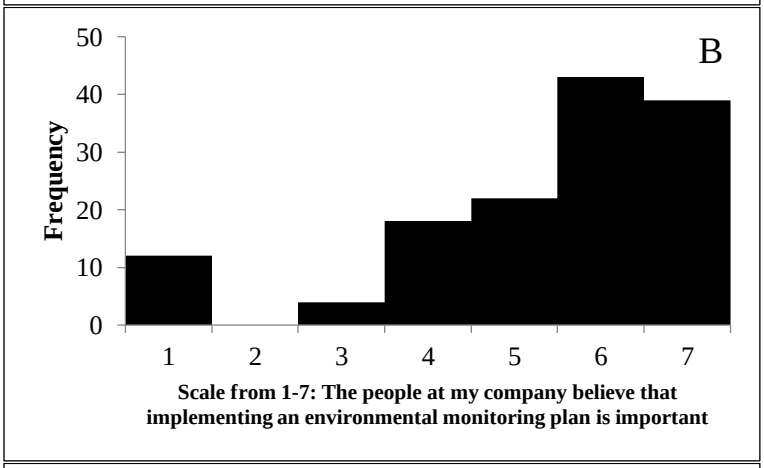
Table 36. Artisan dairy food safety workshop plan participants' identification of sources useful to support the validation of their preventive controls, post-workshop. Participants were told to select all answers that applied. If participants did not answer they were listed under the "don't know/no response" category.

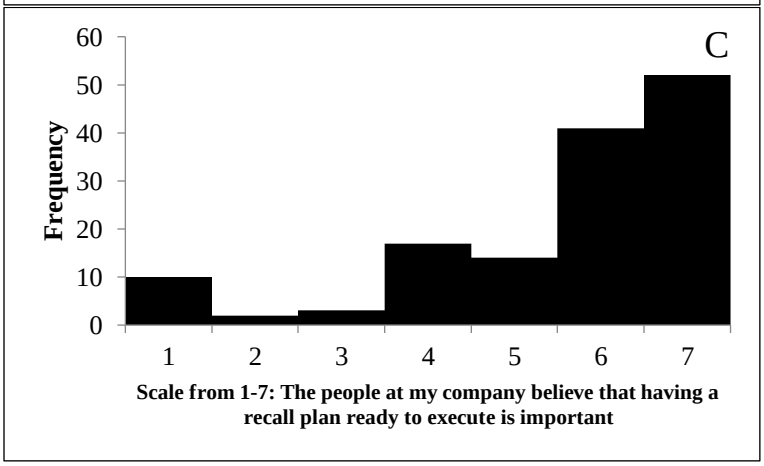
Sources	*(n = 158)*
Peer reviewed scientific literature	124
FDA Hazards Guide	145
Twitter/Facebook	3
Popular magazines	4
Trade association guidance documents (ACS, NDC, etc.)	124
Blogs	4
University cooperative extension	127
websites	10
Don't know/no response	

HISTOGRAMS

Pre-Workshop







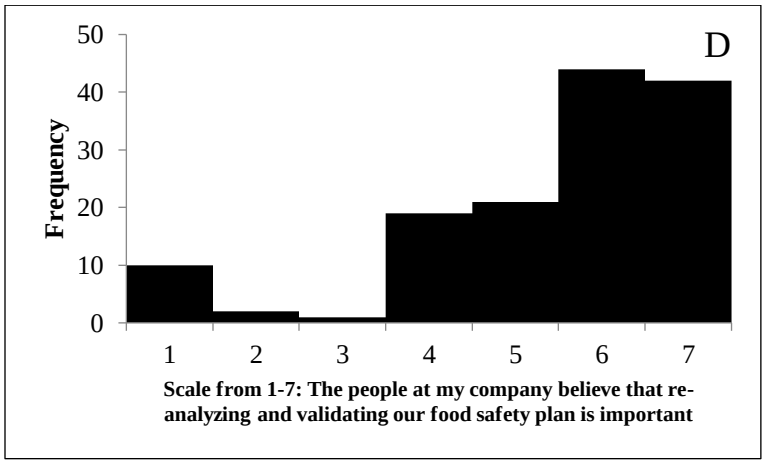


Figure 15. Frequency of how much workshop participants disagreed or agreed with the following statements pre-workshop (1= strongly disagree, 7= strongly agree). (A) the people at my company believe that developing preventive controls is important, (B) the people at my company believe that implementing an environmental monitoring plan is important, (C) the people at my company believe that having a recall plan ready to execute is important, (D) the people at my company believe that re-analyzing and validating our food safety plan is important.

Pre-Workshop and Post-Workshop Histograms

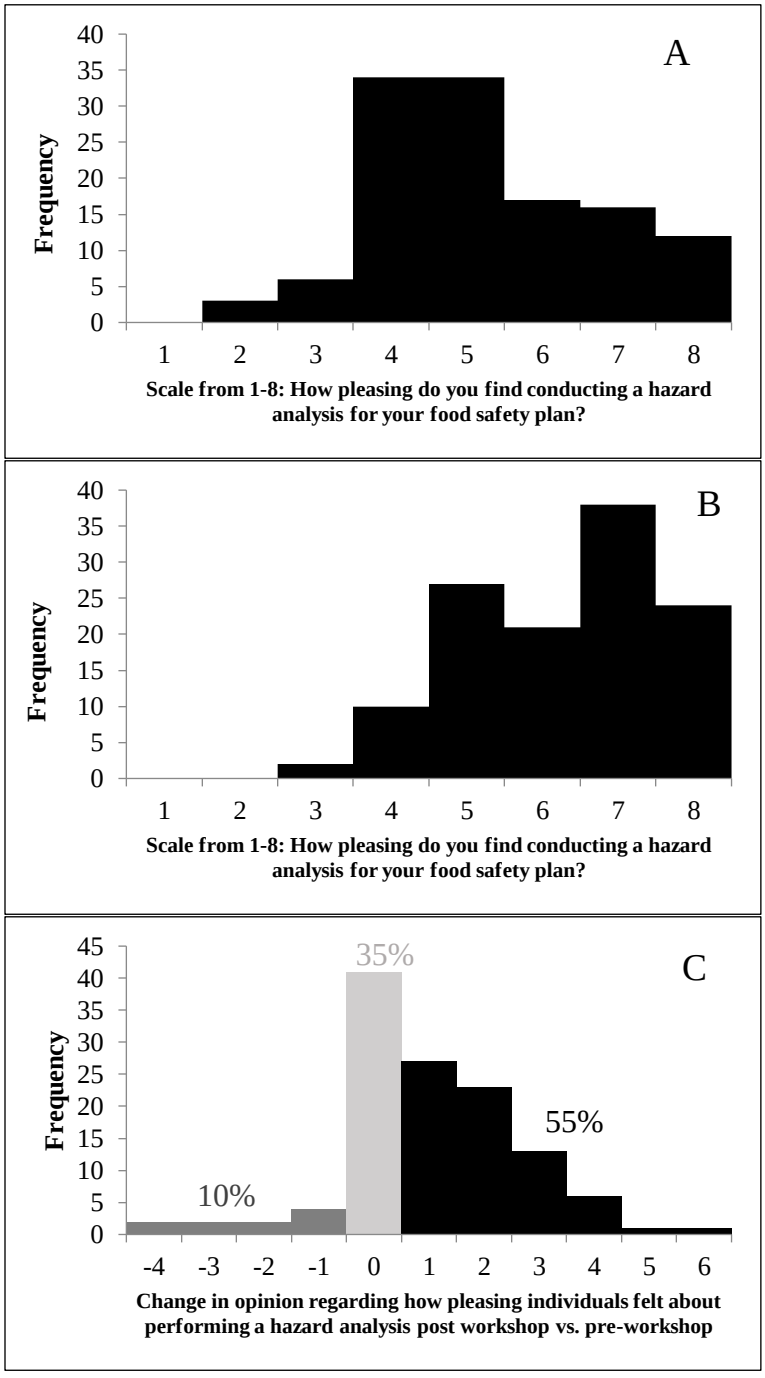


Figure 16. Frequency of how pleasing artisan dairy food safety workshop participants felt about performing a hazard analysis (A) pre-workshop and (B) post-workshop. The

higher the value on the x-axis the more pleasing participants found performing a hazard analysis (1 = extremely displeasing, 7 = extremely pleasing). (C) represents how each individual changed their perspective about performing a hazard analysis when post-workshop values were compared to pre-workshop values. A negative value indicates that individuals perceived completing prerequisite programs as less pleasing post-workshop and a positive value indicates that individuals perceived completing prerequisite programs as more pleasing post-workshop. Percentages indicate the number of individuals who fall into each category (negative change, 0 change, positive change). $p < 0.05$ for A and B. The pre-workshop mean = 5.2 and the post workshop mean = 6.2.

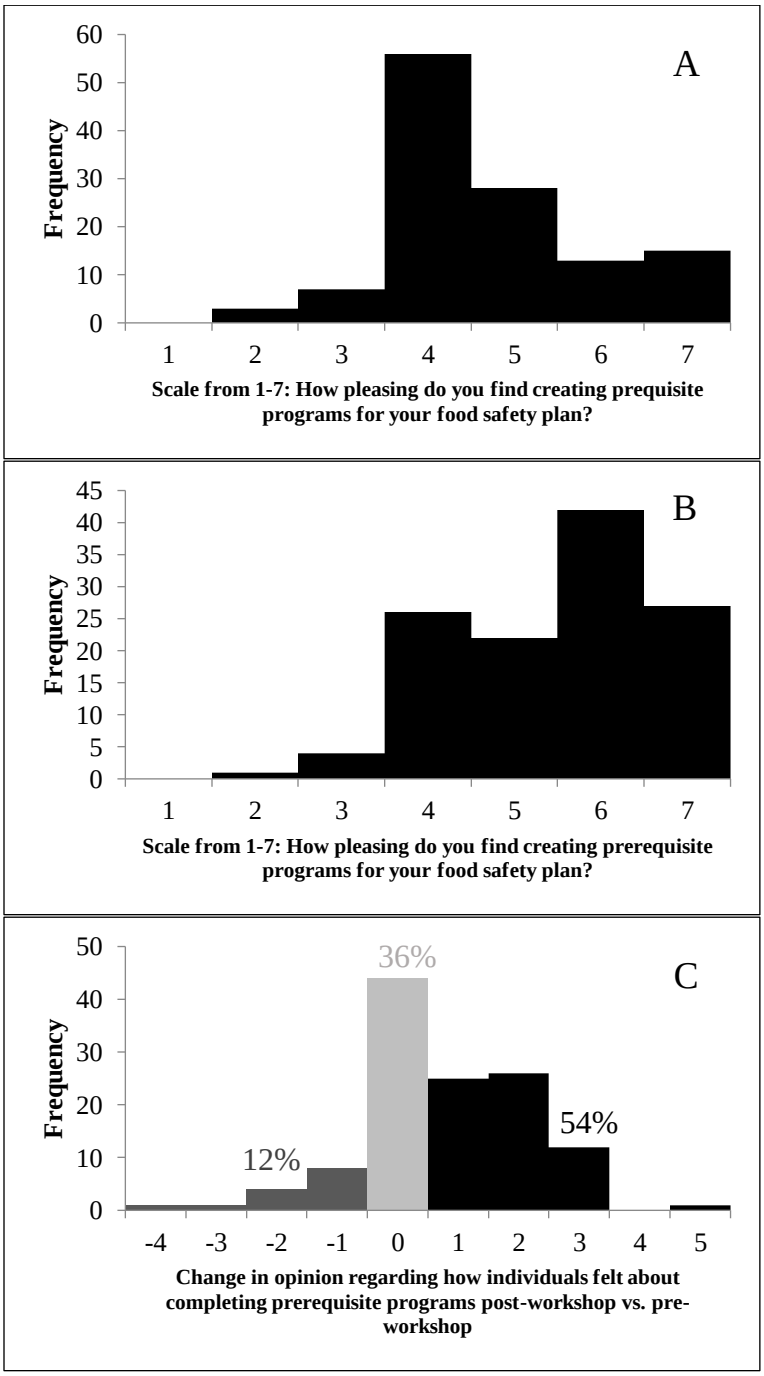


Figure 17. Frequency of how pleasing artisan dairy food safety workshop participants found completing prerequisite programs (A) pre-workshop and (B) post-workshop. The higher the value on the x-axis the more pleasing participants found completing prerequisite program (1= extremely displeasing, 7 = extremely pleasing). (C)

represents how each individual changed their perspective about completing prerequisite programs when post-workshop values were compared to pre-workshop values. A negative value indicates that individuals perceived completing prerequisite programs as less pleasing post-workshop and a positive value indicates that individuals perceived completing prerequisite programs as more pleasing post-workshop. Percentages indicate the number of individuals who fall into each category (negative change, 0 change, positive change). $p < 0.05$ for A and B. The pre-workshop mean = 4.7 and the post workshop mean = 5.5.

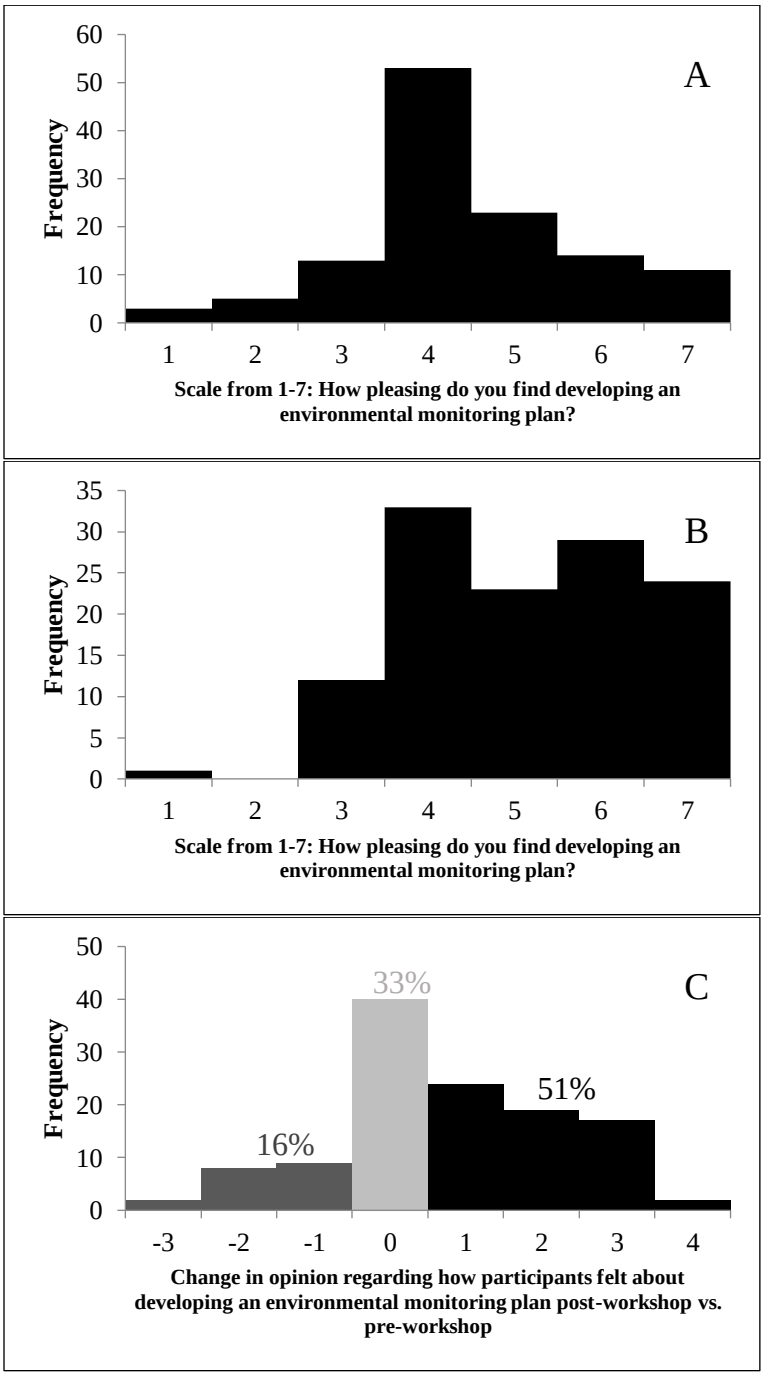


Figure 18. Frequency of how pleasing artisan dairy food safety workshop participants found developing an environmental monitoring program (A) pre-workshop and (B) post-workshop. The higher the value on the x-axis the more pleasing participants found developing an environmental monitoring program (1 = extremely displeasing, 7

= extremely pleasing). (C) represents how each individual changed their perspective about developing an environmental monitoring program when post-workshop values were compared to pre-workshop values. A negative value indicates that individuals perceived developing an environmental monitoring program as less pleasing post-workshop and a positive value indicates that individuals perceived developing an environmental monitoring program as more pleasing post-workshop. Percentages indicate the number of individuals who fall into each category (negative change, 0 change, positive change). $p < 0.05$ for A and B. The pre-workshop mean = 4.4 and the post workshop mean = 5.1.

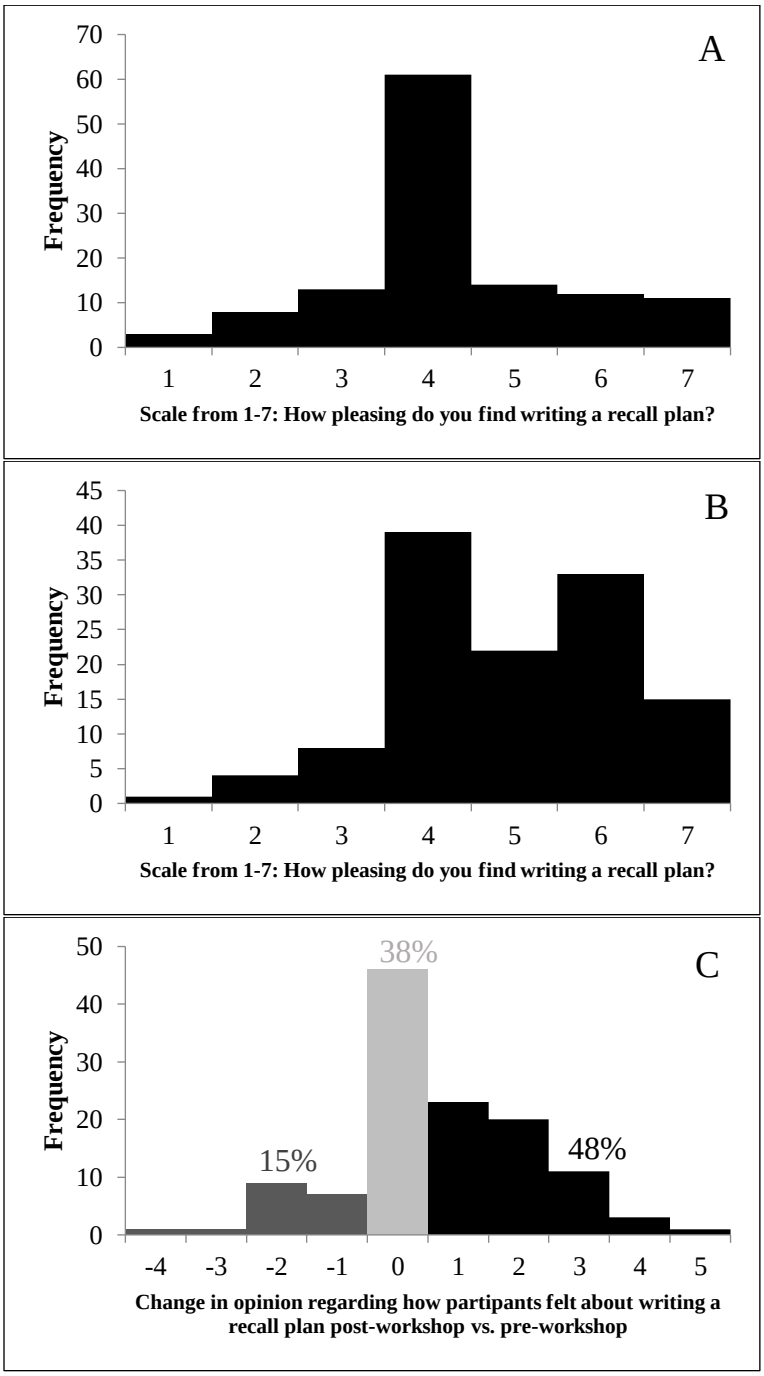


Figure 19. Frequency of how pleasing artisan dairy food safety workshop participants found writing a recall plan (A) pre-workshop and (B) post-workshop. The higher the value on the x-axis the more pleasing participants found writing a recall plan (1 = extremely displeasing, 7 = extremely pleasing). (C) represents how each individual

changed their perspective about writing a recall plan when post-workshop values were compared to pre-workshop values. A negative value indicates that individuals perceived writing a recall plan as less pleasing post-workshop and a positive value indicates that individuals perceived writing a recall plan as more pleasing post-workshop. Percentages indicate the number of individuals who fall into each category (negative change, 0 change, positive change). $p < 0.05$ for A and B. The pre-workshop mean = 4.3 and the post-workshop mean = 4.9.

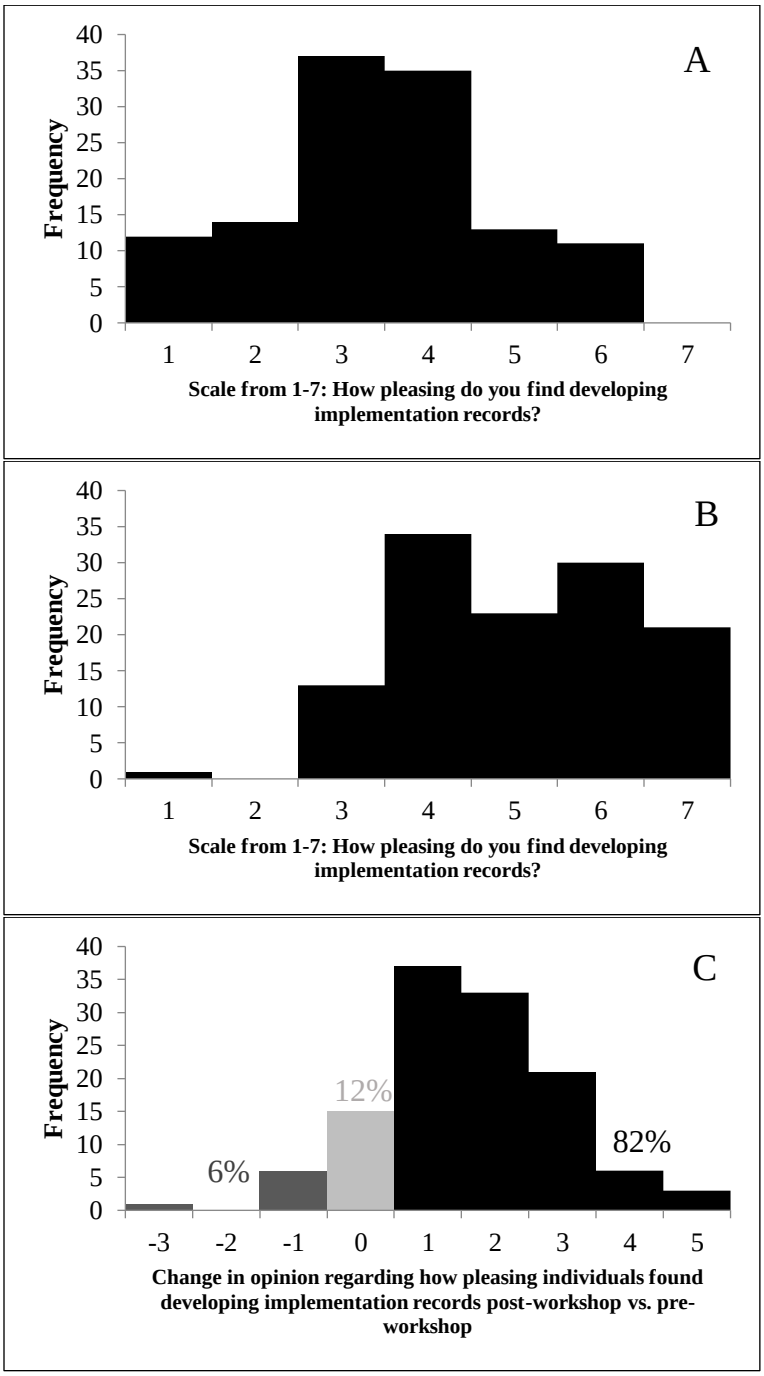


Figure 20. Frequency of how pleasing artisan dairy food safety workshop participants found developing implementation records (A) pre-workshop and (B) post-workshop. The higher the value on the x-axis the more pleasing participants found developing implementation records (1= extremely displeasing, 7 = extremely pleasing). (C)

represents how each individual changed their perspective about developing implementation records when post-workshop values were compared to pre-workshop values. A negative value indicates that individuals perceived developing implementation records as less pleasing post-workshop and a positive value indicates that individuals perceived developing implementation records as more pleasing post-workshop. Percentages indicate the number of individuals who fall into each category (negative change, 0 change, positive change). $p < 0.05$ for A and B. The pre-workshop mean = 3.5 and the post-workshop mean = 5.1.

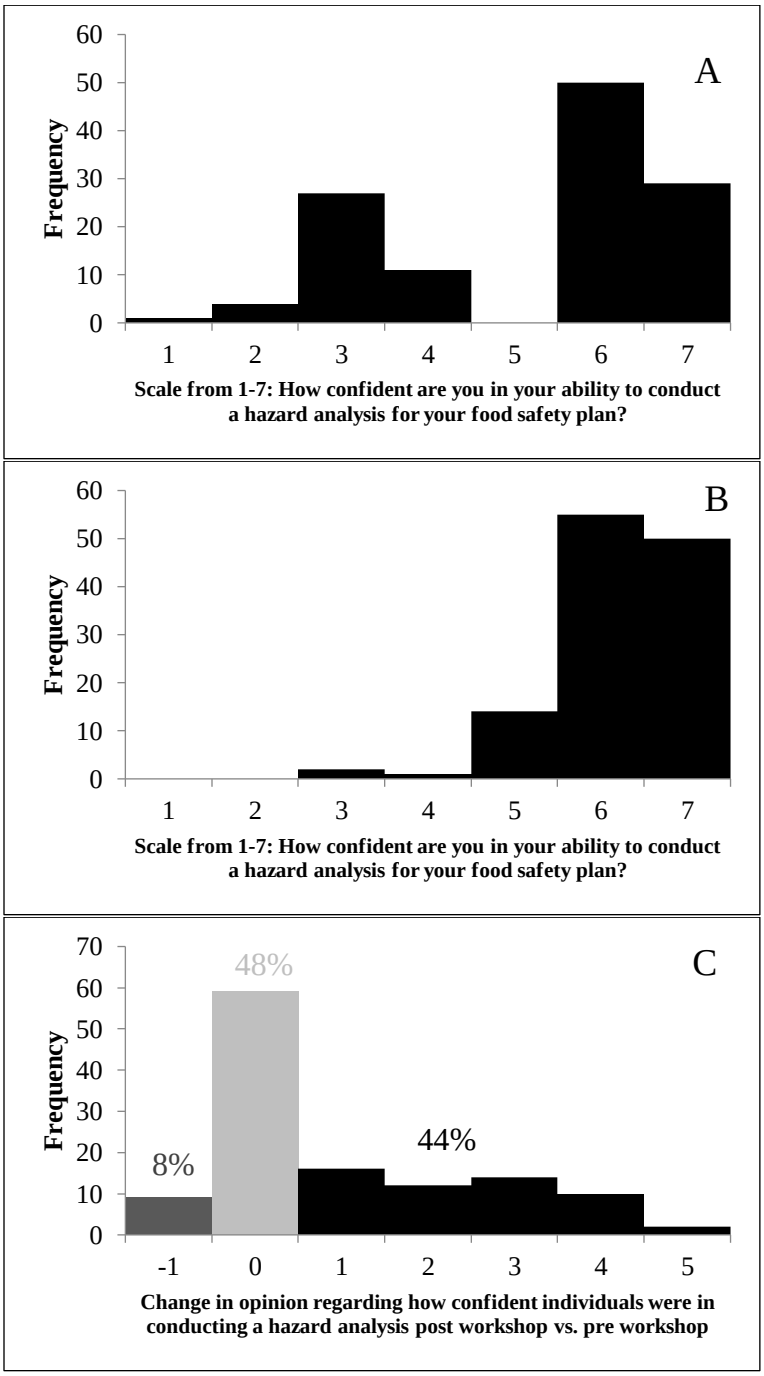


Figure 21. Frequency of how confident dairy food safety workshop participants were in conducting a hazard analysis (A) pre-workshop and (B) post-workshop. The higher the value on the x-axis the more confident participants were in conducting a hazard

analysis (1= extremely uncertain they could, 7 = extremely certain they could). (C) represents how each individual changed their perspective about their confidence in conducting a hazard analysis when post-workshop values were compared to pre-workshop values. A negative value indicates that individuals were less confident about conducting a hazard analysis post-workshop and a positive value indicates that individuals were more confident in conducting a hazard analysis post-workshop. Percentages indicate the number of individuals who fall into each category (negative change, 0 change, positive change). $p < 0.05$ for A and B. The pre-workshop mean = 5.2 and the post-workshop mean = 6.2.

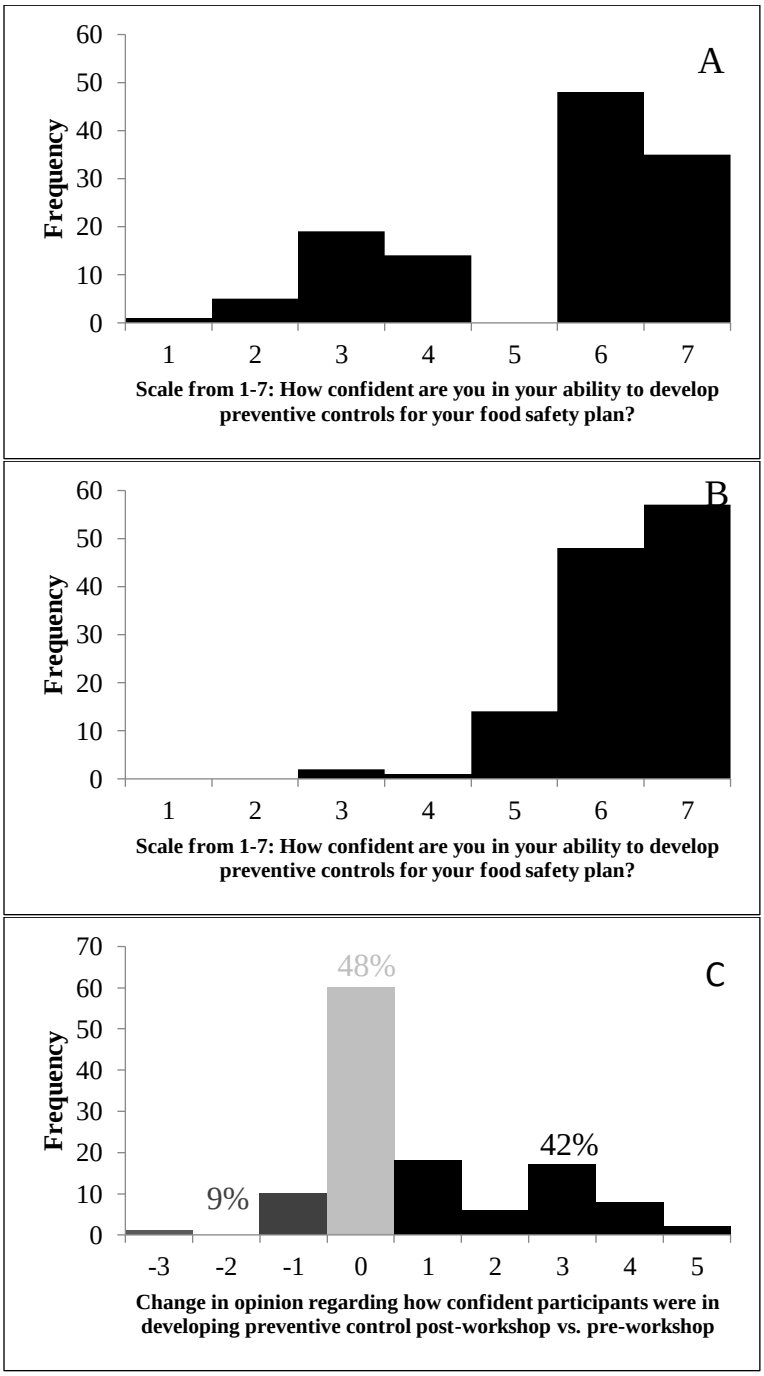


Figure 22. Frequency of how confident dairy food safety workshop participants were in developing preventive controls (A) pre-workshop and (B) post-workshop. The higher the value on the x-axis the more confident participants were in developing

preventive controls (1= extremely uncertain they could, 7 = extremely certain they could). (C) represents how each individual changed their perspective about their confidence in developing preventive controls when post-workshop values were compared to pre-workshop values. A negative value indicates that individuals were less confident about develping preventive controls post-workshop and a positive value indicates that individuals were more confident in developing preventive controls post-workshop. Percentages indicate the number of individuals who fall into each category (negative change, 0 change, positive change). $p < 0.05$ for A and B. The pre-workshop mean = 5.4 and the post-workshop mean = 6.3.

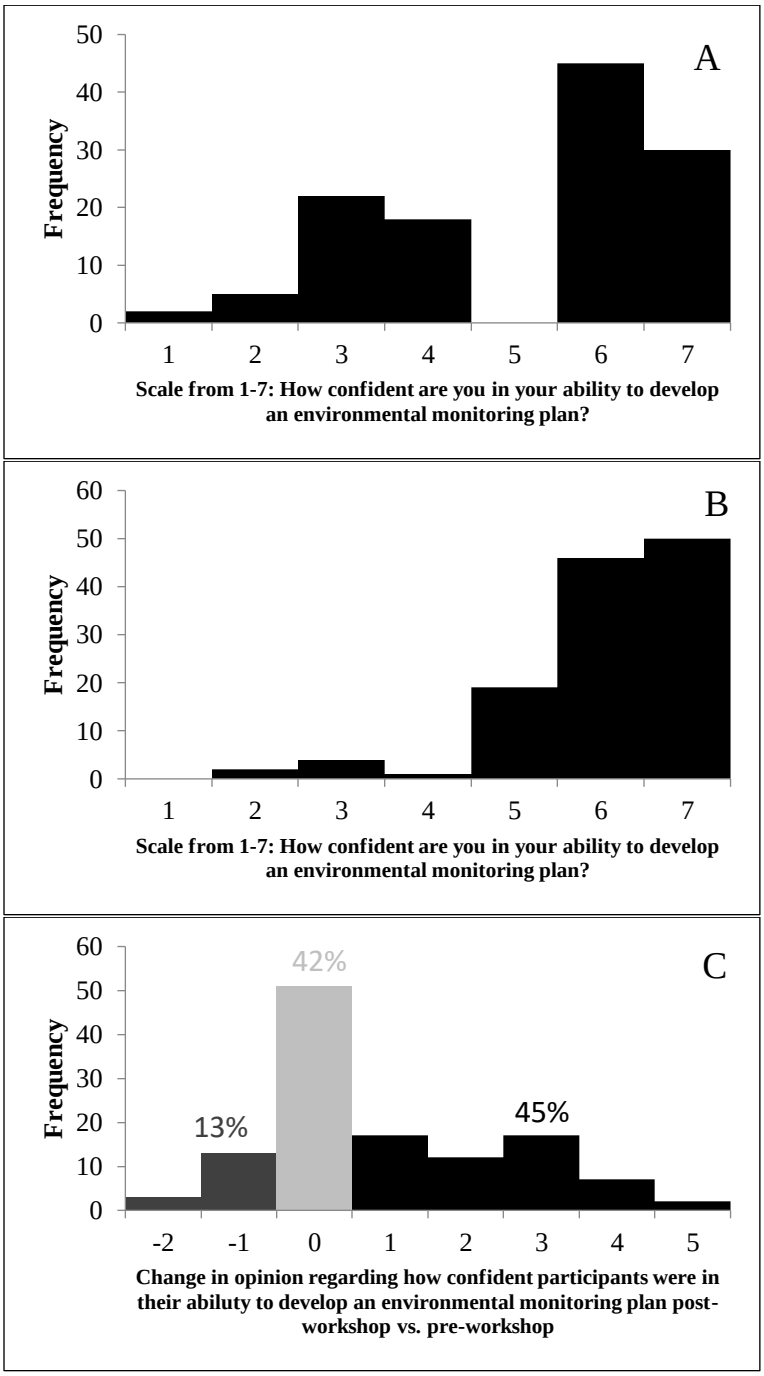


Figure 23. Frequency of how confident dairy food safety workshop participants were in developing an environmental monitoring plan (A) pre-workshop and (B) post-workshop. The higher the value on the x-axis the more confident participants were in

developing an environmental monitoring plan (1= extremely uncertain they could, 7 = extremely certain they could). (C) represents how each individual changed their perspective about their confidence in developing an environmental monitoring plan when post-workshop values were compared to pre-workshop values. A negative value indicates that individuals were less confident about develping an environmental monitoring plan post-workshop and a positive value indicates that individuals were more confident in developing an environmental monitoring plan post-workshop. Percentages indicate the number of individuals who fall into each category (negative change, 0 change, positive change). $p < 0.05$ for A and B. The pre-workshop mean = 5.2 and the post-workshop mean = 6.1.

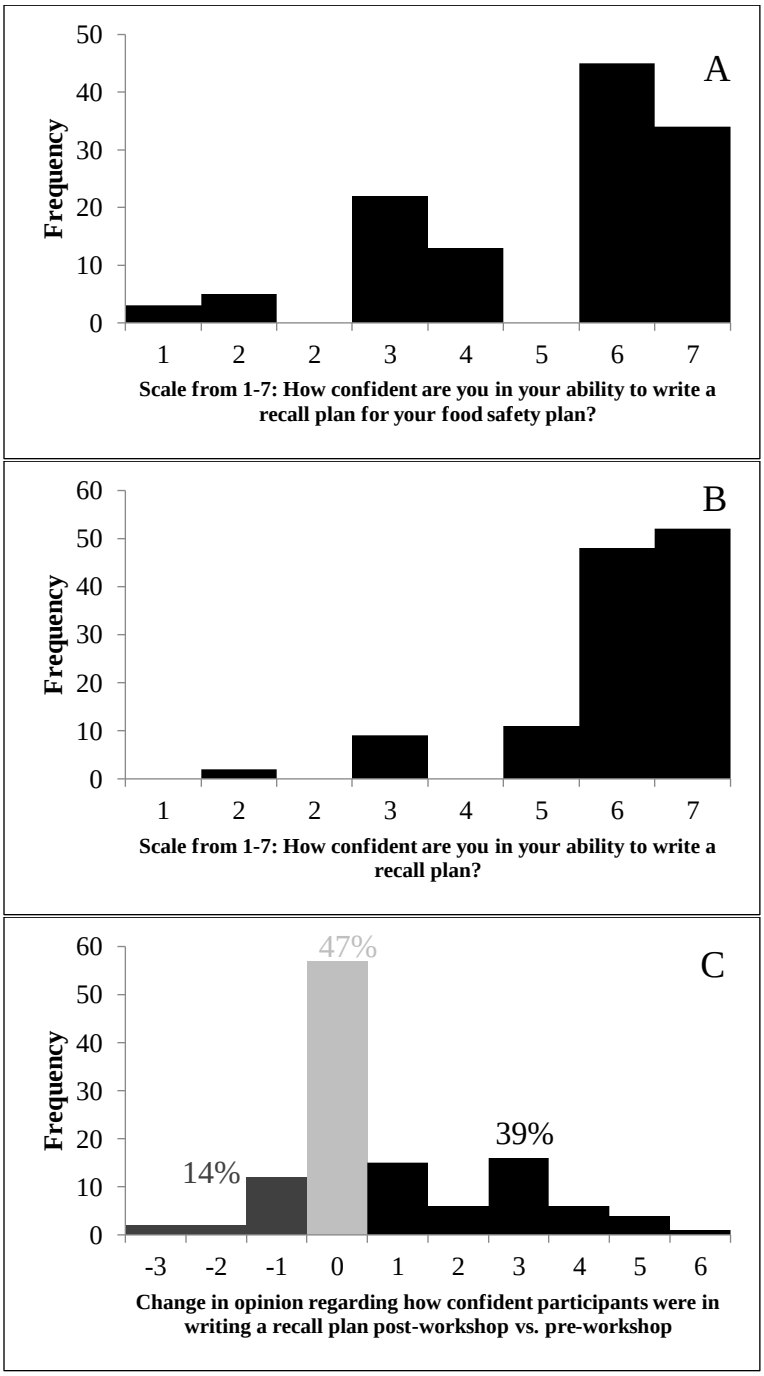


Figure 24. Frequency of how confident dairy food safety workshop participants were in writing a recall plan (A) pre-workshop and (B) post-workshop. The higher the value on the x-axis the more confident participants were in writing a recall plan (1= extremely uncertain they could, 7 = extremely certain they could). (C) represents how

each individual changed their perspective about their confidence in writing a recall plan plan when post-workshop values were compared to pre-workshop values. A negative value indicates that individuals were less confident about writing a recall plan post-workshop and a positive value indicates that individuals were more confident in developing an environmental monitoring plan post-workshop. Percentages indicate the number of individuals who fall into each category (negative change, 0 change, positive change). $p < 0.05$ for A and B. The pre-workshop mean = 5.2 and the post-workshop mean = 6.0.

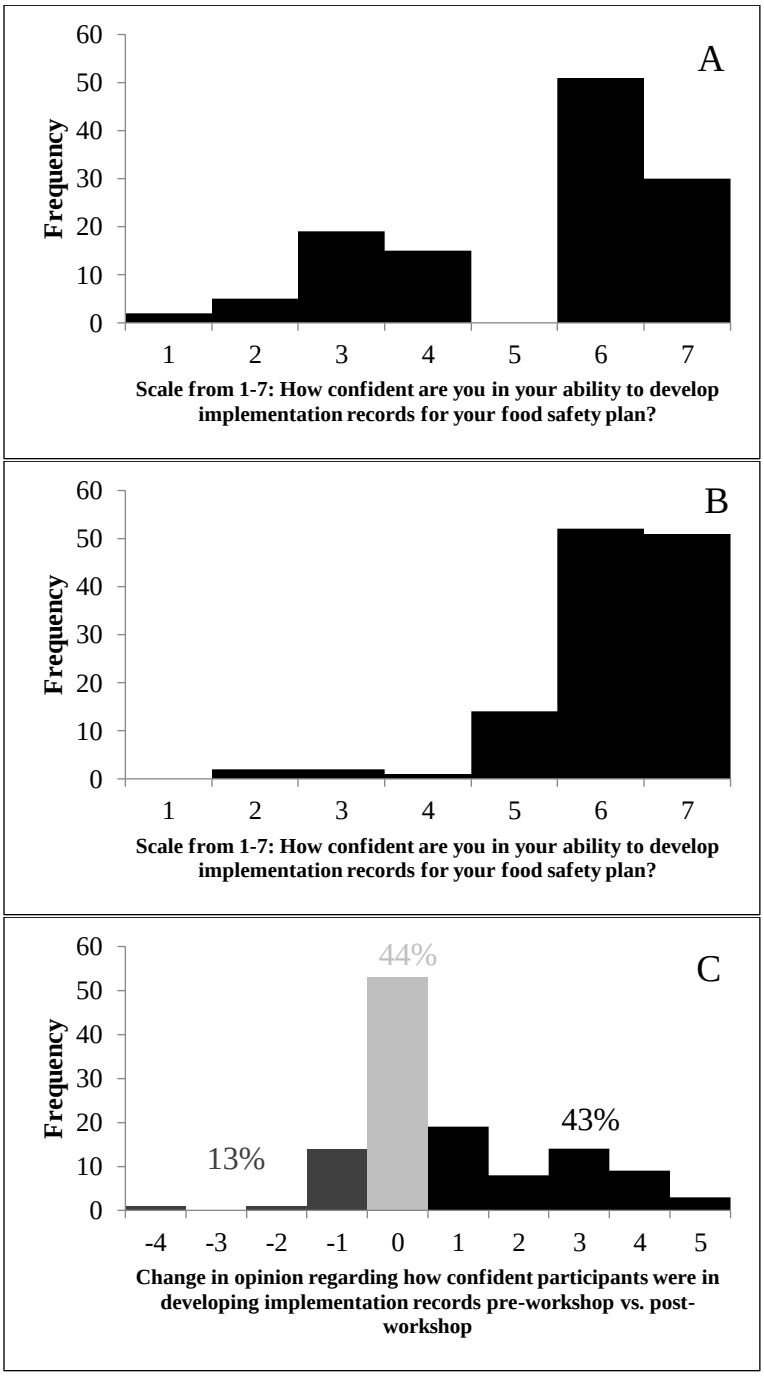


Figure 25. Frequency of how confident dairy food safety workshop participants were in developing implementation records (A) pre-workshop and (B) post-workshop. The higher the value on the x-axis the more confident participants were in developing

implementation records (1= extremely uncertain they could, 7 = extremely certain they could). (C) represents how each individual changed their perspective about their confidence in developing implementation records when post-workshop values were compared to pre-workshop values. A negative value indicates that individuals were less confident about developing implementation records post-workshop and a positive value indicates that individuals were more confident developing implementation records. Percentages indicate the number of individuals who fall into each category (negative change, 0 change, positive change). $p < 0.05$ for A and B. The pre-workshop mean = 5.3 and the post-workshop mean = 6.2.

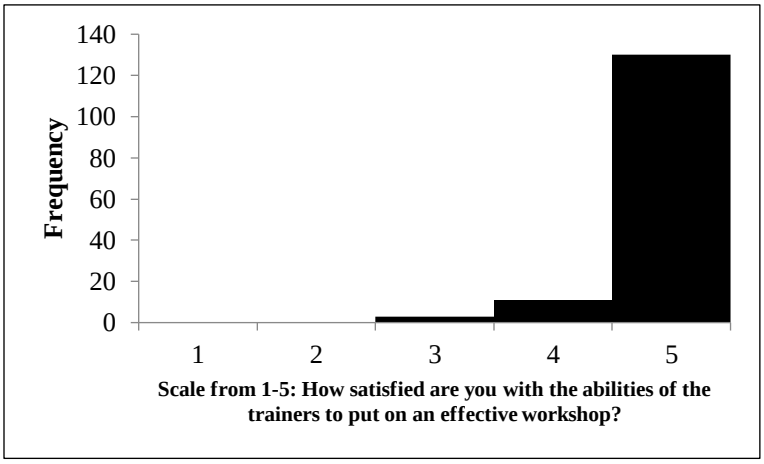


Figure 26. Frequency of how satisfied workshop participants were with the abilities of the trainers to put on an effective workshop. The higher the value on the x-axis the more satisfied the participants were with the abilities of the trainers (1= extremely dissatisfied, 5= extremely satisfied).

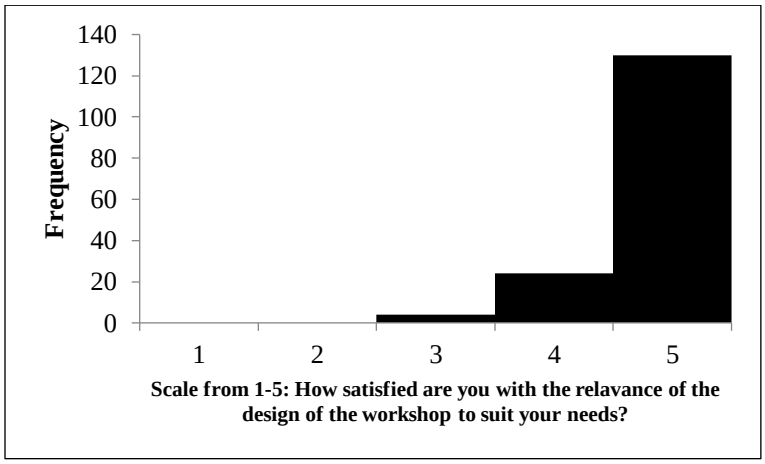


Figure 27. Frequency of how satisfied workshop participants were with the relevance of the design of the workshop to suit their needs. The higher the value on the x-axis the more satisfied the participants were with the design of the workshop to suit their needs (1= extremely dissatisfied, 5= extremely satisfied).

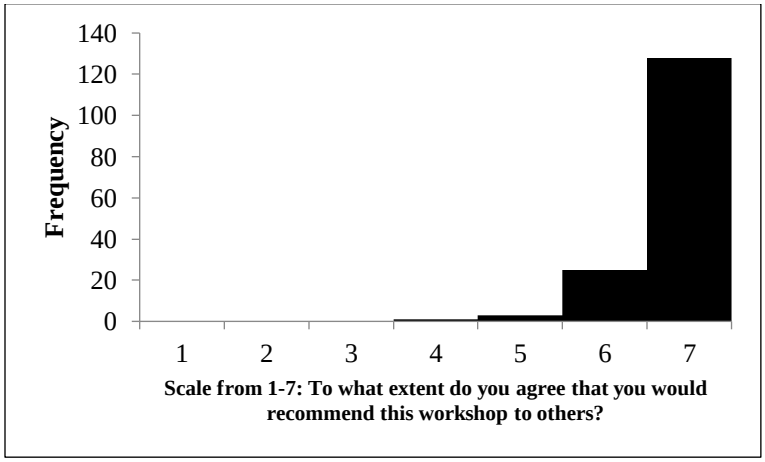


Figure 28. Frequency of the extent workshop participants agreed that they would recommend the workshop to others. The higher the value on the x-axis the more the participants agreed they would recommend the workshop to others (1= strongly disagree, 7= strongly agree).

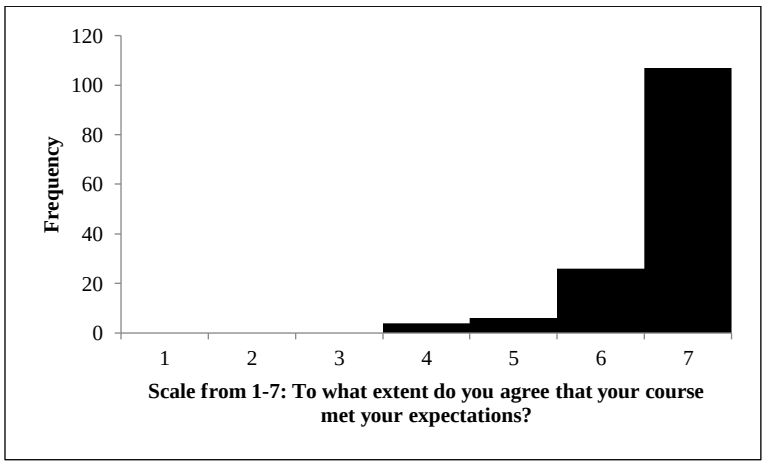


Figure 29. Frequency of the extent workshop participants agreed that the course met their expectations. The higher the value on the x-axis the more the participants agreed that the course met their expectations (1= strongly disagree, 7= strongly agree).

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