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Evaluation of Commercial Protective Cultures for the Control of *Listeria monocytogenes* and Shiga Toxin-Producing *Escherichia coli* in Raw Milk Cheese

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**Evaluation of Commercial Protective Cultures for the Control of *Listeria monocytogenes*
and Shiga Toxin-Producing *Escherichia coli* in Raw Milk Cheese**

Catherine Anne Gensler

B.S. University of Massachusetts-Amherst, 2016

A Thesis

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APPROVAL PAGE

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Evaluation of Commercial Protective Cultures for the Control of *Listeria monocytogenes* and
Shiga Toxin-Producing *Escherichia coli* in Raw Milk Cheese

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LIST OF ABBREVIATIONS

APC	Aerobic Plate Count
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
BLEB	Buffered Listeria Enrichment Broth
CC	Coliform Count
CDC	Centers for Disease Control and Prevention
CFR	Code of Federal Regulations
CFS	Cell Free Supernatant
CFU	Colony Forming Units
EHEC	Enterohemorrhagic <i>Escherichia coli</i>
EIR	Effective Inhibition Ratio
FDA	Food and Drug Administration
FSMA	Food Safety Modernization Act
GRAS	Generally Recognized as Safe
HUS	Hemolytic uremic syndrome
LAB	Lactic Acid producing Bacteria
LM	<i>Listeria monocytogenes</i>
MRS	de Man Rogosa and Sharpe
MSC	Mesophilic Starter Culture
NAHMS	National Animal Health Monitoring System
PC	Protective Culture
PDO	Protected Designation of Origin
SOI	Standard of Identity
STEC	Shiga Toxin-producing <i>Escherichia coli</i>
TSBYE	Tryptic Soy Broth with Yeast Extract
TSC	Thermophilic Starter Culture
UHT	Ultra-high-temperature Pasteurized
USDA	United States Department of Agriculture

ABSTRACT

Recent outbreaks and recalls linked to *Listeria monocytogenes* and shiga toxin-producing *Escherichia coli* in soft-ripened raw milk cheese illustrates an urgent need to identify effective controls compliant with Standards of Identity of cheese. The Food Safety Modernization Act (FSMA) presents an additional hurdle by requiring producers develop and implement science-based preventive controls for biological hazards associated with their product as part of a formal food safety plan, however few strategies have been formally documented in the scientific literature. Protective cultures of lactic acid bacteria (PCs) represent one such actionable and compliant solution, yet laboratory strains which have been documented effective are not available for widespread use. A selection of cultures is commercially available and safe for use in food but their compatibility with common cheese flora and antimicrobial efficacy against pathogens in the presence of raw milk consortia and in raw milk cheese production is unknown. Here, using culture based methods ten protective cultures were tested for inhibition of common cheesemaking starter cultures, ripening bacteria, yeasts and molds. Nine were confirmed compatible with starter cultures. PC inhibition of ripening bacteria and fungi was strain specific. Nine of the protective cultures did not inhibit acid production of the starter cultures tested. A subset of five cultures of these cultures was then tested for activity against *L. monocytogenes* by co-inoculating cultures into raw milk with the pathogen (7:2 log CFU/mL) and then experiencing a temperature profile similar to that of cheesemaking. Two cultures did not impact growth of *L. monocytogenes*, two cultures exerted a bacteriostatic effect and one culture limited *L. monocytogenes* to <1 log CFU/mL throughout testing. Similar testing completed using STEC similarly identified a separate efficacious culture towards that pathogen. Soft-surface mold ripened cheese was then manufactured using raw milk inoculated with the respective pathogens

and challenged with these efficacious cultures. Treatment with Protective Culture did not reduce *L. monocytogenes* counts in milk over ripening. *L. monocytogenes* grew in cheese treated with nisin-producing protective culture. STEC survived in soft-ripened cheese for over 120 days of ripening, however the addition of Protective Culture did not impact rate of survival. These results identify the select compatibility of protective cultures with cheesemaking cultures and the potential for select PCs to control *L. monocytogenes* in raw milk. However further work is needed to optimize application and use parameters for sustained control of pathogens in soft surface mold-ripened cheese.

CHAPTER I
INTRODUCTION

Centers for Disease Control researchers estimate that consumption of unpasteurized dairy products is 150 times more likely to result in food borne illness than pasteurized dairy (6). Among the pathogens associated with raw dairy, *Listeria monocytogenes* and shiga-toxin producing *Escherichia coli* (STEC) each present significant public health risk. Listeriosis is a serious infection caused by *L. monocytogenes* and associated with high rate of hospitalization and mortality (11). Infection with STEC requires a low infectious dose and has the chance to develop into a type of kidney failure known as hemolytic uremic syndrome (HUS) which requires hospitalization (8). Both pathogens have been isolated from the farm environment (1, 7), raw milk (14), and raw milk cheeses (2, 12). A multistate outbreak of listeriosis in 2017 was linked to a raw milk cheese produced in New York and caused 8 hospitalizations and two deaths. This year, 2019, in France soft cheeses made from unpasteurized milk by one manufacturer initiated a multi-country recall after being linked to outbreak of 16 STEC infections of which 14 children all less than four years old developed HUS (4, 5).

Soft-ripened cheese presents a unique food safety hazard because physiochemical changes, notably pH, in the product occur over ripening and mandatory aging allows for growth of *L. monocytogenes* (10). US Food and Drug Administration (FDA) and Health Canada conducted a joint quantitative risk assessment of *L. monocytogenes* in soft-ripened raw milk cheese in 2014. The report identified the heightened risk of listeriosis after the mandated 60 day aging period but failed to identify potential solutions to manage this risk beyond pasteurization (13). The report confirms a 50- to 160- fold increase in listeriosis risk from cheese made from raw milk compared to pasteurized milk in Canada and the United States respectively.

The use of protective cultures (PCs) of lactic acid bacteria (LAB) as a biological control strategy in fermented dairy is promising as it has already been demonstrated an effective strategy

for control of *L. monocytogenes* in other food applications (3). Application of PCs in cheese is advantageous as it is compliant with standards of identity of cheese, follows clean-label desires of consumers, and maintains Generally Regarded as Safe status for use in food by the FDA. Protective cultures exert antimicrobial activity on unwanted spoilage and pathogenic organisms through the production of one or antimicrobial metabolites, chiefly organic acids, hydrogen peroxide, and antimicrobial peptides known as bacteriocins (9). Activity of these metabolites, except for bacteriocins, is non-specific. Bacteriocin activity, in contrast, is frequently limited to bacteria taxonomically similar to the producer. LAB are Gram-positive and exert stronger antimicrobial activity towards similar Gram-positive organisms, notably pathogen *L. monocytogenes*. The impact of the same Gram-positive cultures, especially those commercially available, on Gram-negative STEC is limited. Gram-negative cultures have been also found to exhibit similar antimicrobial potential within other Gram-negative bacteria, however research is far less abundant than that of LAB. In consequence, number commercial protective cultures active against Gram-negative bacteria is also limited.

Successful reports of PC use in cheese in scientific literature employ cultures not available to producers. Further, these studies are frequently conducted in pasteurized milk, so efficacy in the presence of mixed microflora of raw milk is unknown. Commercially available PCs exist but their antimicrobial efficacy, especially in in raw milk, has not been verified. Additionally, non-specific nature of antimicrobial activity suggests that use of PCs as an adjunct culture has the potential to impact activity of desired cheese flora- starter cultures, ripening bacteria, yeasts, and molds. Use of PCs in cheese production would be easy to implement and a low-cost option for cheesemakers.

The hypothesis guiding this work is that PCs exert inhibitory and bactericidal effects against *L. monocytogenes* and STEC without significantly affecting beneficial cheese microbiota and their activity. The specific objectives of this research are as follows:

1. Identify compatible combinations of commercial protective and cheesemaking cultures.
2. Determine the antimicrobial activity of commercial protective cultures against *L. monocytogenes*.
3. Determine the effect of commercial protective cultures on *L. monocytogenes* and STEC during the manufacture and storage of soft-ripened cheese manufactured from raw milk.

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CHAPTER II
REVIEW OF THE LITERATURE

1. MILK FOR CHEESEMAKING

In the United States cheese is defined according to the Code of Federal Regulations (CFR) which reads as follows: “the fresh or matured product obtained by draining after coagulation of milk, cream, skimmed, or partly skimmed milk, or a combination of some or all of these products, and including any cheese that conforms to the requirements of the Food and Drug Administration (FDA) for cheeses and related cheese products” (14). American author Clifton Fadiman went as far to say cheese was “Milk’s leap to immortality” (69). Cheesemaking has been a time honored way to preserve milk to last long after its normal fluid shelf life.

Milk which has not undergone pasteurization, a specific heat treatment to reduce bacterial load in the milk, is considered raw (95). Raw milk, therefore, may contain a combination of bacteria that serve a functional purpose in cheesemaking and those that pose a hazard to human health. Fluid raw milk is not federally legal for sale (95). Cheesemaking with raw milk remains the only exception to this law. The legalization status of fluid raw milk remains a point of contention among public health officials, consumers, and producers because of the hazard to health. Debates regarding raw fluid milk are markedly different than those for raw milk cheese and will not be addressed here. To protect public health and manage potential health risks, several federal regulations present a framework of safety through which raw milk cheeses can be made.

1.1 Motivations for Use of Raw Milk for Cheese

The traditional, historic use of raw milk and the diversity of endogenous microbes in raw milk contribute flavor and texture of cheese are the two primary reasons cheesemakers select raw milk for their cheese. Bacteria enter the milk directly from the lactating animal (Cow, goat,

sheep, buffalo etc.) at the time of production, from the milking method, and from the milking environment (72). For example, *Lactococcus* species, commonly found in raw milk and responsible for acid development from fermentation, are frequently found on the cow's teat (65). Hygienic practices during milking- specifically teat dipping- has been found to reduce the level of lactic species in the milk, which may be a detriment to cheesemakers (58, 100). Commercial starter cultures are comprised of select species of this genera and are used to repopulate milk after pasteurization to precisely restore the activity (84). Before the advent of pasteurization and modern cheesemaking cultures all cheeses were made from raw milk and employed the natural microflora for fermentation. The diverse microbial population contributes a complement of enzymatic activity, enhancing the diversity of flavor compounds produced (3). Studies comparing cheeses made from raw and pasteurized milk find differences in flavor with mixed outcomes on desirability. For example, researchers comparing cheddar cheese made from raw, pasteurized, or microfiltered skim milk observed detrimental flavor differences in cheddar made from raw milk compared to pasteurized (61). Beuvier et al. (1997) found that Swiss cheese made from raw milk had higher scores for overall aroma intensity, pungency, firmness and granular character compared to pasteurized (6). Potential for one-of-a-kind flavor is a marketing point for raw milk cheeses (20).

The rich history of cheese production in Europe, is honored through the continuation of historic methods (by hand, smaller volumes), mechanisms (wooden aging shelves, ripening barrels), and material (raw milk). Specific cheeses are protected by law through Protected Designation of Origin (PDO) or similar national programs. PDO designations ensure the integrity of the cheese, confirming it is from the intended geographic area and follows standards of production, care, and quality. This is both a marketing tactic and boon to particular local

economy. Variation in microbiology across a geographic region can manifest in a terroir, or specific taste for that region (41). Well known cheeses with geographical protected designations include Camembert and Parmigiano-Reggiano. Presently, there is no PDO equivalent in the United States for cheeses; however cheesemakers adopt practices- such as the use of raw milk- with the intent of producing cheese unique to their region (53).

Sources of bacteria in the environment and animal, such as those described earlier, contributing to the microbial population in raw milk are many. The same sources may also harbor and contribute pathogenic organisms such as *Salmonella* ssp, *Campylobacter jejuni*, *Listeria monocytogenes*, and shiga-toxin producing *Escherichia coli* (72). All of these organisms cause serious infection in humans. Vigilant hygiene at all steps of milk production- from husbandry, collection, storage and transport- contributes greatly to microbial quality and safety of the milk. However the best way to eliminate pathogens and manage the risk to public health is pasteurization.

1.2 Pasteurization and Pasteurized Milk Ordinance (PMO)

Introduction of pasteurization reduced the incidence of milk borne disease from ~25% of all foodborne disease outbreaks in 1938 to <1% today (95). Pasteurization is the heating and holding of every particle of milk at a prescribed temperature for a set time, typically 161°F for 15 seconds (95). Approved time-temperature variations, such as High temperature- Short Time and Low Temperature-Long time, are specified in the Pasteurized Milk Ordinance (PMO) document. Stringent adherence to all PMO directives by operators is the cornerstone to a safe milk supply. In addition to the public health benefit, removal of bacteria via pasteurization offers quality advantages. Without batch to batch variation in microbes, consistent cheese is more reliably

made with pasteurized milk. Such removal stands in direct contrast with the philosophy of raw milk cheese makers who rely on and employ these bacteria for their make.

1.3 Standards of Identity

In the United States, Standards of Identity (SOI) are a legal definition of a product outlining the ingredients, composition and key methods of manufacture (14). Not all cheeses have a standard of identity. Unlike PDO mentioned previously, SOI are chemically, not geographically based. If a product is to be legally labeled as a standard cheese type, ingredients allowable in a cheese product are limited to those listed in the SOI. SOI further define if raw milk is allowed in each specific cheese type. Use of raw milk in cheese is not universal and in non-designated cheeses would be illegal.

1.4 60-Day Rule

Public health concern regarding consumption of raw milk and raw milk products stems from the potential for contamination of the product with pathogenic microorganisms. Among these are *Listeria monocytogenes*, shiga-toxin producing *Escherichia coli*, *Salmonella* species, *Campylobacter jejuni*, *Brucella* species, and *Mycobacterium tuberculosis* (13). Observations that younger aged raw milk cheeses were more frequently implicated in outbreaks than older cheeses made from raw milk, lead to state and eventual federal adoption of regulations requiring prolonged aging of raw milk cheeses (39, 79). Text in the federal SOI allowing use of raw milk states that the cheese made from raw milk must be aged for at least 60 days at a temperature greater than 1.6 °C (35 °F). Documented pathogen survival in cheeses of multiple types past the 60 aging period indicates that extensive aging is not a complete guarantee of safety (23).

1.5 Food Safety Modernization Act (FSMA)

The most recent piece of food safety legislation, the Food Safety Modernization Act, responds to the changing hazards in our food landscape including a more globalized food system and growing population of consumers susceptible to illness. Cheesemakers are required to prepare a food safety plan that 1) identifies hazards associated with their product, 2) installs appropriate, science-based preventive controls to manage each hazard, and 3) justifies and documents that such preventive controls adequately manage the risk identified.

2. *LISTERIA MONOCYTOGENES*

2.1 Microbiology

Listeria monocytogenes is a Gram positive non spore-forming rod in the genus *Listeria* (31). The *Listeria* genus contains seven species including *Listeria ivanovii* which infects animals, however *Listeria monocytogenes* is the only species to infect humans (31). Within *L. monocytogenes* there are 13 serotypes, of which 1/2a 1/2b, and 4b cause 95% of infections (11). *L. monocytogenes* is labile to pasteurization and is capable of growing at refrigeration temperatures (31). *L. monocytogenes* may enter raw milk from multiple sources. On the farm, udders with mastitis from *L. monocytogenes* infection can shed the bacteria directly into the milk (87). Poor hygienic practices during milk collection can also transfer *L. monocytogenes* shed in feces into milk (52). In cheesemaking facilities *L. monocytogenes* is commonly isolated from cool and damp niches such as seams in equipment and floor drains (52). *L. monocytogenes* can persist in these areas and on improperly cleaned and sanitized equipment by forming biofilms, leading to post-pasteurization contamination of products (2, 32). For example, *L. monocytogenes*

was found on floors, in floor drains, and plastic crates used for multiple tasks across a Latin-style cheese facility (49).

2.2 Pathogenicity

An infection with the bacteria is known as listeriosis. Ingestion of food contaminated with the organism is the most likely route of exposure. In immune-competent individuals infection is characterized by mild symptoms of general food borne illness, but is rarely encountered and even more rarely diagnosed because of the large infectious dose required (10). In vulnerable populations including the pregnant, elderly, very young and immune compromised; infections can spread beyond the GI tract reaching the bloodstream as sepsis or the brain as meningoencephalitis. In pregnant women, listeriosis infection can cause stillbirth (21, 31). As such, infection in any of these populations results in higher rates of hospitalization (94%) and death (15.9%) than other food borne infections (86). Further, listeriosis symptoms are reported anywhere from 7 to 70 days after exposure, making trace back to implicated food difficult (10). The precise infectious dose and risk of illness modulates based on immune status, volume consumed and cell concentration. For example, Pouillot et al. (2016) estimated contamination levels of ice cream associated with multi-state outbreak of listeriosis ranged from 0.12 to 8 CFU/g of product (71). Serving sizes of each product varied but researchers estimate the general population ingested anywhere from 1.5×10^9 to 1.4×10^{10} cells, but vulnerable populations ingested 7.2×10^6 to 3.3×10^7 (71).

2.3 *L. monocytogenes* Prevalence in Bulk Tank Milk and Cheese

Detection of *L. monocytogenes* in bulk tank raw milk highlights the continued public health risk associated with cheese products made from raw milk. Van Kessel et al (2011) found *Listeria* ssp. isolates in 43 out of 536 bulk tank milk samples (7.1% weighted) , 24 of which were

later confirmed to be *L. monocytogenes* taken from the 2007 National Animal Health Monitoring System (NAHMS) Dairy Survey (98). This data set included both large and small farms. This is a slight increase from detection of *Listeria* spp. in 6.5% of samples five years prior (99). Of bulk tank milk samples collected from 248 dairy herds in Pennsylvania from 2001-2002, only 3 samples returned positive for *L. monocytogenes* (45). In a survey of milk used for small-scale artisan cheesemaking in Vermont, *Listeria* spp. was detected by PCR in only 1 sample of the 101 tested (24). This isolate was confirmed to be *L. innocua*, PCR confirmation for *L. monocytogenes* for this sample was negative. Low prevalence of *L. monocytogenes* in bulk tank milk illustrates a viable, but low risk of pathogenic *L. monocytogenes* for raw milk cheese maker. Potential for environmental contamination mentioned previously further compounds risk for product contamination with *L. monocytogenes*.

Prevalence of *L. monocytogenes* in cheeses in US commerce is low. Recent sampling assignment of raw milk cheeses in the US conducted by the FDA in 2014 found ten of 1,606 samples tested positive for *L. monocytogenes* (96). Nine out of ten of these positive samples were semi-soft raw milk cheeses. The total contamination rate of this cheese type by *L. monocytogenes* was 0.89%.

Despite low prevalence, outbreaks of listeriosis attributed to unpasteurized cheese do occur. Gould et al. (2014) reports that of outbreaks of food borne illness associated with cheese occurring from 1998-2011, *L. monocytogenes* was more often associated with pasteurized cheese (8 out of 44 outbreaks), than unpasteurized (4 out of 38 outbreaks) (40). Soft cheese was most frequently implicated in these outbreaks. Sixty- two percent of the cheeses made from unpasteurized milk were contaminated by animals or the environment, whereas poor worker hygiene and bare hand contact were contributing factors more frequently associated with

outbreaks associated with pasteurized cheese (40). Jackson et al. (2018) and Costard et al. (2017) noted a similar association of pasteurization status and cheese type when analyzing outbreaks of listeriosis from 1998 to 2014 (22, 44). In 2017 a raw milk cheese produced in New York caused a multi-state outbreak of Listeriosis linked which resulted in eight hospitalizations and two deaths (12). An outbreak of listeriosis in France in early 2019 attributed to “l’Explorateur soft ripened cheese” made from raw milk caused two deaths and prompted international recall (35). Since the beginning of 2017, the FDA has issued 21 recalls related to potential or confirmed contamination of cheese products with *L. monocytogenes* (36).

3 SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* (STEC)

3.1 Microbiology

Escherichia coli is a diverse species of bacteria common to the intestines of humans and animals, the environment and foods. *STEC* are Gram-negative bacilli, mesophilic, and aerobic. Most strains of *Escherichia coli* do not cause disease. Of relevance to the food industry are strains that cause gastrointestinal illness on a continuum of severity known collectively as diarrheagenic *E.coli*. These pathogenic strains are grouped by manifestation of illness. These are, enterohemorrhagic/Shiga Toxin *Escherichia coli* (EHEC/STEC) (also called verocytotoxic *Escherichia coli* VTEC), enterotoxigenic *Escherichia coli* (ETEC), enteropathogenic *Escherichia coli* (EPEC), enteroaggregative *Escherichia coli* (EAEC), enteroinvasive *Escherichia coli* (EIEC), and diffusely adherent *Escherichia coli* (DAEC). *Escherichia coli* strains are further distinguished from one another based on surface antigens O (somatic), H (flagellar) and K (capsule), A specific O and H combination or lack thereof defines a serotype (67). Of particular concern to the food industry and the focus of this work are the shiga-toxin

producing *Escherichia coli* (STEC). EHEC are a distinct subgroup of STEC distinguishable by more severe clinical illness characterized by hemorrhagic colitis and hemolytic-uremic syndrome (HUS). Both STEC and EHEC are characterized by the production of one or both shiga-like toxins Stx1 and Stx2 (68), however EHEC strains produce additional virulence factors associated with more severe symptoms including the cell surface protein intimin, and enterohemolysin (27). The combination of proteins allows the bacteria to attach to intestinal cells and excrete toxin, causing more severe symptoms of infection. Historically in the United States EHEC infections have been associated with the serotype O157:H7, however detection of non-O157:H7 serotypes in patients with HUS have prompted the public health community to refer to EHEC infections by their serotype.

3.2 Pathogenicity

Symptoms of STEC infection include severe abdominal pain diarrhea that may be bloody, occasionally a low grade fever, lasting up to 5-7 days (9). The CDC reports that 5-10% of STEC infections develop into a life-threatening acute renal failure known as hemolytic uremic syndrome (HUS) (9, 68). As mentioned previously, HUS is historically associated *Escherichia coli* serotype O157:H7, and this serotype remains the most frequent *Escherichia coli* serotype implicated in food borne infections resulting in HUS. Non-O157:H7 serotypes O26, O111, O103, O121, O45, O145 have been implicated in outbreaks and cases of HUS (7). The infectious dose for STEC is often reported as <100 cells, regardless of serotype (9, 47, 68). STEC can infect individuals at any age, however, children younger than 5 have the highest incidence of HUS (9). Hospitalization rate estimations for STEC are further subdivided to O157 and non-O157 with rates at 46% and 12.8% respectively (86). Death rates for O157 and non O157 STEC are 0.5% and 0.3%, respectively, however patients surviving HUS will require lifelong medical care (86).

An outbreak of STEC infections in spring of 2019 linked to a raw milk surface mold-ripened cheese had 15 pediatric cases (< 4 years old) and 14 of which developed HUS (48).

3.3 Prevalence of STEC in Bulk Tank Milk

STEC has been periodically detected in raw bulk tank milk. Jayarao et al. (2006) reported six isolates out of 248 samples of STEC in bulk tank raw milk in Pennsylvania (45) and 5 out of 131 isolates from South Dakota and Minnesota (46). Van Kessel et al. (2011) detected shiga toxin genes using real time PCR in 78 of 533 bulk tank milk samples, after weighting, this is 2.3% of operations surveyed (98). In all of these surveys, STEC was not the most frequently isolated pathogen, indicating that multiple pathogens contribute to the high risk of the product. Monitoring for STEC in raw milk used for cheesemaking is not required by law, however periodic confirmation of absence in raw milk used for cheesemaking is a recommended practice (1).

3.4 Prevalence and Detection of STEC in Cheese

Detection of the *stx* gene via PCR is the approved method for confirming presence of STEC. This method is more reliable than agar based approaches because of sensitivity and time to results. Madic et al. (2011) screened 400 raw milk soft or smear semi-hard cheeses available for retail sale in France for STEC serotypes using real time PCR (56). 29.8% of samples were positive for *stx*, 55.3% contained at least one of the five EHEC O group markers (O157, O145, O103, O26, O111), and 37.3% contained the *eae* gene; 61 samples contained all three. Fifteen isolates were obtained from a subset of samples showing EHEC associations of markers. Authors reported prevalence of *stx*-positive cheese was mixed in comparison to others studies. In a separate study 136 out of 1039 French raw milk cheese samples, 74 of which were soft cheese, tested positive for *stx* (101). Vernozy-Rozand et al. (2005) recovered 32 strains of STEC from 18

samples of cheese, however none of these isolated contained the combination of three genes (*stx1*, *stx2*, and *eae*) associated with serious infection (101). These studies show that virulence genes *stx* are readily detected in raw milk cheeses of Europe, however, multiple virulence factors are necessary for associated infection.

4 SURFACE-MOLD RIPENED SOFT CHEESE

4.1 Cheese Make

Surface mold ripened soft cheeses include Brie, Camembert and their variants such as Chaource and coulommiers. Also called bloomy rind cheeses, this group is characterized by the fluffy white mold of *Penicillium candidum* and *Geotricum candidum* on the surface. A traditional cheese of French villages, surface mold cheeses are manufactured by ripening the milk with either added starter culture or allowing endogenous Lactic Acid Bacteria to acidify the milk, renneting, and limited cutting and cooking of the curd before ladling into forms. As the story goes, prior to industrialization of cheese cultures, *Penicillium* molds colonized the cheese from the air during the time local women making cheese would attend to other household chores in between making cheese (50). In the United States a standard of identity exists only for soft-ripened cheese category, rather than specific cheeses. 21 CFR §133.182 specifies soft-ripened cheeses shall have 50% fat in dry matter and use of raw milk is allowed with required aging time (14).

4.2 Physiochemical and Microbial Community Changes

Presence and growth of the surface microbial community is a critical component for the ripening of the cheese. Leclercq-Perlat et al. (2004) manufactured pasteurized camembert to observe microflora and biochemical changes of the surface during ripening (54). Growth of the

yeast *Kluyveromyces lactis* occurred rapidly on the cheese surface in the first two days, and slowed in rate until day 7, at which point cell concentrations remained constant out to day 45. Growth of *Geotrichum candidum* at the surface occurred mainly between day 3 and day 12, after which time cell concentrations remained constant. Growth of *P. candidum* was observed between day 7 and 12, with uniform coverage achieved by day 12-14. *Brevibacterium linens*, grew on the surface exponentially starting at day 5 until day 8. pH of the surface of the cheese started at 4.7; at day 6 it began to increase rapidly to 7.7 on day 9. pH of the core increased gradually starting at day 13 to a pH of 7.0 on the last day of ripening. Complete depletion of lactose occurred from day 2 to day 8, accompanied by an increase in lactate concentration peaking at day 6 and decreasing gradually throughout the remainder of ripening. Aligning the microbial growth timelines with those of the biochemical changes describes the dynamic changes occurring on and in the cheese. Growth of *K. lactis* correlates with the depletion of lactose on the rind. Lactose is not a substrate for *G. candidum* growth, therefore growth of *G. candidum* is not observed until its substrate lactate is present in sufficient amounts. Growth of *G. candidum* instigates the increase in pH at the rind through the production of proteases, which upon cleaving proteins release ammonia raising the pH. The rise in pH gives *B. linens* and *P. candidum* an opportunity for growth. The cascade of community development is therefore closely tied to biochemical changes occurring in the cheese as a result of other members; however these changes also support pathogen survival.

4.3 Pathogen Survival and Growth in Soft-Ripened Cheese

Changes in composition through ripening support growth of *Listeria monocytogenes* and survival of STEC in surface mold ripened cheese. In a seminal study of *L. monocytogenes* in camembert cheese, Ryser and Marth (1987) manufactured pasteurized camembert with 2 log

CFU/mL *L. monocytogenes* in the milk, and monitored pathogen counts and pH throughout the cheese make and ripening (80). Ripening and pH changes at surface and interior of these cheeses closely followed that described previously. Fate of *Listeria* was strain specific with strains Scott A, CA and OH experiencing brief 1-2 log reduction in the first two weeks while the pH of the cheese was still low. Then as pH at the surface increased, growth to 8 log CFU/g occurred. Absence of early reduction in counts was the only point of difference in the growth of strain V7 from the other strains in this cheese. Montet et al. (2009) observed a slight increase (<2 log CFU/g) in concentration of cocktails of four acid resistant and non-acid-resistant strains of STEC, throughout manufacture and drying (66). Counts of one non-acid resistant strain decreased to 1 log CFU/g by the end of the 20 days of ripening while the remaining strains showed either slight growth to 4 log CFU/g or mild decrease to 2 log CFU/g from the initial inoculation of 3 log CFU/mL (66). Strain specific survival patterns of STEC O157:H7, O26:H11, O103:H2, and O145:H28 were observed in a lactic cheese made by Miszczycha et al. (2013). Serotype O26:H11 was the only strain to increase in concentration throughout manufacture. At the end of de-hooping, all strains were below the limits of enumeration, but were detectable after enrichment (63). In a follow-up study by Miszczycha et al. (2016) STEC strains O26:H11, O103:H2, O145:H28, and O157:H7 also varied in rate of survival in soft-ripened cheese, indicating the pathogen survival in this cheese type is strain specific.

4.4 FDA/Health Canada Risk Assessment

Association of soft-ripened cheese with sporadic and outbreak related listeriosis infections prompted the FDA and Health Canada to assess the risk posed by this product and identify potential interventions (97). Using a mathematical model based on rates of consumption in the population, health status of the populations and rates of contamination, among other

variables, assessors calculated a 50- to 160- fold increase in the risk of listeriosis from consumption of soft-ripened cheese made from raw milk compared to pasteurized milk in Canada and the United States, respectively. The assessment identified that periodic sampling of individual lots of raw milk cheese would not reduce the risk to below that of pasteurized milk, however sampling *every* lot and removing positive lots would sufficiently reduce the risk. Further, applying a treatment which reduces *L. monocytogenes* population by 6 log CFU/mL would also reduce the risk of illness, however no such intervention has been identified. Removal of the required ripening period of 60 days reduced risk of illness to 62 times that of the pasteurized baseline. Models of an antimicrobial treatment reducing 2 log CFU/g on the surface of the cheese only reduced the rate of listeriosis to 37 times higher than that of pasteurized milk cheese. This model did not investigate reduction of *L. monocytogenes* in the interior of the cheese.

5. PROTECTIVE CULTURES

5.1 Overview of Protective Cultures

Lactic acid bacteria (LAB) serve a multiple roles in successful food fermentations namely, converting substrates to generate desired flavors and textures, and producing of metabolites with antimicrobial properties to extend acceptable shelf-life of the product. Antimicrobial properties of protective cultures stem from production of one or more metabolites chiefly organic acids, but also includes hydrogen peroxide, ethanol, selective enzymes, and of particular interest, bacteriocins (15). Protective cultures have been evaluated for applications in multiple food industries (meat, seafood, produce) and are particularly well suited for fermented

dairy products because of rich growth medium, documented safety, and successful control of pathogens in these products (89).

Bacteriocins are ribosomally produced peptides with antimicrobial potential.

Classification of these compounds is determined by producing organism and structure.

Bacteriocins of Gram positive bacteria fall into three classes. Class I bacteriocins are known as lanthionine-containing lantibiotics and include nisin A and Z. Class II bacteriocins do not contain lanthionine and are further subdivided into 3 subclasses. Class III bacteriocins are larger and unstable to heat. Bacteriocins of Gram negative bacteria are classified according to similarity to the major bacteriocins colicin or microcin. Bacteriocins are most active against competitors similar to the producing strain. Bacteriocins of Gram positive bacteria inhibit competitors through preventing membrane synthesis and forming pores in the membrane (64, 105).

Bacteriocins of Gram negative bacteria exert antimicrobial activity through pore formation, inhibition or digestion of genetic material, or digestion of peptidoglycan precursors (105).

5.2 Control of Pathogens with LAB

Multiple studies have demonstrated antipathogenic activity of LAB strains. Spelhaug et al. (1989) observed inhibition of food borne pathogens *Bacillus cereus*, *Clostridium perfringens*, *Staphylococcus aureus*, select species of the *Listeria* genus, and slight inhibition of strains *Escherichia coli* O157:H7, *Vibrio cholera* 851, *V. parahemolyticus* A865957 by *Lactococcus lactis* subsp. *lactis* 11454, *Pediococcus pentosaceus* FBB61, and *P. pentosaceus* FBB63-DG2, but did not observe inhibition of *Campylobacter jejuni*, *Salmonella enteritidis*, *Yersina* or *Vibrio* species (93). This analysis was completed using agar based assays, giving a broad approximation of antimicrobial potential. This study confirmed strain-specific and narrow spectrum of inhibitory activity of LAB. Similarly, Harris et al. (1989) while testing a panel of 14 LAB strains

identified three cultures that produced proteinaceous compounds that inhibited *L. monocytogenes*. Many studies similar to these are available in the literature with variations in culture origin and intended antimicrobial activity. A limited number of the cultures examined have demonstrated anti-pathogenic activity in dairy, and even fewer have been manufactured for commercial use in dairy applications.

5.3 Commercially Available Protective Cultures

Available information pertaining to the commercialized strains of protective cultures selected for use in the experiments that follow is summarized here. In the absence of published literature using the specific culture, studies using another strain of the same species is presented to illustrate potential for antimicrobial activity. Lack of available and non-codified scientific literature to document validation of process controls is one hurdle processors encounter when drafting a food safety plan.

5.3.1 *Pediococcus acidilactici* B-LC-20

B-LC-20 is a culture of *Pediococcus acidilactici* manufactured by Chr. Hansen (Denmark) for the control of *Listeria monocytogenes* in fermented sausage products (18). Technical document supplied by the manufacturer indicates the culture can be used as an adjunct to normal sausage making, not a surface application (18). This *Pediococcus* strain has an optimal growth temperature at 43°C and does not ferment lactose. Patent application for the product further specifies the strain as *Pediococcus acidilactici* strain DSM 10313 (94). In an evaluation completed by Raimondi et al. (2014) of a *Lactobacillus delbrueckii* strain for potential bio-protective properties, B-LC-20 was used as a benchmarking control for anti-listerial activity (73). Acidification of meat blend was not impacted by the addition of B-LC-20, the final pH of the mixture after incubation at simulated fermentation temperatures was not statistically

different from controls, however the rate of acidification was higher than control. *L. monocytogenes* was inoculated into the meat at 5×10^3 CFU/g and challenged with 5×10^6 CFU/g of the protective culture. At start of fermentation, *L. monocytogenes* numbered $\sim 1 \times 10^4$ CFU/g, and from day 3 onward counts of *L. monocytogenes* in sausages fermented with B-LC-20 were lower than control. At the end of 21 days of fermentation, *L. monocytogenes* counts had decreased by 1.21 log CFU/g, whereas in the same timeframe the control decreased by 0.34 log CFU/g, evidence of the potential for this culture to manage *L. monocytogenes* growth and proliferation in this product. Patent materials also report an average reductions in *Listeria monocytogenes* cocktail in a sausage at 2.71 and 3.06 log CFU/g for low ($\sim 2.9 \times 10^7$ CFU) and high ($\sim 5.6 \times 10^7$ CFU) concentrations of B-LC-20 respectively (94). The application also describes the product produces a class II bacteriocin. Other *Pediococcus* species commonly produce the bacteriocin pediocin, a class II bacteriocin capable of forming pores in target cell membranes (33, 75). Minimal acid development and reported high activity against *L. monocytogenes* in a high-acid fermentation environment prompt investigation of cross-application of this culture for similar use in cheese.

5.3.2 *Lactobacillus curvatus* B-LC-48

B-LC-48 is a frozen pelletized culture of a strain of *Lactobacillus curvatus*, manufactured by Chr. Hansen (Denmark) and marketed with the expressed purpose of ‘minimizing the presence of *Listeria monocytogenes* in cooked, sliced bacon’ according to the manufacturer’s website (19). This culture is speculated to produce the bacteriocin curvatin. Technical documentation for the product also claims the product can be used in “Ready-to-Eat foods which are packed under vacuum or modified atmosphere and cold stored...particularly meat products” (19). This culture does not ferment lactose.

Previous investigations using this culture have reported reductions of *Listeria monocytogenes* in meat applications; however a report of use in cheese exists. Rodriguez et al. (2012) evaluated B-LC-48 to control *Listeria* and *E.coli* O157:H7 in fresh micro cheeses over 21 days of storage, reporting 6 log CFU/g reduction of *E.coli* O157:H7 ATCC 43888 and 2 log CFU/g reduction of *L. monocytogenes* (77). This study is one of the few reported uses of this culture in cheese. In his thesis Frank Dogbatsey investigated the use of protective cultures B-LC-48 and B-LC-20 singularly and in combination as starter cultures in genoa salami production and for the control of *L. monocytogenes* in the same product (29). Application of each PC alone reduced *L. monocytogenes* populations by day seven. However, when combined reduction accelerated and was observed earlier on day 4 (29).

5.3.3 *Lactobacillus plantarum* HOLDBAC

Holdbac is a lyophilized powdered culture of a strain of *Lactobacillus plantarum*, sold with the expressed purpose of controlling *Listeria* contamination in dairy. According to the technical document supplied by the manufacturer, Danisco, the culture is a weak acidifier, targeted for use in cheese products (26). Each laminated foil pouch of Holdbac contains 10 IP units of culture totaling approximately 100g of powder. Technical documents state a cell concentration of $\geq 1 \times 10^9$ total lactobacilli/IP. This culture is not presently available for sale in the United States and was sourced from the producer in France. This culture likely produces pediocin AcH/PA-1, as reported by Roth et al. (2009) (78) and Back et al. (2016) (4), however technical details remain proprietary information with the manufacturer. Pediocin AcH/PA-1 is fairly well characterized class IIa bacteriocin produced by *Pediococcus* and *Lactobacillus* species with demonstrated activity towards gram positive organisms such as *L. monocytogenes* (76).

Effectiveness of Holdbac against *L. monocytogenes* has been mainly assessed in surface applications. Vyřasová et al. (2010) inoculated the surface of three types of cheese with *Listeria innocua* and applied a 3 IP/L Holdbac treatment to the surface either before or after *L. innocua* inoculation (102). Cheeses were stored at two different temperatures, and *L. innocua* were enumerated over 14 days. *L. innocua* counts on cheeses were slightly lower when the Holdbac treatment was applied before contamination. Log reductions at day 14 from initial inoculation levels varied by cheese type and storage temperature, however were reported relative to the initial inoculation level and not an internal *L. innocua* only control (102). In their doctoral thesis Emmanuelle Roth demonstrates *L. innocua* reductions on the surface of semi-hard raclette cheese after treatment with Holdbac as part of a smear brine (78). *L. innocua* was undetectable on the surface of the cheese during the entirety of ripening up to day 37 in one trial. Yet in a separate trial with a higher inoculation level, no appreciable change from control was observed (78). Suitability for *L. innocua* as a surrogate for *L. monocytogenes* behavior in cheese antimicrobial testing is not well established.

5.3.4 *Lactobacillus plantarum* LPAL

LPAL is a powdered culture of *Lactobacillus plantarum* manufactured by Sacco Inc (Italy) for the purpose of inhibiting unwanted bacteria *L. monocytogenes* and other *Listeria* species (83). LPAL is applied as a surface treatment to cheese.

Use of LPAL is not well documented in the literature under its trade name. LPAL was used alongside CNBAL in the study of burrata cheese completed by Minervini et al. (2017). Addition of the protective culture combination reduced early growth rate of the spoilage organisms *Staphylococci*, coliforms, and *Pseudomonas* spp (62), but did not assess anti-listerial activity. Bacteriocin production is common among *L. plantarum* strains with many different

types reported (25). Frequent isolation of *L. plantarum* strains with activity towards *L. monocytogenes* suggests LP-AL likely produces a bacteriocin (25, 104)

5.3.5 *Carnobacterium* ssp. Lyofast CNBAL

Lyofast CNBAL is a powdered culture of Gram-positive *Carnobacterium* species manufactured by Sacco Inc (Italy). Manufacturer technical documents state the culture is designed to control “unwanted bacteria, like *Listeria monocytogenes* and generally *Listeria* spp.” on the surface on cheese (81).

In a series of experiments Spanu et al. (2017, 2018) investigated the used of CNBAL and other commercial protective cultures to control surface spoilage development on cheeses naturally and artificially contaminated with spoilage species (91, 92). In naturally contaminated cheeses treated with CNBAL, lactic acid bacteria counts were lower than other samples. Further, *Pseudomonas* and *Enterobacteriaceae* counts were one log lower than controls at end of shelf life. Difference between treatment and control of counts of these organisms were larger (~>2 log) at earlier time points (92). Leisner et al. (2007) speculates the lower LAB counts observed in these cheeses may be due to poor growth of *Carnobacterium* in media containing acetate (55). MRS, the common media for enumeration of LAB employs sodium acetate as a growth factor for Lactobacilli and inhibitor of other species (5). When artificially contaminated with 2 log CFU/g of *Pseudomonas* spp application of CNBAL reduced pseudomonas counts with respect to control by 1.28 log at day 14 and 0.83 log at day 21, indicating the potential for control of spoilage organisms (91). Both *Pseudomonas* and *Enterobacteriaceae* are Gram negative organisms. Bacteriocins are commonly only active against bacteria with similar cell morphology, indicating a wide range of activity for this culture.

5.3.6 *Enterococcus faecium* SF-68

SF-68 is a pharmaceutical probiotic culture of *Enterococcus faecium*, manufactured by Cerbios Pharma (70). According to the manufacturer's website the SF-68 strain *Enterococcus faecium* NCIMB 10415 was isolated in a healthy newborn baby in Sweden (70). According to analysis completed by Foulquié-Moreno et al. (2003) SF-68 produces an enterocin. Further characterization found no inhibitory activity of SF-68 towards *Listeria innocua* (37). Use of SF-68 directly against foodborne pathogens has not been documented, however the probiotic properties of SF-68 have been studied extensively (42).

Other strains of *Enterococcus faecium* have been investigated in cheese applications for production of enterocin and antimicrobial potential. Cheddar cheese was manufactured with *E. faecium* RZS C5 and *E. faecium* DPC 1146 (38) and bacteriocin activity of cheese throughout ripening was characterized by extracting bacteriocins and challenging the extracts to a well diffusion assay with a lawn of *Listeria innocua* LMG13568. Researchers observed zones of inhibition in all samples from after the scalding step to 12 months in the first trial and 6 months in the second trial, indicating antimicrobial metabolites produced by the culture persisted in the cheese. Sarantinopoulos et al. (2002) evaluated enterocin production of *Enterococcus faecium* FAIR-E 198 in batch fermentations and then produced feta cheese with the culture (85). Researchers did not observe enterocin activity in the cheese, speculating the potential for the enterocin to be adsorbed into the cheese matrix.

5.3.7 *Lactococcus lactis* ssp. *lactis* BioSafe-10

BioSafe-10 is a frozen pelleted culture of *Lactococcus lactis* ssp. *lactis* that is a known producer of the well characterized bacteriocin nisin (17). BS-10 is manufactured by Chr Hansen and is recommended for application in cheese to control Gram positive bacteria *Clostridium* sp. and *Bacillus* sp. The culture has a slow acidification rate and is weakly proteolytic (17).

Nisin producing strains of *Lactococcus lactis* ssp. *lactis* have been used to control pathogens in food and cheese with varying degrees of success, however BS-10 lacks documentation under its trade name in the literature. Maisnier-Patin et al. (1992) produced Camembert cheese with a pair of *Lactococcus lactis* ssp. *lactis* strains that produced nisin (57). *Lactococcus lactis* ssp. *lactis* was inoculated into milk at a concentration of 7 log cfu/g, and grew during manufacture to ~9.5 log cfu/g before declining to ~6.5 log cfu/g by the end of 6 weeks of ripening. During this same time frame nisin titer in both the core and rind of the cheese followed a similar pattern whereby the titer peaked at the end of the first day of manufacture and gradually decreased throughout ripening. Nisin levels at the surface of the cheese were ~100 IU/g lower than the core curd of the cheese. The authors speculated that nisin is labile to proteolytic action of the molds growing at the surface and are thus broken down.

5.3.8 *Lactobacillus rhamnosus* LRB

Lyofast LRB is a powdered culture of *Lactobacillus rhamnosus*, manufactured by Sacco Inc (Italy). Manufacturer technical documents state this culture is intended to inhibit bacteria such as *Listeria* spp., yeasts, and molds in fermented milk and cheese products (82). Technical documents do not specify the exact bacteriocin produced by this organism. The manufacturer also states that this culture may be weakly acidic and may generate aroma from slow citrate fermentation (82). The manufacturer designates that the product may also be used as a generic probiotic for pharmaceutical purpose and in feed, however limited literature is available designating health benefits of this specific strain.

Documented evaluation of LRB's antimicrobial properties in the literature is limited, although some applications as a probiotic have been published. Minervini et al. (2017) reported that burrata cheese manufactured with a combination of LRB and LPAL had lower rates of

acidification and reduced counts of staphylococci, coliforms and *Pseudomonas* spp, (62). Klotz et al (2016) investigated the antifungal properties of LRB observing a 46.9% reduction in growth rate of common dairy mold *Mucor circinelloides* (51). In vitro testing of LRB by Schoster et al. (2013) demonstrated inhibition activity towards *Clostridium difficile* but not *C. perfringens* in broth and agar well diffusion assays (88) .

5.3.9 *Hafnia alvei* B16

B16 is a frozen pelleted culture of *Hafnia alvei* manufactured by Lallemande (France) for the control of Gram-negative organisms in cheese. Presently the mechanism of antagonism is unknown, however colicin production has been documented in other strains of *Hafnia alvei* (103). *Hafnia alvei* is a Gram negative rod of the *Enterobacteriaceae* family commonly found in the environment and intestinal tract of animals and has been isolated from milk (30, 59) and pork (34, 90). Isolated cases of opportunistic infection by the organism have been documented (74) but it is not of high clinical concern for humans or animals. It can form spores which survive the UHT pasteurization, and cause spoilage in UHT milk (16). *H. alvei* is a strong producer of sulfur dioxide gas which raises quality concerns for gas bubbles and early-blowing in the cheese (43). As such, the manufacturer advises use with late-ripening type cheeses.

The use and efficacy of B16 in milk and cheese has been documented. Delbes-Paus et al. (2012) observed 0.8 to 1.4 log reduction of STEC O26 in model cheese made with pasteurized milk was co-inoculated with B16 (28). When co-inoculated in model cheese with additional microbial consortium representing organisms commonly found in raw milk reductions in STEC were enhanced by 0.7 log cfu/g (28). However, no significant reductions in STEC were observed when the same cheese was made with raw milk. Callon et al. (2016) later challenged STEC with B16 on Saint-Nectaire type cheese. Model cheeses were produced using pasteurized milk

inoculated with each of three STEC strains at concentration of 2 log CFU/ml milk and B16 at a concentration of 6 log CFU/mL. Inhibitory power (a metric calculated by subtracting the treatment value from the control value at the same time point) of B16 in model pressed cheeses ranged from 2.6 to 2.0 depending on strain of STEC. When the same cheese was manufactured with raw milk, the IP of B16 against STEC strain O26:H11 21765 decreased from 2.4 to 2 (8).

5.3.10 *Staphylococcus xylosus* XF01

XF01 is a powdered culture of *Staphylococcus xylosus* manufactured by Lallemande for the control of Gram positive organisms in cheese. *Staphylococcus xylosus* is a gram positive, coagulase negative bacteria, it is differentiated from the foodborne pathogen *Staphylococcus aureus* which is coagulase positive. *Staphylococcus xylosus* is a commensal organism on human skin and is considered ubiquitous in the farm environment. Commonly isolated in meat and dairy, it is employed as a starter in meat fermentations. *Staphylococcus xylosus* is not considered a foodborne pathogen of concern, but cases of opportunistic infection have been documented. Antimicrobial work with strains of this species is still emerging. Matikeviciene et al. (2017) isolated a bacteriocin-like peptide from a strain of *Staphylococcus xylosus* which inhibited *L. monocytogenes* in agar based assays (60).

6. HYPOTHESIS AND OBJECTIVES

Based on available published research regarding commercially available protective cultures and their ability to inhibit growth of *L. monocytogenes* and STEC, it was hypothesized that PCs exert inhibitory and bactericidal effects against *L. monocytogenes* and STEC without significantly affecting beneficial cheese microbiota and their activity. The specific objectives of this research are as follows:

1. Identify compatible combinations of commercial protective and cheesemaking cultures.

2. Determine the antimicrobial activity of commercial protective cultures against *L. monocytogenes*
3. Determine the effect of commercial protective cultures on *L. monocytogenes* and STEC during the manufacture and storage of soft-ripened cheese manufactured from raw milk.

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CHAPTER III

COMPATIBILITY OF COMMERCIALY AVAILABLE PROTECTIVE CULTURES

WITH COMMON CHEESEMAKING CULTURES AND THEIR ANTAGONISTIC

EFFECT ON FOODBORNE PATHOGENS

ABSTRACT

The documented survival of food-borne pathogens *Listeria monocytogenes* (LM) or shiga toxin-producing *Escherichia coli* (STEC) during aging of cheeses made from unpasteurized milk highlights the need for additional interventions to enhance food safety. In addition, the Food Safety Modernization Act requires that producers implement preventive controls for such hazards. However, few validated control strategies compliant with Standards of Identity (SOI) of cheese exist. Commercially produced protective cultures (PC) of lactic acid bacteria represent actionable natural interventions to control pathogens in cheese, while maintaining compliance with SOI. The objective of this study was to identify PCs that exert antimicrobial activity towards pathogens with minimal impact on beneficial cheese microflora. Direct antagonism and agar well diffusion assays were used to determine the impact of 10 commercially available PCs on the growth of starter cultures and cultures of ripening bacteria and fungi. Deferred antagonism of PCs towards pathogen cocktails evaluated the potential for use beyond those intended by the manufacturer. PCs and starter cultures were co-cultured at 10^7 CFU/mL each, in UHT milk to determine changes in starter culture acid production. Co-cultures were incubated according to a simulated cheese-making temperature profile (4 h at 35°C followed by 20 h at 20°C) and the pH was measured at 2, 4, 6, 18 and 24 h. Compatibility assays found PC antagonism to be microbe and strain specific. A nisin-producing culture of *Lactococcus lactis* was more antagonistic toward growth of thermophilic starters than mesophilic. Antagonism against ripening bacteria and fungi was consistent among similar species. One PC negatively impacted the acidification of all starters tested. All PCs displayed deferred inhibition of either LM or STEC. This study provides data for the compatibility of commercialized PCs with cheesemaking cultures, permitting future investigations of their efficacy in cheesemaking scenarios.

HIGHLIGHTS

- Identified nine protective cultures that did not inhibit starter culture growth.
- Identified seven protective cultures did not impact starter acidification.
- Inhibition of ripening bacteria and fungi was strain specific.
- Deferred antagonism of pathogen growth highlights antimicrobial potential.

Unpasteurized (raw) milk and cheeses made from raw milk are linked to a greater number of illnesses on a per-consumption basis than pasteurized milk products (21, 28, 38). Detection of *Listeria monocytogenes* (LM) and shiga toxin-producing *Escherichia coli* (STEC) in cheeses made from raw milk (52), as well as recalls (54), and outbreaks of foodborne infection resulting in hospitalizations and death (7, 11, 26), highlights the need for additional pathogen control strategies for fermented dairy products and cheeses made from raw milk. Current regulation specifies cheeses made from unpasteurized milk be aged for at least 60 days at $\leq 1.67^{\circ}\text{C}$ (35°F) as an alternative to pasteurization. Documentation of pathogen survival in cheese beyond 60 days of aging suggests additional controls would enhance the safety of these products (13). Requirements introduced by the Food Safety Modernization Act (FSMA) set a higher standard of accountability for the safety of food products. FSMA requires food processors, including cheesemakers, to develop food safety plans that identify and manage hazards associated with their product. The use of protective bacterial cultures is one potential approach.

Protective cultures (PCs) of lactic acid bacteria (LAB) have been isolated from a variety of foods and shown to produce a host of antimicrobial metabolites such as organic acids and bacteriocins as non-specific defense against competitors (12). Cultures of LAB are generally recognized as safe (GRAS) based on their historic use in food fermentations whereas purified bacteriocin preparations require additional testing before GRAS approval (29, 36). Furthermore, the Standards of Identity (SOI) for Cheese and Cheese Products outlined in 21 CFR §133 (8), which protect the product from adulteration and misbranding, exclude the use of most antimicrobial agents allowed in other food products. Although the SOI limit the use of bacteriocin preparations to select cheeses (20), the same active compounds are allowed if produced in situ. The antimicrobial activity of laboratory strains of PCs for the control of

pathogens and spoilage organisms has been demonstrated both in culture media as well as in dairy and other food applications (35, 40). However, data on the antimicrobial activity and compatibility of commercially available cultures are limited. Commercial cultures are available to producers and are often screened for desirable attributes including flavor or aroma production, acidification rate, resistance to food enzymes, and substrate compatibility. Adjunct cultures have long been used in cheesemaking to provide additional flavor and physical properties to a cheese (2, 46), however such adjuncts must be compatible with starter and ripening cultures (22). Commercialized PCs currently available to producers are limited with specific intended applications in meat (51), dairy (19, 47), and seafood (43). The potential for cross-product (e.g. salami and cheese) and cross-pathogen (e.g. *L. monocytogenes* and *Salmonella*) is not well documented.

The objectives of this study were to screen commercially available protective cultures against foodborne pathogens of concern and to characterize their compatibility with common starter and ripening cultures of bacteria and fungi used in cheesemaking.

MATERIALS AND METHODS

Culture preparation. Starter cultures, ripening bacteria, yeasts and molds were selected to include those commonly used in the cheese industry to produce a variety of cheese types. Mesophilic starter cultures (MSC) (RA21, MA11, MA4001, MM100, and KAZU), thermophilic starter cultures (TSC) (TA54, TA61, and TM8), ripening bacteria (MGE, LR, SR1, and SR3), ripening yeast (CUM, KL, and DH), and both surface (GEO17, ABL, HP5, Neige, and VB) and internal (PA, PJ, PS, and PV) ripening molds were all produced by Danisco-Dupont (New Century, KS) and purchased through Dairy Connection Inc. (Madison, WI) (Table S1). Commercially produced protective cultures used in the present study are intended for controlling STEC in

cheese, LM in dairy (e.g., cheese surface) or meats, and spoilage microorganisms in dairy including cheese. Strains included *Lactococcus lactis* subsp. *lactis* DVS BS-10 (LLN), *Pediococcus acidilacticii* B-LC-20 (PA), *Lactobacillus curvatus* B-LC-48 (LC) (Chr. Hansen Inc., Milwaukee, WI), *Lactobacillus plantarum* (LPP) Holdbac Listeria (Danisco USA Inc, New Century, KS), *Lactobacillus rhamnosus* Lyofast LRB (LR), *Lactobacillus plantarum* Lyofast LPAL (LP), *Carnobacterium* spp. Lyofast CNBAL (CD) (Sacco, Cadorago CO, Italy), LALCULT Protect *Hafnia alvei* B16 (HA), LALCULT Protect *Staphylococcus xylosum* XF01 (Lallemand Specialty Cultures, Rhinelander, WI), and *Enterococcus faecium* SF68 (EF) (NCIMB 10415, Cerbios-Pharma SA, Barbengo, Switzerland) (Table S2). All cultures were stored according to manufacturer recommendations. Working PC cultures were prepared for use by inoculating cultures directly in either 0.1% peptone water (BD, Franklin Lakes, NJ) or UHT milk to achieve the desired counts (CFU/mL) for use in the assays below. Working cultures were mixed for 15 s then allowed to hydrate at room temperature for 30 m before use. Counts were confirmed following serial dilution in 0.1% peptone water, plating on de Man Rogosa Sharpe (MRS) agar (BD) and incubation at 30°C for 48 h. *Hafnia alvei* counts were confirmed using plate count agar supplemented with 1% skim milk powder (Price Chopper New York, NY), 5mg/L vancomycin (Fisher Scientific, Hampton, NH), and 5mg/L crystal violet (Fisher Scientific) after incubation at 30°C for 48 h as described by Delbus et al. (2013) (17). *S. xylosum* (SX) counts were confirmed on Baird Parker agar (BD) incubated at 37° for 48 h. Working cheese cultures were prepared in 0.1% peptone. MSC were enumerated on MRS as described for PCs whereas TSC were incubated anaerobically (10% CO₂, 90% N₂) at 42°C for 48 h. Ripening bacteria were enumerated on tryptic soy agar (BD) supplemented with 0.6% yeast extract (BD). Fungal cultures were propagated in sterile UHT milk (Natrell, Price Chopper, Schenectady, NY)

at 25°C for five days. Then, 100 µL were spread onto Sabouraud dextrose agar (SDA) (BD) and incubated at 25°C for 7 days. Spore suspensions were harvested by applying 10 mL of sterile deionized water with 0.5% Tween 80 (Fisher Scientific) and dislodging spores from mycelia with a sterile glass rod (Corning, Corning, NY). Spore concentrations were confirmed with a hemocytometer (Hausser Scientific, Horsham, PA).

A cocktail containing six strains of LM selected from milk and cheese-related outbreaks was prepared (CWD 675-3: Hispanic-style cheese-related outbreak, DJD1: washed rind cheese-related outbreak, ATCC 51414: milk-related outbreak, Scott A: milk-related outbreak, 2012L-5323: ricotta salata cheese-related outbreak, 2014L-6025: Hispanic style cheese-related outbreak). The cocktail of non-O157 STEC included six strains representing each of the six major serogroups (O26:H11 NRRL B-59821, O103:H2 NRRL B-59827, O45:H2 NRRL B-59823, O121:H19 03-2832 NRRL B-59834, O145:NM 03-4699 NRRL B-59836, and O111:NM 94-0961). The cocktail of STEC O157:H7 included six strains from various sources (EC2:Gouda, 7927: Apple cider, 0016: milk, ATCC 700599: Salami, Odwalla, EDL933: ground beef outbreak). Frozen (-80°C) stock cultures of each pathogen were inoculated individually into 9 mL of tryptic soy broth with 0.6% yeast extract added (TSBYE) (BD) for *E. coli* or brain heart infusion broth (BHI) (BD) for *Listeria*. Cultures were incubated at 37°C for 18 h for two subsequent transfers before use. For each pathogen, equal proportions of cells from each culture were combined as a cocktail. The resulting cocktail was pelleted through centrifugation (4,000 × g for 15 min at 4°C) (Thermo Scientific Sorvall Legend X1R, ThermoFisher Scientific, Waltham, MA), and re-suspended in an equal volume of 0.1% peptone.

Direct antagonism of starter and ripening bacteria. Direct antagonism was determined according to the method described by Barefoot and Klaenhammer (1983) (3) with minor

modification. Briefly, 1 mL of MRS agar (1.5% agar) was added to individual wells of 12-well tissue-culture treated plates (Corning Costar, Corning, NY). Bacterial cultures were prepared as previously described to achieve 9 log CFU/mL. Wells were spotted with 3 μ L of PC and the plates were allowed to dry for 15 m in a biosafety cabinet. Soft MRS agar (0.75% agar), seeded with starter, ripening, or protective cultures at 7 log CFU/mL, was then applied (750 μ L) as an overlay. Plates were covered and incubated aerobically at 35°C for 48 h for MSC and 42°C for 48 h for TSC. Definitive zones of inhibition were measured using electronic calipers (Fisher Scientific) and antagonism was defined as follows: (-) no antagonism observed, no zone; (+) slight antagonism, diffuse clearing or 0-3 mm zone; (++) moderate antagonism, 3-6.25 mm zone; (+++) significant antagonism, 6.25+ mm zone (48).

Deferred antagonism of pathogens. Deferred antagonism was determined according to the method described by Coeuret et al. (2004) with minor modification (10). Briefly, 5 μ L of PC in MRS at 9 log CFU/mL were spotted onto MRS agar and allowed to dry in a biosafety cabinet for 30 m. Then 7 mL of soft (0.3%) MRS agar was overlaid on the surface and plates were incubated at 30°C for 24 h. Following incubation, plates were overlaid with 7 mL of soft (0.3%) TSBYE seeded with a target pathogen at 7 log CFU/mL. Plates were incubated at 37°C for 24 h. Following incubation, definitive zones of inhibition and final spot diameters were measured using electronic calipers. The effective inhibition ratio (EIR) was calculated to compare relative antimicrobial activity following the equation provided by Coeuret et al. (2004). The difference between the diameters of the zone of clearing and the PC spot was divided by the diameter of the PC spot (10). An EIR value above 1 indicates the diameter of the zone of clearing exceeded the diameter of the spot which produced it. The EIR value is zero in the absence of a zone of clearing.

Direct antagonism of fungi. Direct antagonism of fungi was determined according to the method described by Inglin et al. (2015) (25). Briefly, 24-well cell-culture plates (Corning Costar) containing 300 μ l of either BHI (for cultures HA and SX) or MRS (for all other PCs) were spot-inoculated with 0.75 μ l of PC cell suspensions as previously described and incubated for 48 h at 35°C. Wells were then overlaid with 100 μ l of soft SDA (0.5% agar) seeded with 3-4 log fungal spores/mL. Plates were incubated at 25°C and inhibition relative to control was visually recorded daily for 7 days on a four-point scale as follows: (-) no antagonism observed; (+) slight antagonism, growth less dense than control; (++) moderate antagonism, growth markedly weaker or less dense than control but still present; (+++) significant antagonism, complete inhibition of growth.

Agar well diffusion assay. Indirect antagonism was determined using a standard well diffusion assay as described by Ryan et al. (1996) (44). Overnight cultures of PCs incubated in MRS broth at 35°C were centrifuged (15 min, 4000 x g, 4°C) and supernatants were collected. Subsamples of each PC supernatant were neutralized to pH 7 with 1N NaOH (Fisher Scientific). Both un-neutralized and neutralized supernatants were filter-sterilized (0.22 μ m, Fisher Scientific) to obtain cell-free supernatants (CFS). Cheese cultures were serially diluted in 0.1% peptone and added to molten soft (0.5% agar) MRS agar (for starter cultures) or TSAYE (for ripening bacteria) to achieve 7 log CFU/mL and poured into sterile petri plates. Once the agar had solidified, 8 mm diameter wells were aseptically removed and 50 μ L of neutralized or un-neutralized CFS was added in duplicate. Zones of inhibition were measured using electronic calipers after 24 h of incubation at either 42°C (TSC), 35°C (MSC), or 30°C (ripening bacteria).

Co-culture acidification. Stock cultures of PCs and SCs were prepared in UHT whole milk as previously described. Equal volumes (5 mL) of cultures were combined such that each

was present at 7 log CFU/mL. Un-inoculated milk was used as a negative control and individual PCs or SCs alone served as positive controls. Cultures were incubated statically at 35°C for 4 h, then 20°C for 20 h to simulate the temperature profile of a cheesemaking process. Samples were removed for pH measurements prior to incubation (0) and then during incubation at 2, 4, 18, and 24 h.

Statistical analysis. The antagonism and agar well diffusion assays were performed in triplicate on three different days using independently propagated cultures with technical replicates per treatment. For agar well diffusion assay, radii of zones of inhibition measured from technical duplicates were averaged for each trial. Co-culture acidification assays were performed in triplicate on three different days using independently propagated cultures with technical replicates per treatment. pH measurements from technical replicates were averaged for each treatment-time point. Data were analyzed using the PROC MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC) to determine the effects of treatment, time, and treatment \times time interactions on pH. Covariance structure AR (1) was used for repeated measures. Pairwise comparisons were performed using LSMEANS with the Tukey-Kramer method.

RESULTS AND DISCUSSION

Inhibition of starter culture growth. Nine of the 10 PCs tested did not inhibit growth of MSC or TSC in direct antagonism assays (Table 2). LLN was the only PC that inhibited growth of both MSC and TSC, with TSC being the most sensitive. The mesophilic LLN culture is a known producer of nisin, which is a well characterized bacteriocin of the class I lantibiotics. Optimal temperatures for cell growth and nisin production differ (37 and 30°C, respectively) and are strain dependent (9). Despite incubation at non-optimal temperature for nisin production by

LLN, thermophiles were still markedly inhibited. This suggests that *S. thermophilus* has an increased susceptibility to nisin. This agrees with the results of previous studies that demonstrated enhanced antimicrobial activity of nisin and nisin variants against TSC compared to MSC (18, 42). *S. thermophilus* and other TSC are acid sensitive and intracellular activity responsible for cell growth and metabolism is inhibited or lost at or below pH of <5.5 (4). Therefore, co-production of organic acids by LLN could enhance the overall antimicrobial effect. Agar well diffusion results agreed with those of the direct antagonism assay, whereby LLN demonstrated an inhibitory effect against all the starter cultures tested (Table 3). Both the neutralized and un-neutralized supernatants from LLN were inhibitory to all starter cultures tested, indicating that antimicrobial activity is not entirely pH dependent and is most likely attributed to nisin production.

Starter cultures can contain mixtures of both thermophilic and mesophilic bacteria in proprietary proportions (Supplemental Table 1). Therefore, the degree of antagonism may not be uniform across all strains in the mixture. This may limit the ability of this assay to identify potential effects of PC antagonism on cheese technology. For example, *S. thermophilus*, identified as sensitive to nisin, is added to many mixed cultures including MM4001 for flavor production late in cheese aging due to autolysis of the culture. Inhibition of *S. thermophilus* in a mixed culture by nisin might not be detected by this assay but could impact final cheese flavor by arresting growth and activity of this specific culture. Additional studies on individual strains in mixtures would be needed to identify more specific culture compatibility.

The absence of direct antagonism of starter cultures in the presence of PCs could partially be explained by production of acid by the starters, a low relative abundance of antimicrobial metabolites produced by the PC, or limits to the range of bacteriocin activity. Lactic acid

bacteria, while isolated from high-acid environments and known for their ability to produce lactic acid, are considered neutrophiles and are not well adapted for growth at low pH (24). Acid tolerance systems have been observed to abate antagonism of *Lactococcus lactis* from multiple stresses (39), but induction of these systems would not be expected within the short timeframe of cheesemaking. As such, production and accumulation of organic acids by starter cultures or PCs could inhibit starter culture growth. Additionally, most starter cultures, with the exception of select strains of lactobacilli and lactococci, do not produce the enzyme catalase to neutralize hydrogen peroxide (14) and would be susceptible to any localized peroxide production by PCs. Finally, the range of bacteriocin activity is narrow. Bacteriocins produced by Gram positive bacteria exert antimicrobial activity by disrupting the cell membrane either through inhibiting membrane synthesis or forming pores causing cell contents to leak. While limited reports of bacteriocin activity towards both Gram negative and Gram positive bacteria exist (15, 32), bacteriocin activity is typically limited to the cell wall type of the bacteriocin producer (1). This may partially explain the absence of activity of HA on Gram positive starter cultures observed here.

Inhibition of ripening bacteria. Growth of *Arthrobacter nicotianae* (MGE) was affected by all PCs except LR, with the largest zones of inhibition observed when grown with LLN. Sensitivity of MGE to the SX culture is notable as these two species are frequently found co-inhabiting cheese rind communities (6). The CFS from six PCs, including LLN, inhibited MGE in the agar well diffusion assay, indicating inhibition was not dependent on PCs directly, but rather their production of inhibitory metabolites. *Brevibacterium linens* cultures (LR, SR1, and SR3) were only mildly inhibited by several PCs including PA, LC, LPP, and LP. Variation among these cultures allows cheesemakers to optimize color, growth rate, and proteolytic

activity for flavor production in the final cheese. However, similarities in sensitivity to PCs suggests that specific trial-and-error testing in each cheese and specific ripening bacteria may be necessary to ensure ripening is not inhibited in cheese made with these PCs. *B. linens* strains have been found to produce the bacteriocins linecin or linocin, but susceptibility to other bacteriocins has not been well characterized (34, 53). Ripening cultures MGE, SR1, and SR3 were less sensitive to the un-neutralized CFS of LR, suggesting a partial effect of acid produced by this culture. None of the ripening cultures tested, including Gram negative MGE, were sensitive to supernatants of Gram negative HA, despite inhibition in the direct assay. This is in agreement with data from the manufacturer that the mechanism of action is not believed to be a function of bacteriocin or acid production. Together, these results suggest potential compatibility of all PCs except LLN with these commercial ripening cultures. Tables 2 and 4 provide reference for the compatibility of commercially produced PCs and common cheesemaking cultures.

Inhibition of protective cultures. Previous studies have identified the potential for synergy among laboratory strains of protective cultures (37) but not those that are commercially produced. Screening this panel for inhibition between cultures is the first step towards identifying the potential for synergistic combinations. Protective cultures LLN and HA inhibited the growth of nearly all other PCs except themselves and each other. The probiotic culture EF was inhibited by all cultures except itself and SX. The SX culture was inhibited by PA, LC, LLN and HA and was the second most sensitive of the PCs tested. These results indicate that LLN, HA, SX, and EF are not good candidates for combinatory approaches.

Inhibition of pathogens. Deferred antagonism, as measured by a zone of clearing and calculated effective inhibition ratio (EIR) value, was observed for all PC-pathogen combinations (Table 3). *Lb. plantarum* LPP demonstrated the highest measurable activity towards both Gram

negative and Gram positive pathogens with EIRs >2.6. Similar results were reported by Coeurt et al. (2004) (10), for laboratory strains of probiotic lactobacilli against strains of *L. monocytogenes*. While LPP had the largest EIR value, CD, and LR had statistically similar values to LPP. All cultures with the exception of LPP shared a statistical grouping, indicating the pronounced activity of LPP. Antimicrobial activity against *E. coli* was strain dependent with EIRs ranging from 0 to 2.8. EIR values of multiple PCs were statistically similar for both O157 and non-O157 STEC, indicating similarity in extent of inhibition. Culture HA and EF had the lowest values of the panel and shared a statistical grouping while *L. plantarum* cultures LP and LPP, as well as CD and LR or LC and SX had the largest EIR values and shared groupings for non-O157 and O157 STEC respectively. EIR values reported in the present study for O157 and non-O157 STEC all exceeded 1, suggesting potential antimicrobial activity for all PCs against these pathogens. As mentioned previously, antagonistic activity can in part be attributed to the production of multiple metabolites including organic acids, hydrogen peroxide, and bacteriocins that diffuse into the agar layer prior to the growth of the indicator. Steps to neutralize hydrogen peroxide or acidity were not taken (23). Given the cell wall specificity of bacteriocins mentioned previously, antagonism observed could be partially attributed to these metabolites.

Inhibition of fungi. Antagonism of fungi was strain dependent (T), whereby nine of the 10 PCs demonstrated antifungal activity against at least one of the 12 fungal cultures.

Debaryomyces hansenii (DH) was the only yeast tested whose growth was visibly affected by PCs. Substantial inhibition was observed on day four with PA, LPP, LP, and LR and slight inhibition with CD and EF. Strom et al. (2001) observed inhibition of *D. hansenii* when treated with the supernatant of *L. plantarum* MiLAB393 that they attributed to specific cyclo-dipeptides (49). A study by Magnusson et al. (2003), reported on the selective antifungal activity of

multiple *L. plantarum* isolates towards some, but not all, yeasts or molds (33, 45). Yeasts neutralize the pH at the surface of the cheese to allow for subsequent succession by a community of molds and bacteria, depending on the cheese type (50). Reduced growth in response to the presence of PCs could disrupt community development. As a result delays in ripening or development of niches for less-desirable organisms could impact both quality and safety.

Surface ripening molds, including *Penicillium candidum*, are responsible for flavor and texture development of cheese (27, 30). PCs PA, LPP, CD, LP, LR showed slight or intermediate inhibitory effects against most *P. candidum* cultures. Cultures ABL and Neige were both completely inhibited by LPP, but ABL recovered with slight growth observed through the remaining days of observation. This suggests that use of LPP could impact rind development and ripening if used in conjunction with these cultures. One intended use for LR is for the control of unwanted yeast and mold. This culture was partially inhibitory to five of the 12 fungal cultures tested and did not completely inhibit any. *Geotricum candidum* GEO17 was the only surface culture not susceptible to any of the PCs tested. This agrees with the results reported by Salas et al. (2018) on the resistance of *G. candidum* to a panel of LAB isolates (31).

P. roqueforti imparts characteristic color, flavor, and texture to blue cheeses. Because *P. roqueforti* cultures can differ in growth rate, salt tolerance, enzyme production and color, multiple mold cultures are commonly used to modulate ripening and produce desirable qualities in the final cheese. Only HA and SX showed slight antagonism towards *P. roqueforti* cultures PA, PJ, and PV. The degree of inhibition reported was often due to the density of the culture when compared to control rather than color or absence of growth. Slight inhibition observed did not advance in extent beyond day four, suggesting metabolites produced by the PC persisted in the media preventing mold regrowth or recovery. The resulting matrix presented in T could be

used to anticipate potential antifungal activity when selecting cultures to avoid negative impacts on in surface community development and ripening.

Impact of PCs on acidification activity of starters. A starter culture's primary role in cheesemaking is the conversion of lactose into lactic acid, which aids curd development and contributes to the safety of the product. Even mild inhibition of acidification by PCs endangers product quality, consistency, and safety if pH-time targets are not met. Overall, no significant treatment effects or treatment and time interactions were identified for 56 of 80 co-culture combinations. In other words, seven of the 10 PCs did not impact the acidification of common starters at all, while LP and LLN only impacted the acidification of one starter each. Significant treatment and time interactions and significant treatment effects were observed for 22 of the remaining combinations. Mean pH values were not significantly different at any time point for 15 of this 22 combination subset. The remaining seven combinations for which pH values at one or more time points differed from control are displayed in Table 6. Significant treatment effects were identified for the combinations of MA11 and LPP and MA4001 and LP. LPP impacted the acidification of all MSC and TSC with pH values higher than control as incubation time progressed (Table 6). This suggests that products may fail to meet acidification targets when used with this culture at the recommended dose of ~ 7 log CFU/mL. Initial decreases in pH following the addition of LPP could be attributed to the lower cell counts per unit of culture that necessitates the addition of more product by weight. The greater amount of added material (carrier/fermentate) in the same volume of milk also contributed to visual and textural differences in the stock solution compared to other PCs. Oumer et al. (2001) reported that the acidification potential of TSCs was more readily impacted in the presence of PCs than that of

MSCs (42). They concluded that using PCs as an adjunct to MSCs is more feasible than with TSCs. Data presented here follow a similar pattern and support this conclusion.

Acid production by PCs in UHT milk alone was strain specific, but most did not decrease pH by more than 1 unit over 24 h incubation through the cheesemaking gradient. Cultures LPP, LP, and LLN produced the greatest reductions in pH at 1.47, 0.95, and 1.4 units, respectively. The difference in pH from the starting value of each PC treatment was less than 0.3 pH unit at the four hour time point for all PCs except LPP (-0.35 lower than control), LP (-0.35), LLN (-0.52), and HA (-0.33). For comparison, cheesemaking starter cultures had reduced pH of UHT milk by ~1.5 units at the 4h time point and total decrease of ~2 units by the 24 h point. These data suggest that while select PCs can contribute to the acidification of milk, it is neither at the same rate nor to the same extent as the starter cultures. Starter culture activity is vital throughout the cheese make process, but is especially critical early on. Based on these data, acidification is most likely attributed to the starter at early time points and that acid development by select PCs will be additive, potentially accelerating a cheese make provided PC activity does not inhibit growth of the starter when combined.

Manufacturer technical documentation, direct antagonism and agar well diffusion assays suggested the potential for LLN to prevent growth of starter cultures. However, co-culture pH data indicate that there is little impact of LLN on the acidification potential of most starter cultures at this inoculation level. Overall, these results suggest LLN could be safely used in a cheese application and therefore will be a candidate in further testing. In contrast, co-cultures with LPP had a significant effect on the acidification of several starter cultures at the recommended inoculation level despite no indication of inhibition in agar-based assays. Previous studies in milk (42), yogurt (16) and cheese (5, 41, 42) have demonstrated a strain-dependent

impact of PC on starter acidification. Although cultures are generally compatible, the potential for strain specific interactions underscores the importance of screening new cultures for compatibility and to determine acceptable inoculation levels.

In summary, most of the commercially available PCs screened in the present study did not inhibit cheesemaking starter growth or acidification potential. Inhibition of ripening bacteria, yeasts and molds by PCs was mixed and strain specific. More testing is needed in cheese models to confirm compatible use of PCs in specific applications. Limited cross-reactivity between PCs identifies combinations of PCs that could be used in combination for potential synergistic effects. Additional studies are needed to confirm the antimicrobial activity of PCs against target pathogens and optimize inoculation parameters for antimicrobial effect without disrupting culture activity under specific conditions of use.

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TABLES

TABLE 1. Direct antagonism of cheesemaking cultures by protective cultures

Culture		Degree of Inhibition by Protective Cultures									
		PA	LC	LPP	LP	CD	EF	LLN	LR	HA	SX
Mesophilic Starter Cultures	RA21	-	-	-	-	-	-	+	-	-	-
	MA11	-	-	-	-	-	-	+	-	-	-
	MA4001	-	-	-	-	-	-	+	-	-	-
	MM100	-	-	-	-	-	-	+	-	-	-
	KAZU	-	-	-	-	-	-	+++	-	-	-
Thermophilic Starter Cultures	TA54	-	-	-	-	-	-	+++	-	-	-
	TA61	-	-	-	-	-	-	+++	-	-	-
	TM81	-	-	-	-	-	-	+++	-	-	-
Ripening Bacteria	MGE	+	+	+	+	+	+	+++	-	+	+
	LR	++	+	++	++	-	-	-	-	+++	-
	SR1	++	+	++	++	-	-	-	-	-	-
	SR3	+	+	++	+	+	-	-	-	-	-

Degree of Inhibition: (-) No Inhibition Observed– No Zone; (+) Slight Inhibition – Diffuse Clearing, or 0-3 mm Zone ; (++) Moderate Inhibition- 3-6.25 mm Zone; (+++) Significant Inhibition- 6.25+ mm Zone.

TABLE 2. Direct antagonism of protective cultures

Culture	Degree of Inhibition by Protective Cultures									
	PA	LC	LPP	LP	CD	EF	LLN	LR	HA	SX
PA	-	-	-	-	-	-	++	-	++	-
LC	-	-	-	-	-	-	++	-	++	-
LPP	-	-	-	-	-	-	+	-	++	-
LP	-	-	-	-	-	-	+	-	++	-
CD	-	-	-	-	-	-	++	-	++	-
EF	+	+	+	+	+	-	+	+	+	-
LLN	-	-	-	-	-	-	-	-	-	-
LR	-	-	-	-	-	-	+	-	+	-
HA	-	-	-	-	-	-	-	-	-	-
SX	+	+	+	-	+	-	+	-	+	-

Degree of Inhibition: (-) No Inhibition Observed– No Zone; (+) Slight Inhibition – Diffuse

Clearing, or 0-3 mm Zone; (++) Moderate Inhibition- 3-6.25 mm Zone; (+++) Significant

Inhibition- 6.25+ mm Zone.

PA: *Pediococcus acidilactici* B-LC-20, LC: *Lactobacillus curvatus* BLC-48, LPP: *Lb. plantarum*

Holdbac, LP: *Lactobacillus plantarum* Lyofast LPAL, CD: *Carnobacterium divergens* CNBAL,

EF: *Enterococcus faecium* SF68, LLN: *Lactococcus lactis* ssp. *lactis* BS-10, LR: *Lb. rhamnosus*

Lyofast LRB, HA: *Hafnia alvei* B16, SX: *Staphylococcus xylosus* XF01

TABLE 3. Inhibition of cheesemaking cultures by neutralized (N) and un-neutralized (U) cell free supernatants of protective cultures

Starter Culture/ Ripening Bacteria		Degree of Inhibition by Protective Cultures																				
		PA		LC		LPP		LP		CD		EF		LLN		LR		HA		SX		
		U	N	U	N	U	N	U	N	U	N	U	N	U	N	U	N	U	N	U	N	
Mesophilic Starter Cultures	RA21	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
	MA11	-	-	-	-	-	-	-	-	-	-	-	-	-	++	++	-	-	-	-	-	-
	MA4001	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
	MM100	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
	KAZU	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
Thermophilic Starter Cultures	TA54	-	-	-	-	-	-	-	-	-	-	-	-	++	++	-	-	-	-	-	-	
	TA61	-	-	-	-	-	-	-	-	-	-	-	-	++	++	-	-	-	-	-	-	
	TM81	-	-	-	-	-	-	-	-	-	-	-	-	++	++	-	-	-	-	-	-	
Ripening Bacteria	MGE	+	+	-	-	++	+	+	+	+	+	-	-	+	+	+	-	-	-	-	-	
	LR	-	-	-	-	-	-	-	-	-	-	-	-	++	++	+	-	-	-	-	-	
	SR1	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	
	SR3	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	

Degree of Inhibition: (-) no zone; (+) 1 to 5mm; (++) 5 to 15mm; (+++) 15 mm and over.

TABLE 4. Direct antagonism of pathogens by protective cultures

Pathogen	Effective Inhibition Ratio ¹									
	PA	LC	LPP	LP	CD	EF	LLN	LR	HA	SX
Non-O157 STEC	1.92 ± 0.11 ^b	1.74 ± 0.30 ^{bc}	2.62 ± 0.62 ^a	1.98 ± 0.22 ^{ab}	1.64 ± 0.08 ^{ab}	1.46 ± 0.04 ^c	1.32 ± 0.14 ^{bc}	1.97 ± 0.13 ^{ab}	1.39 ± 0.15 ^c	1.86 ± 0.14 ^b
STEC O157	1.77 ± 0.33 ^{bc}	2.13 ± 0.22 ^{ab}	2.65 ± 0.08 ^a	2.09 ± 0.21 ^{ab}	1.72 ± 0.24 ^{bc}	1.38 ± 0.13 ^c	1.64 ± 0.41 ^{bc}	1.86 ± 0.34 ^{bc}	1.35 ± 0.10 ^c	1.96 ± 0.14 ^{abc}
LM ²	2.31 ± 0.19 ^b	1.87 ± 0.04 ^b	3.14 ± 0.19 ^a	2.28 ± 0.15 ^b	2.41 ± 0.13 ^{ab}	1.36 ± 0.32 ^b	1.86 ± 0.17 ^b	2.60 ± 0.22 ^{ab}	1.29 ± 0.32 ^b	2.26 ± 0.56 ^b

Means within a row with the same letter are not significantly different

¹Effective Inhibition Ratio (EIR) was calculated as the difference between the diameters of the zone of clearing and the PC spot, divided by the diameter of the PC spot.

²LM: *Listeria monocytogenes*

TABLE 5 Degree of inhibition of fungi by protective cultures relative to control

Fungal Culture		Degree of inhibition by Protective Culture									
		PA	LC	LPP	LP	CD	EF	LLN	LR	HA	SX
Yeasts	CUM	-	-	-	-	-	-	-	-	-	-
	KL71	-	-	-	-	-	-	-	-	-	-
Surface Molds	DH	++	-	++	++	+	+	-	++	-	-
	GEO17	++	-	+++	++	+	-	-	++	+	-
	ABL	-	-	-	-	-	-	-	-	-	-
	HP6	++	-	++	++	+	-	-	+	-	-
	Neige	++	-	+++	++	-	-	-	++	+	-
	VB	++	-	++	++	+	-	-	++	-	-
Internal Molds	PA	-	-	-	-	-	-	-	-	+	+
	PJ	-	-	-	-	-	-	-	-	+	+
	PS	-	-	-	-	-	-	-	-	-	-
	PV	-	-	-	-	-	-	-	-	+	+

Degree of Inhibition: (-) Uninhibited- Same Amount of Growth (Density of coverage) as control;

(+) Slight Inhibition- Weakened Growth relative to control; (++) Substantial Inhibition- Slight

amount of growth relative to control; (+++) Complete Inhibition- No Growth compared to

control.

TABLE 6. pH Values (mean +/- SD) of UHT milk incubated at a cheesemaking temperature profile with cheesemaking starter cultures alone or with protective cultures.

Treatment	Mean pH							
	2h		4h		18h		24h	
RA21	4.86	± 0.29	4.56	± 0.13	4.27	± 0.15	4.26	± 0.12
RA21 + LPP	5.59	± 0.20	5.48	± 0.19	*	4.99 ± 0.08	4.82	± 0.05
MA11	4.68	± 0.03	4.65	± 0.24	4.21	± 0.09	4.23	± 0.13
MA11 + LPP	5.49	± 0.12	5.32	± 0.10	4.96	± 0.03	4.92	± 0.14
MA4001	5.01	± 0.10	4.79	± 0.05	4.36	± 0.07	4.35	± 0.03
MA4001 + LPP	5.55	± 0.13	*	5.46 ± 0.17	*	5.03 ± 0.08	*	4.85 ± 0.01
MA4001 + LP	5.02	± 0.11	4.73	± 0.05	4.22	± 0.03	4.15	± 0.01
MM100	4.94	± 0.07	4.72	± 0.09	4.33	± 0.09	4.36	± 0.07
MM100 + LPP	5.53	± 0.13	*	5.40 ± 0.14	*	4.97 ± 0.06	*	4.82 ± 0.02
KAZU	5.51	± 0.08	4.97	± 0.21	4.36	± 0.03	4.28	± 0.03
KAZU + LPP	5.61	± 0.11	5.48	± 0.13	*	4.96 ± 0.07	*	4.78 ± 0.03
TA54	5.36	± 0.33	4.87	± 0.31	4.49	± 0.19	4.43	± 0.26
TA54 + LPP	5.61	± 0.26	5.37	± 0.29	4.79	± 0.12	4.66	± 0.06
TA54 + LLN	5.90	± 0.27	5.30	± 0.27	*	4.71 ± 0.15	4.61	± 0.19
TA61	5.17	± 0.31	4.64	± 0.25	4.27	± 0.07	4.22	± 0.14
TA61 + LPP	5.67	± 0.24	5.42	± 0.20	*	4.73 ± 0.10	4.56	± 0.08
TM81	5.23	± 0.22	4.60	± 0.16	4.32	± 0.02	4.30	± 0.05
TM81 + LPP	5.78	± 0.16	*	5.45 ± 0.24	*	4.82 ± 0.12	*	4.67 ± 0.04

Within a column* designates value is significantly different (P <0.05) from respective control

LPP: *Lb. plantarum* Holdbac, LP: *Lb. plantarum* LPAL, LLN: *Lc. lactis ssp lactis* BS-10

SUPPLEMENTARY TABLES

TABLE S1. Danisco cheesemaking cultures used, Sourced from Dairy Connection

Culture Name	Species	Culture Details
RA21	<i>Lactococcus lactis</i> ssp. <i>lactis</i> <i>Lc. lactis</i> ssp. <i>cremoris</i> <i>Streptococcus salivarius</i> ssp. <i>thermophilus</i>	Mesophilic culture, fast acidification
MA11	<i>Lc. lactis</i> ssp. <i>lactis</i> <i>Lc. lactis</i> ssp. <i>cremoris</i>	Mesophilic heterofermentative cultures
MA4001	<i>Lc. lactis</i> ssp. <i>lactis</i> <i>Lc. lactis</i> ssp. <i>cremoris</i> <i>Lc. lactis</i> ssp. <i>lactis</i> biovar <i>diacetylactis</i> <i>S. thermophilus</i>	Heterofermentative farmhouse cultures with a mesophilic predominance
MM100	<i>Lc. lactis</i> ssp. <i>lactis</i> <i>Lc. lactis</i> ssp. <i>cremoris</i> <i>Lc. lactis</i> ssp. <i>lactis</i> biovar <i>diacetylactis</i>	Aromatic acidifying mesophilic culture,
KAZU	<i>Lc. lactis</i> ssp. <i>lactis</i> <i>Lc. lactis</i> ssp. <i>cremoris</i> <i>Lc. lactis</i> ssp. <i>lactis</i> biovar <i>diacetylactis</i> <i>Lactobacillus helveticus</i>	Mesophilic heterofermentative farmhouse cultures adapted to farmer productions, equal balance of strains
TA54	<i>S. thermophilus</i>	Thermophilic culture
TA61	<i>S. thermophilus</i>	Thermophilic “cooked cheese” culture
TM81	<i>S. salivarius</i> ssp. <i>thermophilus</i> <i>Lb. delbrueckii</i> ssp. <i>bulgaricus</i>	Thermophilic, rapid acidification for cheese with high dry matter
MGE	<i>Arthrobacter nicotianae</i>	Ripening Bacteria
LR	<i>Brevibacterium linens</i>	Ripening Bacteria
SR1	<i>B. linens</i>	Ripening Bacteria
SR3	<i>B. linens</i>	Ripening Bacteria
CUM	<i>Candida utilis</i>	Yeast
KL71	<i>Kluyvermyces lactis</i>	Yeast
DH	<i>Debaromyces hansenii</i>	Yeast
GEO17	<i>Geotrichum candidum</i>	Surface Yeast/mold hybrid
ABL	<i>Penicillium candidum</i>	Surface Mold
HP6	<i>P. candidum</i>	Surface Mold
NEIGE	<i>P. candidum</i>	Surface Mold

VB	<i>P. candidum</i>	Surface Mold
PA	<i>P. roqueforti</i>	Internal Mold
PJ	<i>P. roqueforti</i>	Internal Mold
PS	<i>P. roqueforti</i>	Internal Mold
PV	<i>P. roqueforti</i>	Internal Mold

TABLE S2: Commercially available protective cultures

Manufacturer	Trade Name	Internal Code	Organism (s)	Manufacturer Intended Purpose
Chr. Hansen	BLC-20	PA	<i>Pediococcus acidilactici</i>	LM ¹ control in meat
Chr. Hansen	BLC 48	LC	<i>Lactobacillus curvatus</i>	Minimize LM on bacon
Danisco	Holdbac	LPP	<i>Lactobacillus plantarum</i>	LM control in dairy
SACCO	LPAL	LP	<i>Lactobacillus plantarum</i>	Surface treatment for LM on cheese
SACCO	CNBAL	CD	<i>Carnobacterium divergens ssp.</i>	Surface treatment for LM on cheese
Cerbios Pharma	SF68	EF	<i>Enterococcus faecium</i>	Pharmaceutical probiotic
Chr. Hansen	BS-10	LLN	<i>Lactococcus lactis ssp lactis</i>	Control of Gram + : <i>Clostridium</i> sp, <i>Bacillus</i> sp.
SACCO	LRB	LR	<i>Lactobacillus rhamnosus</i>	Inhibit LM, yeast, molds
Lallemand Lalccult	B16	HA	<i>Hafnia alvei</i>	STEC control in milk
Lallemand Lalccult	XF01	SX	<i>Staphylococcus xylosum</i>	LM control in milk

¹-*Listeria monocytogenes*

TABLE S3: pH (mean +/- SD) of UHT milk incubated at a cheesemaking temperature profile with cheesemaking starter cultures alone or with protective cultures.

Treatment		Mean pH				
		0h	2h	4h	18h	24h
RA21	Control	5.96 ± 0.62	4.86 ± 0.29	4.56 ± 0.13	4.27 ± 0.15	4.26 ± 0.12
	PA	6.37 ± 0.15	4.89 ± 0.28	4.59 ± 0.21	4.31 ± 0.17	4.26 ± 0.04
	LC	6.35 ± 0.19	4.95 ± 0.35	4.61 ± 0.24	4.34 ± 0.16	4.23 ± 0.02
	LPP	6.01 ± 0.21	5.59 ± 0.20	5.48 ± 0.19	4.99 ± 0.08	4.82 ± 0.05
	LP	6.34 ± 0.21	5.03 ± 0.42	4.61 ± 0.27	4.25 ± 0.12	4.12 ± 0.03
	CD	6.35 ± 0.23	4.98 ± 0.39	4.62 ± 0.25	4.36 ± 0.16	4.31 ± 0.05
	EF	6.34 ± 0.21	5.00 ± 0.35	4.62 ± 0.25	4.33 ± 0.15	4.28 ± 0.05
	LLN	6.26 ± 0.29	4.94 ± 0.29	4.57 ± 0.21	4.31 ± 0.12	4.29 ± 0.09
	LR	6.29 ± 0.29	5.03 ± 0.39	4.61 ± 0.24	4.30 ± 0.13	4.27 ± 0.09
	HA	6.28 ± 0.30	5.04 ± 0.44	4.64 ± 0.27	4.39 ± 0.15	4.30 ± 0.11
	SX	6.35 ± 0.33	5.06 ± 0.46	4.61 ± 0.25	4.34 ± 0.11	4.28 ± 0.08
MA11	Control	5.89 ± 0.68	4.68 ± 0.03	4.65 ± 0.24	4.21 ± 0.09	4.23 ± 0.13
	PA	5.94 ± 0.46	4.84 ± 0.22	4.47 ± 0.06	4.22 ± 0.05	4.14 ± 0.01
	LC	5.93 ± 0.36	4.69 ± 0.03	4.49 ± 0.01	4.27 ± 0.08	4.19 ± 0.01
	LPP	5.86 ± 0.26	5.49 ± 0.12	5.32 ± 0.10	4.96 ± 0.03	4.92 ± 0.14
	LP	5.94 ± 0.48	4.74 ± 0.07	4.47 ± 0.05	4.12 ± 0.05	4.10 ± 0.04
	CD	5.90 ± 0.52	4.74 ± 0.06	4.49 ± 0.04	4.25 ± 0.06	4.21 ± 0.04
	EF	5.97 ± 0.46	4.74 ± 0.05	4.50 ± 0.05	4.24 ± 0.02	4.21 ± 0.07
	LLN	5.86 ± 0.52	4.72 ± 0.05	4.45 ± 0.06	4.26 ± 0.02	4.24 ± 0.07
	LR	5.88 ± 0.44	4.73 ± 0.03	4.48 ± 0.08	4.21 ± 0.03	4.21 ± 0.08
	HA	5.94 ± 0.42	4.70 ± 0.07	4.47 ± 0.08	4.24 ± 0.03	4.24 ± 0.03
	SX	5.88 ± 0.46	4.67 ± 0.06	4.48 ± 0.05	4.24 ± 0.01	4.24 ± 0.06
MA4001	Control	6.24 ± 0.16	5.01 ± 0.10	4.79 ± 0.05	4.36 ± 0.07	4.35 ± 0.03
	PA	6.23 ± 0.04	4.97 ± 0.15	4.74 ± 0.13	4.35 ± 0.04	4.31 ± 0.05
	LC	6.26 ± 0.12	5.02 ± 0.09	4.76 ± 0.04	4.38 ± 0.03	4.33 ± 0.05
	LPP	5.96 ± 0.17	5.55 ± 0.13	5.46 ± 0.17	5.03 ± 0.08	4.85 ± 0.01
	LP	6.20 ± 0.09	5.02 ± 0.11	4.73 ± 0.05	4.22 ± 0.03	4.15 ± 0.01
	CD	6.25 ± 0.15	5.02 ± 0.11	4.76 ± 0.05	4.38 ± 0.03	4.37 ± 0.06
	EF	6.24 ± 0.10	5.02 ± 0.14	4.75 ± 0.06	4.36 ± 0.03	4.34 ± 0.02
	LLN	6.15 ± 0.19	4.95 ± 0.10	4.71 ± 0.06	4.38 ± 0.03	4.35 ± 0.04
	LR	6.20 ± 0.15	5.03 ± 0.10	4.75 ± 0.05	4.35 ± 0.02	4.31 ± 0.05
	HA	6.16 ± 0.13	5.02 ± 0.12	4.75 ± 0.04	4.38 ± 0.02	4.36 ± 0.01
	SX	6.30 ± 0.20	4.99 ± 0.10	4.75 ± 0.04	4.39 ± 0.02	4.36 ± 0.03
MM100	Control	6.17 ± 0.31	4.94 ± 0.07	4.72 ± 0.09	4.33 ± 0.09	4.36 ± 0.07

Treatment	Mean pH				
	0h	2h	4h	18h	24h
PA	6.18 ± 0.15	4.91 ± 0.10	4.67 ± 0.04	4.34 ± 0.03	4.35 ± 0.08
LC	6.15 ± 0.05	4.91 ± 0.11	4.67 ± 0.04	4.32 ± 0.07	4.32 ± 0.08
LPP	5.95 ± 0.19	5.53 ± 0.13	5.40 ± 0.14	4.97 ± 0.06	4.82 ± 0.02
LP	6.16 ± 0.20	4.98 ± 0.09	4.68 ± 0.04	4.20 ± 0.03	4.15 ± 0.02
CD	6.09 ± 0.15	4.92 ± 0.10	4.66 ± 0.09	4.32 ± 0.06	4.31 ± 0.04
EF	6.14 ± 0.22	4.96 ± 0.05	4.69 ± 0.02	4.33 ± 0.04	4.31 ± 0.03
LLN	6.10 ± 0.28	4.95 ± 0.09	4.68 ± 0.04	4.37 ± 0.03	4.33 ± 0.03
LR	6.17 ± 0.24	4.99 ± 0.10	4.71 ± 0.03	4.34 ± 0.02	4.31 ± 0.02
HA	6.11 ± 0.25	4.98 ± 0.13	4.71 ± 0.04	4.37 ± 0.02	4.34 ± 0.01
SX	6.17 ± 0.33	5.00 ± 0.17	4.72 ± 0.05	4.39 ± 0.02	4.35 ± 0.03
KAZU Control	6.47 ± 0.10	5.51 ± 0.08	4.97 ± 0.21	4.36 ± 0.03	4.28 ± 0.03
PA	6.50 ± 0.07	5.40 ± 0.10	4.89 ± 0.02	4.34 ± 0.05	4.28 ± 0.02
LC	6.39 ± 0.07	5.38 ± 0.21	4.88 ± 0.03	4.33 ± 0.05	4.28 ± 0.06
LPP	6.06 ± 0.04	5.61 ± 0.11	5.48 ± 0.13	4.96 ± 0.07	4.78 ± 0.03
LP	6.39 ± 0.04	5.48 ± 0.04	4.90 ± 0.08	4.23 ± 0.05	4.18 ± 0.06
CD	6.45 ± 0.06	5.53 ± 0.03	4.93 ± 0.09	4.36 ± 0.03	4.36 ± 0.05
EF	6.44 ± 0.07	5.52 ± 0.08	4.94 ± 0.12	4.38 ± 0.03	4.31 ± 0.02
LLN	6.33 ± 0.04	5.36 ± 0.13	4.84 ± 0.05	4.44 ± 0.01	4.34 ± 0.01
LR	6.40 ± 0.04	5.44 ± 0.04	4.90 ± 0.04	4.33 ± 0.03	4.26 ± 0.04
HA	6.35 ± 0.07	5.39 ± 0.12	4.93 ± 0.06	4.38 ± 0.03	4.31 ± 0.03
SX	6.48 ± 0.11	5.56 ± 0.33	4.99 ± 0.22	4.38 ± 0.00	4.31 ± 0.06
TA54 Control	6.71 ± 0.23	5.36 ± 0.33	4.87 ± 0.31	4.49 ± 0.19	4.43 ± 0.26
PA	6.64 ± 0.26	5.47 ± 0.51	5.01 ± 0.32	4.54 ± 0.26	4.42 ± 0.22
LC	6.66 ± 0.14	5.62 ± 0.37	5.11 ± 0.23	4.61 ± 0.22	4.51 ± 0.24
LPP	6.16 ± 0.10	5.61 ± 0.26	5.37 ± 0.29	4.79 ± 0.12	4.66 ± 0.06
LP	6.70 ± 0.14	5.71 ± 0.20	5.18 ± 0.24	4.54 ± 0.05	4.46 ± 0.08
CD	6.70 ± 0.17	5.90 ± 0.27	5.30 ± 0.27	4.71 ± 0.15	4.61 ± 0.19
EF	6.67 ± 0.12	5.84 ± 0.21	5.19 ± 0.18	4.68 ± 0.15	4.60 ± 0.13
LLN	6.49 ± 0.04	5.95 ± 0.16	5.50 ± 0.01	4.87 ± 0.02	4.69 ± 0.14
LR	6.64 ± 0.15	5.72 ± 0.30	5.03 ± 0.19	4.50 ± 0.11	4.52 ± 0.19
HA	6.62 ± 0.14	5.64 ± 0.19	5.13 ± 0.15	4.73 ± 0.17	4.61 ± 0.23
SX	6.60 ± 0.14	5.56 ± 0.22	5.06 ± 0.18	4.68 ± 0.17	4.64 ± 0.14
TA61 Control	6.76 ± 0.22	5.17 ± 0.31	4.64 ± 0.25	4.27 ± 0.07	4.22 ± 0.14
PA	6.72 ± 0.33	5.30 ± 0.24	4.71 ± 0.16	4.30 ± 0.10	4.27 ± 0.08
LC	6.60 ± 0.17	5.47 ± 0.39	4.87 ± 0.18	4.35 ± 0.14	4.34 ± 0.13
LPP	6.13 ± 0.04	5.67 ± 0.24	5.42 ± 0.20	4.73 ± 0.10	4.56 ± 0.08
LP	6.60 ± 0.28	5.63 ± 0.51	4.78 ± 0.30	4.27 ± 0.11	4.20 ± 0.06
CD	6.66 ± 0.37	5.72 ± 0.71	4.89 ± 0.38	4.37 ± 0.16	4.31 ± 0.11

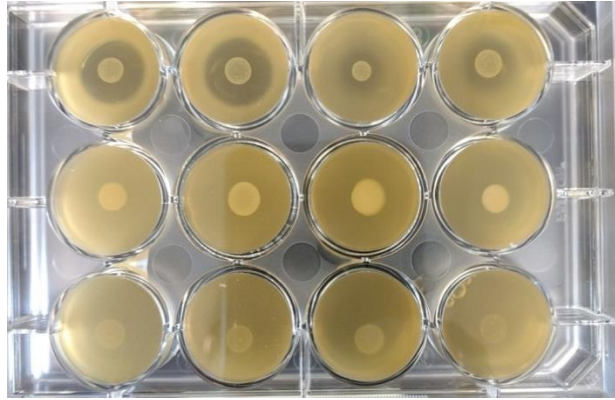
Treatment	Mean pH					
	0h	2h	4h	18h	24h	
EF	6.61 ± 0.37	5.58 ± 0.49	4.78 ± 0.25	4.32 ± 0.11	4.30 ± 0.09	
LLN	6.56 ± 0.33	5.39 ± 0.33	4.77 ± 0.22	4.34 ± 0.12	4.29 ± 0.10	
LR	6.60 ± 0.36	5.53 ± 0.56	4.75 ± 0.20	4.29 ± 0.09	4.31 ± 0.09	
HA	6.57 ± 0.38	5.36 ± 0.41	4.70 ± 0.22	4.33 ± 0.13	4.32 ± 0.17	
SX	6.56 ± 0.37	5.21 ± 0.40	4.58 ± 0.22	4.31 ± 0.08	4.29 ± 0.10	
TM81	Control	6.91 ± 0.19	5.23 ± 0.22	4.60 ± 0.16	4.32 ± 0.02	4.30 ± 0.05
	PA	6.93 ± 0.20	5.28 ± 0.29	4.61 ± 0.15	4.32 ± 0.03	4.36 ± 0.07
	LC	6.85 ± 0.17	5.41 ± 0.22	4.72 ± 0.13	4.38 ± 0.04	4.37 ± 0.05
	LPP	6.25 ± 0.06	5.78 ± 0.16	5.45 ± 0.24	4.82 ± 0.12	4.67 ± 0.04
	LP	6.84 ± 0.17	5.52 ± 0.33	4.67 ± 0.20	4.21 ± 0.04	4.15 ± 0.03
	CD	6.90 ± 0.19	5.58 ± 0.50	4.74 ± 0.21	4.35 ± 0.05	4.34 ± 0.06
	EF	6.92 ± 0.18	5.49 ± 0.40	4.69 ± 0.18	4.34 ± 0.03	4.42 ± 0.11
	LLN	6.72 ± 0.16	5.46 ± 0.19	4.84 ± 0.24	4.43 ± 0.08	4.41 ± 0.09
	LR	6.87 ± 0.19	5.43 ± 0.40	4.66 ± 0.16	4.30 ± 0.02	4.31 ± 0.06
	HA	6.82 ± 0.20	5.39 ± 0.31	4.68 ± 0.16	4.37 ± 0.04	4.33 ± 0.07
	SX	6.84 ± 0.15	5.24 ± 0.36	4.60 ± 0.14	4.34 ± 0.02	4.32 ± 0.03

SUPPLEMENTAL FIGURE S1: Representative images of inhibition in culture based compatibility assays completed

Direct Antagonism Assay

Top Row: Measureable Inhibition (Zones of Clearing)

Middle/Bottom Rows: No Zones

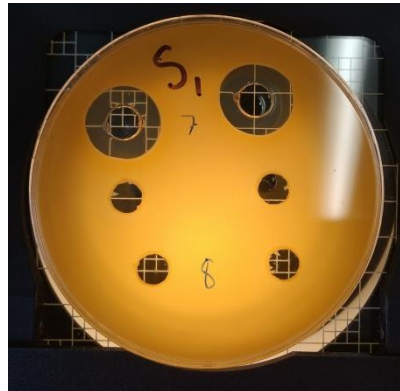


Agar Well Diffusion

Top Row: Measureable Inhibition (Zones of Clearing)

Middle Row: Control Wells (No Addition, No Zone)

Bottom Row: No Zones



Anti-Fungal Assay

Top Row Score:

+++/-/+++//++/-/-

Second Row Score:

+++/-/+++//++/-/none

Third Row Score:

-/-/++//+/-/-

Fourth Row Score:

-/-/++//+/-/none

Last column alternate untreated positive controls and media negative controls



CHAPTER IV

**FATE OF *LISTERIA MONOCYTOGENES* IN RAW MILK CO-INOCULATED WITH
COMMERCIALY AVAILABLE PROTECTIVE CULTURES**

ABSTRACT

Listeria monocytogenes (LM) is a significant hazard in the production of cheese when using unpasteurized milk. Commercially produced protective cultures (PC) of lactic acid bacteria represent actionable natural interventions to control this and other pathogens in cheese. The objective of this study was to determine the efficacy of five PCs to control the growth of *L. monocytogenes* in raw milk when co-inoculated and exposed to a typical cheese-making temperature profile. Raw milk inoculated with a six-strain cocktail of LM at ~2 log CFU/mL was incubated overnight at 4°C. Freeze dried protective cultures were added directly to contaminated raw milk the following morning at ~7 log CFU/mL. Raw milk containing both pathogen and PC were then incubated under a simulated cheesemaking temperature profile consisting of 4 h at 35°C followed by 18 h at 20°C, and an additional five days at 12°C. LM growth in the control exceeded 4 log CFU/mL after the 24h of the cheesemaking gradient and reached a high of 5.44 log CFU/mL 120h after inoculation. Impact of cultures on LM growth varied. Two cultures did not impact LM growth while two cultures exerted a bacteriostatic effect and one culture reduced and maintained LM concentration in milk to <1 log CFU/mL starting 24h after addition. This work demonstrates the effectiveness of select PCs for the control of LM in an intended dairy matrix (raw milk) and under simulated use parameters (temperature and time). Differences in effectiveness based on pathogen and culture inoculation method inform future applications in the production of raw milk cheese and other fermented dairy products made from raw milk.

Human pathogen *Listeria monocytogenes* (LM) has been isolated from raw milk (25), detected in the farm dairy processing environment (1, 14), and finished raw milk cheese products (13, 23). The organism is capable of growing at refrigerator temperatures and is of significant public health concern due to high case fatality rates (4). Implementation of the Food Safety Modernization Act now mandates that cheesemakers anticipate and manage biological hazards such as LM in their product using preventive controls as part of a comprehensive food safety plan. However, scientifically validated controls compliant with the Standards of Identity of Cheese are limited, impeding compliance with FSMA.

Potential for biological control of LM using laboratory strains of protective cultures (PCs) has been well established in the scientific literature (5, 20); however validation of commercially available cultures as a process control in raw milk dairy applications is limited as validation studies are infrequently published with trade names. PCs are considered Generally Recognized as Safe (GRAS) for use in food products, due to historic use in safe and successful food fermentations. Protective cultures of lactic acid bacteria exert protective antimicrobial activity in a food product through the production of metabolites such as organic acids, hydrogen peroxide, and antimicrobial peptides known as bacteriocins (5). Metabolite production is a bacterial defense mechanism towards similar bacteria attempting to occupy the same ecological niche. Due to the generic nature, specificity of metabolite activity is not limited to a single species; however bacteriocin activity is frequently limited to bacteria sharing the same cell wall morphology as the producer (7). In addition to application in dairy, commercially available strains of protective cultures are available for quality and safety enhancement in meat (22), seafood (11), and produce (19). Commercialized cultures are selected for desirable antimicrobial activity in intended matrix, however cross protection is possible (6). Therefore, validation of

commercially available cultures (with both intended and undocumented use in dairy) under expected use conditions of cheesemaking is a critical first step towards expanding process control options available to raw milk cheese makers to manage pathogen risk.

Here, a panel of five commercially available protective cultures intended for LM control in dairy and meat applications were assessed for anti-listerial potential in the presence of raw milk consortia over time-temperature conditions emulating of cheesemaking. Cultures were selected based on results from previous anti-listerial activity testing in our lab using BHI broth and UHT milk (data not shown) in combination with compatibility results discussed previously. Culture selections had demonstrated anti-listerial activity in the milk and culture media and did not inhibit starter culture acidification. The most effective culture(s) identified from this proof-of-concept screen will be used for further validation of activity in raw milk cheese.

MATERIALS AND METHODS

Bacterial cultures and cocktail preparation. Five commercial cultures were selected from the panel used in compatibility analysis (Chapter III) using results of previous anti-pathogenic screening completed in our lab. Cultures were of *Lactococcus lactis* ssp. *lactis* BioSafe-10 (LLN), *Pediococcus acidilactici* B-LC-20 (PA) (Chr. Hansen Inc., Milwaukee, WI), *Lactobacillus plantarum* Lyofast LPAL (LP), *Lactobacillus rhamnosus* Lyofast LRB (LR) (Sacco, Cadorago CO, Italy), and *Lb. plantarum* Holdbac (LPP) (Danisco USA Inc, New Century, KS). As per manufacturer recommendations, direct vat set (DVS) format culture LLN was stored at -80°C prior to use, powdered lyophilized cultures were stored at -20°C. Cultures were added directly to milk to achieve a target count of ~7 log CFU/mL. Cell counts for all PCs

were confirmed following serial dilution in 0.1% peptone (BD-Difco, Franklin Lakes, NJ), plating onto de Man Rogosa Sharpe agar (MRS) (BD), and incubation at 30°C for 48 h.

A six strain cocktail of *Listeria monocytogenes* was prepared with strains associated with dairy-related outbreaks of listeriosis including: ATCC 51414 (raw milk); CWD 675-3 (raw milk); Scott A; 2012L-5323 (Ricotta Salata); 2014L-6025 (Hispanic-style cheese); and DJD 1 (washed-rind cheese) (10). Frozen (-80°C) stock cultures were inoculated individually into 9 mL of brain heart infusion broth (BHI) (BD). Cultures were incubated at 37°C for 18 h for two subsequent transfers before use. Equal proportions of cells from each culture were combined as a cocktail. The resulting cocktail was pelleted through centrifugation (4,000 × g for 15 min at 4°C) (Thermo Scientific Sorvall Legend X1R, ThermoFisher Scientific, Waltham, MA), and re-suspended in an equal volume of 0.1% peptone yielding ~9 log CFU/mL.

Milk Collection. Raw milk was aseptically collected from the bulk tank at the Kellogg Dairy center at the University of Connecticut in sterilized 1L Duran flasks (Fisher Scientific), transported to the lab within 30m of collection and stored at 4°C for use within 72h. Each batch of milk was screened for presence of *L. monocytogenes* (LM) according to the methods outlined in the FDA Bacterial Analytical Manual (9). Briefly, 25mL samples were enriched in 225mL Buffered Listeria Enrichment Broth at 30°C for 4 h followed by 44h of selective enrichment at 30°C. Aliquots of 100µL were streaked onto CHROMagar Listeria, incubated for 48h at 37°C, and examined for the presence of typical colonies (teal in color surrounded by a halo).

Co-Culture Assay. LM cocktail prepared as described previously was serially diluted in raw milk and added to 30 mL of raw milk in dilution bottles (Corning, Corning NY) to a target count of 2 log CFU/mL. Bottles were stored at 4°C for ~18h to mimic storage of contaminated milk prior to cheesemaking. The following morning, PC stocks were prepared in raw milk as

described and inoculated in milk to a final count of 7 log CFU/mL. Duplicate bottles of each PC were prepared. Duplicate bottles of raw milk as well as raw milk inoculated with LM alone or PC alone were included as controls.

Bottles were mixed at the time of each addition and prior to sampling by shaking the bottle in a vertical 10 inch arc for 10 seconds. Bottles were incubated statically at 35°C for 4h then were equilibrated to room temperature (~22°C) for 20h prior to storage at 12°C for another 5 days. This time and temperature profile was selected to emulate the time-temperature exposure conditions for cheese making, and pressing and ripening (24). The pH of samples were determined at the time of LM inoculation, PC inoculation (0 h), at each temperature change (4, 24, and 48h), and then daily (72, 96, 120, and 144h) thereafter.

Bacterial Enumeration. Samples of milk were removed for enumeration at the same time points described for pH. Peptone water (0.1%) was used for all serial dilutions. Samples were plated onto CHROMagar Listeria for enumeration of LM and MRS for the enumeration of total mesophilic lactic acid bacteria. Typical colonies were counted after incubation at 30°C or 37°C for 48h for MRS and CHROMagar Listeria, respectively.

Statistical Analysis. Mean values of pH, mesophilic LAB counts and pathogen counts were generated from three independent replicates with technical duplicates. Data were analyzed using the PROC MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC) with repeated measures to determine the effects of treatment, time, and treatment × time interactions for each variable. Pairwise comparisons were performed using LSMEANS with the Tukey-Kramer method. Compound Symmetry was used as a covariance structure for repeated measures. Significance was assessed at $P \leq 0.05$.

RESULTS & DISCUSSION

Population of mesophilic lactic acid bacteria in co-culture. Mean counts of mesophilic lactic acid bacteria (Table 1) were determined to confirm PC inoculation levels and observe changes over time. Concentration of mesophilic LAB of the raw milk did not change throughout overnight storage then increased from 1.37 log CFU/mL to 6.78 log CFU/mL over the first 24h. LAB counts of raw milk continued to increase throughout refrigerated storage until a population of 8.8 log CFU/mL was reached at 96h after which point the population did not change. No differences in lactic counts between LM and RM controls were observed. PC growth was not inhibited by endogenous microflora, likely a result of their predominance in the mixture (7 log CFU/mL). Lactic counts in co-cultures and respective PC controls did not differ at any time point after PC addition. Lactic counts in co-cultures with LR, PA, or LPP decreased by <1 log CFU/mL from the time of inoculation through four hours of incubation at 35°C. In contrast, LM counts in co-cultures of LLN and LP increased slightly. This suggests that cultures vary in their ability to quickly adapt to, and grow in, the milk environment. Total LAB counts in all treatments exceeded 8 log CFU/mL after 24h of incubation and did not differ from one another through the remainder of the storage.

Behavior of *Listeria monocytogenes* in co-culture. All raw milk samples were negative for the presence of LM. Inoculated LM counts did not increase from the time of inoculation through overnight storage (Time 0) at 4°C (Figure 1). Similarly, counts did not increase during the initial 4h incubation at 35°C. LM counts in control increased by 2 log CFU/mL during the subsequent 20h at room temperature, and reached a mean count of 5.44 log CFU/mL by the end of observation (144h). These changes in counts are similar to those reported for LM growth in raw milk under a similar time-temperature scheme (12). Absence of growth during the 4 h

incubation at 35°C to emulate “cheesemaking” could be attributed to the lag phase of *Listeria monocytogenes*. Reports indicate strain dependent lag phases as short as 24h in autoclaved skim milk (18) and as long as 7 h in TSBYE (2). Competitive exclusion (16) or antimicrobial compounds (15, 21) produced by autochthonous microorganisms could also extend the lag phase of LM when in a mixed culture such as raw milk and limit overall growth.

LM counts in co-culture with LR or PA did not differ from control at any time point. LR is intended for use to control LM, yeasts, and mold in cheese. Previous work in our lab identified LR as an effective LM control (3), absence of activity here could indicate interaction with raw milk microbiota. Under conditions tested here, these data suggest that this culture would not be effective in controlling LM during the manufacture of raw milk cheese. This, however, would have to be confirmed in a cheese model. Although the culture of PA is intended for the control of LM in meat fermentations, our results suggest that this application may not extend to dairy fermentations.

Mean counts of LM in the LP and LPP treatments were similar at each time point indicating that these different cultures of *Lb. plantarum* are similarly effective in inhibiting the growth of LM under the conditions tested. *Lb. plantarum* strains have been shown to produce both plantaricins and pediocins that have bacteriostatic to bactericidal potential (8). LPP is a pediocin producing culture according to the manufacturer. The inhibition of LM by LPP could also be attributed to pH as the pH of LPP co-cultures was significantly lower than other treatments from inoculation of the culture through 48 h of incubation (Table 2). Differences in culture concentration necessitated that a greater quantity of LPP culture to be added compared to other treatments. An immediate reduction in milk pH was observed upon addition of the larger quantity of culture. The difference in quantity of culture required to produce the intended

antimicrobial benefit could be costly for a cheesemaker and is likely to significantly impact cheesemaking technology.

LLN reduced LM counts to levels below that of the initial inoculation within the first 24 h of incubation. Regrowth of LM to levels above inoculation was not observed. This culture of *Lc. lactis* is intended for the control Gram positive spoilage microorganisms, namely spore-forming species of *Bacillus* and *Clostridium*, LLN has been identified by its manufacturer as a producer of the bacteriocin nisin. Nisin activity is enhanced at low pH (17). Consistent with results observed in pH co-culture experiments in chapter III of this work, incubation of milk with LLN resulted in slight reductions in milk pH over time (Table 2). However, reductions in pH were similar to those observed with less efficacious cultures, suggesting that pH alone was not responsible for LM reductions, and LM reductions were likely due to strong nisin production. While the LLN culture was observed to inhibit growth of cheesemaking starter cultures in culture media, it was not excluded from further testing here because culture addition did not impact starter culture acid production. This characteristic combined with anti-listerial activity observed make it a viable candidate for further testing in cheese manufacture.

These results demonstrate the varying efficacy of commercial PCs to control *L. monocytogenes* in raw milk incubated through a cheesemaking time and temperature profile. While acidity provides a hurdle, the ability of PCs to control LM growth is dependent upon the cultures ability to produce antimicrobial metabolites in the presence of raw milk consortia. While previous work identified the potential for LLN culture to inhibit starter culture growth, that same work showed it did not impact acidification activity of the starters. This suggests that it can be used in cheesemaking. Positive compatibility results in combination with the strong anti-listerial activity suggests the LLN culture is the most promising PC for future studies in cheese.

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TABLES AND FIGURES

TABLE 1. Mean mesophilic LAB counts (\pm SD) in raw milk co-inoculated with LM (2 log CFU/mL) and Protective Cultures (7 log CFU/mL).

Treatment	Inoculation with LM	Mesophilic LAB count							
		0 h	4 h	24 h	48 h	72 h	96 h	120 h	144 h
RM	1.37 \pm 0.35	1.37 \pm 0.35 ^a	3.38 \pm 0.84 ^a	6.72 \pm 1.54	7.30 \pm 1.51	8.32 \pm 0.52	8.80 \pm 0.24	8.82 \pm 0.18	8.86 \pm 0.15
LM	1.72 \pm 0.46	1.67 \pm 0.56 ^a	3.47 \pm 0.77 ^a	6.65 \pm 1.36	7.19 \pm 1.69	8.51 \pm 0.21	8.86 \pm 0.25	8.72 \pm 0.19	8.79 \pm 0.17
PA	1.40 \pm 0.09	6.97 \pm 0.13 ^{bc}	6.86 \pm 0.28 ^{bc}	7.98 \pm 0.17	8.33 \pm 0.41	8.62 \pm 0.12	8.74 \pm 0.16	8.80 \pm 0.19	8.78 \pm 0.04
PA+LM	1.94 \pm 0.27	7.10 \pm 0.15 ^{bc}	7.04 \pm 0.24 ^{bc}	8.08 \pm 0.21	8.38 \pm 0.43	8.62 \pm 0.21	8.79 \pm 0.10	8.90 \pm 0.11	8.86 \pm 0.11
LR	1.36 \pm 0.24	6.65 \pm 0.25 ^{bc}	6.65 \pm 1.05 ^{bc}	8.03 \pm 0.11	8.50 \pm 0.23	9.01 \pm 0.40	8.87 \pm 0.20	8.84 \pm 0.05	8.90 \pm 0.18
LR+LM	1.92 \pm 0.42	6.53 \pm 0.18 ^b	5.70 \pm 0.69 ^c	8.31 \pm 0.31	8.51 \pm 0.21	8.58 \pm 0.25	8.87 \pm 0.08	8.88 \pm 0.09	8.94 \pm 0.22
LP	1.48 \pm 0.18	7.27 \pm 0.08 ^{bc}	7.47 \pm 0.57 ^{bc}	7.95 \pm 0.10	8.54 \pm 0.25	8.66 \pm 0.44	8.89 \pm 0.19	8.90 \pm 0.04	8.87 \pm 0.18
LP+LM	1.91 \pm 0.39	7.24 \pm 0.09 ^{bc}	7.53 \pm 0.39 ^{bc}	7.99 \pm 0.09	8.54 \pm 0.22	8.40 \pm 0.39	9.02 \pm 0.15	8.85 \pm 0.14	8.83 \pm 0.30
LPP	1.42 \pm 0.25	6.54 \pm 0.10 ^b	6.24 \pm 0.67 ^{bc}	8.44 \pm 0.23	8.86 \pm 0.10	8.94 \pm 0.10	8.99 \pm 0.14	8.79 \pm 0.08	8.92 \pm 0.07
LPP + LM	1.92 \pm 0.38	6.55 \pm 0.02 ^b	5.96 \pm 0.15 ^c	8.38 \pm 0.18	8.76 \pm 0.34	8.91 \pm 0.15	8.97 \pm 0.19	8.89 \pm 0.08	9.03 \pm 0.06
LLN	1.28 \pm 0.47	7.56 \pm 0.37 ^{bc}	8.06 \pm 0.36 ^b	8.11 \pm 0.11	8.38 \pm 0.18	8.63 \pm 0.15	8.89 \pm 0.16	8.91 \pm 0.08	8.92 \pm 0.06
LLN + LM	1.85 \pm 0.26	7.44 \pm 0.35 ^{bc}	8.01 \pm 0.21 ^b	8.28 \pm 0.13	8.37 \pm 0.24	8.65 \pm 0.28	9.00 \pm 0.08	9.04 \pm 0.05	8.91 \pm 0.03

Means within a column with the same letter are not significantly different; columns without letters were not significantly different

RM: Raw milk control, LM: *L. monocytogenes* cocktail control, PA: *Pediococcus acidilactici* B-LC-20, LR: *Lactobacillus rhamnosus*

Lyofast LRB, LP: *Lactobacillus plantarum* Lyofast LPAL, LPP: *Lb. plantarum* Holdbac, LLN: *Lactococcus lactis* ssp. *lactis* BS-10.

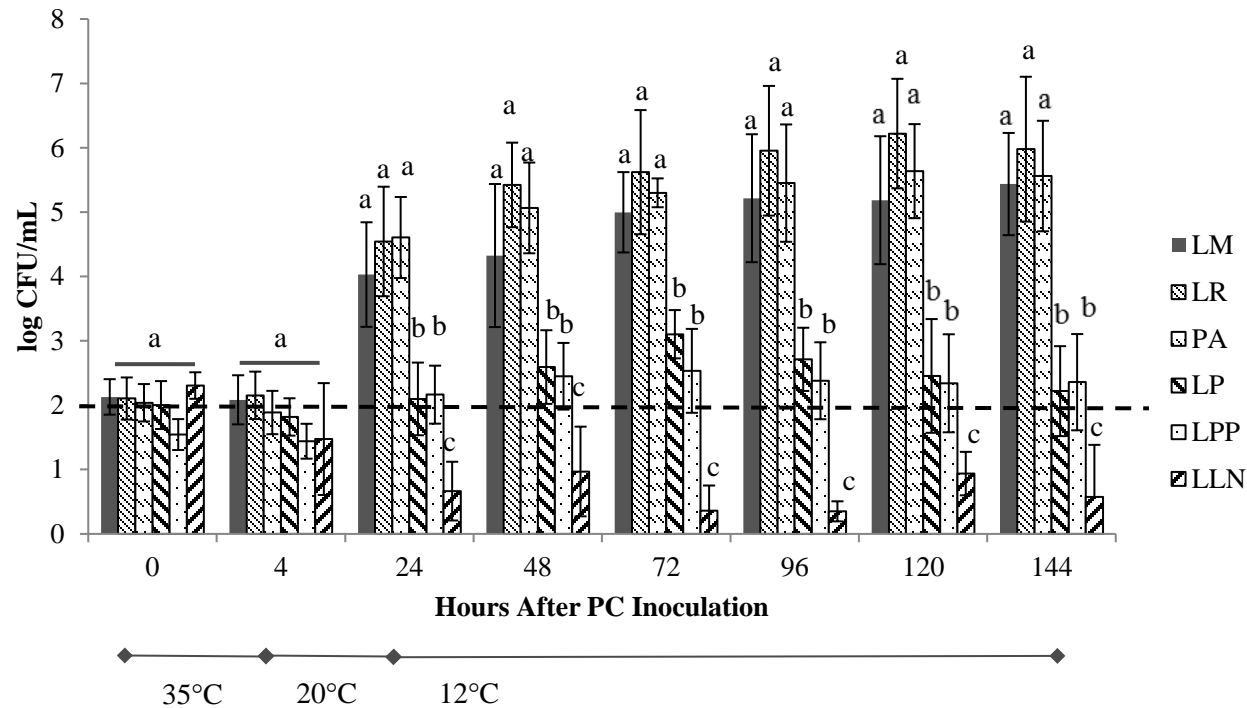
TABLE 2. pH (\pm SD) of raw milk co-cultured with LM and Protective Cultures.

	pH								
	Inoculation with LM	0 h	4 h	24 h	48 h	72 h	96 h	120 h	144 h
RM	6.73 \pm 0.07	6.76 \pm 0.03 ^a	6.73 \pm 0.03 ^{ab}	6.56 \pm 0.09 ^a	6.16 \pm 0.14 ^a	5.99 \pm 0.40 ^{ab}	6.00 \pm 0.21 ^a	5.85 \pm 0.21 ^{ab}	5.51 \pm 0.46 ^a
LM	6.73 \pm 0.05	6.77 \pm 0.05 ^a	6.74 \pm 0.03 ^a	6.56 \pm 0.09 ^a	6.16 \pm 0.14 ^a	6.11 \pm 0.25 ^a	6.04 \pm 0.26 ^a	5.88 \pm 0.22 ^a	5.62 \pm 0.36 ^a
PA+LM	6.76 \pm 0.06	6.72 \pm 0.03 ^a	6.61 \pm 0.03 ^{abcde}	6.38 \pm 0.10 ^{ab}	5.51 \pm 0.45 ^{bdc}	4.99 \pm 0.42 ^{ab}	4.94 \pm 0.55 ^{abc}	4.91 \pm 0.80 ^{abc}	4.48 \pm 0.15 ^{ab}
LR+LM	6.75 \pm 0.05	6.75 \pm 0.03 ^a	6.59 \pm 0.21 ^{ace}	6.48 \pm 0.06 ^{ab}	6.07 \pm 0.03 ^{ab}	6.06 \pm 0.09 ^{ab}	5.88 \pm 0.13 ^{ab}	5.64 \pm 0.45 ^{bc}	5.51 \pm 0.59 ^a
LP+LM	6.75 \pm 0.08	6.77 \pm 0.02 ^a	6.55 \pm 0.03 ^{abcd}	6.24 \pm 0.04 ^{abc}	5.96 \pm 0.06 ^{dc}	5.55 \pm 0.32 ^{dc}	5.20 \pm 0.53 ^{cd}	4.91 \pm 0.41 ^c	4.58 \pm 0.08 ^c
LPP + LM	6.75 \pm 0.04	6.36 \pm 0.03 ^b	6.44 \pm 0.24 ^f	5.93 \pm 0.15 ^d	5.08 \pm 0.06 ^e	4.92 \pm 0.19 ^d	4.91 \pm 0.41 ^d	4.93 \pm 0.66 ^c	4.62 \pm 0.31 ^{bc}
LLN + LM	6.72 \pm 0.07	6.75 \pm 0.02 ^a	6.65 \pm 0.07 ^{ef}	6.48 \pm 0.09 ^{bc}	5.82 \pm 0.41 ^{abc}	5.78 \pm 0.52 ^{bc}	5.68 \pm 0.57 ^{bcd}	5.52 \pm 0.74 ^c	5.42 \pm 0.81 ^{bc}

Means within a column with the same letter did not differ ($P > 0.05$).

RM: Raw milk control, LM: *L. monocytogenes* cocktail control, PA: *Pediococcus acidilactici* B-LC-20, LR: *Lactobacillus rhamnosus* Lyofast LRB, LP: *Lactobacillus plantarum* Lyofast LPAL, LPP: *Lb. plantarum* Holdbac, LLN: *Lactococcus lactis* ssp. *lactis* BS-10.

FIGURE 1 *Listeria monocytogenes* counts (Log CFU/mL \pm SD) in raw milk following refrigerated storage overnight, co-culture with PCs the following morning, and following a cheesemaking time and temperature profile.



Dotted line indicates LM inoculation level prior to overnight refrigeration.

^{a-c} Columns within a time point with different letters differ at $P < 0.05$.

LM: *L. monocytogenes* cocktail, LR: *Lactobacillus rhamnosus* Lyofast LRB, PA: *Pediococcus acidilactici* B-LC-20, LP: *Lactobacillus plantarum* Lyofast LPAL, LPP: *Lb. plantarum* Holdbac, LLN: *Lactococcus lactis* ssp. *lactis* BS-10.

CHAPTER V

**FATE OF *LISTERIA MONOCYTOGENES* AND SHIGA TOXIN-PRODUCING
ESCHERICHIA COLI DURING MANUFACTURE AND AGING OF A SURFACE-
MOLD-RIPENED SOFT CHEESE MANUFACTURED WITH PROTECTIVE
CULTURES AS ADJUNCTS.**

ABSTRACT

Soft-ripened cheese supports survival and growth of pathogens *Listeria monocytogenes* (LM) and shiga-toxin producing *Escherichia coli* (STEC). The potential presence of these pathogens in unpasteurized milk elevates the risk of foodborne illness upon consumption of cheese manufactured with unpasteurized milk. Protective bacterial cultures (PCs), producing non-specific defenses such as bacteriocins, represent a potential biological control strategy compliant with the Standard of Identity of cheese. The purpose of this study was to evaluate two commercially available protective cultures for the control of LM or STEC in a surface-mold-ripened soft cheese. Cheese was manufactured in the lab from raw milk intentionally contaminated with 6 and 7 strain cocktails of either LM or STEC, respectively, at a concentration of ~2 log CFU/mL prior to manufacture. Commercially available PCs previously identified as effective against each pathogen were added to contaminated milk at a concentration of ~6 log CFU/mL according to manufacturer instructions. After de-hooping and dry salting, cheeses were ripened in a controlled environmental chamber (12°C, 93% RH) for 14 days prior to storage at 4°C up to day 63 and 7°C for another 8 weeks. Pathogens were enumerated throughout the cheesemaking process and bi-weekly throughout ripening and storage. Two independent batches of each control and treatment were used for analysis. STEC, but not LM, concentration increased over milk ripening. Both pathogens were concentrated in the cheese curd over the course of manufacture and pressing. Mean LM concentration in control and treatment cheeses decreased slightly by day 35, relative to starting inoculation, and increased gradually out to day 120. LM counts were associated with the extent of cheese ripening, which varied between trials. STEC was detectable in cheese out to day 120.

HIGHLIGHTS

- Treatment with Protective Culture did not reduce LM counts in milk during ripening
- LM grew in cheese treated with Nisin-producing protective culture
- STEC populations decreased but remained enumerable for over 120 days of ripening

Recent analysis of historical outbreak data has shown that unpasteurized dairy products cause 840 times more illnesses and 45 times more hospitalizations than pasteurized products (11). The prevalence of pathogens *Listeria monocytogenes* (LM) and Shiga toxin-producing *Escherichia coli* (STEC) in cheese made from raw milk is higher than that of cheese made from pasteurized milk, elevating the risk of associated infection and illness upon consumption (32, 38). LM and STEC infections are rank among the highest rates of death and hospitalization respectively of all food borne illnesses (50). In 2017 an outbreak of listeriosis resulting in eight hospitalizations and two deaths was linked to a washed rind raw milk cheese produced by a New York Creamery (6). Multiple international recalls since the beginning of 2019 (21, 22) further emphasizes continued public health risk associated with raw milk cheese. Without pasteurization, pathogens such as LM and STEC are not eliminated from raw milk prior to cheesemaking. Physicochemical characteristics such as the high moisture content and the increases in pH in surface ripened soft cheeses during ripening and storage allow for survival of pathogens, if present, compounding the risk of food borne illness. FDA regulations require that cheeses made using raw milk be aged for a minimum of 60 days at a temperature no less than 1.6°C (9). Both STEC and LM have been documented to survive past the mandatory 60-day aging period in the United States in these and other cheeses (12). Globally, soft-ripened cheeses made from raw milk have caused numerous infections, hospitalizations (3, 30), deaths (20), and prompted international recalls (24), establishing an urgent need for validated control strategies for these pathogens in this specific cheese type.

The use of metabolites produced in situ from protective cultures (PCs) of lactic acid bacteria is a promising naturally-derived antimicrobial strategy allowable by the standard of identity of cheese (21 CFR 133) (2, 18). Pervious work using PCs and their bacteriocins has

supported their potential for pathogen control in the production of cheese (4, 36, 55). Many of these studies were completed in pasteurized milk using laboratory strains of PCs or their bacteriocin preparations, both of which are not approved yet for use in food, nor available for widespread use. Select cultures with anti-microbial properties are commercially available to producers, each targeting one of many safety and quality issues in dairy products. Presently, protective cultures are used in cheese for control of *Clostridia* spores to prevent gas-blowing defects, and reduce yeast and mold contaminants. Documentation of anti-pathogenic activity of commercially available strains is minimal in the literature, despite multiple options presently for sale. The present work aims to evaluate two commercially available cultures for control of LM or STEC in a surface mold-ripened soft cheese made from raw milk.

MATERIALS & METHODS

Bacterial cultures and cocktail preparation. Previous work in our lab identified top-performing commercial protective cultures against each LM (Chapter IV) and STEC that did not inhibit acidification activity of common cheesemaking starter cultures (Chapter III). Direct vat set cultures of *Lactococcus lactis* ssp. *lactis* Bio-Safe-10 (LLN) (Chr. Hansen Inc., Milwaukee, WI) and *Hafnia alvei* LALCULT Protect B16 (HA) (Lallemand Specialty Cultures, Rhinelander, WI) were found effective against LM and STEC, respectively. Cultures were added directly to milk to achieve a target count of ~6 log CFU/mL. Cell counts for LLN confirmed by enumeration following serial dilution in 0.1% peptone (BD- Difco, Franklin Lakes, NJ), plating onto de Man Rogosa Sharpe (MRS) agar (BD) and incubation at 30°C for 48 h. HA counts were confirmed by enumeration on plate count agar supplemented with 1% skim milk powder (Price Chopper, Schenectady, NY), 5mg/L vancomycin (Fisher Scientific, Waltham, MA), and 5mg/L

crystal violet (Fisher Scientific) following incubation at 30°C for 48 h as described by Delbus et al. (2013) (16). A six strain cocktail of LM was prepared with strains associated with dairy-related outbreaks of listeriosis including: ATCC 51414 (raw milk); CWD 675-3 (raw milk); Scott A; 2012L-5323 (Ricotta Salata); 2014L-6025 (Hispanic-style cheese); and DJD 1 (washed-rind cheese) (33). A seven-strain cocktail of Shiga toxin-producing *Escherichia coli* (STEC) was prepared to include non-O157:H7 *E. coli* strains representing the six major serogroups (O26:H11 NRRL B-59821, O103:H2 NRRL B-59827, O45:H2 NRRL B-59823, O121:H19 03-2832 NRRL B-59834, O145:NM 03-4699 NRRL B-59836, and O111:NM 94-0961) and an acid tolerant strain of *E. coli* O157:H7 (ATCC 700599) isolated from salami. Frozen (-80°C) stock cultures of each pathogen were inoculated individually into 9 mL of tryptic soy broth with 0.6% yeast extract added (TSBYE) (BD) for STEC or brain heart infusion broth (BHI) (BD) for *Listeria*. Cultures were incubated at 37°C for 18 h for two subsequent transfers before use. For each pathogen, equal proportions of cells from each culture were combined as a cocktail. The resulting cocktail was pelleted through centrifugation (4,000 × g for 15 min at 4°C) (Thermo Scientific Sorvall Legend X1R, ThermoFisher Scientific, Waltham, MA), and re-suspended in an equal volume of 0.1% peptone.

Milk Collection. Raw milk was aseptically collected from the bulk tank at the Kellogg Dairy center at the University of Connecticut in sterilized carboys (Thermo Scientific 22500050 20L Nalgene, Fisher Scientific) using a sanitized siphon (Beckson Marine Siphon-Mate Transfer Pump, Grainger Industries, Miami, FL) and sterile tubing (Tygon B-44-4X, Fisher Scientific). Milk was transported to the lab within one hour and stored at 4°C prior to use the following morning. Each batch of milk was screened for presence of *L. monocytogenes* and STEC

according to the methods outlined in the FDA Bacterial Analytical Manual (19, 26). Briefly, 25 mL samples were enriched in 225 mL Buffered Listeria Enrichment Broth at 30°C for 4 h, followed by 44 hours of selective enrichment at 30°C. Aliquots of 100µL were streaked onto CHROMagar Listeria (DRG International Springfield, NJ), incubated for 48h at 37°C, and examined for the presence of typical colonies (teal in color surrounded by a halo). For STEC, 25 mL samples of milk were enriched in 225 mL of modified buffered peptone water with pyruvate (mBPWp) (Fisher Scientific) at 37°C for 5 h followed by selective enrichment overnight at 42°C. Aliquots of 100uL were then streaked onto CHROMagar STEC (DRG International), incubated 24h at 37°C, and examined for typical colonies (mauve).

Cheese Manufacture. Small format surface-mold-ripened soft cheeses were manufactured following a traditional Camembert cheese technology in a laboratory-scale cheese vat (Labtronix Inc., Philomath, OR). On the day prior to cheese manufacture, a 50 mL portion of raw milk was inoculated with the appropriate pathogen cocktail to achieve 2 log CFU/mL in the 6 gallon batch the next day and stored at 4°C overnight to simulate contaminated milk being held prior to cheesemaking. On the day of manufacture, the vat, hoops and ripening and draining mats were cleaned and then sanitized using 200 ppm sodium hypochlorite (Low Temp Sanitizer, Alpha Chemical Services, Stoughton, MA). Twenty-three liters of raw milk was added to the vat prior to the addition of the 50 mL portion of raw milk inoculated with the pathogen. Treatments included LM alone (control), LM with LLN, STEC alone (control), and STEC with HA. Calcium chloride (New England Cheesemaking, Deerfield, MA) was added at 10 mL/100 L and the milk was warmed to 34°C. The starter and ripening cultures were added as follows: MM100 (4.5 DCU/100 L), TA54 (2 DCU/100 L), KL71, GEO17, SAM3 (each 0.10 DCU/100 L) (Danisco-Dupont, New Century, KS). Protective cultures were added to milk at a concentration of 6 log

CFU/mL. HA culture was added to STEC contaminated milk according the manufacturer's instructions prior to the addition of starters. The addition of LLN was delayed 15 minutes after the addition of the starter cultures based on the manufacturer's recommendation and previous results in our lab to minimize potential inhibition of starter cultures. Milk was mixed at approximately 45 rpm using a pitched-blade overhead paddle assembly. Temperature was maintained at 34°C for ~4 hours until a target pH of 6.3 was attained. Milk was stirred for 45 seconds following the addition of coagulant (DCI Star Coagulant Double strength microbial coagulant, Dairy Connection Inc., Madison, WI) at 4.4 mL/100 L diluted 1:80 in deionized water. Cutting time was determined by multiplying the time to flocculation by 6. Once the desired firmness was reached, heat to the vat was removed and the coagulum was cut into ~1 inch cubes. Curds were stirred gently for five minutes followed by five minutes of rest. Approximately 15% of the whey (3.5 L) was drained off and curds were ladled into hoops (M608MTS185, 80mm diameter, Fromagex, Quebec, Canada) on sanitized racks with draining mats (M603STO010, Fromagex) over four additions. Cheeses were transferred to a 25°C incubator and flipped after 2.5 hours of draining. The temperature of the incubator was then reduced to ambient (20-22°C) and the cheeses were flipped hourly for 2-3 hours, and then allowed to drain overnight.

Salting, Drying, Ripening and Holding. The following morning, cheeses were de-hooped and dry salted (Diamond Crystal Kosher salt, Cargill, Minneapolis, MN) at 1.95% of the cheese weight. Cheeses were then placed on stainless-steel aging racks (Fromagex) and dried in a climate controlled environmental chamber (Caron Products Inc, Marietta, OH) at 12°C and 85% relative humidity for 48 h. The humidity was then increased to 93% and cheeses were aged for 10-14 days until mold growth sufficiently covered the cheese. Cheeses were turned daily

throughout the drying and aging phases. At the end of aging cheeses were wrapped (White Mold Paper, Fromagex), stored at 4°C for 63 days, and then stored at 7°C through 120 days.

Temperature (Oakton Temp300 data logging thermocouple, OAKTON Instruments, Vernon Hills, IL, USA) and pH (Accumet AB150, Fisher Scientific) were measured throughout manufacture.

Bacterial Enumeration. Duplicate 25mL samples of raw milk were removed at the time of milk collection to assess milk microbial quality. Milk standard plate counts (SPC) were determined using the Petrifilm Aerobic Count Plate (3M, St.Paul, MN) method (45). Counts were rounded to two significant figures at time of conversion to SPC. Total coliform counts were determined using 3M Petrifilm *E.coli*/Coliform count plates following the manufacturer's instructions. Plates were incubated at $32 \pm 1^\circ\text{C}$ for 24 ± 1 h. Mesophilic lactic acid bacteria were enumerated on MRS under the same incubation conditions described earlier for PC enumeration. During cheese manufacture, duplicate 10 mL samples of un-inoculated milk, inoculated milk, milk after ripening and whey after stirring were removed for pathogen enumeration. Samples were serially diluted in 0.1% peptone before plating onto MRS, CHROMagar Listeria or CHROMagar STEC and incubated as previously described. Gram negative agar described by Callon et al. (2016) was used to approximate the total Gram negative counts for cheeses inoculated with HA (5). Typical cream colored colonies were enumerated after incubation at $30 \pm 1^\circ\text{C}$ for 48 ± 1 h.

Duplicate samples of curd (25g) were removed at the time of cutting and first flipping. Duplicate cheese samples (25g) were collected prior to salting, on days 1 and 7 of aging, and then bi-weekly through 120 days. At each sampling time point, a single cheese was randomly selected from each batch, cut in half using a sterile knife, transferred to sterile bags (24oz Whirl-

pak, Fisher Scientific) and macerated by hand until a homogenous paste was obtained. Subsamples of 25g were homogenized (Smasher stomacher, Biomerieux, Marcy-l'Étoile, France) for 1 min at 560 strokes/min) in 100mL of BLEB or mBPWp for LM and STEC, respectively. Following serial dilutions in 0.1% peptone, homogenates were plated onto their respective chromogenic agar. When applicable, 1 mL of homogenate was plated over 4 agar plates (250 μ L per plate) to achieve a detection limit of ≥ 5 CFU/g. When colony counts fell below the limit of detection, an additional 125mL of the enrichment broth was added and standard enrichment procedures were followed as described earlier.

Physiochemical Analysis. Physiochemical analyses conducted on representative samples from each independent batch of cheese, in duplicate, at the day 7 time point, included dry matter (DM) (forced draft oven method; Hooi et al., 2004), fat (Babcock method; Hooi et al., 2004), and salt (Quanttab Chloride, Hach, Loveland, CO). Duplicate pH readings were taken at the rind, just below the surface of the cheese, and at the center of each cheese before homogenization. Salt in moisture (SM), moisture in the non- fat substance (MNFS), and fat in dry matter (FDM) were determined using the following formulae: % SM = [% salt/ (100 - % DM)] \times 100; % MNFS = [100 - % DM/ (100 - % fat)] \times 100; and % FDM = (% fat/% DM) \times 100. Compositional targets were dry matter ~50%, fat ~22-23%, salt ~1.7%.

Statistical Analysis. Eight batches of cheese were independently manufactured on separate days including two batches for each pathogen with and without the respective PC. For each pathogen, counts were pooled and means were analyzed using the GLM procedure of SAS 9.4 with the interaction of time and treatment. Least square means of the interaction were calculated and compared using Tukey post-hoc testing. In the absence of an interaction, Least

Square Difference of main effect means was used to assess differences. Significant differences were considered at $P < 0.05$.

RESULTS AND DISCUSSION

Raw milk microbial quality. There is no federal limit established for coliforms in raw milk used for cheesemaking. Since coliforms can enter the milk from multiple environmental sources, high coliform counts are associated with loss of hygienic control during milk collection, milking of mastitic cows or improper temperature control during storage. However, a correlation between pathogen presence and coliform counts has not been established (14, 28, 53). Recommended counts range from <10 CFU/mL to <100 CFU/mL (1). Coliform count in the present study ranged from 18 to 56 CFU/mL with a mean of 38 CFU/mL. The mean SPC for raw milk (420 CFU/mL) was low in the context of current limits for Grade A milk ($<100,000$ CFU/mL) and recommendations for cheese ($<10,000$ CFU/mL) (1, 51). All batches of raw milk used for cheesemaking were negative for the presence of LM and STEC.

Cheese Composition. US federal regulation requires that soft-ripened cheese made from raw milk must be held for a minimum of 60 days at a temperature no less than 1.67°C (35°F) (9). The federal Standard of Identity for soft-ripened cheese also specifies a minimum FDM of 50% (8). The FDM for all cheeses in the present study were above this limit except those manufactured with LLN, which were lower ($45.48 \pm 3.31\%$ FDM) (Table 1). Dry matter, fat and salt content were all within acceptable range of targets and means of all compositional metrics of both LLN and HA treated cheeses were not significantly different from respective controls. Cheeses were manufactured with lower target moisture content at dehooping, to slow the rate of ripening to achieve a desirable texture following the mandatory 60 day aging. Desired extent of

ripening for salable quality of all cheeses was subjectively reached by day 70. Lower measured fat content for cheeses treated with LLN, contributing to a calculated lower FDM, was likely a result of incomplete digestion of sample by sulfuric acid during analysis. Fat content is only determined by the milk used and cannot be modified with the addition of protective culture. Inaccurate fat analysis is a more likely explanation of differences observed.

Changes in pH of Cheese. Target pH of 4.65 in finished cheese at time of dehooping was met. Change in pH over time at the center of individual cheeses treated with LM was not impacted by the addition of LLN. pH of the center of the cheeses treated with HA only differed from control only on day 7 ($P = 0.049$) and day 63 ($P = 0.011$). The pH of the rind of all cheeses increased rapidly from 4.6 to >6 within the 21 days of aging (Figure 1) as a result of proteolytic activity of surface molds, consistent with observations of other researchers (49). pH did not differ at any time point between treatment and control for either pathogen.

Population of lactic acid bacteria and *Hafnia alvei* during cheese manufacture and ripening. Mesophilic LAB counts exceeded 8 log CFU/mL in milk after addition of starter cultures and remained near that level (>7 log CFU/g) in all cheeses through the duration of sampling. Total Gram negative counts, a proxy for HA concentration, increased by ~ 1 log from inoculation initial inoculation levels throughout pressing then decreased over the course of ripening and aging from 7.92 log CFU/g on day 1 to 6.46 log CFU/g on day 120 (Data not shown). Other studies using this culture in a semi-hard model cheese observed a similar increase in *Hafnia* count early in manufacture followed by gradual decrease over 40 days of cheese ripening (16). Plausible mechanisms of antimicrobial activity of HA (e.g. pH or bacteriocin production, competitive exclusion) are all dependent on high prevalence of the organism. Reduction in counts over time could limit extent of control.

Survival of STEC during cheese manufacture. Overall, STEC counts increased in the milk and curd over the course of manufacture (Table 2). Mean STEC counts during manufacture between cheeses made with and without PCs did not differ. Increase in STEC counts in curd after cutting and further increase in STEC counts during draining and flipping were consistent with previous reports of STEC growth and capture in the curd (40, 43). STEC ($< 3 \log \text{CFU/mL}$) was also detected in drained whey, indicating an incomplete capture in the curd. This value is greater than that reported in studies of cheeses of other types (13, 23, 46), however differences in factors that drive curd strength and contraction (curd cut size, stirring, cooking time and temperature) may contribute to equilibrium of the pathogen in the whey and cheese resulting in higher values observed here. This is a concern since previous work has shown acid tolerance of STEC allows for survival and growth in cheddar cheese whey (37). This highlights the potential for cross-contamination of the cheesemaking environment and the farm environment if untreated whey is applied as an agricultural amendment. There is also the potential for animal infections if used as animal feed supplement, amplifying the potential for foodborne illness of other non-dairy food products.

Behavior of STEC during drying and aging. Mean STEC count in both treated and untreated cheeses averaged $3.8 \log \text{CFU/g}$ on the first day of aging, and decreased to $1.99 \log \text{CFU/g}$ and $2.3 \log \text{CFU/g}$ in control and treated cheeses, respectively, by day 120 (Figure 2B). STEC counts in PC treated cheeses was higher than control later in aging on days 63 ($P = 0.046$), 77 ($P = 0.03$), 91 ($P = 0.001$), 105 ($P = 0.004$) and 120 ($P = 0.043$). However, counts only differed by $< 0.5 \log \text{CFU/g}$, which may not be biologically significant. Average weight of cheeses manufactured with HA did not differ from control, however gas production by HA produced small eyes in these cheese paste that reduced in size as cheeses ripened.

Low pH during cheese manufacture may have a greater impact on STEC behavior than HA activity alone. As mentioned previously, pH of soft-ripened cheeses decreases rapidly over the course of manufacture then gradually increases over ripening and storage. Miszczycha et al. (2013) demonstrated that when STEC growth was observed during the first step of manufacture, as seen here, STEC concentration in a lactic cheese decreased over 60 days of ripening, in part due to low pH and storage temperature (41). Multiple authors document similar patterns of STEC survival: persistence, albeit with reduction in concentration, in cheeses with pH as low as 4.3 (43, 54). Among these, Montet et al. (2009) observed reduction, but not complete elimination, of both acid resistant and non-acid resistant strains of STEC in camembert cheese after 20 days of ripening as the pH rose from 4.66 at the end of molding to 5.11 on day 20 (43). STEC behavior and pH profile is similar to that seen of this present study, although acid-resistance status of strains used here is mixed. Previous work using HA in an uncooked pressed model cheese made from raw milk reported a ~1 log CFU/g reduction in *E.coli* O26:H11 compared to control one day after manufacture and maintained this difference out to day 28 of observation (16). The authors hypothesized that acetic acid production by HA may synergize with acid produced by milk consortium contributing to STEC reduction. The pH of the curd over this time frame did not drop below 5.33. However, STEC concentration was not reduced upon treatment with HA at the reduced pH of the cheese here. Key differences in composition between the pressed cheese and soft-ripened cheese might explain some differences, however, moisture or other metrics were not presented for comparison. Optimal pH range for this culture is unknown but a low tolerance for high-acid environments might explain gradual decrease in total Gram negative count from initial inoculation observed in treated cheeses, which may contribute to the lack of inhibition observed.

Recent evidence has shown that survival of O157:H7 and non-O157:H7 strains, when adapted to acidic environments, is dependent on other food conditions such as water activity and food matrix (48). However, under select conditions, survival of O157:H7 and non-O157:H7 serogroups were not significantly different regardless of acid adaption (48). Other researchers have confirmed differences in growth behavior of *E. coli* strains in raw milk cheeses, demonstrating that select non- O157:H7 serotypes may be better competitors than O157:H7. However this is dependent on cheese type (41, 42). As both O157:H7 and non-O157:H7 strains have been linked to outbreaks and recalls in raw milk cheese and commercial PC cultures have not been validated against these strains in this cheese type, a cocktail with representatives of each type was used to approximate the antimicrobial activity towards STEC in general. Therefore, survival of only a single or a few serotypes is possible. The selective and differential agar media used in the present study did not allow for differentiation of colonies based on serotype.

It is important to note here, as in other studies, immediate regrowth of STEC is not observed in either treatment or control as pH increases over ripening. Additional compositional hurdles in the cheese or delayed recovery from acid adaption, if any, are plausible explanations. Hsin-Yi and Chou (2001) proposed that the benefit of *E.coli* O157:H7 acid-adaption in fermented milk products might be outweighed by an increase in susceptibility to antimicrobials of LAB and protective cultures, if present (27). Here, the rate of STEC reduction was not enhanced by PC presence. In fact, the addition of PC seemed to enhance STEC survival at later time points. Others have observed enhanced STEC survival in the presence of additional consortia when in culture media (17). Until the mode of action of HA is more precisely known, it does not appear that it enhances control of STEC in this cheese type. Further, the gas production

defect typical of *Enterobacteriaceae* as a result of HA addition means cheese visual appeal at time of sale may be reduced without a benefit to safety.

Survival of *L. monocytogenes* during cheese manufacture. Mean LM counts in milk and curd samples of treated cheese were not significantly different from control throughout manufacture. A slight decrease in LM concentration was observed at the end of the 4 h ripening period (Table 2) followed by an increase at time of cutting, which is consistent with limited growth during ripening followed by concentration and entrapment in the curd described by others (35, 36). Limited duration at optimal growth temperature and high concentration of competing organisms can limit LM growth. LM was detected in whey at levels similar to that of other types of cheese (35), indicating an incomplete capture in the curd.

Behavior of *L. monocytogenes* in cheese during drying and aging. Mean LM counts in cheeses treated with LLN were not significantly different from control at any time point (Figure 2A). Variation in LM counts between replicates at later time points in both treatment and control cheeses is a major fault of this data set. Subjective visual observations at time of sample collection noted delayed ripening of one batch of LLN treated cheeses. pH values at the rind of these cheeses rose as anticipated, but the total volume of the cheese at the pH amenable to LM growth was estimated to be less than that of other cheeses over storage. Efficiently distributing curds during hooping limited premature moisture loss and development of a rubbery curd texture. However, uneven distribution of denser curds among forms may have resulted in cheeses of slightly different curd composition within a batch. Cheeses with a denser, lower moisture curd, may have inhibited diffusion and activity of proteolytic enzymes over the duration of storage of select cheeses.

Previous work suggested that the LLN culture would not impact acidification of mesophilic starter culture. However, there was the potential for inhibition of thermophilic culture TA54 (Chapter III). One batch of cheese produced with LLN took 75 minutes longer to reach the target pH for cutting compared to the average ripening time of the comparative LM control. Apart from this delay, pH values through the remainder of manufacture did not differ from anticipated values. There was also no indication that this delay affected LM counts and did not impact final cheese pH as differences in core and rind pH of final cheeses were not observed. Thermophilic cultures are used to complement the activity of mesophilic cultures by extending acid development late in the cheese make process. The delay in acidification early in the process during milk ripening suggests a potential inhibition of the mesophilic culture.

Microbiological community activity and mold growth at the surface of the cheese result in key physiochemical changes over the course of ripening, allowing for *Listeria* growth and survival. After dry salting, colonization of the rind with ripening bacteria, yeast and mold, quickly metabolizes available lactose resulting in the diffusion of lactate and lactose from the center of the cheese. Activity of proteases produced by *Penicillium candidum* metabolizes proteins into component amines generating ammonia and raising the pH locally at the rind (34). Essentially, ripening activity and resulting changes in pH modulates both the ripeness of the cheese and environment available for LM growth. Concentration of LM increases in areas of cheese with a pH above 5.5 and high moisture content, as these conditions supports LM growth (49). Ryser and Marth (1987) reported strain-specific differences in survival of LM in cheese at pH levels >5.5 (49). As mentioned previously, cheeses in which LM did not grow did not ripen in the same manner as the others. This illustrates the key role of pH (as a consequence of protease diffusion and ripening activity) in controlling LM growth in this cheese environment.

The efficacy of protective cultures as adjuncts for controlling pathogens in surface-mold-ripened soft cheeses varies. Protective cultures used as primary starter cultures tend to be more effective. Maisnier-Patain et al (1992) observed a 1.5 log decrease in LM counts after the first day of production with a *Lc. lactis* subsp *lactis* CNRZ 150 as a starter. Limited regrowth was observed in the treated cheese after 2 weeks of aging compared control, indicating that nisin production by this culture was essential for LM control (22). Bacteriocin production is suggested to be induced by the presence of other bacteria (10), however absence of competition from starter cultures or background microflora did not prevent nisin production in the aforementioned study. Suzler et al. (1991) observed an anti-listerial effect when *Lactococcus lactis* 1881 was used as a starter alone in the production of camembert cheese, but not when added alongside another starter culture (52). Favorable levels of gene expression of *nisA* in milk and curd during Graciera cheese manufacture were measured in a separate study (44). However *nisA* transcript levels decreased post-fermentation, potentially due to prevalence of other cultures, transcript degradation or inactivation, or depletion of nutrients required for transcription, illustrating the potential impact of other cultures on potential for antimicrobial activity on the molecular level (44). Previous work in our lab demonstrated that LLN reduced LM counts in raw milk as early as four hours into incubation at 35°C. While it attempted to emulate cheesemaking, this experiment did not account for high concentration of starter culture or background microflora as variables potentially impacting PC activity and nisin production. It is also possible that nisin, if produced in cheese, was diluted or enzymatically degraded under cheesemaking conditions. Absence of anti-listerial activity and lack of replication of our work from raw milk, could be attributed to a combination of one or more of the many factors influence nisin production, persistence, and ultimate degradation in cheese.

Nisin production by *Lc. lactis* occurs throughout exponential growth and ceases when the growth rate decreases (31). Production is influenced heavily by environmental factors such as pH and available carbon source, milk being a suitable medium for consistent Nisin production (15, 47). Previous work both in our lab has shown that the anti-listerial activity of LLN was retained in the presence of proteolytic coagulants used for cheese making (data not shown). Other labs have demonstrated mixed susceptibility of nisin with other common food proteases (39), and susceptibility to select enzymes of the gastrointestinal tract (25, 29). Proteolysis and associated ripening of surface-mold-ripened soft cheese is the result of enzymes predominantly produced by *P. candidum*, with small contribution by *Geotrichum* species and LAB. Evidence of nisin susceptibility to the aspartyl protease, metalloprotease, and exopeptidase produced by *P. camemberti* (7), and auxiliary proteinases produced by LAB and other has not been exclusively tested. Others have observed nisin titer decreased in ripened cheeses after mold development, suggesting that this bacteriocin might be labile to enzymatic activity of the molds (36). In the absence of commercialized nisin-producing starter culture blends, more cheesemaking trials are needed to identify process parameters and application methods necessary to balance commercially available Nisin-producing protective cultures and existing starter cultures such that neither inhibits the activity of the other.

In summary, aging alone was not sufficient to eliminate *L. monocytogenes* or STEC in a surface-mold-ripened soft cheese made with raw milk. Both pathogens were detectable in cheeses 120 days after manufacture. As cheeses ripened as a result of proteolytic activity from surface molds LM increased in concentration, while STEC decreased. The addition of protective bacterial cultures with demonstrated efficacy in raw milk did not provide additional control of these pathogens under the conditions tested. Additional research studies are needed to optimize

use of commercially available protective cultures in high-risk cheese types prior to validating their use for the control of pathogens.

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TABLES AND FIGURES

TABLE 1. Composition (% +/- SD) of surface-mold-ripened soft cheese on day 7 of ripening when manufactured with and without protective cultures

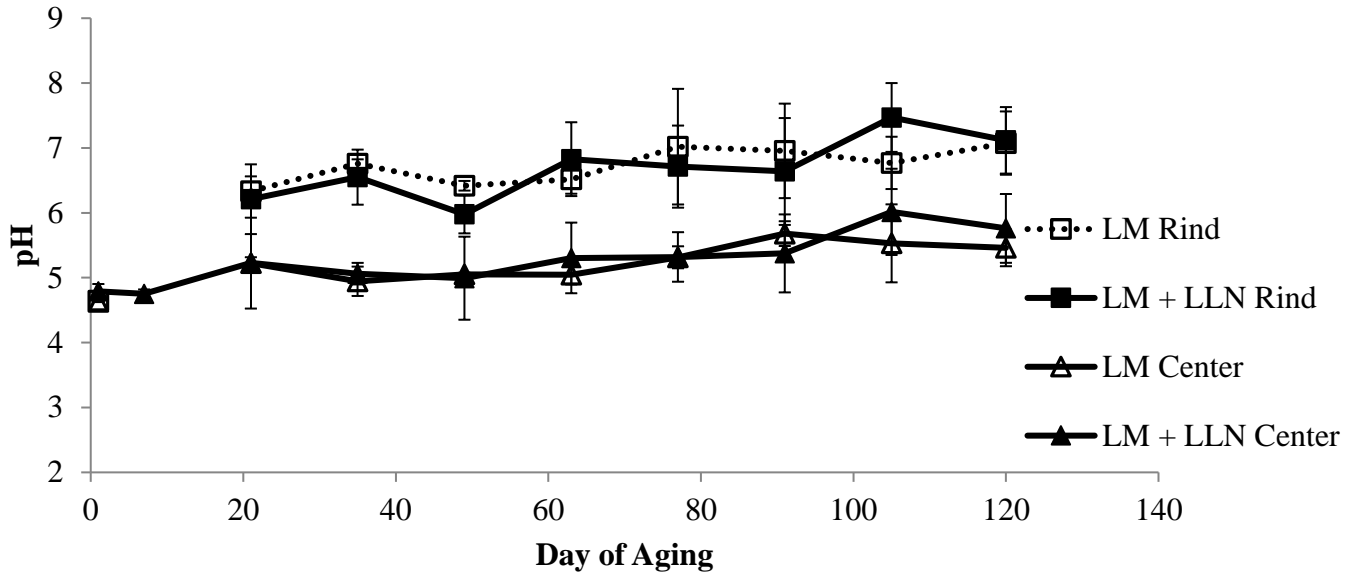
	DM	Moisture	Fat	Salt	FDM
LM	53.89 ± 4.54	46.11 ± 4.54	29.63 ± 4.07	1.87 ± 0.22	54.85 ± 2.92
LM + LLN	52.91 ± 3.53	47.09 ± 3.53	24.13 ± 3.36	1.86 ± 0.15	45.48 ± 3.31
STEC	51.32 ± 3.31	48.68 ± 3.31	28.88 ± 4.07	1.96 ± 0.29	56.12 ± 4.30
STEC + HA	48.96 ± 0.08	51.04 ± 0.08	27.25 ± 1.06	1.84 ± 0.22	55.66 ± 2.26

DM: dry matter; FDM: fat in dry matter; LM: *Listeria monocytogenes*; STEC: shiga toxin-producing *Escherichia coli*; LLN: *Lc. lactis ssp lactis*; HA: *Hafnia alvei*.

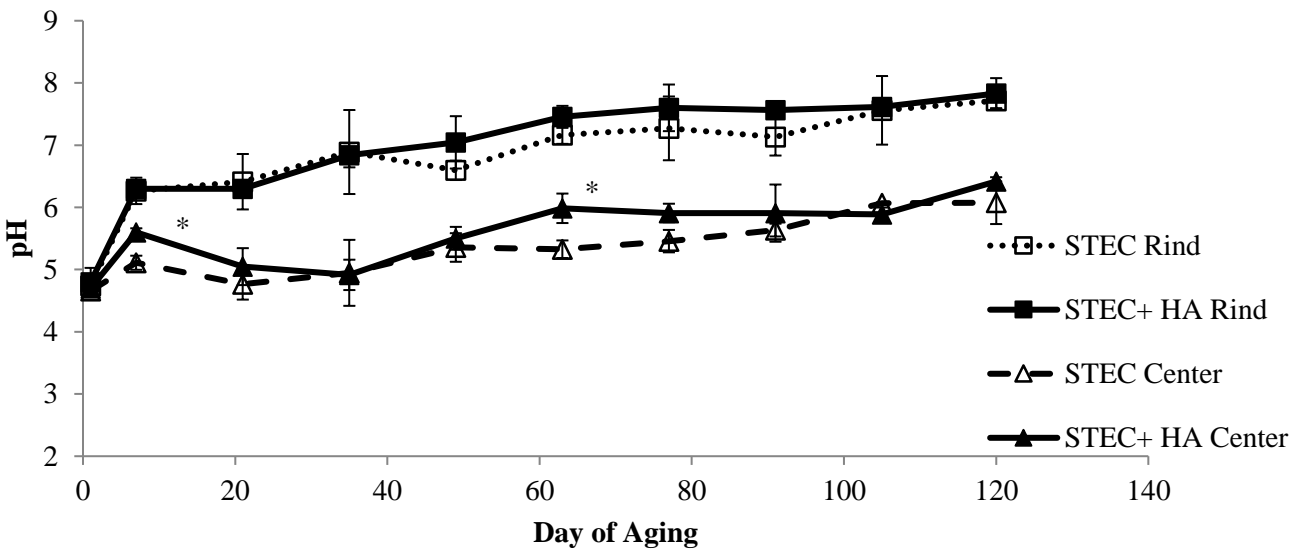
Treatment and control means were not significantly different from one another (P< 0.05) for any composition measurement

FIGURE 1. Change in cheese pH over time when manufactured from raw milk contaminated with *Listeria monocytogenes* (LM) (A) or shiga toxin-producing *Escherichia coli* (STEC) (B)

A



B



Squares represent the pH at the rind (□ Pathogen, ■PC) and triangles represent pH in the cheese center (△ Pathogen, ▲PC). Error bars show standard deviation, * indicates significant difference ($P < 0.05$) between treatment and control.

1

TABLE 2. Mean counts (log CFU/ml or g \pm SEM) of *Listeria monocytogenes* (LM) and shiga toxin-producing *Escherichia coli* (STEC) in milk and curd during the manufacture of cheese with and without protective cultures.

	Inoculated milk	Ripened milk	Whey	Curd at cutting	Cheese at first flip	Cheese before salting
LM	2.43 \pm 0.24	2.00 \pm 0.34	0.96 \pm 0.58	1.96 \pm 0.40	2.23 \pm 0.61	2.79 \pm 0.47
LM +LLN	2.25 \pm 0.31	2.22 \pm 0.15	0.82 \pm 0.12	2.02 \pm 0.25	2.26 \pm 0.10	2.90 \pm 0.15
STEC	1.99 \pm 0.21	3.09 \pm 0.02	2.50 \pm 0.46	3.76 \pm 0.08	4.08 \pm 0.004	3.84 \pm 0.34
STEC +HA	2.09 \pm 0.24	2.88 \pm 0.11	1.97 \pm 0.23	3.14 \pm 0.26	3.34 *	3.57 \pm 0.13

LLN: *Lc. Lactis ssp lactis*, HA: *Hafnia alvei*

Counts in treatments were not significantly different ($P < 0.05$) from controls at all sampling points.

*- Value from single trial, SEM and variability were not calculated

FIGURE 2A. Change in counts (log CFU/g) of *Listeria monocytogenes* (LM) in cheese manufactured with and without protective cultures during aging. Emboldened lines trace the mean of two independent cheeses made with pathogen only (dotted) and pathogen and treatment (solid). Pathogen counts from individual replicates of control (○) and PC treated (◆) cheeses are also displayed. LLN: *Lc. Lactis ssp lactis*, nisin producer.

A

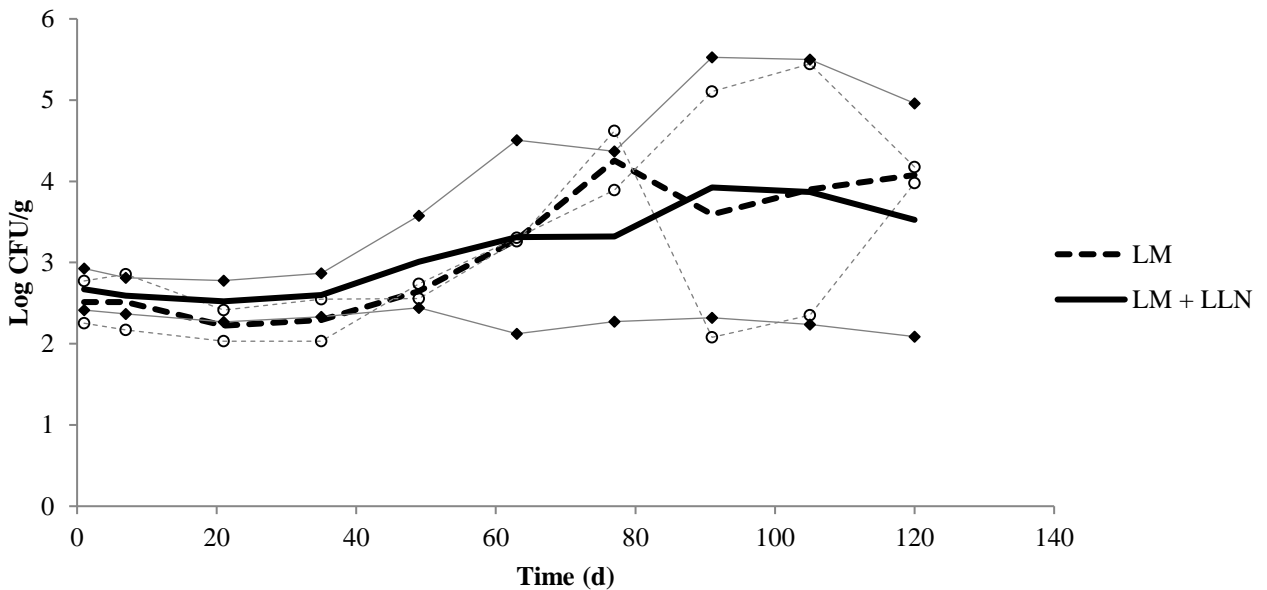
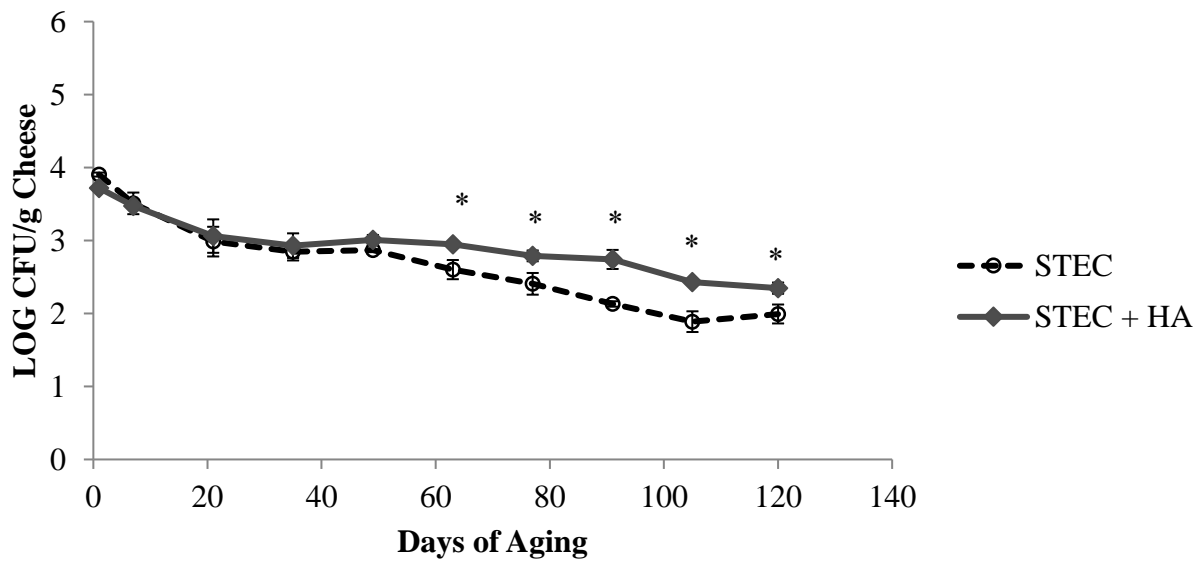


FIGURE 2B. Change in counts (log CFU/g \pm SD) of shiga toxin-producing *Escherichia coli* (STEC) in cheese manufactured with and without protective cultures during aging. Lines trace the mean of two independent cheeses made with pathogen only (dotted) and pathogen and treatment (solid). HA: *Hafnia alvei*.

B



* means differed significantly ($P < 0.05$).

SUPPLEMENT

FIGURE S1. Gas defect in cheese treated with HA protective culture at day 1 (A), 63 (B) and 105 (C) of aging.

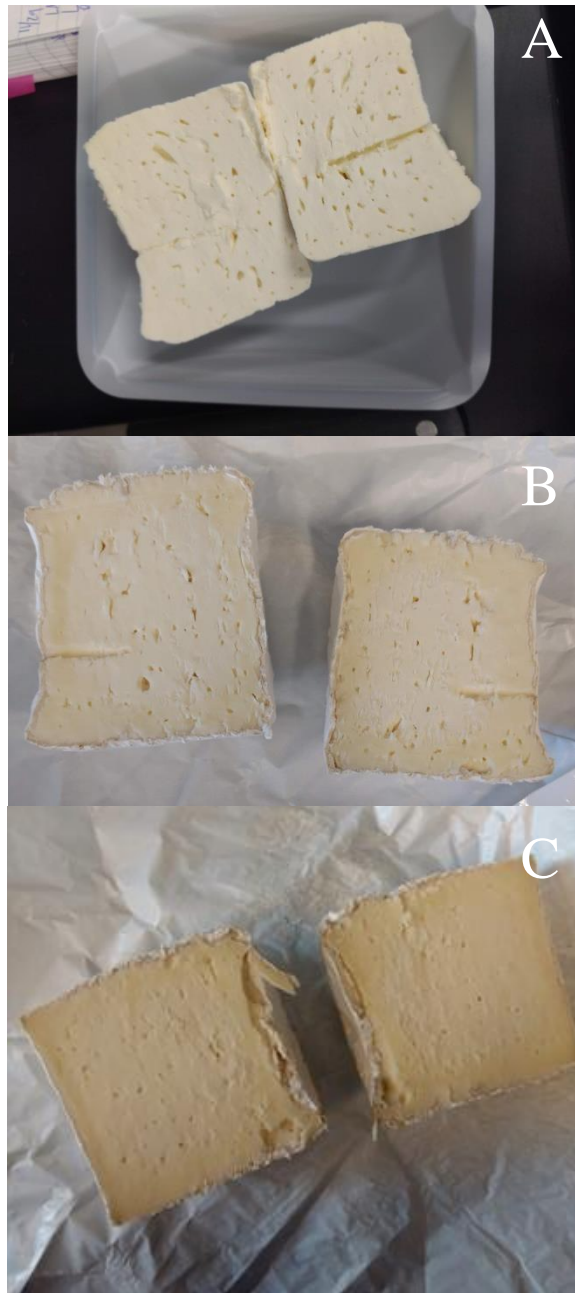
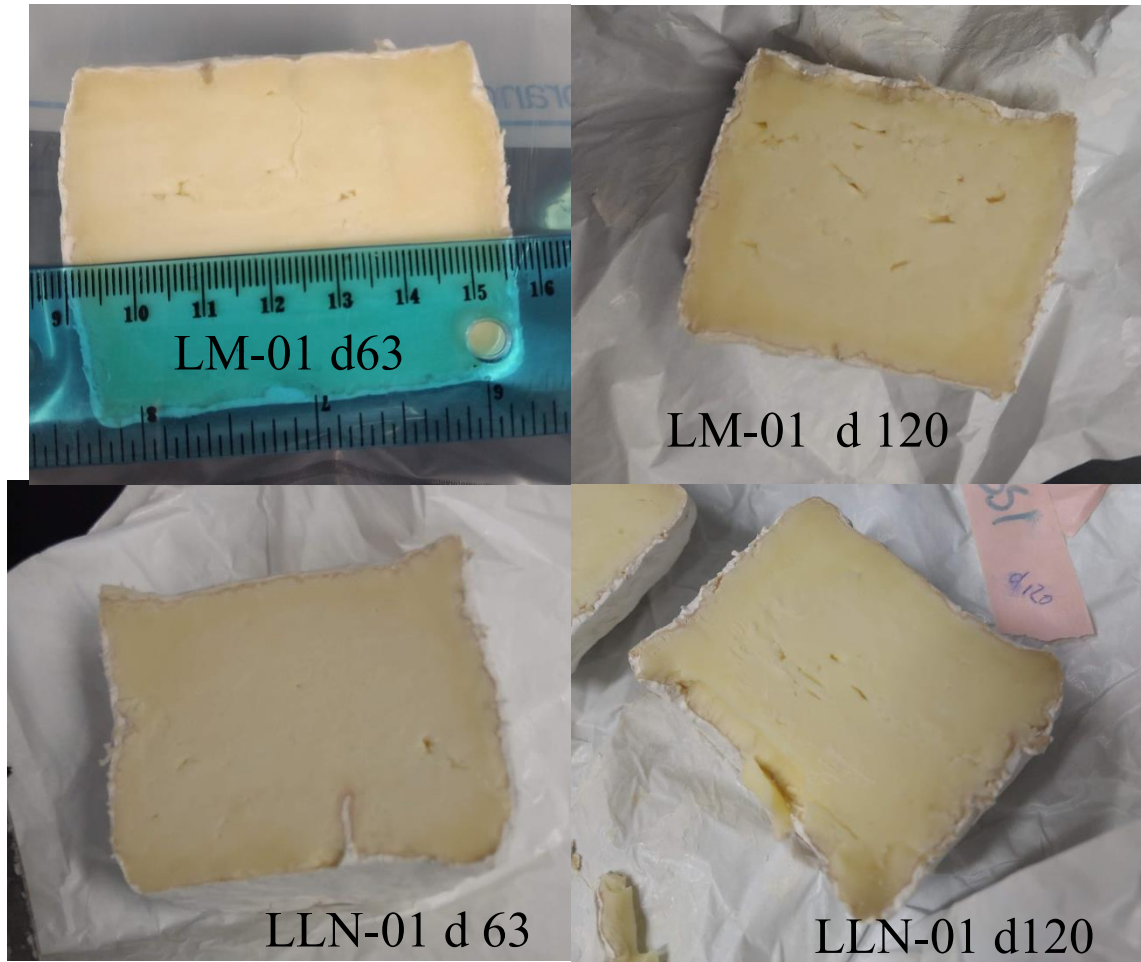


FIGURE S2. Ripening observations of LM and LLN treated cheeses. Degree of ripening in trial 01 was not the same extent as LM control at the same timepoint.



CHAPTER VI

SUMMARY

Listeria monocytogenes and shiga toxin-producing *Escherichia coli* pose significant risk to public health. Documented survival in raw milk cheese, in part due to favorable physiochemical changes over the course of cheese ripening, underscores the urgency to identify an easy-to-implement control strategy compliant with the standard of identity of cheese. This research examined the potential use of commercially available protective cultures for the control of *Listeria monocytogenes* and shiga toxin-producing *Escherichia coli* in soft surface mold ripened raw milk cheese. The first objective of this study was to assess compatibility of protective cultures with common cheesemaking starter cultures, ripening bacteria, yeasts and molds. Nine PCs did not inhibit growth or acid production of the starter cultures tested. PC inhibition of ripening bacteria and fungi was strain specific. This work created reference matrices for future use by cheesemakers to anticipate potential incompatibility with intended cheese cultures prior to selecting and using a PC.

The second objective of this study was to determine the antimicrobial activity of commercial protective cultures against *L. monocytogenes* when in raw milk. Five members of the original panel were tested. Two cultures (LR, PA) did not impact growth of *L. monocytogenes*, two cultures (LP, LPP) exerted a bacteriostatic effect and one culture (LLN) limited *L. monocytogenes* to <1 log CFU/ml throughout testing. This culture was selected for use in cheesemaking, despite previous observations of potential starter culture antagonism.

The third objective of this study was to determine the effect of commercial protective cultures on *L. monocytogenes* or STEC during the manufacture and storage of soft-ripened cheese made with raw milk. Treatment with nisin-producing LLN protective culture did not reduce *L. monocytogenes* counts in milk over ripening. Further, *L. monocytogenes* grew in cheese treated with this protective culture; however variation between trials inhibits any

definitive conclusion about the impact of the PC on *L. monocytogenes* growth in this cheese. STEC survived in soft-ripened cheese for over 120 days of ripening. Addition of HA protective culture did not impact STEC survival during manufacture or early in ripening, however may slightly enhanced survival late in ripening, as such this culture might not be the appropriate for this cheese type. Suspected impact of starter culture on protective culture performance prompts the need for additional testing to optimize application.

These results together identify the select compatibility of protective cultures with cheesemaking cultures and the potential for select PCs to control *L. monocytogenes* in raw milk. However further work is needed to optimize application and use parameters for control of pathogens in soft surface mold-ripened cheese.