Short communication: Evaluation of commercial meat cultures to inhibit *Listeria monocytogenes* in a fresh cheese laboratory model

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ABSTRACT

Control of *Listeria monocytogenes* in queso fresco and other fresh cheeses continues to be a challenge in the United States. These cheese types are particularly challenging due to their high moisture and high pH, which provide favorable conditions for the growth of *L. monocytogenes*. Protective cultures (i.e., viable strains of lactic acid bacteria that inhibit other microorganisms) have been investigated in foods such as meat as an alternative, clean-label control strategy for *L. monocytogenes*. However, the efficacy of protective cultures can vary by food matrix. In this study, we were interested in whether protective cultures used to control *L. monocytogenes* in meats could be applied to control the pathogen in queso fresco. We selected 4 commercially available bacterial cultures used for the control of *L. monocytogenes* in meat: *Lactobacillus curvatus*, *Lactobacillus sakei*, *Pediococcus acidilactici*, and *Leuconostoc carnosum*. We incorporated these cultures into batches of queso fresco during manufacturing and evaluated them for their ability to inhibit the growth of surface-applied *L. monocytogenes* at levels of 1×10^2 and 1×10^4 cfu/g. We stored the queso fresco at 6 and 21°C for up to 21 d. After 14 d, *Listeria* was able to grow to 1×10^7 cfu/g on the cheese. Our data show that the bacterial cultures did not significantly inhibit the growth of *L. monocytogenes* in queso fresco. The results from this study highlight the complexity of antagonistic bacterial interactions and their potential variability across food matrices. Protective cultures represent an important, clean-label tool for the control of *L. monocytogenes* in foods, but each strain must be evaluated in the food environment it is intended for to ensure its efficacy.

Key words: *Listeria*, lactic acid bacteria, biopreservation, cheese

Short Communication

Listeria monocytogenes is prevalent in the environment and has regularly been isolated in food manufacturing facilities (Kabuki et al., 2004; Ibarra-Sánchez et al., 2017). This organism is an important cause of foodborne outbreaks and can be introduced into ready-to-eat food during post-processing if proper hygienic practices are not in place (Kabuki et al., 2004). As well, *L. monocytogenes* can grow readily in certain ready-to-eat dairy products such as fresh cheeses because of their low salt levels, high moisture content, and near-neutral pH (Lin et al., 2006). Eighteen deaths were linked to *L. monocytogenes* in cheese between 1998 and 2017, according to the United States Centers for Disease Control (CDC) National Outbreak Reporting System. In particular, Latin or Hispanic-style cheeses (e.g., queso fresco) have been the source of many outbreaks (CDC, 2018). Demand for these types of cheeses is growing: the USDA reported the production of over 264 million pounds of Hispanic cheese in 2017 (USDA, 2018). The increasing popularity and consumption of these cheeses highlights the need for improved strategies to control or prevent the contamination and growth of *L. monocytogenes* in these food products.

The use of lactic acid bacteria (**LAB**) in food products has been investigated as a clean-label biopreservation method against spoilage and pathogenic microorganisms (Devlieghere et al., 2004). Lactic acid bacteria are naturally present in traditionally fermented foods and have been shown to have antagonistic interactions with other microorganisms present in the food, through direct competition for nutrients, production of antimicrobial products such as bacteriocins or organic acids, or both (Tichaczek et al., 1992; Vogel et al., 1993; Devlieghere et al., 2004). The ability of LAB to inhibit undesirable microorganisms in food has led to some strains being described as “protective cultures.”

Protective cultures have been used increasingly in foods as specific controls for *L. monocytogenes*, particularly in meat products. Several strains of LAB—*Lactobacillus curvatus*, *Lactobacillus sakei*, *Pediococcus acidilactici*, and *Leuconostoc carnosum*—have been

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evaluated for their antilisterial properties in various meat applications, including frankfurters, cooked ham, fermented sausages, chicken cold cuts, and cold-smoked salmon, and have been shown to be effective in reducing or preventing the growth of *L. monocytogenes* without noticeable sensory defects in the products (Amézquita and Brashears, 2002; Katla et al., 2002; Budde et al., 2003; Vermeiren et al., 2004; Benkerroum et al., 2005). Some of these strains, particularly *P. acidilactici*, *Lb. sakei*, and *Lb. curvatus*, have been shown to produce bacteriocins with antilisterial activity (Pucci et al., 1988; Nielsen et al., 1990; Tichaczek et al., 1992; Katla et al., 2002).

Given the wealth of available research on these protective cultures, it is perhaps not surprising that strains of *Lb. curvatus*, *Lb. sakei*, *P. acidilactici*, and *Leu. carnosum* are commercially available for use in high-risk ready-to-eat meat applications. As for many of these meats, controlling the post-processing contamination of high-risk cheese such as queso fresco by *L. monocytogenes* is a challenge (Moreno-Enriquez et al., 2007; Jackson et al., 2011). Interestingly, published work investigating the ability of these meat cultures to protect high-risk cheese is scarce. In this study, we took 4 commercial protective cultures available for the control of *L. monocytogenes* in meats, each representing 1 of the 4 species (*Lb. curvatus*, *Lb. sakei*, *P. acidilactici*, and *Leu. carnosum*), and evaluated their ability to inhibit the growth of surface-applied *L. monocytogenes* during storage at 6°C to reflect average consumer refrigeration temperature, and 21°C to reflect accelerated shelf life.

We obtained strains of each culture (*Lb. curvatus*, *Lb. sakei*, *P. acidilactici*, and *Leu. carnosum*) from a commercial supplier. Each strain was propagated on de Man, Rogosa, and Sharpe (MRS) agar (Becton, Dickinson, and Co., Franklin Lakes, NJ) at 30°C (*Lb. curvatus*, *Lb. sakei*, and *Leu. carnosum*) or 40°C (*P. acidilactici*) for 48 h from a frozen glycerol stock. Individual colonies of each strain were inoculated into 5 mL of MRS broth (Becton, Dickinson, and Co.) and incubated at 30 or 40°C for 24 h. Then, 1 mL of each culture optical density = 1.5) was centrifuged at $1,538 \times g$ for 4 min, and the resulting pellet was resuspended in 1 mL of UHT pasteurized skim milk (Parmalat USA Corp., Grand Rapids, MI). The suspension was then transferred to a culture tube containing 9 mL of UHT milk. Cultures were further incubated at 30 or 40°C for 48 h to achieve a final concentration of 7 to 8 log cfu/mL, which was confirmed with dilution and plating on MRS agar during each experiment.

Five strains of *L. monocytogenes* representing 4 outbreak strains linked to fresh cheese (FSL R9-5621, FSL R9-5623, FSL R9-5624, and FSL R9-5625) and 1 internal laboratory strain (FSL X1-0001), were ob-

tained from Dr. Wiedmann's Food Safety Laboratory at Cornell University (Ithaca, NY); they can be accessed through www.foodmicrobetracker.com. Each strain was propagated on brain heart infusion (BHI) agar (Becton, Dickinson and Co.) at 37°C for 24 h. We then used an individual colony from each strain to separately inoculate 5 mL of BHI broth (Becton, Dickinson and Co.). The cultures were grown overnight with shaking (200 rpm) at 37°C. Then, each culture was diluted 1:10 and 1:100, and both dilutions were grown at 30 and 37°C with shaking (200 rpm) for approximately 8 h. The cultures closest to and optical density at 600 nm = 1.00 (9 log cfu/mL based on previous standard curves for these strains) were used to create a cocktail containing approximately equal amounts of each strain. The cocktail was then serially diluted to achieve the desired inoculum concentration of either 2 log cfu/g or 4 log cfu/g. The initial inoculum cocktail was also diluted and spread-plated on BHI and modified Oxford (MOX) agar (Becton, Dickinson, and Co.) for enumeration to confirm the starting concentration. Plates were incubated at 30°C for 48 h.

Cheese was prepared on a laboratory scale as follows, with guidance from previously established protocols (Van Tassel et al., 2015; Henderson et al., 2019). For each treatment, 600 mL of nonhomogenized whole milk was brought to 35°C in 1 L sterile bottles. To each bottle, the following were added: 804 µL of a 32 to 33% CaCl₂ solution (Dairy Connection Inc., Madison, WI); 6 mL of a 7 to 8 log cfu/mL LAB culture; and 90 µL of double-strength rennet (Chy-Max Extra; Chr. Hansen, Milwaukee, WI) brought to a final volume of 6 mL in deionized water. The bottles were swirled to mix, and then the mixture was poured into individual plastic cheese vats. The vats were then incubated for 45 min in a 35°C water bath. The curd was cut and the vats were incubated for 15 min at 35°C, with a gradual increase to a final temperature of 40°C. Then, 60 mL of whey was taken out of each vat and replaced with 60 mL of an aqueous NaCl solution (0.16 g/mL) and stirred to mix. The vats were incubated at 40°C for another 20 min. The whey was then drained from the vats with cheesecloth for 1 h. The curd was then scooped into 12-well plates (Corning Inc., Corning, NY), adding 6 g of curd to each well. The curd was then pressed for 2 h, giving a final cheese with a weight of approximately 4 g. The pH of each treatment was measured using an InLab Surface pH probe (Mettler Toledo LLC, Columbus, OH) and the moisture content was measured using a Smart Turbo Moisture Analyzer (CEM Corp., Matthews, NC). Each measurement was taken in duplicate for each treatment and each batch. Cheeses were prepared in duplicate for each time point and treatment in at least 3 replicates on separate days.

After pressing, cheeses (4 g) were surface-inoculated with 100 μ L of the prepared *L. monocytogenes* cocktail, achieving a final concentration of either 2 or 4 log cfu/g. Positive controls (inoculated with *L. monocytogenes* only) and negative controls (no inoculum) were included. Each set of cheeses was incubated at 21°C or 6°C for 4 or 21 d, respectively.

We enumerated samples on d 0, 1, 2, and 4 (21°C incubation) or d 0, 1, 7, 14, and 21 (6°C incubation) for *L. monocytogenes* and LAB. Each cheese (4 g) was placed in a sterile Whirl-Pak filter bag (Nasco, Fort Atkinson, WI) and diluted 1:10 (wt/vol) with sterile PBS. Samples were then homogenized using a stomacher set at normal speed for 60 s, and then serially diluted in PBS. Appropriate dilutions were spread-plated on MRS and MOX agar for enumeration. Plates were incubated at 30°C for 48 h. A total LAB count was counted on MRS agar and *Listeria* (round, concave, black colonies) were counted on MOX agar.

All statistical analyses were performed using R (version 3.5.2; R Development Core Team, Vienna, Austria). We performed ANOVA and Tukey's honest significant difference test individually for each time point to compare the log differences in *Listeria* counts between all LAB treatments and the positive control.

The total MRS counts on d 0 and 1 were 5 to 6 log cfu/g, and *L. monocytogenes* counts were 2 log or 4 log cfu/g, indicating that the LAB strains were present and viable in the cheese. For each treatment, *L. monocytogenes* levels increased throughout the incubation period, resulting in a final concentration of approximately 7 to 8 log cfu/g for each temperature and inoculation level. For the higher *L. monocytogenes* inoculation level of 4 log cfu/g at 21°C (Figure 1A), no treatment resulted in any significant difference ($P > 0.05$) in *L. monocytogenes* levels compared with the control for any time point. At 6°C (Figure 1B) at the same inoculation level, the samples treated with *Lb. curvatus* and *Lb. sakei* showed a significant difference ($P < 0.05$) in *L. monocytogenes* counts on d 14. However, these counts were only about 0.5 log cfu/g lower than the control and did not continue to be significant through the 21 d of storage. When the cheese was inoculated with a lower concentration of *L. monocytogenes* (2 log cfu/g), at 21°C (Figure 2A) the *L. monocytogenes* count of the samples treated with *Lb. curvatus* displayed a significant difference ($P < 0.05$) from all other treatments and the control on d 1. However, the difference was only about 0.5 log cfu/g. All treatments showed similar *L. monocytogenes* counts to the control, with no significant differences throughout incubation at 21°C. At 6°C (Figure 2B), *Lb. curvatus* showed counts about 0.5 log cfu/g lower than the control ($P < 0.05$) on d

7, but by d 21 we found no significant difference ($P > 0.05$) in *L. monocytogenes* levels.

The pH of the queso fresco, regardless of treatment, was slightly less than a neutral pH of 7.0 (Table 1). Typical queso fresco cheeses made in the United States have a pH above 6.0, and traditionally the pH can range from 5.3 to 6.5, but there is no standard of identity for queso fresco as regulated by the US Food and Drug Administration (Van Hekken et al., 2006; Clark et al., 2009; Ibarra-Sánchez et al., 2017). The negative control and treatments containing LAB were of similar pH even with the addition of LAB, indicating that the cultures did not produce sufficient amounts of acid to affect pH during cheesemaking. The moisture content of queso fresco is typically high relative to other cheeses and can range from 41 to 59% (Clark et al., 2009). This variability in moisture content is the result of manufacturer and consumer preference (Clark et al., 2001; Tunick and Van Hekken, 2010). The queso fresco samples from this study were at the higher end of this range (Table 1). Although the moisture content varied among treatments, the low standard deviation between microbial counts for each treatment and the positive control suggested that the moisture content did not influence microbial growth in this experiment.

Our results suggest that the selected LAB are unable to inhibit the growth of *L. monocytogenes* in a fresh cheese matrix. Although *L. monocytogenes* counts were reduced compared with the control on certain days, the difference was only about 0.5 log cfu/g. This reduction was not significant from a biological standpoint and would not be effective as a control strategy. Each of the LAB species have been shown to have antagonistic activity toward *L. monocytogenes* in media and in meat matrices (Tichaczek et al., 1992; Amézquita and Brashears, 2002; Budde et al., 2003; Vermeiren et al., 2004). However, certain factors could contribute to their contrasting antagonistic behavior in queso fresco. The effectiveness of a strain depends on its ability to adapt to the food matrix and relies on aspects such as pH, moisture, and water activity (Holzapfel et al., 1995). In a study that reported antagonistic activity of *Lb. sakei* against *L. monocytogenes* in cooked ham, the ham had a pH close to 6.0, lower than that of the queso fresco used in this study (Vermeiren et al., 2004). Additionally, antimicrobial compounds produced by the LAB, such as bacteriocins, can have limitations depending on the food matrix. For example, binding and interactions to certain food components (such as lipids) can render the bacteriocin ineffective (Holzapfel et al., 1995). A previous study investigating other protective cultures in queso fresco also had unpromising results for the inhibition of *L. monocytogenes*, showing that after

Figure 1. *Listeria monocytogenes* counts in a laboratory model of queso fresco prepared with protective cultures (*Lactobacillus curvatus*, *Lactobacillus sakei*, *Pediococcus acidilactici*, and *Leuconostoc carnosum*) and an initial *L. monocytogenes* load of 10^4 cfu/g at (a) 21°C, and (b) 6°C. Bars with different letters (a, b) indicate significant differences ($P < 0.05$) between protective culture treatments on the same day. Error bars represent SD.

Figure 2. *Listeria monocytogenes* counts in a laboratory model of queso fresco prepared with protective cultures (*Lactobacillus curvatus*, *Lactobacillus sakei*, *Pediococcus acidilactici*, and *Leuconostoc carnosum*) and an initial *L. monocytogenes* load of 10^2 cfu/g at (a) 21°C and (b) 6°C. Bars with different letters (a, b) indicate significant difference ($P < 0.05$) between protective culture treatments on the same day. Error bars represent SD.

Table 1. Composition of queso fresco samples ($\pm$ SD)

Culture treatment ¹	Moisture content (%)	pH
No culture	57.19 $\pm$ 3.02	6.82 $\pm$ 0.29
<i>Lactobacillus curvatus</i>	57.72 $\pm$ 1.45	6.68 $\pm$ 0.16
<i>Lactobacillus sakei</i>	58.76 $\pm$ 2.22	6.71 $\pm$ 0.16
<i>Pediococcus acidilactici</i>	57.95 $\pm$ 2.79	6.68 $\pm$ 0.19
<i>Leuconostoc carnosum</i>	58.51 $\pm$ 2.37	6.72 $\pm$ 0.24
Negative control	58.30 $\pm$ 2.44	6.74 $\pm$ 0.24

¹Indicates the protective culture added to the queso fresco. The negative control had no protective culture added and was not challenged with *Listeria monocytogenes*.

21 d, *L. monocytogenes* counts in a cheese containing a strain of *Lactococcus lactis* were only 0.7 log cfu/g lower than the control (Lourenço et al., 2017).

Some studies using other fresh cheeses have shown successful inhibition of *L. monocytogenes* by LAB. For example, several strains of *Enterococcus* spp. and a bacteriocin-producing *Lactococcus lactis* strain were evaluated for their antagonistic activity toward *L. monocytogenes* in Pico cheese, a type of Latin-style fresh cheese (Coelho et al., 2014). In that study, several combinations of different strains led to a 5 log cfu/g reduction of *L. monocytogenes* after 7 d, and no sensory differences were detected when the cheese inoculated with these strains was given to consumers. Another study found a bacteriostatic effect against *L. monocytogenes* when several strains of *Enterococcus* spp. were applied in fresh Minas cheese (Vera Pingitore et al., 2012). Those cultures resulted in a 3 log inhibition in final *L. monocytogenes* levels and a decrease in pH to 5.2 after 12 d. These studies suggest that cultures may be more effective when multiple strains are used in combination or if they produce enough acid to lower the pH.

Protective cultures have been effective at inhibiting *L. monocytogenes* in other cheese types. A study of the antagonistic activity of a bacteriocin-producing *Lactococcus lactis* strain against *L. monocytogenes* in cottage cheese saw an initial 1 log cfu/mL reduction of *L. monocytogenes* after 24 h, and after 5 d the levels of *L. monocytogenes* were reduced to below detectable limits (McAuliffe et al., 1999). Although cottage cheese is also considered a fresh cheese, it traditionally has a starter culture for acidification and therefore has a lower pH (5.2) than queso fresco (McAuliffe et al., 1999). Although similar to queso fresco, cottage cheese may be better suited for inhibition of *L. monocytogenes* with protective cultures because of compositional characteristics such as moisture content, salt concentration (which affects water activity), and pH. These parameters are known to play an important role as hurdles for *L. monocytogenes* outgrowth (Schvartzman et al., 2011),

potentially more so when used in combination with hurdles such as protective cultures. The salt, moisture, and pH of queso fresco is reported to vary widely (Guo et al., 2011), and the composition of the queso fresco in this study (Table 1) was at the high end of those ranges for both moisture and pH, likely representing a worst-case scenario for inhibiting *L. monocytogenes*.

Control of *L. monocytogenes* contamination continues to be a challenge in queso fresco and other Latin-style fresh cheeses because of their high pH and high moisture content. When working with these types of cheeses, control strategies such as protective cultures may not provide a high level of protection; therefore, good manufacturing practices should continue to be a focus to prevent contamination. Compositional traits such as moisture levels, salt content, and pH are also critical for *L. monocytogenes* control, and future research is needed to understand the interplay between those factors and protective cultures to more effectively combine these hurdles to control *L. monocytogenes* in high-risk cheeses. Certain cultures, such as the ones tested here, may be suited for protection against *L. monocytogenes* in applications such as meat, but their efficacy was clearly challenged under the queso fresco conditions in this study.

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