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Anti-listerial activity of thermophilin 110 and pediocin in fermented milk and whey

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ABSTRACT

Listeria monocytogenes is a pathogenic bacterium responsible for foodborne illness worldwide. Antimicrobial peptides, or bacteriocins, produced by food-grade lactic acid bacteria can serve as preservatives to prevent *Listeria*'s growth in various foods, including dairy products. This study investigated the anti-listerial activities of bacteriocin-producing lactic acid bacteria, *Streptococcus thermophilus* B59671, and *Lactobacillus plantarum* 076. *In vitro* studies showed that the concentration of pediocin produced by *L. plantarum* 076 (2560 AU/mL) inhibited the growth of a six-strain cocktail of *L. monocytogenes*. However, the concentration of thermophilin 110 produced by *S. thermophilus* B59671 (320 AU/mL) only delayed the growth by ~2 h. Higher concentrations of thermophilin 110 (≥640 AU/mL) suppressed *Listeria* growth for up to 22 h. Pasteurized skim milk fermented with a co-culture of *S. thermophilus* B59671 and *L. plantarum* 076 reduced the number of *L. monocytogenes* cells by > 4 Log CFU/mL due mainly to the activity of pediocin. The anti-listerial activity was not observed in whey samples collected from pasteurized skim milk fermented with this co-culture but was detected when raw milk was the substrate. Two additional whey preparations, the by-products from commercial bovine and goat raw-milk cheeses, also inhibited *Listeria* growth and reduced the number of cells following storage at 4 °C for one week. This study showed that a concentrated preparation of thermophilin 110 has potential as an anti-listerial compound. It demonstrated the prospect of using a co-culture of *S. thermophilus* B59671 and *L. plantarum* 076 to prevent *Listeria* contamination in dairy foods. Additionally, results showed that metabolites with antimicrobial activities may be generated during the fermentation of raw milk due to indigenous microflora.

1. Introduction

Listeria monocytogenes is a robust psychrotrophic foodborne pathogen. It is estimated to cause about 1600 illnesses each year in the United States, with more than 1500 related hospitalizations and 260 associated deaths (Center for Disease Control, 2016; Liu et al., 2012; Scallan et al., 2011). Additionally, European (EU) countries reported 2480 cases of invasive listeriosis and 227 related deaths in 2017 (EFSA & ECDC, 2018). Dairy products are a potential source for *L. monocytogenes* and have been implicated in past listeriosis outbreaks worldwide (European Commission, 2018; EFSA & ECDC, 2018; Jackson, Gould, Hunter, Kucerova, & Jackson, 2018). Specifically, fresh and soft cheeses have been investigated for their role in *Listeria* outbreaks, as their high moisture content and neutral pH are suitable for the growth and survival of *L. monocytogenes* (Leggett et al., 2012; Tilocca et al., 2020; Tomasula et al., 2014).

Consumers' demand for natural products has led the food industry to investigate alternatives to traditional preservatives to prevent the growth of microbial contaminants. Whey, a co-product of the dairy industry, was previously considered a waste material and an environmental burden. However, its rich nutrient content, including lactose, soluble proteins, lipids, minerals, vitamins, and organic acids, has resulted in its expanding valorization in the last few decades. Sweet whey, the by-product of rennet set cheese, has been increasingly used in

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a variety of food and health-promoting products as a high-quality protein source for its well-balanced essential amino acid profile and possible underlying effects on gut microbiota and on nutrient absorption (Master & Macedo, 2020; Turgeon & Brisson, 2020). A range of bioactivities of whey has also been reported, including antimicrobial, prebiotic, immunomodulatory, anti-hypertensive, anti-obesity, and anti-oxidative properties (Benkerroum, 2010; Corrochano, Buckin, Kelly, & Giblin, 2018; Gupta & Prakash, 2017; Marshall, 2004; Pihlanto, 2006).

Applications for acid whey, generated during Greek-style yogurt and cottage cheese production, have thus far remained limited due to its higher concentration of lactic acid and lower levels of protein, fat, and lactose than sweet whey (Chandrapala et al., 2015; Menchik, Zuber, Zuber, & Moraru, 2019). These shortcomings, compounded by the rapidly rising market demand for acid-coagulated dairy products (yogurt, cream cheese, etc.), are becoming serious economic and environmental challenges (Chandrapala et al., 2015). However, like sweet whey, acid whey can be a source of antimicrobials, including lactic acid, lactoferrin, and lactoperoxidase (Marshall, 2004). Lactic acid bacteria (LAB) could also produce antimicrobial peptides when used as a starter or adjunct cultures during fermentation. These LAB-induced antimicrobial peptides, termed bacteriocins, have been investigated for potential food safety applications. However, their activity spectrum is typically limited to other Gram-positive species, including *Listeria monocytogenes* (Anyogu, Awamaria, Sutherland, & Ouoba, 2014; Cotter, Hill, & Ross, 2005). Considerable attention has been paid to broadening their activity spectrum to include Gram-negative pathogens. These efforts included using additional antimicrobial measures or "hurdles," such as chelators, high pressure, temperature shock, and eukaryotic antimicrobial peptides, to weaken the bacterial outer membrane (Boziaris & Adams, 2001; Lüders, Birkemo, Fimland, Nissen-Meyer, & Nes, 2003; Masschalck, Deckers, & Michiels, 2003; Stevens, Sheldon, Klapes, & Klaenhammer, 1991).

Our laboratory has characterized bacteriocins produced by several *Streptococcus thermophilus* strains (Somkuti & Renye, 2015). *S. thermophilus* B59671 is one of these strains shown to secrete thermophilin 110, a broad-spectrum antimicrobial peptide with demonstrated anti-listerial activity (Gilbreth & Somkuti, 2005). In this study, we determined the concentration of thermophilin 110 required to inhibit *L. monocytogenes* growth. We also investigated the potential for using a co-culture of *S. thermophilus* B59671 and *L. plantarum* 076, a natural pediocin-producing culture, to prevent *Listeria* survival in fermented milk. Additionally, this co-culture was used to ferment both raw and pasteurized milk to obtain novel whey preparations. These whey preparations, along with two additional whey samples recovered from commercial bovine and goat raw-milk cheese productions, were assessed for their anti-listerial activity.

2. Materials and methods

2.1. Bacterial strains and whey samples

Streptococcus thermophilus strains B59671, ST13, and ST29 were maintained in Tryptone-Yeast Extract-Lactose (TYL) broth (Somkuti & Steinberg, 1986). *Lactobacillus plantarum* 076 (Danisco, Niebüll, Germany) and *Pediococcus acidilactici* PAF (gift from B. Ray, Univ. of Wyoming) were regularly passaged in Lactobacilli MRS broth (Difco, MI). *L. innocua*, *L. monocytogenes* Scott A (ATCC49594), and a multi-strain cocktail (Table 1) containing six *L. monocytogenes* strains (from the ERRC collection, prepared as described below in Section 2.4.) were maintained in Brain Heart Infusion (BHI) broth. The stocks of each culture were stored in their respective media and 20% glycerol (v/v) at −80 °C. All *Listeria* strains used in the cocktail were originally isolated from foods, including hot dogs, milk, and cheese.

A total of five whey preparations were evaluated in this study. Three whey samples were generated by fermenting raw milk, pasteurized cream-line (non-homogenized) milk, and pasteurized skim milk with a co-culture of *S. thermophilus* B59671 and *L. plantarum* 076. Whey was generated using a modified yogurt-making procedure. The mixed starter culture (*S. thermophilus* B59671/*L. plantarum* 076) was pre-incubated in 5 mL of skim milk at 37 °C for 3 h. For each yogurt preparation, 500 mL of milk was heated to 80 °C and then immediately cooled to 40 °C. The starter culture (5 mL) was added directly to the preheated milk and incubated at 40 °C overnight (~21 h). Fermented milk samples were initially filtered through cheesecloth (4 layers). The collected whey was then centrifuged at 12,000×*g* for 30 min. Supernatants were decanted into sterile conical tubes, and the resulting whey samples were stored at −20 °C. The remaining two whey samples were collected following the production of hard cheeses (Biodynamic Farms LLC, Fleetwood, PA): one from raw bovine milk (RBM) and the other from raw goat milk (RGM).

Table 1
Listeria monocytogenes strains in the cocktail used in this study.

NO	Strain	Serotype
1	*Listeria monocytogenes* F2365	4b
2	*Listeria monocytogenes* H7858	4b
3	*Listeria monocytogenes* ATCC19115	4b
4	*Listeria monocytogenes* F4260	1/2b
5	*Listeria monocytogenes* V7	1/2a
6	*Listeria monocytogenes* MFS53	4b

2.2. Partial purification of thermophilin 110

Thermophilin 110 was extracted using the chloroform extraction method, as previously described (Gilbreth & Somkuti, 2005). Briefly, cell-free supernatant (CFS) from an overnight culture of *S. thermophilus* B59671 (1 L) was mixed with a 0.5 volume of chloroform and stirred vigorously for 45 min. Following centrifugation, the aqueous and solvent phases were removed. The interface layer and sediment were dispersed in 15 mL of sterile water. The suspension was centrifuged, and the supernatant was discarded. A stream of air was passed over the pellet to remove trace amounts of solvent. The pellet was resuspended in 5 mL of sterile water and stored overnight at 4 °C. Following centrifugation, the pellet was again resuspended in 5 mL of sterile water. Serial 2-fold dilutions of the sample were prepared. Fifty microliters (50 μL) of each dilution were tested for antimicrobial activity by a well-diffusion assay using *S. thermophilus* ST113 as the target bacterium. Arbitrary units (AU/mL) were calculated as the reciprocal of the highest dilution showing an inhibition zone multiplied by 20. Thermophilin 110 stock was diluted in water to obtain a concentration of 5120 AU/mL.

Pediocin was not purified from the cell-free supernatant of *L. plantarum* 076 because the concentration produced could suppress *Listeria* growth. Arbitrary units of pediocin present in LP 076 CFS were determined using *L. innocua* as the surrogate for *L. monocytogenes*.

2.3. Anti-listerial activities of thermophilin 110 and pediocin in fermented milk

Cells from an overnight culture (5 mL) of *S. thermophilus* B59671 and *L. plantarum* 076 were collected, washed 2X in sterile peptone water, and finally resuspended in 5 mL of peptone water. Pasteurized skim milk was inoculated with either *S. thermophilus* or *L. plantarum* (1% v/v) or the co-culture, a combined bacteria species at 0.5% of each strain. Fermentation was performed at 42 °C for 4.5 h, at which point a culture sample was collected to measure the pH. Fermented milk samples were then inoculated with an overnight culture of *L. monocytogenes* Scott A (1% v/v) and immediately placed at 4 °C. *Listeria* survival was measured by viable counts on Modified Oxford *Listeria* agar (Becton, Dickinson and Co.), following serial dilutions of the fermented milk samples. The plates were incubated at 37 °C for 24 h. Results were the mean of the duplicate

measurements with the initial *Listeria* inoculum averaged at 6.2 Log CFU/mL.

2.4. Anti-listerial activities of bacteriocins produced by S. thermophilus B59671 and L. plantarum 076 against a six-strain L. monocytogenes cocktail

The six-strain *L. monocytogenes* cocktail was prepared as follows. Each strain was streaked onto BHI agar plates and incubated at 37 °C for 24 h. A single colony was then harvested to inoculate 5 mL BHI broth to obtain the pure cultures (Dias et al., 2018). The cultures were normalized to the same optical density (OD_{600nm}). An equal volume of each culture was used to obtain the six-strain cocktail.

Two-fold serial dilutions of the partially purified thermophilin 110 and the cell-free supernatant (CFS) from *L. plantarum* 076 (containing pediocin) were prepared in BHI broth and tested for activity against the six-strain *Listeria* cocktail. Briefly, the *Listeria* cocktail was grown overnight in BHI and then diluted 1:100 in fresh BHI broth. Serial 2-fold dilutions (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128) of thermophilin 110 (at 5120 AU/mL) stock or LP 076 CFS were prepared in BHI broth, with 100 μL remaining in wells (column) of a 96 well plate. One hundred microliters (100 μL) of the diluted *L. monocytogenes* cocktail were added to each well, resulting in an additional 2-fold dilution of the testing antimicrobial sample. *Listeria* growth was monitored hourly at 37 °C for 24 h by optical density (OD_{600nm}) using an Epoch2 multi-plate reader (Biotek, Winooski, VT, USA). *Listeria,* incubated and monitored in fresh BHI, was used as a negative control. Olive leaf extract (OLE, 3.9 mg/mL stock; a gift from EuroMed Inc., Barcelona, Spain) was used as a positive control for anti-listerial activity. A minimum of three replicates was performed for each antimicrobial test.

2.5. Anti-listerial activity of whey

The *Listeria* cocktail (600 μL) was aliquoted into eight microcentrifuge tubes and pelleted (9000×*g* and 4 °C for 7 min). A bacterial pellet was gently resuspended in each of the five whey samples (600 μL) or BHI as a control. *Listeria* survival was assayed immediately after the pellet was resuspended (T^0) and followed by a 24-h incubation at 37 °C. The suspensions were then stored at 4 °C. *Listeria* survival was accessed after storage at 24 h, 72 h, and one week using the 6 × 6 plating method on BHI agar (C.-Y. Chen, Nace, & Irwin, 2003). Each experiment was conducted in triplicate.

2.6. Scanning electron microscopy (SEM)

SEM was used to determine the morphological effects of thermophilin 110 or pediocin (from LP 076) on *Listeria*, specifically the cell surface and flagellum. The samples were prepared as described previously (Ceruso et al., 2020) with the following modifications: a 5 mL culture of *Listeria monocytogenes* cocktail was exposed to varying concentrations of thermophin 110 or LP 076 CFS. The culture was incubated overnight at 30 °C with shaking at 200 rpm. One hundred microliters (100 μL) of the culture were pipetted onto a 12 mm micro-cover glass slide (Thermo Scientific Portsmouth NH, USA) and allowed to adhere for 10 min. Four hundred microliters (400 μL) of 2.5% glutaraldehyde were used to cover the bacterial sample. After 30 min fixation, the slides were washed with 2–3 mL of the following solutions: 0.1 M imidazole (2 consecutive washes for 30 min each); 50% ethanol; 80% ethanol; and 90% ethanol. Lastly, the samples were washed three times, with 100% ethanol. The prepared slides were stacked into a wire basket, separated by cloth, and placed into a critical point drying apparatus (Denton DCP-1) to dry completely using liquid carbon dioxide. They were then removed from the critical dryer, fixed on SEM support, and sputter-coated with gold for 1 min.

Sample slides were viewed with the FEI Quanta 200 F Scanning Electron Microscope (FEI Co., INC., Hillsboro, OR), with an accelerating voltage of 10 kV in high vacuum mode. Instrumental magnification was set at 1,000x, 10,000x and 50,000x for imaging purposes.

2.7. Statistical analysis

All results were represented as the means of a set of triple or quintuple measurements. A one-way analysis of variance (ANOVA) was performed using the Igor Pro software (version 8.03, WaveMatrics, Inc., Oregon, CA). *P* values of <0.05 were considered statistically significant.

3. Results and discussion

3.1. Anti-listerial activities of thermophilin 110 and pediocin produced in complex medium and fermented milk

It was previously reported that cell-free supernatant (CFS) from an overnight culture of *S. thermophilus* B59671 in TYL broth contained thermophilin 110 (Gilbreth & Somkuti, 2005). In this study, the concentration of thermophilin 110 was estimated to be 320 AU/mL using *S. thermophilus* ST113 as the target bacterium (Fig. 1a). Thermophilin 110 was also reported to inhibit the growth of *L. monocytogenes* (Gilbreth & Somkuti, 2005). An inhibition zone was only observed against *L. monocytogenes* Scott A when exposed to undiluted CFS in this work (data not shown). In comparison, pediocin present in the CFS from an overnight culture of *L. plantarum* 076 showed more potent anti-listerial activity, estimated at 2560 AU/mL using *Listeria innocua* as the target bacterium (Fig. 1b). Similar size inhibition zones were observed when *L. monocytogenes* Scott A was used as the target bacterium (data not shown). The well diffusion assays also showed that the CFS from *L. plantarum* 076 did not inhibit the growth of *S. thermophilus* strains ST13, ST29, or B59671. The CFS from *S. thermophilus* B59671 did not inhibit *L. plantarum* 076 (data not shown). These results suggested that *S. thermophilus* B59671 and *L. plantarum* 076 could be explored as a co-culture for inhibiting *Listeria*'s growth. *Lactococcus lactis* ATCC 11454, known to produce nisin, was also considered a candidate for co-culture studies, but this was not pursued as nisin inhibited the growth of both *S. thermophilus* B59671 and *L. plantarum* 076 (data not shown).

Thermophilin 110 production in milk was initially assessed by well diffusion assay using *S. thermophilus* ST113 as the target bacterium (data not shown). When grown in skim milk, *S. thermophilus* B59671 was shown to produce thermophilin 110 at 320 AU/mL, equivalent to the concentration determined for TYL growth (see above). However, the concentration of thermophilin 110 generated in pasteurized whole milk only ranged from 20 to 80 AU/mL. It increased to 80 and 160 AU/mL when *S. thermophilus* B59671 was grown in pasteurized cream-line (non-homogenized) milk and raw milk, respectively. This suggested that homogenization negatively affected the antimicrobial activity of thermophilin 110 in milk, agreeing with a previous study that reported a homogenization-induced reduction of nisin's anti-listerial activity (Bhatti, Veeramachaneni, & Shelef, 2004).

Production of pediocin by *L. plantarum* 076 in different milk varieties was not assessed as growth was not observed following incubation at 37 °C for 24 h. Lack of growth was evidenced by the absence of milk protein precipitation and no change in the culture's pH (remained ~6.5). However, when *L. plantarum* 076 was co-cultured with *S. thermophilus* B59671 the culture pH was reduced to ~4.0, and inhibition zones were observed against *L. innocua* and *S. thermophilus* ST113, suggesting that both pediocin and thermophilin 110 were produced (data not shown). A similar size inhibition zone was detected against *L. innocua* when *L. plantarum* 076 was co-cultured with *S. thermophilus* ST29, which does not produce a thermophilin. This result indicated that the anti-listeria activity observed from both co-cultures was primarily due to the production of pediocin.

S. thermophilus B59671 and *L. plantarum* 076, as a single and a co-culture, were further evaluated for inhibiting the survival of *L. monocytogenes* Scott A in fermented pasteurized skim milk stored at

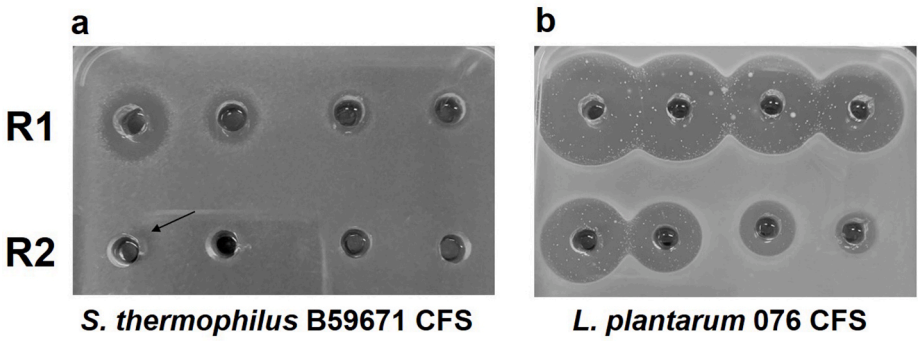


Fig. 1. Antimicrobial activity in CFS from an overnight culture (37 °C for 24 h) of (a) *S. thermophilus* B59671 and (b) *L. plantarum* 076. CFS was serially diluted 2-fold in sterile water, and 50 μL was added to precast wells with agar medium inoculated with *S. thermophilus* 113 (a) *or Listeria innocua* (b) used as the target bacterium. The lowest concentration showing antimicrobial activity for thermophilin 110 is indicated with an arrow. Dilutions of CFS (left to right) were made as follows: Row 1 (R1): undiluted, 2, 4, 8-fold; Row 2 (R2): 16, 32, 64, 128-fold.

refrigeration temperature. Fig. 2 shows the anti-listerial activity of skim milk fermented with *S. thermophilus* B59671, *L. plantarum* 076, and a co-culture of *S. thermophilus* B59671 and *L. plantarum* 076. Fermentations resulted in a final pH of 4.71 (±0.08) and 4.75 (±0.02) using *S. thermophilus* B59671 alone and the co-culture, respectively, after 4.5 h at 42 °C. However, the use of *L. plantarum* 076 alone did not result in a precipitation of milk proteins nor a drop in the culture's final pH (6.43 ± 0.06). Increasing the fermentation time to 6 h also failed to lower the pH value. Although fermentation with *L. plantarum* 076 was not successful, the sample was still evaluated (Fig. 2) for *L. monocytogenes* survival to determine if any pediocin was produced.

Listeria monocytogenes Scott A counts increased greater than 3 Log CFU/mL in skim milk following storage for 6 weeks at 4 °C (Fig. 2). Fermentation with a single LAB culture resulted in slightly increased and comparable ($P > 0.05$) *Listeria* counts during early (1–2 days) and mid (1–2 weeks) storage times. However, the *Listeria* counts were reduced by an average of 4.5 Log CFU in the co-cultured sample after 1–2 weeks of storage. When stored for six weeks, the co-culture continued to suppress *Listeria* growth at the same level ($P > 0.05$) with counts below the detection level (1 Log CFU/mL).

To test if thermophilin 110 was required for the observed anti-listerial activity of the co-culture, we performed another fermentation using a co-culture of *S. thermophilus* ST113, which does not produce a bacteriocin, and *L. plantarum* 076. After six weeks of storage, this culture also showed a 4.2 (±1.1) Log reduction of *Listeria*. This result (not shown for reasons of brevity) suggested that *S. thermophilus* may be conditioning the fermentation substrate to allow for the growth of *L. plantarum* 076 and subsequent production of pediocin, which is primarily responsible for inhibiting *Listeria*. Furthermore, it also showed that a concentrated dose of thermophilin 110 would be required to be used as an anti-listerial ingredient in foods.

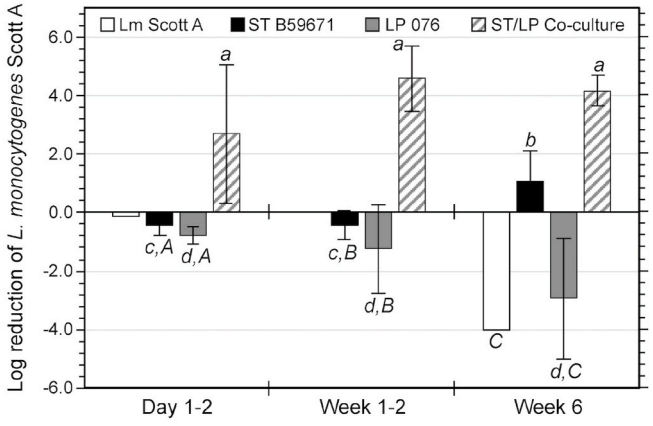


Fig. 2. Anti-listerial activity of skim milk fermented with *S. thermophilus* B59671 (black bars), *L. plantarum* 076 (grey bars), and *S. thermophilus* B59671/*L. plantarum* 076 co-culture (striped bars). Milk was fermented for 4.5 h and then inoculated with *L. monocytogenes* Scott A (1% v/v) and immediately stored at 4 °C. *L. monocytogenes* Scott A survival was measured by colony count at early (1–2 days), mid (1–2 weeks), and late (6 weeks) storage times. *L. monocytogenes* Scott A inoculated in heated skim milk (no cultures) was used as a control (white bars). Data sharing the same letter are not significantly different ($P > 0.05$). Lower case letters represent comparisons among the same sample at varying storage times. Upper case letters compare different samples at the same storage time.

3.2. Anti-listerial activity of whey generated from the co-culture of *S. thermophilus B59671 and L. plantarum 076*

Whey samples from skim, cream-line and raw milk fermented with a co-culture of *S. thermophilus* B59671 and L. *plantarum* 076 were collected and assessed for anti-listerial activity. For this experiment, a six-strain cocktail of *L. monocytogenes* was used to ensure that the bacteriocins were effective against a variety of *L. monocytogenes* strains. Following inoculation with the *Listeria* cocktail, the whey samples were incubated at 37 °C for 24 h to assess the killing of the *L. monocytogenes* strains (Fig. 3a). Results showed that whey recovered from skim milk fermentation displayed weak anti-listerial activity, as the cocktail remained at a cell density of 8.8 Log CFU/mL, slightly lower than ($P < 0.05$) the control culture grown in BHI broth (9.2 Log CFU/mL). This differed substantially from the anti-listerial activity in fermented skim milk described above (Fig. 2), suggesting the bacteriocins may remain trapped within the coagulated milk proteins. As milk processing has been reported to affect bacteriocin activity (Bhatti et al., 2004), we also studied anti-listerial activity in whey generated from pasteurized cream-line milk (non-homogenized) and raw milk. Fig. 3a showed slightly improved ($P < 0.05$) anti-listerial activity in whey from the cream-line milk fermentation compared to skim milk. However, raw milk whey reduced the *Listeria* counts by > 2 Log CFU/mL, the most considerable reduction level among all whey samples. The pH of all whey samples generated using the co-culture of *S. thermophilus* B59671 and *L. plantarum* 076 reached ~4.0, suggesting that anti-listerial activity associated with the raw milk whey was not the result of acid production alone. As discussed above, the highest level of thermophilin 110 activity was achieved in skim milk (320 AU/mL) but not in raw milk (160 AU/mL), indicating the anti-listerial activity was not due to the presence of thermophilin 110 in raw milk whey. It was possible that the pediocin produced by *L. plantarum* 076 during fermentation was transferred to the whey or that the indigenous microflora within raw milk produced an additional anti-listerial compound during fermentation, which remains to be investigated.

To assess the potential of generating anti-listerial compounds using raw milk fermentation, we also tested the anti-listerial activity of two additional raw milk whey samples. These whey samples were collected during the production of raw bovine (RBM) or goat (RGM) milk cheeses using a commercial starter culture containing *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris* (CHOOZIT® MA 11 LYO 50 DCU, Danisco), which was not marketed for its bioprotective properties. These

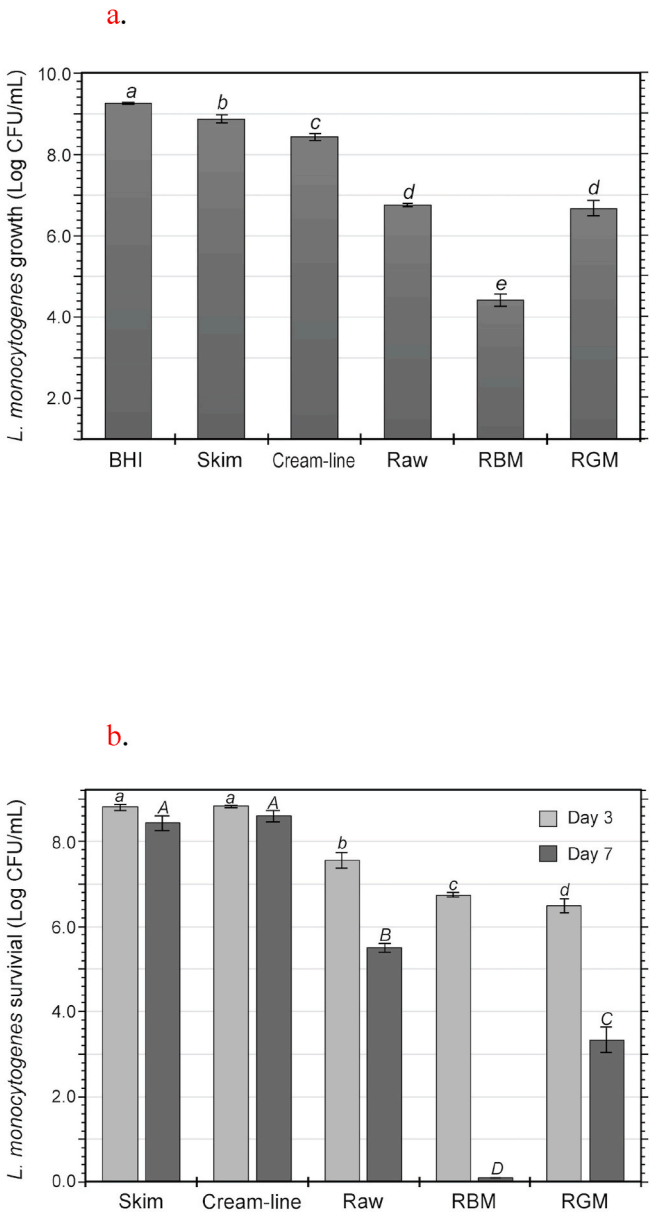


Fig. 3. Effect of whey preparations on the killing of a six-strain *L. monocytogenes* cocktail at (a) 37 °C for 24 h and (b) 4 °C for 3 and 7 days. Bacterial cells grown in BHI were used as a positive control for *Listeria* growth. Samples were obtained by fermenting pasteurized skim milk, cream-line milk, and raw milk using a co-culture of *S. thermophilus* B59671 and *L. plantarum* 076. Raw milk whey recovered from bovine (RBM) and goat (RGM) cheeses were also tested. *L. monocytogenes* cells were resuspended in BHI and the whey samples with growth and survival measured by colony counts (CFU/mL). Data sharing the same letter are not significantly different ($P > 0.05$).

whey samples also reduced the *Listeria* counts significantly, with only 4.8 and 2.5 Log CFU/mL remaining in RBM and RGM, respectively (Fig. 3a). Additionally, Fig. 3a also revealed similar anti-listerial activity ($P > 0.05$) for the whey recovered from RGM cheese and the raw milk fermented with the ST B59671/LP076 co-culture. The pH values for RBM and RGM whey samples were identical to the laboratory-produced whey samples, further evidencing that acid production was not responsible for the observed anti-listerial activity. These anti-listerial activities may likely be caused by the metabolites produced during fermentation by the unique indigenous microflora present in the raw milk samples.

The anti-listerial properties of whey were further assessed on the *L. monocytogenes* cocktail following storage at 4 °C. *Listeria* counts remained between 8.9 and 9.5 Log CFU/mL in all whey samples and the BHI broth control after 24 h of storage (data not shown). However, after 72 h of storage, *Listeria* counts dropped by 1.9–3.0 Log CFU/mL for all three raw milk whey samples (raw, RBM, and RGM), and by 3.92–7.43 Log CFU/mL after one week of storage (Fig. 3b). *Listeria* counts remained above 8.5 Log CFU/mL in whey samples prepared from pasteurized milk, demonstrating that antimicrobial activity was unique to raw milk fermentations.

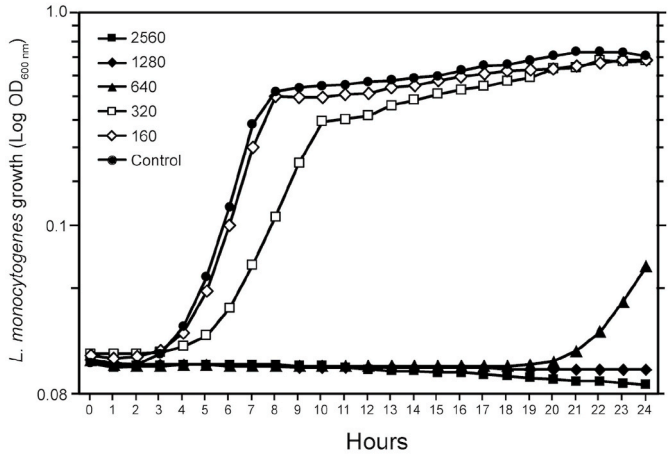
Results from this study suggested that the anti-listerial activities associated with whey recovered from the fermentation of raw milk may be due to the production of novel anti-listerial compounds. However, it is evident that the anti-listerial activity observed in skim milk fermented with the co-culture of *S. thermophilus* B59671 and *L. plantarum* 076 was the result of bacteriocin production, demonstrating the potential for using this co-culture to prevent the growth of *Listeria* (Fig. 2). Pediocin has been investigated as a natural antimicrobial for controlling the growth of foodborne pathogens in foods. ALTA 2341, a pediocin-containing fermentate, is commercially available (Quest International, Sarasota, FL). This fermentate has been reported to inhibit *L. monocytogenes* in meat products when used in combination with other antimicrobial hurdles, *i.e.*, sodium diacetate and thermal processing (Calderon-Miranda, Barbosa-Canovas, & Swanson, 1999; C. M.; Chen, Sebranek, Dickson, & Mendonca, 2004). Previous studies have also shown that *in situ* pediocin production (Loessner, Guenther, Steffan, & Scherer, 2003; Somkuti & Steinberg, 2010) or pediocin-containing fermentates (Huang, Lacroix, Daba, & Simard, 1994; Loessner et al., 2003; Motlagh, Holla, Johnson, Ray, & Field, 1992; Pucci, Vedamuthu, Kunka, & Vandenbergh, 1988; Verma, Sood, Saini, & Saini, 2017) have the potential to protect milk and dairy products from *L. monocytogenes* contamination. In most studies, optimal activity was obtained with a low *Listeria* inoculum (≤2 Log CFU/mL), as pediocin works via a single-hit mechanism and is inactivated after binding to the target cells (Tagg, Dajani, & Wannamaker, 1976). Thus, higher contamination levels can overwhelm the concentration of pediocin present. Also, pediocin resistant strains of *L. monocytogenes* have been reported (Loessner et al., 2003), suggesting a single bacteriocin is insufficient for preventing contamination with foodborne pathogens. This study indicated that *in situ* production of thermophilin 110 in milk is not sufficient to inhibit the growth of *Listeria,* but potentially higher concentrations of the bacteriocin could be used as an additional hurdle with pediocin to prevent the growth of *L. monocytogenes* in food.

3.3. Concentration of thermophilin 110 and pediocin required to inhibit the growth of a six-strain L. monocytogenes cocktail

The effectiveness of each bacteriocin at various concentrations was further investigated against the six-strain cocktail of *L. monocytogenes*. OLE, at a concentration of 0.49 mg/mL, was used as a positive control to inhibit *L. monocytogenes* (Liu, McKeever, & Malik, 2017). The inhibition lasted up to 24 h (data not shown). To determine an effective concentration of thermophilin 110 against *Listeria*, we partially purified the peptide from CFS and standardized a stock solution to 5120 AU/mL. The two highest thermophilin 110 concentrations, 2560 and 1280 AU/mL suppressed *Listeria* growth for 24 h. However, a concentration of ≤320 AU/mL allowed the cultures to reach a similar final density as the control (BHI alone) (Fig. 4a). The lag in listerial growth observed in the presence of 320 AU/mL of thermophilin 110 may explain why an inhibition zone was observed for the undiluted CFS using the well diffusion assay, where target plates were incubated for 6–8 h prior to analysis.

For *L. plantarum* 076, a 2-fold dilution of CFS (1280 AU/mL) collected from an overnight culture in MRS prevented the growth of *Listeria* for 24 h. However, a further serial dilution of the CFS permitted *Listeria* growth after an initial lag (Fig. 4b). Surprisingly, cultures containing 320 AU/mL or 160 AU/mL of pediocin reached a final cell density comparable to that of the control culture. More studies are

a.



b.

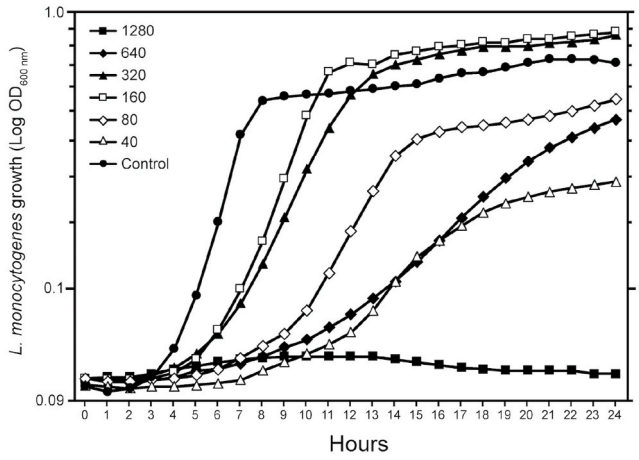


Fig. 4. Anti-listerial activity of (a) partially purified thermophilin 110 and (b) cell-free supernatant from *L. plantarum* 076 containing pediocin. Serial 2-fold dilutions of a thermophilin 110 stock (5120 AU/mL) and *L. plantarum* 076 CFS (2560 AU/mL) were prepared in BHI broth and inoculated with a six-strain cocktail of *L. monocytogenes*. *L. monocytogenes* growth in BHI alone was used as the control. Growth was monitored at OD 600 nm.

needed to explain this observation. It is possible that a component that existed in the CFS stimulated *Listeria* growth as the bacteriocin concentration decreased. Subsequent dilutions of the CFS may have reduced this component allowing for the lower concentrations of pediocin (80 and 40 AU/mL) to impair Listeria growth.

Analysis of *L. monocytogenes* cells by SEM showed cells with an expected morphology of short rods with an intact cell structure, a smooth and compact surface (Fig. 5a). Following the exposure to 2560 AU/mL of thermophilin 110, cells showed significant morphological damage, including the detachment of the cytoplasmic membrane from the cell wall, leakage of intracellular components, and cell collapse (Fig. 5c). This was expected as it was previously reported that thermophilin 110

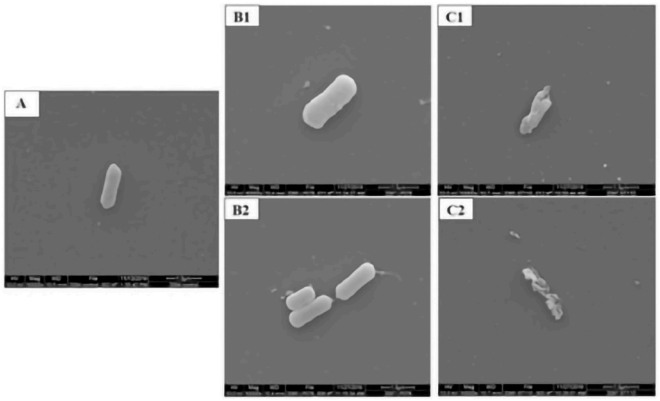


Fig. 5. Morphology of bacterial cells (A) untreated *L. monocytogenes*, (B1 and B2) exposed to LP 076 CFS, and (C1 and C2) partially purified thermophilin 110. Images were captured using SEM at 50,000 X magnification.

activity resulted in pore formation in the target cell membrane resulting in leakage of intracellular material (Gilbreth & Somkuti, 2005). However, following the exposure to a concentration of the *L. plantarum* 076 CFS (1280 AU/mL), which inhibited *Listeria* growth, only minor cell damage was observed (Fig. 5b). Although unexpected, these results agreed with a previous study that reported pediocin produced by *Pediococcus acidilactici* H inhibited the growth of several *L. monocytogenes* strains, yet cell lysis was only observed for some strains (Motlagh et al., 1992).

4. Conclusions

This study showed that thermophilin 110 has the potential to serve as a broad spectrum anti-listerial agent. However, it requires the use of concentrated fermentates for optimal activity. Further studies are needed to assess the potential for using higher concentrations of thermophilin 110 and pediocin as a hurdle technology to prevent the growth of *Listeria* in dairy foods. Additionally, further studies are needed to characterize the anti-listerial activity observed in raw whey samples. This activity could be attributed to the production of an anti-listerial bacteriocin, other than thermophilin 110 or pediocin, by a member of the indigenous microflora. It is also possible that raw milk fermentation released a milk-derived compound (peptide) with broad-spectrum antimicrobial activity. Further studies are needed to characterize the anti-listerial compound in the raw whey samples and assess the potential for using this dairy co-product for food safety applications.

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CRediT authorship contribution statement

Marina Ceruso: Investigation, Writing - original draft, Writing - review & editing, Revised draft. **Yanhong Liu:** Investigation, Writing - original draft, Writing - review & editing, Revised draft. **Nereus W. Gunther:** Conceptualization, Investigation, Writing - original draft, Writing - review & editing, Revised draft. **Tiziana Pepe:** Supervision. **Aniello Anastasio:** Supervision. **Phoebe X. Qi:** Investigation, Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Revised draft. **Peggy M. Tomasula:** Writing - review & editing, Orginal draft, Supervision. **John A. Renye:** Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review &

editing, Revised Draft.

Declaration of competing interest

None.

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