Final Report to American Meat Institute Foundation

Project Title:

Control of *Listeria monocytogenes* in food processing facilities by competitive inhibition microorganisms

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Project Summary

The objective of this project was to develop a method to prevent contamination of *L. monocytogenes* in foods by eliminating/reducing the occurrence of *L. monocytogenes* in the environment of food processing facilities. The project was completed according to the original schedule. One patent application "Control of *Listeria monocytogenes* in food processing facilities by competitive exclusion microorganisms" will be filed. One paper "Control of *Listeria monocytogenes* in food processing plants by administration of probiotic bacteria" is ready for submission for publication and a Masters student's thesis "Competitive inhibition of *Listeria monocytogenes* by lactic acid bacteria isolated from food processing facilities" was completed.

Biofilms from drains in food processing facilities with a recent history of no detectable *Listeria monocytogenes* in floor drains were cultured for microorganisms producing anti-listerial metabolites. A total of 1102 microbial isolates obtained from 12 drain biofilm samples were assayed at 15°C and 37°C for antagonistic activity to *L. monocytogenes* by two agar plate assays. Seventy-six of 689 presumptive lactic acid isolates, twenty-one of 257 general bacterial isolates and 3 of 156 yeast isolates had anti-listerial activity. All 100 isolates, which produced metabolites inhibitory to *L. monocytogenes* were assayed for anti-listerial activity in co-inoculated broth cultures containing tryptic soy broth with yeast extract (TSB-YE). A 5-strain mixture of 10^3 *L. monocytogenes*/ml and 10^5 cfu of the competitive microorganism/ml were combined in TSB-YE and incubated at 37°C for 24 h, 15°C for 14 d, 8°C for 21 d and 4°C for 28 d. Substantial inhibition of *L. monocytogenes* growth (4 to 5 log cfu/ml) was observed for 9 isolates at 37°C, 2 at 15°C and 8°C, and 3 at 4°C. None of the 3 yeast isolates was even moderately inhibitory to *L. monocytogenes* ($\leq$ 1 $\log_{10}$ cfu/ml growth inhibition) at 37°C. The nine inhibitory bacterial isolates were identified as *Enterococcus durans* (6 isolates), *Lactococcus lactis* subsp. *lactis* (2 isolates), and *Lactobacillus plantarum* (1 isolate). The anti-*L. monocytogenes* properties of these isolates were determined in biofilms of *L. monocytogenes* on stainless steel coupons at 37°, 15°, 8° and 4°C. Results revealed that all of nine isolates were highly inhibitory (more than 5 $\log_{10}$ *L. monocytogenes* cfu/cm^2 growth inhibition to a undetectable level as determined by a direct plating method) at 37°C for 24 h when compared with a control biofilm of *L. monocytogenes* only. At 4° and 8ºC, three isolates (*E. durans* 141-1 and 152; *Lc. lactis subsp. lactis* C-1-92) were highly inhibitory to *L. monocytogenes* through 35 days (>4 $\log_{10}$ *L. monocytogenes*/cm^2 growth inhibition). These three bacterial isolates appear to be excellent competitive inhibition candidates to control *L. monocytogenes* in biofilms at environmental temperatures of 4°, 8º and 37°C.

A manuscript "Control of *Listeria monocytogenes* in a biofilm by competitive inhibition bacteria," which provides details of the studies, is attached.

Control of *Listeria monocytogenes* in a biofilm by competitive inhibition bacteria

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Running head: Competitive inhibition of *L. monocytogenes*

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Abstract

Biofilms from floor drains in food processing facilities with a recent history of no detectable *Listeria monocytogenes* were cultured for microorganisms producing anti-listerial metabolites. A total of 413 microbial isolates obtained from 12 drain biofilm samples were assayed at 15°C and 37°C for antagonistic activity to *L. monocytogenes* by two agar plate detection methods. Twenty-one of 257 bacterial isolates and 3 of 156 yeast isolates had anti-listerial activity. All 24 microbial isolates, which produced metabolites inhibitory to *L. monocytogenes,* were assayed for anti-listerial activity in co-inoculated broth cultures containing tryptic soy broth with 0.6% yeast extract (TSB-YE). A 5-strain mixture of 10^3 *L. monocytogenes*/ml and 10^5 cfu of the candidate microorganism/ml were combined in TSB-YE and incubated at 37°C for 24 h, 15°C for 14 d, 8°C for 21 d and 4°C for 28 d. Substantial inhibition of *L. monocytogenes* growth (4 to 5 log cfu/ml) was observed for 9 isolates at 37°C, 2 at 15°C and 8°C, and 3 at 4°C. None of the 3 yeast isolates was even moderately inhibitory to *L. monocytogenes* (≤ 1 $\log_{10}$ cfu/ml growth inhibition) at 37°C. The nine inhibitory bacterial isolates were identified as *Enterococcus durans* (6 isolates), *Lactococcus lactis* subsp. *lactis* (2 isolates), and *Lactobacillus plantarum* (1 isolate). The anti-*L. monocytogenes* properties of these isolates were evaluated in biofilms of *L. monocytogenes* on stainless steel coupons at 37°, 15°, 8° and 4°C. Results revealed that all of nine isolates were highly inhibitory (>5 $\log_{10}$ *L. monocytogenes* cfu/cm^2 growth inhibition to a undetectable level as determined by a direct plating method) at 37°C for 24 h when compared with a control biofilm of *L. monocytogenes* only. At 4° and 8ºC, three isolates, *E. durans* 141-1 and 152 and *Lc. lactis subsp. lactis* C-1-92, were highly inhibitory to *L. monocytogenes*

through 35 days (>4 $\log_{10}$ *L. monocytogenes*/cm^2 growth inhibition). These three bacterial isolates appear to be especially promising competitive inhibition candidates to control *L. monocytogenes* in biofilms at environmental temperatures of 4°, 8º and 37°C.

Investigations of several outbreaks of listeriosis have revealed that contamination during or after processing was the primary source of *L. monocytogenes* in many commercially prepared ready-to-eat (RTE) foods (4, 8, 11, 13). Studies indicate that certain strains of *L. monocytogenes* can become well-established in a food processing facility and remain members of the resident microbial flora for months or years (19). Although major improvements in plant layout and processing equipment design and in procedures for cleaning and sanitizing have been made, environmental testing results indicate that *L. monocytogenes* often continues to persist in or be introduced into the environment in which RTE foods are exposed for further processing and packaging (19)

The objectives of this study was to isolate from biofilms of floor drains of food processing facilities microorganisms that produce metabolites inhibitory to *L. monocytogenes* and to determine their ability to inactivate or suppress growth of *L. monocytogenes* in a biofilm.

Materials and Methods

***Bacterial strains*:** A five-strain mixture of *L. monocytogenes*, including LM101 (serotype 4, salami isolate), LM112 (serotype 4, salami isolate), LM113 (serotype 4, pepperoni isolate), H9666 (serotype 1/2c, human isolate) and ATCC 5779 (serotype 1/2c, cheese isolate) from the UGA Center for Food Safety were used. Each strain was individually grown in tryptic soy broth with 0.6% yeast extract (TSBYE; Becton Dickinson, Sparks, MD) at 37°C for 16 h. The cultures were sedimented by centrifugation at 8,000 x g for 20 min and resuspended in 0.1% peptone. The optical density of each strain was adjusted in a spectrophotometer with 0.1% peptone to an OD reading of 0.5 (ca. 10^8 cfu/ml) at 630

nm. Approximately the same cell number of each strain was combined to prepare a 5-strain mixture before each experiment.

Isolation and screening of microorganisms for metabolites antagonistic to L. monocytogenes Biofilm samples collected from floor drains at different food processing plants having a recent history of no detectable *L. monocytogenes* in floor drains were used to obtain isolates of bacteria and yeasts. Two methods, which included a direct plating and an enrichment culture procedure, were used to isolate these microorganisms. Tryptic soy broth (TSB, 10 ml) was added to each biofilm sample (ca. 1 g) and biofilm preparations were serially diluted (1:10) in 0.1% peptone to 10^{-3}. A volume of 0.1 ml of each dilution was plated on dichloran rose bengal chloramphenicol agar (DRBC) and tryptic soy agar (TSA) plates in duplicate, with DRBC plates incubated at 30°C for 72 h and TSA plates incubated at 37°C for 24 h. Biofilm preparation (1 ml) also was added to 9 ml of TSB and incubated at 37°C for 24 h. Enrichment cultures were en-fold serially diluted in 0.1% peptone and 0.1-ml portions from dilutions of 10^{-5} to 10^{-8} were plated onto TSA and DRBC plates, and incubated according to the conditions described above. Ten colonies per biofilm specimen were selected randomly from plates and streaked for isolation.

Two methods, including a spot on lawn assay and a double layer assay, and two temperatures (37°C and 15°C) were used to screen isolates for anti-listerial activity. For the spot on lawn assay, 0.1 ml of ca 10^7 cells of the 5-strain mixture of *L. monocytogenes* was plated onto each of duplicate TSA plates. Candidate competitive inhibition isolates were grown individually in TSB at 37°C for 24 h, cells were sedimented by centrifugation (4,000 x g for 20 min), and the supernatant fluid of each culture was filter-

sterilized (0.22-µm-pore-size cellulose acetate membrane; Nalgene Co., Rochester, N.Y.). A 12-mm disc (Dispens-O-Disc; Difco Laboratories, Detroit, MI.) was placed onto the surface of each TSA plate, and 0.1 ml of filter-sterilized supernatant fluid from a single culture was applied to the surface of the disc. The plates were incubated at 37°C for 24 h and observed for zones of inhibition. In addition, a disc with nisin (3.125 µg, Sigma, St. Louis, MO) was used as the positive control and a disc with 0.1 M phosphate-buffered saline (PBS), pH 7.2, was used as the negative control.

The double layer assay, also a two-step procedure, involved first growing a spot-inoculated candidate competitive inhibition isolate on TSA and then applying a second layer of growth medium containing the 5-strain mixture of *L. monocytogenes*. Specifically, an individual colony of competitive bacteria was inoculated in the center of each of two TSA plates and incubated for 24 h at 37°C. A 5-strain mixture of *L. monocytogenes* was added at 10^6 cfu/ml to brain heart infusion with 0.4% agar (BHIA, Difco) at 50°C and mixed for 1 min at 200 rpm with a magnetic stir bar. The mixture (8 ml) was poured onto each TSA plate as a second layer and allowed to cool to room temperature. The cultures were incubated for 24 h at 37°C and observed for zones of inhibition. Nisin-producing *Lc. Lactis* subsp. *lactis* ATCC 11454 was used as the positive control and a yeast isolate, which was isolated from this study and confirmed to have no inhibitory effect on the growth of *L. monocytogenes*, was used as the negative control.

Competitive inhibition in broth cultures at different temperatures All isolates with anti-*L. monocytogenes* activity were further tested in TSBYE for competitive growth at 4°, 8º, 15º, or 37ºC. A culture (0.1 ml) of ca 10^7 to 10^9 cfu (range $10^{6.7}$ to $10^{4.0}$ cfu) of

competitive microorganism having anti-*L. monocytogenes* activity and 0.1 ml of ca. 10^5 to 10^6 cfu (range $10^{5.2}$ to $10^{6.6}$) of the 5-strain mixture of *L. monocytogenes* were added to 10 ml of TSBYE and incubated at 4º, 8º, 15º,or 37ºC. Cultures (1 ml) were sampled at 0, 8 and 24 h for incubation at 37°C, at 0, 1, 2, 3, 7, 10 and 14 days for 15°C, at 0, 1, 7, 14 and 21 days for 8°C, and at 0, 2, 7, 14 21 and 28 days for 4°C incubation, and enumerated for *L. monocytogenes* on modified Oxford agar (MOX, Difco) at 37°C for 48 h. An equivalent inoculum of the 5-strain mixture of *L. monocytogenes* or the competitive microorganism/bacterium was added to each of duplicate tubes and incubated under the same conditions to serve as *L. monocytogenes*-only and competitive microorganism/bacteria-only controls, respectively. *L. monocytogenes* counts were determined on MOX at 37º for 48 h and competitive microorganism/bacteria counts in TSA at 37°C for 48 h.

Identification of competitive microorganisms Bacterial isolates having antagonistic activity to *L. monocytogenes* at all four temperatures were characterized by Gram stain, biochemical assays (API CHB and API CHL kits; bioMérieux Industry, I'Etoile, France), and 16S rRNA gene alignment profile analysis (Midi Labs, Newark, DE) for identification of genus and species.

Preparation of stainless steel coupons Stainless steel (T-304, Tull Metals Company, Atlanta, GA) coupons (4 cm x 2.5 cm) were washed by a 10-min immersion with agitation (150 rpm) in 1000 ml of an aqueous 2% RBS 35 Detergent solution (20 ml of RBS 35 Concentrate per liter of tap water at 50°C; Pierce, Rockford, IL), and rinsed by immersion in 1000 ml of tap water (initially at 50°C) with agitation (150 rpm) for 25 min. Five additional 1-min immersions with agitation (150 rpm) in 1000 ml of distilled water

at ambient temperature were performed. The coupons were dried and a hydrophobic marker was used to encircle an area of 1.13 cm in diameter. The coupons were then individually wrapped in aluminum foil and autoclaved at 121°C for 30 min.

Competitive inhibition of L. monocytogenes in a biofilm Biofilms were grown using a modification of the protocols described by Leriche and Carpentier (10) and Chae and Schraft (5). An inoculum of 0.1 ml of ca. $10^{6.0}$–$10^{8.4}$ cfu of candidate competitive microorganisms and 0.1 ml of ca. $10^{2.6}$–$10^{4.6}$ cfu of the 5-strain mixture of *L. monocytogenes* were deposited within the marked area of each stainless steel coupon, and then placed in a humidity-controlled incubator (approx. 95% relative humidity) at 4º, 8º, 15º, or 37°C for 6 h. Non-adherent bacteria were removed by vacuum aspiration after 6 h of incubation and replaced with 0.1 ml of fresh TSB. The stainless steel coupons were reincubated at the same temperature and the media were replaced every 7, 3, 3 and 1 days for incubation at 4º, 8º, 15º, and 37°C, respectively. At each sampling time, selected coupons in duplicate were transferred to a laminar flow hood in which weakly adherent bacteria were removed by washing the marked area of each coupon 3 times with PBS, then removing the remaining liquid from the marked area by vacuum aspiration. Each coupon was placed in a 50-ml centrifuge tube containing 9.9 ml of PBS and ca. 30 glass beads (5 mm, Fisher Scientific, Norcross, GA) and agitated by a Vortex mixer (Fisher Scientific) for 2 min to disrupt bacteria in the adherent biofilm. The suspended bacteria were serially diluted (1:10) in 0.1% peptone and plated in duplicate on TSA for enumerating competitive microorganisms or total bacteria (if *L. monocytogenes* counts on MOX were greater than or equal to the bacterial counts on TSA), and on MOX for enumeration of *L. monocytogenes*. The plates were incubated for 48 h at 37°C and

competitive microorganism and *L. monocytogenes* counts were determined. Coupons inoculated with only $10^{2.6}$-$10^{4.6}$ *L. monocytogenes* served as positive controls, whereas coupons inoculated only with $10^{6.0}$-$10^{8.4}$ competitive microorganisms served as negative controls. Results reported were the average of duplicate determinations.

Identification of nisA and nisB competitive microorganisms A polymerase chain reaction (PCR) method was used to determine if the competitive microorganisms encoded *nisA* and *nisB*. Bacterial DNA was extracted using a microbial genomic DNA isolation kit according to the protocol described by the manufacturer (Mo Bio Laboratories, Solana Beach, CA). The oligonucleotide sequences of the primers used were *nisA* 5-CGGCTCTGATTAAATTCTGAAG and 5-CGGTTGAGCTTTAAATGAAC); and for *nisB* were 5-AGAGAAGTTATTTACGATCAAC and 5-ATCTGACAACAAATCTTTTTGT (15). PCR was performed with an Icycler 96 Well Reaction Module (Bio-Rad Laboratories, Hercules, CA) according to the procedure described by Olasupo et al. (15).

Results

A total of 12 biofilms from floor drains of four different food processing facilities were screened for microorganisms inhibitory to *L. monocytogenes*. A total of 156 yeast and 257 bacterial isolates were obtained from the biofilms and assayed for antagonistic activity against *L. monocytogenes*. Twenty-four isolates, including 3 yeasts and 21 bacteria, were inhibitory to *L. monocytogenes* (0.5- to 3.5-mm zones of inhibition), with no bacteria and 3 yeasts identified by the spot on lawn assay, and 21 bacteria and no yeasts identified by the double layer assay.

All isolates antagonistic to *L. monocytogenes* were evaluated individually for their ability to inhibit growth or inactivate a 5-strain mixture of *L. monocytogenes* in TSB at 37°C. Under these conditions, two yeast isolates were weakly antagonistic to *L. monocytogenes*, repressing growth of listeriae by 0.7 $\log_{10}$ cfu/ml compared to the positive control of *L. monocytogenes* only. In contrast, nine of the bacterial isolates were strongly antagonistic to *L. monocytogenes*, with each providing a greater than 5 $\log_{10}$ cfu/ml differential within 24 h when compared with the positive control (Table 1).

Twelve isolates were assayed under the same conditions, but at 15°C. Three of the isolates were highly antagonistic to *L. monocytogenes*, with greater than a 4 $\log_{10}$ *L. monocytogenes*/ml differential at day 7 compared to the positive control, and one isolate, no. C-1-92, was exceptionally bactericidal, with no detectable *L. monocytogenes* present (>8 $\log_{10}$ *L. monocytogenes*/ml differential compared with positive control) at 7 and 14 days (Table 2).

These same twelve isolates were assayed for antagonistic activity to *L. monocytogenes* in TSB at 8°C. Six of isolates were highly inhibitory, with greater than a 4 $\log_{10}$ *L. monocytogenes*/ml differential at 14 days compared to the positive control, and one isolate, no. 152, was exceptionally antimicrobial, with a 6.3 $\log_{10}$ *L. monocytogenes*/ml differential at 21 days (Table 3).

Nine isolates with antagonistic activity to *L. monocytogenes* at all three temperatures were assayed for their activity against *L. monocytogenes* at 4°C. Three isolates were highly antagonistic, with greater than a 4 $\log_{10}$ *L. monocytogenes*/ml differential at 28 days compared to the positive control, and one isolate, no. 152, was

exceptionally antimicrobial, with a 6 $\log_{10}$ *L monocytogenes*/ml differential at 28 days (Table 4).

Identification of the nine most antagonistic cultures revealed that six (isolate nos. 141-1, 141-2, 143-2, 152, 375-1 and 375-2) were *Enterococcus durans* and 16S rRNA analysis indicated all were indistinguishable; two (isolate nos. C-1-92 and C-1-152) were *Lactococcus lactis* subsp. *lactis*; and one (isolate no. 143-1) was *Lactobacillus plantarum*. *Lc. lactis* subsp. *lactis* C-1-92 encoded both *nisA* and *nisB,* but none of the other competitive microorganisms evaluated encoded either *nisA* or *nisB* (Fig. 1).

The nine antagonistic bacterial isolates and two yeast isolates were evaluated at 37ºC at two different cell number combinations (highest level at 6.9 (for yeasts) or 8.3-8.4 (for baceria) $\log_{10}$ competitive microorganisms/cm^2 and 4.6 $\log_{10}$ *L. monocytogenes*/cm^2; and lower level at 6.4-6.5 $\log_{10}$ competitive microorganisms/cm^2 and 2.9 $\log_{10}$ *L. monocytogenes*/cm^2) for their ability to control *L. monocytogenes* in biofilms on stainless steel coupons. Results of studies with the highest combination of microbial populations revealed a more than 6 $\log_{10}$ *L. monocytogenes*/cm^2 (to an undetectable level by a direct plating method, <1.7 $\log_{10}$ cfu/cm^2) differential compared to the positive control for eight isolates at 37°C for 24 h and a 3 to 5 $\log_{10}$ *L. monocytogenes*/cm^2 differential for one isolate (Table 5). There was only a 0.2 to 0.9 $\log_{10}$ *L. monocytogenes*/cm^2 differential for the two yeast isolates (Table 5). Studies with a lower combination of microbial populations resulted in all nine competitive bacterial isolates providing a greater than 6 $\log_{10}$ *L. monocytogenes*/cm^2 (to an undetectable level by a direct plating method) differential compared to the positive control (Table 6).

Six competitive bacterial isolates were evaluated under similar conditions (initial cell populations of 3.7 $\log_{10}$ *L. monocytogenes*/cm^2 and 6.3-6.5 $\log_{10}$ competitive microorganisms) at 15°C, of which two isolates (*Lc. lactis* subsp. *lactis* C-1-92 and C-1-152) controlled *L. monocytogenes* to an undetectable level (>7.8 $\log_{10}$ *L. monocytogenes*/cm^2 differential) through 28 days which was the end of the study (Table 7). The same six competitive isolates were assayed in biofilms at 8ºC for their antagonistic activity to *L. monocytogenes.* *E. durans* 141-1 and *Lc. lactis* subsp. *lactis* C-1-92, were highly inhibitory to *L. monocytogenes*, with no listeriae detected (>6.8 $\log_{10}$ cfu/cm^2 differential) at 21 and 28 days (Tables 8 and 9).

Five and six competitive bacterial isolates (at initial populations of 6.3-6.6 $\log_{10}$ cfu/cm^2) were evaluated in combination with two initial populations of *L. monocytogenes*, i.e., 2.6 and 4.3 $\log_{10}$ *L. monocytogenes* /cm^2 on stainless coupons held at 4°C. One competitive isolate, *Lc. lactis* subsp. *lactis* C-1-92, was especially effective in controlling *L. monocytogenes*, with no detectable *L. monocytogenes* (differentials of >5.1 and >7.0 $\log_{10}$ *L. monocytogenes*/cm^2 compared to the control) at 35 days when either initial population of *L. monocytogenes* was used (Tables 10 and 11). Interestingly, *Lc. lactis* subsp. *lactis* C-1-92 did not grow but rather declined in cell numbers (3.6-3.8 $\log_{10}$ cfu/cm^2 reduction) during 35 days at 4°C, whereas cell populations of all five other competitive bacteria increased by 1 to 2 $\log_{10}$ cfu/cm^2 under the same conditions. The other five competitive bacteria also were inhibitory to *L. monocytogenes* through 35 days at 4°C, with differentials of *L. monocytogenes* cell populations in biofilms compared to positive controls ranging from 2.0 to >7.0 $\log_{10}$ *L. monocytogenes*/cm^2.

Discussion

Controlling the widely occurring psychrotrophic *L. monocytogenes* in food processing facilities has been a formidable challenge for the entire food industry, from the smallest to the largest food processor (19). Besides the pathogen's widespread occurrence in nature, it is nonfastidious, grows at refrigeration temperature, forms protective biofilms, and thrives in moist environments (14, 17). Floor drains in food processing facilities are a particularly important niche for persistence of *L monocytogenes* and are a point of contamination for the processing plant environment and food products (19, 20).

Decontaminating floor drains of listerae is especially challenging because, when entrapped in a biofilm, *L. monocytogenes* is afforded unusual protection against disinfectants and treatments available to control pathogens on environmental surfaces (19). *L. monocytogenes* can attach through biofilms to various types of surfaces including stainless steel, glass, and rubber. Biofilms, which entrap and protect *L. monocytogenes* from disinfectants, have been documented in meat and dairy processing plant environments (6, 7, 14). It is well documented that strains of *L. monocytogenes* can become well established in a food processing environment and remain members of the resident microbial flora for many years (19). Our goal was to isolate and characterize microorganisms that would thrive in combination with *L. monocytogenes* within its biofilm at a wide range of temperatures that occur in food processing facilities (especially under refrigeration conditions) and would compete to control listerial growth and possibly eliminate the pathogen.

Using biofilms from floor drains on food processing facilities having a history of no *L. monocytogenes* contamination as the source of candidate competitive microorganisms, 413 microbial isolates were obtained for evaluation. Initial screening identified 24 promising candidates with anti-listerial activity. Further competitive testing between the candidate microorganisms and *L. monocytogenes* in broth and in biofilms at different temperatures identified nine bacterial isolates that effectively reduced, controlled, or eliminated detectable *L. monocytogenes* depending on environmental conditions. One strain in particular, *Lc. lactis* subsp. *lactis* C-1-92, was especially effective in controlling *L. monocytogenes* when in biofilms for extended periods of time, including at 4ºC. This strain uniquely produced nisin A and nisin B, which are inhibitory to *L. monocytogenes* (15). Interestingly, this isolate did not grow at 4ºC but apparently produced anti-listerial metabolites at this temperature to keep *L. monocytogenes* populations in biofilms below the detectable limit. Similarly, Amézquita and Brashears (1) observed that some lactic acid bacteria can competitively inhibit *L. monocytogenes* in ready-to-eat meats at refrigeration temperature even though the competitive bacteria did not grow.

To other isolates, *Enterococcus durans* 141-1 and 152, also were very effective in controlling *L. monocytogenes* in biofilms. Enterococci are sometimes used as starter cultures for meat fermentations when acid production is of primary important (12). Metabolites, including enterocins, that are bactericidal to *L. monocytogenes* are produced by *Enterococcus spp*. (2, 3, 9, 18). The isolates (*E. durans* 141-1 and 152) we obtained can grow at refrigeration temperature, and also have antagonistic activity to *L. monocytogenes* under refrigeration conditions. These strains would be especially useful

in food processing locations that require a low temperature environment such as for processing ready-to-eat foods.

Lactic acid bacteria and their metabolites have been well documented for their antimicrobial activity against *L. monocytogenes* (1, 15, 16). However, their application in the food industry to control *L. monocytogenes* has been limited because of their cost, variable effect, restrictive growth requirement and potential for spoilage of foods. The lactic acid bacteria we isolated from biofilms formed in floor drains, in order to persist, would have had to adapt to the environmental conditions that occur in food processing facilities. Hence, they can grow or compete at a wide range of temperatures, including refrigeration temperature and also form a biofilm to enable attachment to equipment and drain surfaces. We obtained several lactic acid bacteria isolates, but two in particular, that were highly effective in controlling, and perhaps eliminating, *L. monocytogenes* in biofilm at different temperatures. Additional studies are needed to determine the efficiency of these strains in controlling listeriae when different cell numbers (including exceptionally high numbers, e.g., 10^8 cfu/cm^2) of *L. monocytogenes* are present in biofilms, the effect of different nutrients and environmental conditions on listeriae inactivation, and the anti-listerial activity of the different competitive bacteria when used in combination, particularly in the food processing plant environment. However, our initial studies are very encouraging and indicate that several of the selected lactic acid bacteria we have isolated and characterized are promising candidates for controlling *L. monocytogenes* in biofilms in food processing facilities.

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FIG. 1. PCR amplication of *nisA* (lane 1 to 7) and *nisB* (lane 8 to 15) of the isolates from floor drains, Lanes: 1. negative control (water), 2. DNA ladder (100 bp), 3. positive control (*Lc. Lactis* ATCC11454), 4. *Lc.Lactis* (C-1-92), 5. *Lc. Lactis* (C-1-152), 6. *Lacto. Plantarum* (143), 7. negative control (water); 8. DNA ladder (100 bp), 9. positive control (*Lc. Lactis* ATCC11454), 10. *Lc. Lactis* (C-1-92), 11. *Lc. Lactis* (C-1-152), 12. *Lacto. Plantarum* (143), and 13-15. negative control (water)

Table 1. Inhibition at 37°C of *L. monocytogenes* (LM) by competitive microorganisms (CM) in tryptic soy broth

Isolate No.	*L. monocytogenes* or competitive microorganism count (log_{10} cfu/ml)								
	0 time			8 h			24 h		
	LM only[a]	LM +CM[b]	CM only[c]	LM only	LM +CM	CM only	LM only	LM +CM	CM only
Bacteria									
C-2-101	3.0	3.0	5.7	6.7	6.4	7.4	9.4	9.0	8.4
C-1-152	3.0	2.8	6.3	6.4	5.3	9.1	9.8	7.8	9.2
C-2-188	3.5	2.7	6.5	6.4	6.7	6.5	9.1	9.3	7.7
C-1-92	3.3	3.2	6.0	6.3	<0.7[d]	9.5	9.3	0.7	9.3
143	3.2	2.9	6.1	6.5	1.7	9.1	8.8	<0.7	9.3
375-1	3.0	2.9	5.8	6.7	2.2	9.4	9.5	2.8	9.4
129	2.8	3.0	6.4	6.6	5.2	9.2	9.4	8.5	9.4
123	3.5	3.3	6.3	6.7	6.5	8.2	9.2	9.0	9.6
B-2-18	3.3	3.2	5.7	6.7	5.8	9.1	9.4	7.6	9.6
152	3.6	3.4	6.2	6.7	2.4	9.1	8.7	3.3	9.5
141	3.5	3.4	6.0	7.9	3.5	8.8	9.5	3.1	9.1
123	3.2	3.2	6.0	6.4	6.6	8.5	9.0	9.2	9.7
147	3.2	3.2	6.1	7.6	2.7	9.4	9.4	3.6	9.3
375-3	2.9	2.8	5.8	5.7	5.9	7.7	9.3	6.9	9.4
107 (tiny)	3.0	3.1	6.1	6.7	6.1	8.6	9.6	6.7	9.0
123	3.2	3.2	6.3	6.7	6.2	8.2	9.6	8.3	9.6
375-2	3.3	3.1	6.3	6.4	2.0	9.4	9.4	3.1	9.3
107	3.3	3.2	6.0	7.2	6.4	7.8	9.4	7.7	9.2
141 (2)	3.1	3.0	6.3	7.4	3.1	9.1	9.6	3.8	9.6
143 (2)	3.2	3.2	7.0	7.5	3.4	9.3	9.3	4.0	9.3
107 (2)	3.2	3.2	6.0	7.3	6.8	8.2	9.0	6.7	9.4
Yeasts									
C-2-45	2.6	2.6	4.5	6.7	5.5	6.6	9.6	9.6	7.6
C-2-187	2.7	2.7	4.4	5.7	5.3	6.9	9.9	9.2	7.8
C-3-53	2.9	2.7	5.6	7.0	5.6	7.2	10.0	9.3	7.8

[a] LM only=*L. monocytogenes* count
[b] LM+CM=*L. monocytogenes* count
[c] CM only=Competitive microorganisms count
[d] Minimum detection limit 0.7 log_{10} cfu/ml

Table 2. Inhibition at 15°C of *L. monocytogenes* (LM) by competitive bacteria (CB) in tryptic soy broth

Isolate No.	*L. monocytogenes* or competitive bacteria count (log_{10} cfu/ml)													
	0 time			1 day			2 days			7 days			14 days		
	LM Only[a]	LM +CB[b]	CB only[c]	LM only	LM +CB	CB only	LM only	LM +CB	CB only	LM only	LM +CB	CB only	LM only	LM +CB	CB only
141	3.4	3.4	6.2	6.8	5.4	8.8	9.8	2.9	9.6	9.7	8.4	9.6	9.0	9.1	8.4
141-2	3.4	3.4	6.2	7.3	2.7	9.6	9.7	2.8	9.6	9.8	7.2	9.8	9.0	7.6	9.8
B-2-18	3.5	3.3	6.7	7.2	6.3	9.6	9.8	7.2	9.9	9.4	8.0	10.2	9.6	8.0	10.2
152	3.4	3.4	6.2	7.2	6.0	9.1	9.8	4.1	9.4	9.7	7.3	9.4	9.1	8.6	9.4
143-2	3.5	3.4	5.5	7.4	7.2	8.8	9.8	4.7	9.5	9.6	8.5	9.7	8.8	8.1	9.6
147	3.5	3.4	6.3	7.5	2.6	9.4	9.9	2.4	9.6	9.7	7.4	9.7	9.3	8.5	9.7
375-2	3.6	3.3	6.4	7.8	2.3	9.5	9.9	3.0	9.8	9.9	6.6	9.5	8.7	7.7	9.9
129	3.5	3.2	5.5	6.6	5.5	9.8	10.3	5.1	9.8	9.6	6.9	9.3	9.0	8.6	6.9
C-1-92	3.4	3.3	6.4	8.0	1.7	9.9	10.2	1.0	9.8	9.2	<0.7[d]	9.2	9.0	<0.7	7.8
375-1	3.4	3.4	6.3	6.5	6.2	9.1	9.7	3.0	9.7	9.3	3.8	9.4	9.1	7.2	9.8
C-1-152	3.5	3.4	6.3	7.9	7.1	9.4	9.9	8.0	9.5	9.7	8.2	9.3	9.0	9.5	9.4
143	3.6	3.4	6.4	7.2	7.0	8.0	9.7	3.5	9.5	8.3	4.1	9.3	8.5	7.6	9.5

[a] LM only=*L. monocytogenes* count
[b] LM+CB=*L. monocytogenes* count
[c] CB only=Competitive bacteria count
[d] Minimum detection limit 0.7 log_{10} cfu/ml

Table 3. Inhibition at 8°C of *L. monocytogenes* (LM) by competitive microorganisms (CM) in tryptic soy broth

Isolate No.	*L. monocytogenes* or competitive microorganism count (log_{10} cfu/ml)														
	0 time			1 day			7 days			14 days			21 days		
	LM only[a]	LM +CM[b]	CM only[c]	LM only	LM +CM	CM only	LM only	LM +CM	CM only	LM only	LM +CM	CM only	LM only	LM +CM	CM only
Bacteria															
141	3.3	3.3	6.1	4.0	4.0	6.4	8.9	4.4	9.3	9.8	5.2	9.5	9.8	6.4	9.4
141-2	3.4	3.3	6.3	3.7	3.8	6.3	8.5	5.0	9.2	9.7	5.3	9.4	9.8	7.6	9.5
B-2-18	3.3	3.5	6.7	4.2	4.2	7.0	8.9	7.4	9.1	9.8	9.0	9.0	9.3	8.7	9.4
152	3.3	3.2	6.2	4.2	4.0	6.6	9.0	2.7	9.1	9.9	2.9	9.3	9.8	3.5	9.4
143-2	3.2	3.1	6.4	3.7	3.5	6.4	8.0	6.0	8.3	9.4	6.4	8.9	9.5	8.0	9.1
147	3.5	3.5	6.4	3.7	3.8	6.2	8.5	7.3	9.2	9.4	9.2	9.4	9.5	8.9	9.5
375-2	3.3	3.2	6.3	4.2	3.5	6.4	9.3	3.2	9.1	9.8	5.5	9.2	9.8	7.5	9.5
129	3.3	3.1	5.2	3.5	3.8	6.3	9.3	6.9	8.9	9.8	6.9	9.2	9.8	7.5	9.2
C-1-92	3.5	3.4	6.3	3.7	3.5	6.0	9.1	5.7	8.3	9.8	5.3	8.8	9.9	5.1	8.8
375-1	3.5	3.5	6.3	3.9	3.9	6.4	9.5	4.6	9.1	9.7	4.7	9.1	9.8	6.5	9.3
C-1-152	3.2	3.3	6.4	4.0	4.0	6.4	8.9	5.6	9.4	9.7	6.7	9.3	9.8	7.9	9.4
143	3.2	2.6	6.1	3.7	3.5	7.0	9.5	5.2	8.4	9.8	6.9	9.2	9.7	7.9	9.4
Yeasts															
C-2-45	2.3	2.5	5.7	3.2	3.1	5.4	8.1	8.1	7.7	9.6	9.1	7.8	9.8	9.4	7.9
C-3-53	2.5	2.7	5.8	3.4	3.2	5.6	8.1	8.2	8.0	9.9	7.8	6.3	9.8	9.2	8.1
C-2-187	2.2	2.3	4.7	2.7	2.8	4.4	8.5	7.3	5.1	9.7	9.3	7.7	9.8	9.7	6.5

[a] LM only=*L. monocytogenes* count
[b] LM+CM=*L. monocytogenes* count
[c] CM only=Competitive microorganisms count

Table 4. Inhibition at 4°C of *L. monocytogenes* (LM) by competitive bacteria (CB) in tryptic soy broth

Isolate No.	*L. monocytogenes* or competitive bacteria count (log_{10} cfu/ml)														
	0 time			7 days			14 days			21 days			28 days		
	LM only[a]	LM +CB[b]	CB only[c]	LM only	LM +CB	CB only	LM only	LM +CB	CB only	LM only	LM +CB	CB only	LM only	LM +CB	CB only
141-1	2.7	2.6	5.0	4.2	4.1	5.6	6.0	6.5	6.5	8.6	6.6	8.2	9.5	6.3	8.7
152	3.4	3.4	6.4	4.1	3.7	6.6	7.0	4.3	7.5	8.5	4.5	8.7	9.9	3.9	9.2
C-1-92	3.5	3.0	6.5	4.1	3.7	5.9	6.9	5.8	5.7	8.2	6.7	5.0	9.9	8.0	5.3
143-1	2.9	2.5	5.0	3.3	3.3	5.2	6.7	5.9	5.2	8.0	7.5	6.4	9.5	8.3	6.9
C-1-152	2.7	2.9	5.9	3.7	3.5	5.6	6.5	6.1	6.6	8.3	7.1	7.3	9.5	7.4	8.4
375-1	2.8	2.4	5.2	4.2	3.8	5.4	7.0	6.4	6.7	8.4	7.2	8.0	9.7	7.3	8.6
143-2	3.3	3.2	5.9	4.2	3.3	6.4	6.8	4.9	7.3	8.3	5.0	8.2	9.3	5.4	8.8
141-2	3.2	3.2	6.1	4.0	3.7	6.4	6.8	4.9	7.2	8.0	5.2	8.0	9.4	5.4	8.9
375-2	3.4	3.3	6.4	4.1	3.6	6.4	6.3	4.5	6.8	8.0	5.0	8.4	9.0	5.0	9.0

[a] LM only=*L. monocytogenes* count
[b] LM+CB=*L. monocytogenes* count
[c] CB only=Competitive bacteria count

Table 5. Inhibition at 37°C of *L. monocytogenes* (LM) by competitive microorganisms (CM) in biofilms formed on stainless steel coupons

Isolate No.	*L. monocytogenes* or competitive microorganism count (log_{10} cfu/cm^2)											
	Trial No. 1						Trial No. 2					
	0 time			24 h			0 time			24 h		
	LM only[a]	LM +CM[b]	CM only[c]	LM only	LM +CM	CM only	LM only	LM +CM	CM only	LM only	LM +CM	CM only
Bacteria												
141-1	4.6	4.6	8.3	7.8	<1.7[d]	7.5	4.6	4.6	8.3	7.8	<1.7[d]	7.2
152	4.6	4.6	8.4	7.8	<1.7	7.6	4.6	4.6	8.3	7.8	<1.7	7.7
C-1-92	4.6	4.6	8.4	7.8	<1.7	6.6	4.6	4.6	8.4	7.8	<1.7	7.3
143-1	4.6	4.6	8.3	7.8	2.7	7.0	4.6	4.6	8.3	7.8	4.2	7.1
C-1-152	4.6	4.6	8.4	7.8	<1.7	7.1	4.6	4.6	8.4	7.8	<1.7	7.4
375-1	4.6	4.6	8.3	7.8	<1.7	7.3	4.6	4.6	8.3	7.8	<1.7	7.7
143-2	4.6	4.6	8.4	7.8	<1.7	6.9	4.6	4.6	8.4	7.8	<1.7	7.4
141-2	4.6	4.6	8.4	7.8	<1.7	7.4	4.6	4.6	8.4	7.8	<1.7	7.3
375-2	4.6	4.6	8.4	7.8	<1.7	7.4	4.6	4.6	8.4	7.8	<1.7	7.5
Yeasts												
C-2-45	4.6	4.6	6.9	7.8	7.3	5.0	4.6	4.6	6.9	7.8	7.4	7.2
C-3-53	4.6	4.6	6.9	7.8	7.6	7.3	4.6	4.6	6.9	7.8	6.9	6.3

[a] LM only=*L. monocytogenes* count
[b] LM+CM=*L. monocytogenes* count
[c] CM only=Competitive microorganism count
[d] Minimum detection limit 1.7 log_{10} cfu/cm^2

Table 6. Inhibition at 37°C of *L. monocytogenes* (LM) by competitive bacteria (CB) in biofilms formed on stainless steel coupons

Isolate No.	*L. monocytogenes* or competitive bacteria count (log_{10} cfu/cm^2)					
	0 time			24 h		
	LM only[a]	LM +CB[b]	CB only[c]	LM only	LM +CB	CB only
141-1	2.9	2.9	6.5	7.7	<1.7[d]	7.9
152	2.9	2.9	6.6	7.7	<1.7	7.8
143-1	2.9	2.9	6.4	7.7	<1.7	7.3
C-1-152	2.9	2.9	6.4	7.7	<1.7	7.8
375-1	2.9	2.9	6.4	7.7	<1.7	8.0
143-2	2.9	2.9	6.5	7.7	<1.7	8.0
141-2	2.9	2.9	6.5	7.7	<1.7	7.8
375-2	2.9	2.9	6.6	7.7	<1.7	7.9
C-1-92	2.6	2.6	6.4	7.1	<1.7	7.2

[a] LM only=*L. monocytogenes* count
[b] LM+CB=*L. monocytogenes* count
[c] CB only=Competitive bacteria count
[d] Minimum detection limit 1.7 log_{10} cfu/cm^2

Table 7. Inhibition at 15°C of *L. monocytogenes* (LM) by competitive bacteria (CB) in biofilms formed on stainless steel coupons

Isolate No.	*L. monocytogenes* or competitive bacteria count (log_{10} cfu/cm^2)											
	0 time			7 days			14 days			28 days		
	LM only[a]	LM +CB[b]	CB only[c]	LM only	LM +CB	CB only	LM only	LM +CB	CB only	LM only	LM +CB	CB only
141-1	3.7	3.7	6.5	8.5	<1.7[d]	8.1	9.2	5.3	8.2	9.5	6.6	8.8
152	3.7	3.7	6.3	8.5	1.7	7.9	9.2	4.3	8.7	9.5	6.5	8.4
375-1	3.7	3.7	6.4	8.5	2.7	7.7	9.2	5.3	8.7	9.5	6.6	9.0
C-1-92	3.7	3.7	6.6	8.5	<1.7	6.7	9.2	<1.7	8.6	9.5	<1.7	8.5
143-1	3.7	3.7	6.5	8.5	4.7	7.7	9.2	2.6	7.8	9.5	6.4	9.0
C-1-152	3.7	3.7	6.3	8.5	<1.7	7.5	9.2	<1.7	6.7	9.5	<1.7	8.3

[a] LM only=*L. monocytogenes* count
[b] LM+CB=*L. monocytogenes* count
[c] CB only=Competitive bacteria count
[d] Minimum detection limit 1.7 log_{10} cfu/cm^2

Table 8. Inhibition at 8°C of *L. monocytogenes* (LM) by competitive bacteria (CB) in biofilms formed on stainless steel coupons

	L. monocytogenes or competitive bacteria count (log_{10} cfu/cm^2)														
	0 time			7 days			14 days			21 days			28 days		
Isolate No.	LM[a] only	LM[b] +CB	CB[c] only	LM only	LM +CB	CB only	LM only	LM +CB	CB only	LM only	LM +CB	CB only	LM only	LM +CB	CB only
141-1	3.7	3.7	6.0	6.8	3.7	7.4	8.5	3.3	8.4	8.5	<1.7[d]	8.0	8.8	<1.7	7.7
152	3.7	3.7	6.4	6.8	3.9	7.5	8.5	3.5	8.2	8.5	<1.7	7.6	8.8	<1.7	7.8
375-1	3.7	3.7	6.5	6.8	3.7	7.6	8.5	3.0	8.5	8.5	<1.7	7.8	8.8	<1.7	7.8
C-1-92	3.7	3.7	6.7	6.8	<1.7	4.7	8.5	<1.7	6.5	8.5	<1.7	5.4	8.8	<1.7	4.7
143-1	3.7	3.7	6.5	6.8	3.9	7.2	8.5	4.4	8.1	8.5	5.9	7.5	8.8	3.3	7.7
C-1-152	3.7	3.7	6.4	6.8	3.1	7.4	8.5	4.5	8.1	8.5	4.9	7.8	8.8	6.4	7.7

[a] LM only=*L. monocytogenes* count
[b] LM+CB=*L. monocytogenes* count
[c] CB only=Competitive bacteria count
[d] Minimum detection limit 1.7 log_{10} cfu/cm^2

Table 10. Inhibition at 4°C of *L. monocytogenes* (LM) by competitive bacteria (CB) in biofilms formed on stainless steel coupons

Isolate No.	*L. monocytogenes* or competitive bacteria count (log_{10} cfu/cm^2)														
	0 time			14 days			21 days			28 days			35 days		
	LM only[a]	LM +CB[b]	CB only[c]	LM only	LM +CB	CB only	LM only	LM +CB	CB only	LM only	LM +CB	CB only	LM only	LM +CB	CB only
141-1	2.6	2.6	6.4	3.7	2.2	6.4	4.5	2.3	7.0	6.0	2.3	7.6	6.8	3.2	7.9
152	2.6	2.6	6.6	3.7	<1.7	6.6	4.5	<1.7	7.3	6.0	2.7	7.8	6.8	2.5	7.8
375-1	2.6	2.6	6.5	3.7	2.2	6.2	4.5	2.2	7.0	6.0	<1.7	7.3	6.8	2.4	7.8
C-1-92	2.6	2.6	6.3	3.7	<1.7	3.8	4.5	<1.7	3.9	6.0	<1.7	3.5	6.8	<1.7	2.5
143-1	2.6	2.6	6.5	3.7	<1.7	5.9	4.5	2.2	6.5	6.0	<1.7	6.9	6.8	3.0	7.5
C-1-152	2.6	2.6	6.5	3.7	2.0	6.1	4.5	<1.7	6.4	6.0	2.5	7.2	6.8	3.4	7.4

[a] LM only=*L. monocytogenes* count
[b] LM+CB=*L. monocytogenes* count
[c] CB only=Competitive bacteria count
[d] Minimum detection limit 1.7 log_{10} cfu/cm^2

Table 11. Inhibition at 4°C of *L. monocytogenes* (LM) by competitive bacteria (CB) in biofilms formed on stainless steel coupons

Isolate No.	*L. monocytogenes* or competitive bacteria count (log_{10} cfu/cm^2)														
	0 time			14 days			21 days			28 days			35days		
	LM only[a]	LM +CB[b]	CB only[c]	LM only	LM +CB	CB only	LM only	LM +CB	CB only	LM only	LM +CB	CB only	LM only	LM +CB	CB only
141-1	4.3	4.3	6.4	3.6	1.7	6.1	5.1	3.2	6.9	6.6	2.6	7.3	8.7	2.5	8.0
152	4.3	4.3	6.5	3.6	3.0	6.4	5.1	<1.7[d]	6.9	6.6	<1.7	7.6	8.7	<1.7	8.3
C-1-92	4.3	4.3	6.5	3.6	<1.7	4.3	5.1	2.9	3.9	6.6	2.0	3.5	8.7	<1.7	2.9
143-1	4.3	4.3	6.5	3.6	3.0	6.1	5.1	1.7	6.6	6.6	4.5	7.0	8.7	4.8	7.4
C-1-152	4.3	4.3	6.6	3.6	<1.7	6.0	5.1	3.1	6.8	6.6	1.7	7.1	8.7	6.7	7.0

[a] LM only=*L. monocytogenes* count
[b] LM+CB=*L. monocytogenes* count
[c] CB only=Competitive bacteria count
[d] Minimum detection limit 1.7 log_{10} cfu/cm^2

Table 9. Inhibition at 8°C of *L. monocytogenes* (LM) by competitive bacteria (CB) in biofilms formed on stainless steel coupons

	L. monocytogenes or competitive bacteria count (log_{10} cfu/cm^2)														
	0 time			7 days			14 days			21 days			28 days		
Isolate No.	LM[a] only	LM[b] +CB	CB[c] only	LM only	LM +CB	CB only	LM only	LM +CB	CB only	LM only	LM +CB	CB only	LM only	LM +CB	CB only
141-1	3.8	3.8	6.2	6.4	4.6	7.7	7.9	4.8	7.7	8.5	<1.7[d]	8.4	8.9	<1.7	8.4
152	3.8	3.8	6.5	6.4	4.1	7.4	7.9	5.3	7.9	8.5	3.5	8.0	8.9	5.0	8.6
375-1	3.8	3.8	6.6	6.4	4.2	7.3	7.9	4.7	8.0	8.5	3.0	8.0	8.9	4.1	8.0
C-1-92	3.8	3.8	6.2	6.4	<1.7	6.3	7.9	<1.7	5.0	8.5	<1.7	5.4	8.9	<1.7	4.6
143-1	3.8	3.8	6.0	6.4	4.2	6.9	7.9	5.4	7.0	8.5	5.9	8.0	8.9	3.9	7.9
C-1-152	3.8	3.8	6.4	6.4	3.8	7.4	7.9	5.3	7.9	8.5	4.9	7.9	8.9	6.3	8.3

[a] LM only=*L. monocytogenes* count
[b] LM+CB=*L. monocytogenes* count
[c] CB only=Competitive bacteria counts
[d] Minimum detection limit 1.7 log_{10} cfu/cm^2