

**Title:** Competitive Inhibition of *Listeria monocytogenes* in Ready-to-Eat Meat Products, Phase II - NPB #01-114

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### ABSTRACT

Forty-nine strains of lactic acid bacteria (LAB), isolated from commercially available ready-to-eat (RTE) meat products, were screened for their ability to inhibit the growth of *Listeria monocytogenes* at refrigeration (5°C) temperatures on agar spot tests. The three most inhibitory strains were identified as *Pediococcus acidilactici*, *Lactobacillus casei*, and *Lactobacillus paracasei* by 16S rDNA sequence analysis. Their antilisterial activity was quantified in associative cultures in MRS broth at 5°C for 28 days, resulting in a pathogen reduction of 3.5 log<sub>10</sub> cycles compared to its initial level. A combined culture of these strains was added to frankfurters and cooked ham co-inoculated with *L. monocytogenes*, vacuum packaged and stored at 5°C for 28 days. Bacteriostatic activity was observed in cooked ham, whereas bactericidal activity was observed in frankfurters. Numbers of *L. monocytogenes* were 4.2 – 4.7 log<sub>10</sub> and 2.6 log<sub>10</sub> cycles lower than controls in frankfurters and cooked ham, respectively, after the 28-day refrigerated storage. In all cases, numbers of LAB increased by only 1 log<sub>10</sub> cycle. The strain identified as *P. acidilactici* was possibly a bacteriocin producer, while the antilisterial activity of the other two strains was due to production of organic acids. There was no significant difference (P>0.05) in the antilisterial activity detected in frankfurters by using the LAB strains individually or as combined cultures. Further studies over a 56-day period indicated that there was no impact on the quality of the product. This method represents a potential antilisterial intervention in RTE meats because it inhibited the growth of the pathogen at refrigeration temperatures without causing sensory changes.

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## INTRODUCTION

*Listeria monocytogenes* is a food-borne pathogen of particular concern in ready-to-eat (RTE) meat products (13, 14, 26, 48) due to its ability to grow at refrigeration temperatures (15, 30, 43, 51, 74), its ubiquitous character (27, 28, 60), and its capacity to tolerate high concentrations of salt and levels of sodium nitrite typically utilized in these products (11, 12, 23, 49). This pathogenic microorganism characteristically gets into RTE foods from the plant environment after cooking or thermal processing steps (26, 60), and survives for long periods of time in different environments. Refrigeration temperatures do not inhibit the growth of the microorganism during cold storage (15, 24, 51, 74). Outbreaks of food-borne listeriosis have been caused by ingestion of coleslaw (63), pasteurized milk (29), Mexican-style cheese (46), pate (50), jellied pork tongue (39) and RTE meat products mainly frankfurters and cold cuts (13, 14).

A recent outbreak of food-borne listeriosis occurred between August of 1998 and February of 1999 (13, 14). The Centers for Disease Control and Prevention reported that 100 illnesses and 21 deaths caused by *L. monocytogenes* (serotype 4b) had occurred in 22 U.S. states (14). The vehicle for the transmission of this pathogen was identified as RTE meat products, including hot dogs and deli meats produced under different brands in the same manufacturing facility. This outbreak prompted the Food Safety and Inspection Service (USDA-FSIS) to call for a reassessment of HACCP plans in meat processing plants to ensure that *L. monocytogenes* was adequately addressed in the process (2).

Lactic acid bacteria (LAB) are inhibitory towards various pathogenic bacteria and spoilage microorganisms during growth and refrigerated storage in associative cultures (7, 8, 34, 41, 62) and in food products (9, 10, 11, 20, 21, 22, 35, 44, 56, 57, 64, 71). The concept of microbial antagonism is well known and refers to the inhibition of other microorganisms by competition for nutrients or by the production of microbial metabolites (33, 34, 37, 41, 65). In addition to lactic acid and other organic acids, LAB produce other metabolites with antimicrobial activity, mainly hydrogen peroxide (17, 58, 70, 73), diacetyl (33), reuterin (4, 25), bacteriocins (5, 38, 45, 53, 54, 66), and other low molecular weight products (55). Numerous reviews (31, 33, 34, 37, 65, 69) have suggested that pathogens and spoilage microorganisms in fermented foods such as summer sausage and yogurt may be inhibited during growth of LAB. Additionally, inhibition can occur during refrigerated storage. Current studies up to this time have focused on isolating strains of LAB that grow rapidly at refrigeration temperatures so that more inhibition is observed. While the pathogens may be inhibited, this would not be practical in an actual food processing environment.

Growth of LAB in RTE meat products would not be desirable since they may cause spoilage. Nevertheless, adding cells to the meat product held at refrigeration temperature could still give an inhibitory effect due to the continuous production of metabolites by the cells during the storage. In general, LAB do not grow at refrigeration temperatures, but if the product is temperature abused, LAB cells can serve as an indication of the abuse by spoiling the product (33, 34, 41, 65). For this reason, it is fundamental that the strains selected exhibit inhibitory activity toward the target pathogen survive during the storage at refrigeration temperatures and do not alter the sensory properties of the food except under temperature abuse conditions.

The objectives of this study were, (i) to isolate, select and identify strains of LAB from RTE meat products with inhibitory activity toward *L. monocytogenes* at refrigeration temperatures, (ii) to quantify the antagonistic activity of the selected LAB isolates toward *L. monocytogenes* in associative cultures at refrigeration temperatures, (iii) to examine the antilisterial activity of the selected LAB strains applied directly on to RTE meat products during refrigerated storage, (iv) to identify the predominant inhibitory substances produced by the selected LAB strains at refrigeration temperatures, and (v) to determine the impact of the LAB on the sensory properties of the product during extended storage.

## OBJECTIVES

The objectives of this project were as follows: 1) identify inhibitory compounds produced by the LAB and alter growth conditions to maximize production of inhibitory substances, 2) quantify the amount of inhibition of LM in RTE products by LAB grown under optimum conditions, and 3) determine the effect of the LAB on the sensory properties of the products.

## PROCEDURES

**Bacterial strains and culture media.** Four strains of *Listeria monocytogenes* were used for this study: Scott A, Murray B, Brie 1 and ATCC 7644 (all human isolates). These cultures were obtained from the stock culture collection in the Department of Food Science and Technology of the University of Nebraska Lincoln (UNL). To facilitate enumeration on non-selective media in the presence of LAB, spontaneous mutants of the four *L. monocytogenes* strains, with resistance to streptomycin sulfate (Sigma Chemical Co., St. Louis, MO, S-6501) at a concentration of 1000 µg/ml, were isolated by sequential selection on tryptic soy agar (Difco Becton Dickinson Microbiology Systems, Sparks, MD) and tryptic soy broth (Difco) containing increasing concentrations of the antibiotic. Both streptomycin resistant (strep<sup>R</sup>) and non-resistant strains were maintained as frozen (-70°C) stocks in tryptic soy broth (TSB) plus 10% (v/v) sterile glycerol as cryoprotectant. For further experiments, the strains were subcultured in TSB or TSB + 1000 µg/ml streptomycin according to antibiotic resistance, and passed twice (at 37°C for 24 h) before use. Cocktail mixtures of the 4 strains were prepared by mixing and homogenizing equal volumetric parts of each freshly cultured strain into a sterile container and used in further studies.

**Isolation of inhibitory organisms from RTE meat products.** Five commercial samples of cooked ham and five commercial samples of frankfurters were obtained from a local supermarket and from the UNL meat laboratory. A 1-gram sample of each product was added to 99 ml of Lactobacillus Selection broth (LBS broth, BBL® Becton Dickinson Microbiology Systems, Cockeysville, MD), homogenized in a stomacher for 1 min and allowed to grow at 32°C for 18-24 h. The resulting cultures were streaked onto LBS agar (BBL®) and incubated at 32°C for 48 h (CO<sub>2</sub> flushed) to obtain isolated colonies. Individual colonies were picked from the LBS plates, grown in MRS broth at 32°C for 18-24 h, and re-streaked onto MRS agar (Difco) plates incubated at 32°C for 24-48 h until obtain pure colonies on the plates. Isolated colonies were re-inoculated in MRS broth and the procedure was repeated as necessary until pure cultures were obtained.

**Screening and identification of LAB strains with antilisterial activity at refrigeration temperatures.** Screening and selection of isolates with potential antagonistic activity toward *L. monocytogenes* at refrigeration temperatures in laboratory media was accomplished by using the agar spot test described by Harris et al. (32). Three different incubation conditions were studied, as follows: 37°C for 24 h, 12°C for 28 days, and 5°C for 45 days. Antilisterial activity on these tests was determined by measuring the clear or translucent zone around the colonies, considering a diameter of 0.5 mm or greater inhibitory toward the pathogen. Agar spot tests were done in triplicate. Six LAB strains active against *L. monocytogenes* at refrigeration temperatures were selected for identification procedures and further studies.

Gram stains and catalase tests were conducted to identify strains. The fermentation pattern of each isolate was also determined by using the API system (bioMérieux, Inc., Hazelwood, MO) with API 50CHL medium for lactic acid bacteria.

Three selected isolates were sent to the Laboratory for Molecular Typing at Cornell University (Ithaca, NY) for identification purposes by 16S rDNA sequence analysis.

### **Preparation of a frozen concentrated culture (FCC) of the selected LAB strains.**

Frozen concentrated cultures of the selected strains were prepared as described by Brashears et al. (8) and combined into a cocktail mixture for further studies. The cocktail was transferred into 2-ml cryogenic vials and quickly frozen in liquid nitrogen. Frozen vials were stored at  $-70^{\circ}\text{C}$ .

**Quantification of antilisterial activity in associative cultures.** A 4-strain cocktail mixture of streptomycin resistant (strep<sup>R</sup>) *L. monocytogenes* was prepared as described earlier and diluted in buffered peptone water (Difco) to obtain approximately  $1 \times 10^6$  CFU/ml. The diluted *L. monocytogenes* suspension was added to sterile MRS broth to obtain approximately  $1 \times 10^5$  CFU/ml. The inoculated broth was split into two equal portions, one was inoculated with LAB frozen concentrated culture (FCC) to obtain approximately  $1 \times 10^7$  CFU/ml, and the other portion was used as control by adding 2.5 ml of a 10% solution of NFMS. Treatments were stored at  $5^{\circ}\text{C}$  and samples were collected on days 0, 7, 14, 21, and 28 to determine the numbers of LAB and *L. monocytogenes*. The total number of *L. monocytogenes* present was determined by using the pour plate method (67) with appropriate dilutions onto TSA supplemented with  $1000 \mu\text{g/ml}$  streptomycin sulfate. Samples were also plated onto MRS agar to determine if the selected LAB grew under refrigeration conditions. The pH of each treatment was determined at each sampling time. Experiments were done in triplicate.

### **Inhibition of *L. monocytogenes* in RTE meat products by the selected LAB strains.**

This study was conducted as a completely randomized design with 4 treatments and 5 sampling intervals over time. The treatments were, (i) products containing only the pathogen (control), (ii) products containing both the pathogen and the LAB, (iii) products containing only the LAB, and (iv) a background control containing neither the pathogen nor the LAB. Experiments were done in triplicate.

Two different formulations of frankfurters and one of cooked ham were evaluated in this study. The two formulations of frankfurters consisted of, (a) a commercial brand obtained in a local supermarket, and (b) a pork-based formulation with no smoke added especially prepared for this study by the University of Nebraska Lincoln Meat Science Laboratory. The one formulation of cooked ham evaluated was a commercial product obtained from a local supermarket. Both frankfurters and ham were cut into pieces and boiled in water for 60 sec to eliminate the effect of background microflora in the study. Pieces were cut such that the final weight of each piece was approximately  $11 \pm 0.2$  grams after the boiling treatment.

A fresh concentrated culture of the selected strains of LAB was prepared using sterile MRS broth to resuspend the pellets. The concentrated culture was diluted in fresh sterile MRS broth to obtain approximately  $1 \times 10^7$  CFU/ml of the selected LAB strains. A 4-strain cocktail mixture of strep<sup>R</sup> *L. monocytogenes* was prepared as previously described, and diluted appropriately in sterile buffered peptone water to obtain approximately  $1 \times 10^5$  CFU/ml.

For treatments (i) and (ii), to surface inoculate the products with strep<sup>R</sup> *L. monocytogenes*, pieces of frankfurters and ham were aseptically dipped into the previously prepared diluted mixture of the pathogen for 7 minutes. Excess fluid was drained into a sterile container and the products were allowed to air dry for 15 minutes under a laminar flow hood to allow *L. monocytogenes* cells to attach. Air-dried inoculated products were aseptically placed individually into 6 x 8 in. vacuum package bags (3 Mil standard barrier Nylon/PE vacuum pouches, permeability for  $\text{O}_2 = 3.5 \text{ cm}^3/100 \text{ in}^2/24 \text{ h}$  at  $21.1^{\circ}\text{C}$  and for water vapor =  $0.6 \text{ g}/100 \text{ in}^2/24 \text{ h}$  at  $37.8^{\circ}\text{C}$  and 100% relative humidity; Koch Supplies Inc., Kansas City, MO). For treatment (ii), 1 ml of the fresh concentrated culture of LAB containing a population of  $1 \times 10^7$  CFU/ml was added and distributed with a dropper onto the surface of the products. To prepare the control (treatment (i)), 1 ml of sterile water was added in a similar way.

Samples corresponding to treatment (iii) were prepared by adding only 1 ml of the fresh concentrated culture of LAB as described before, without being previously dipped into the pathogen mixture. Similarly, background controls (treatment (iv)) containing no isolates or pathogen were prepared by simply adding 1 ml of sterile water.

All samples were vacuum packaged using an Easy Pack (Koch Manufacturing, Kansas City, MO) packaging machine. Vacuum packaged samples were stored for 28 days at 5°C. Samples were collected on days 0, 7, 14, 21, and 28 and the total number of strep<sup>R</sup> *L. monocytogenes* and LAB present determined by pour plating appropriate dilutions onto TSA + 1000 µg/ml streptomycin sulfate and MRS agar respectively, as previously described. The pH values on the surface of the products were also determined at each sampling time.

**Characterization of antilisterial activity.** The selected LAB strains were allowed to metabolize the inhibitory compounds at 5°C for 14 days in MRS broth. After 14 days of storage, samples were centrifuged (7,000 x g, 10 min, 1°C) and supernatants were collected and filter sterilized using 0.45 µm pore-size Acrodisc® filters (Pall Gelman Laboratory, Ann Arbor, MI) into sterile containers. The pH value of each cell-free supernatant was measured.

A total of 8 treatments and 4 sampling intervals over time were used for this study. In all cases, a 4-strain cocktail mixture of *L. monocytogenes* was prepared as described earlier and inoculated at a level of approximately  $1 \times 10^5$  CFU/ml. The treatments studied were, (i) control of MRS broth, (ii) control of cell-free supernatant of each LAB strain, (iii) cell-free supernatants neutralized to pH 6.50 to eliminate the effect of weak organic acids, (iv) cell-free supernatants supplemented with catalase (Sigma, C-40) to exclude the effect of hydrogen peroxide; the remaining 4 treatments consisted of the addition of 4 different proteolytic enzymes to cell-free supernatants to eliminate any possible inhibitory effect of bacteriocins or proteinaceous compounds. The proteolytic enzymes evaluated were, (v) trypsin (Sigma, T-4665), (vi) pepsin (Sigma, P-6887), (vii) protease type XIV (Sigma, P-5147), and (viii) proteinase K (Sigma, P-6556). Experiments were done in triplicate.

All inoculated treatments were incubated at 37°C. Samples were collected on times 0, 4, 8, and 12 hours. The total number of *L. monocytogenes* present was determined by pour plating appropriate dilutions onto TSA + 1000 µg/ml streptomycin sulfate as described earlier.

**Synergistic effect among LAB strains.** One of the RTE meat products previously used (a commercial brand of frankfurters) was selected for this study. Samples were prepared and evaluated as previously described except the frankfurters were inoculated with a 3 strain mixture of the LAB. Two additional treatments were studied in which one of the LAB strains, previously determined to be a bacteriocin-producer, was combined with each one of the other two strains (non-bacteriocin producers) separately, and these combined cultures were added ( $\sim 1 \times 10^7$  CFU/ml) to the frankfurters pre-inoculated with the pathogen. A control sample was also studied with no LAB cells added.

**Sensory Evaluation.** One of the selected LAB strains (isolate code D3), a possible bacteriocin producer, was selected for this part of the study. This strain was selected because it did not change the pH of the samples significantly.

Frankfurters were manufactured at the University of Nebraska Lincoln Meat Laboratory under standard manufacturing procedures. Chilled and peeled hot dogs were surface inoculated with a fresh culture of the selected LAB strain at a level of approximately  $1 \times 10^7$  CFU/ml of rinsate, vacuum packaged, and stored at 4°C. The LAB culture was prepared as described previously (FCC culture) and distilled sterile water was used to resuspend the cells. All the steps involved in the preparation of this culture were done in food grade laboratories. Control samples were prepared by vacuum packaging the frankfurters with no LAB cells added.

A triangle test with a balanced reference was used to determine if a difference existed between the control and the LAB-treated frankfurters. Prior to serving, the hot dogs were heated in hot water ( $90^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ) for 90-120 seconds depending on how many (2-4) were being heated at the time. After heating each hot dog was cut into 1.5 inch pieces, placed into a 2 oz plastic cup that had been coded with a three digit random number. A lid was placed on the cup and samples were held under a heat lamp (less than 5 min) until served. Samples were presented in a random order to each panelist who were instructed to taste beginning with the sample to their left. Evaluations were conducted so that each panelist was seated in an individual lighted partitioned booth; a red light was used to mask color irregularities (3). Samples were tested weekly for a total of 8 weeks, and the number of panelists that participated ranged from 46-54; panelists were students and staff from the Department of Food Science and Technology.

Samples of both control and LAB-treated frankfurters were also aseptically collected at each evaluation period and pour plated on MRS agar and Plate Count Agar (PCA) (Difco) and incubated at  $37^{\circ}\text{C}$  for 24 hours to determine the numbers of *P. acidilactici* and background microbiota, respectively.

**Statistical Analysis.** All the experiments related to quantification of antilisterial activity in co-inoculated samples, both in laboratory media and in RTE meat products, as well as the experiments concerning the characterization of substances consisted of repeated measurements over time. The results of these experiments were all analyzed based on the mixed model for repeated measures (SAS PROC MIXED utilizing the repeated statement) in the Statistical Analysis System (SAS Institute Inc., Cary, NC). Least squares means were used to evaluate the effect of treatment at various times in the four experiments related to the inhibition of *L. monocytogenes* in associative cultures and RTE meat products. Additionally, in the experiments related to the characterization of antilisterial activity, least squares means were used to compare the various treatments with the control. In that case, Dunnett's correction was used as the appropriate *P*-value adjustment for this situation. All tests were conducted with a significance level of 0.05 ( $P < 0.05$ ).

## RESULTS

**Isolation, screening and identification of LAB strains from RTE meat products with antilisterial activity at refrigeration temperatures.** Isolation procedures led to a total of 49 isolates from the 10 commercially available RTE meat products sampled. Fifteen of the isolates showed significant inhibition (inhibitory zone  $> 0.5$  mm) towards *L. monocytogenes* on agar spot tests after 24 h at  $37^{\circ}\text{C}$ . The 6 most inhibitory isolates (isolate codes D3, I6, D6, I2, J11, I5) were selected for screening at  $12^{\circ}\text{C}$  and  $5^{\circ}\text{C}$ . All six isolates inhibited *L. monocytogenes* on the agar spot test at  $12^{\circ}\text{C}$  and  $5^{\circ}\text{C}$  for 28 and 45 days, respectively, showing areas of inhibition greater than 0.5 mm (data not illustrated).

The six isolates were identified by Gram staining, catalase tests, and the API system with API 50CHL medium for lactic acid bacteria. All six isolates were catalase negative and Gram-positive. As to the microscopy morphology of the isolates, strain D3 was cocci and the other 5 strains were rods. Three LAB strains had identical profiles in the API test and one more resulted in a doubtful profile, hence only three strains (isolate codes D3, D6, and I5) were positively identified and selected for further studies. Results of 16S rDNA sequence analysis compared to sequences contained in the public database GenBank, showed significant homology of the selected strains to the following microorganisms: strain D3 aligned most closely with *P. acidilactici* being the closest one, strain D6 closely aligned with *L. casei*, and strain I5 aligned closely with mainly two species of *Lactobacillus*: *L. paracasei* and *L. casei*.

**Quantification of antilisterial activity in associative cultures.** There was significant ( $P < 0.05$ ) reduction in populations of *L. monocytogenes* during storage at  $5^{\circ}\text{C}$  by the selected LAB

strains (Fig. 1). After 7 days of storage, the numbers of *L. monocytogenes* in the control samples (no LAB added) increased by approximately 2.5 log<sub>10</sub> cycles. Numbers of the pathogen in samples containing LAB slightly decreased during these first 7 days of storage. Over the 28 day storage period, the counts of *L. monocytogenes* in the control samples continued to increase to 8.46 log<sub>10</sub> CFU/ml while those that contained LAB decreased significantly ( $P < 0.05$ ), with a total reduction of approximately 3.48 log<sub>10</sub> cycles, demonstrating bactericidal activity. By day 28 of storage, there was more than a 6.4-log<sub>10</sub> CFU/ml difference in controls and treated samples. There was less than 1 log<sub>10</sub> cycle increase in the numbers of LAB in the broth during the storage period.

The changes in pH were significantly different ( $P < 0.05$ ) between the control sample with no LAB added and the treatment containing LAB cells over the 28-day storage period. The pH in the control sample decreased during storage from 6.50 to 4.70 on day 28. Change in pH in the sample that contained LAB changed from 5.93 on day 0 to 3.87 after 28 days of storage.

**Inhibition of *Listeria monocytogenes* in RTE meat products by the selected LAB strains.** In all of the RTE meat products evaluated, there was significant inhibition ( $P < 0.05$ ) of the growth of *L. monocytogenes* when the selected LAB strains were added on the surface of the products. In control samples with no LAB added, the pathogen exhibited gradual growth of approximately 3 log<sub>10</sub> cycles in all cases as compared to its initial numbers.

In “frankfurters 1”, a commercially available brand, the numbers of *L. monocytogenes* in the control samples increased almost 3 log cycles during storage (Fig. 2A). Numbers of the pathogen showed no significant increases or reductions for the first 14 days of storage in samples containing LAB. However, after 21 days of storage, there was a small but significant reduction ( $P < 0.05$ ) in these counts as compared to their initial level on day 0, reaching a final level of 3.25 log<sub>10</sub> CFU/ml on day 28. This indicates an overall slight bactericidal activity by the LAB cells considering the entire storage period. Numbers of LAB increased from 6.92 to 8.01 log<sub>10</sub> cycles during the storage period (Table 1). Although the increment in numbers of LAB over time was significant ( $P < 0.05$ ) when compared with their initial value (day 0), the overall difference of approximately 1 log<sub>10</sub> cycle over the 28-storage period does not represent serious concern because the sensory quality of the frankfurters was not affected.

Background controls showed no counts of any microorganisms on both TSA + 1000 µg/ml streptomycin sulfate and MRS agar plates over the 28-day storage period, demonstrating that there was no influence of background microbiota on the inhibition of the pathogen (data not shown). Changes in pH were significantly different ( $P < 0.05$ ) between the control samples and the treatments containing LAB (Table 2) over the 28-day storage period. The pH in the control samples decreased gradually to 5.30 on day 28. On the other hand, the pH of samples co-inoculated with *L. monocytogenes* and LAB decreased to 4.57 after 28 days.

Similar results were observed for, “frankfurters 2”, a pork-based formulation especially prepared for this study by the UNL Meat Laboratory (Fig. 2B). These were prepared so that there would be no inhibitory effect of smoke on the *L. monocytogenes*.

Conversely, in the case of cooked ham, the behavior of both pathogen growth in co-inoculated samples, and pH-change over the time, was different from that observed in frankfurters. In control samples (no LAB added), the numbers of *L. monocytogenes* increased rapidly by more than 2 log cycles after only 7 days. After that, the increment in the numbers of the pathogen was slower and gradual (Fig. 2C). On the other hand, on co-inoculated samples with LAB, the numbers of *L. monocytogenes* increased only by approximately 0.4 log<sub>10</sub> cycles over the 28-day storage period. Overall, the growth inhibition was significantly different ( $P < 0.05$ ) when compared to control samples at the end of the storage period, hence the inhibitory activity of LAB against *L. monocytogenes* in this case can be considered bacteriostatic. Again, numbers of LAB increased by less than 1 log<sub>10</sub> cycle over the 28-day storage period (Table 1). Background controls demonstrated no influence of background microbiota on the inhibition of

the pathogen (data not shown). Changes in pH were significantly different ( $P < 0.05$ ) between the control samples and the treatments containing LAB (Table 2) over the 28-day storage. However, the change in pH in co-inoculated samples was not as dramatic as the one observed in frankfurters. In this case, the pH decreased from 6.50 on day 0 to a minimum value of 5.63 on day 21. In control samples the change in pH was even less dramatic, decreasing from 6.60 on day 0 to a minimum of 6.03 on day 21, and this value remained constant during the last 7 days of storage.

**Characterization of antilisterial activity by the selected LAB strains.** Analysis of the differences of least squares means with a level of significance of 0.05 ( $P < 0.05$ ) showed that the antagonistic effect of isolate D3 (*P. acidilactici*) against *L. monocytogenes* was due to the production of a proteinaceous compound. This potential bacteriocin was produced by this strain over a period of 14 days at 5°C, having sterile MRS broth as substrate, and effectively inhibited the growth of *L. monocytogenes* at 37°C during the first 8 h of incubation (Fig. 3A). This was demonstrated since the numbers of the pathogen in the supernatant that was adjusted to a pH = 6.50 and in the supernatant that was treated with catalase, were significantly different ( $P < 0.05$ ) from the counts of *L. monocytogenes* in the MRS control, but showed a similar behavior to the numbers of *L. monocytogenes* in the untreated cell-free supernatant for the first 8 h of incubation at 37°C. In the untreated cell-free supernatant, numbers of viable cells of *L. monocytogenes* decreased from 5.32 log<sub>10</sub> CFU/ml at 0 h to 5.08 log<sub>10</sub> CFU/ml at 8 h, while these counts decreased from 5.31 to 5.14 log<sub>10</sub> CFU/ml and 5.36 to 5.27 log<sub>10</sub> CFU/ml in neutralized supernatant (pH adjusted to 6.50) and catalase-treated supernatant, respectively, at the same sampling times. Conversely, numbers of the pathogen in cell-free supernatants that were treated with either trypsin, protease type XIV or proteinase K were not significantly different ( $P > 0.05$ ) from the numbers in the MRS control (Fig. 3A) over the 12-h incubation period, showing that the inhibitory substance was susceptible to proteolysis. This proteinaceous compound was not sensitive to the action of pepsin.

Conversely, the antilisterial activity of cell-free supernatants obtained from strains D6 and I5 (*L. casei* and *L. paracasei*, respectively) was due to the production of organic acids, and there was no evidence of production of bacteriocins or hydrogen peroxide (Figs. 3B and 3C).

**Synergistic effect among LAB strains.** There was no significant difference in the reduction of *L. monocytogenes* counts in frankfurters co-inoculated with LAB when each one of the selected strains was studied individually as compared to combined cultures. Furthermore, we studied the possibility of a synergistic effect between the strong acid-producing strains (*L. casei* and *L. paracasei*) and the bacteriocin-producing strain (*P. acidilactici*), since most of the LAB bacteriocins are active at acidic pH values (38, 45). However, no significant difference was observed in the antilisterial activity when the LAB strains were inoculated individually as compared to combinations of *P. acidilactici*-*L. casei* or *P. acidilactici*-*L. paracasei* (Fig. 4).

Nevertheless, there were significant differences ( $P < 0.05$ ) in the pH change over the time among those samples containing each one of the LAB strains individually (Fig. 5).

**Sensory evaluation.** The results of the triangle test showed that no significant differences ( $P < 0.05$ ) were found during the study (Table 3) over the 8-week evaluation period. Hence, application of cells of the selected strain (*P. acidilactici*, strain D3) to the surface of frankfurters at a level of approximately  $1 \times 10^7$  CFU/ml of rinsate neither have any significant effect on the sensory quality of the products during a storage period of 56 days at 5°C, nor it caused any signs of spoilage.

The numbers of *P. acidilactici* (strain D3) remained constant during the evaluation period at a level of approximately 6.80 log<sub>10</sub> CFU/ml of rinsate on MRS agar plates. Background controls started at a level of  $< 10$  CFU/ml of rinsate on day 0, and increased gradually to a level



of  $4.5 \log_{10}$  CFU/ml of rinsate on day 56, on PCA plates. After the heating step, LAB-treated frankfurters that were given to the panelists had a level of approximately  $3.50 \log_{10}$  CFU/ml of rinsate.

There were no obvious sign of spoilage observed for frankfurters in the package. The liquid in the package remained clear for the duration of the study.

## DISCUSSION

There exist alternate control measures that have been proposed or are in practice to prevent and control the growth of *L. monocytogenes* in meat systems. Some of them include the use of various additives in the formulation, such as sodium lactate and sodium acetate (6), liquid smoke (52), monoglycerides (72), and lysozyme (36). Despite the use of these non-traditional additives in recent years, outbreaks of food-borne listeriosis associated with RTE meat products have continued to occur (13, 14). Other approaches include physical methods, mainly post-processing pasteurization (59), irradiation (68), and high hydrostatic pressure (1). These approaches have proven to be effective in the control of the pathogen in laboratory media and in some specific applications; however, they represent a high-cost alternative. Furthermore, these technologies are effective only on the external surface of the products, which limits their utilization in some RTE meat products such as sliced products or double-layer frankfurter packages.

Biopreservation systems using lactic acid bacteria as a method of control of the pathogen have also been investigated. Several studies have reported the inhibition of *L. monocytogenes* by microbial antagonism of LAB in laboratory media (10, 21, 32) as well as in different meat systems, (20, 44, 71, 22, 56, 12, 64, 9, 42, 35).

In some of these studies, the LAB strains that have been used are characterized for having fast growth rates at refrigeration temperatures, so that they can overcome the growth of *L. monocytogenes* by nutrient depletion or by enhancement of production of bacteriocins. Buchanan and Bagi (10) suggested that the inhibition of *L. monocytogenes* by two bacteriocin-producing strains of *C. piscicola* in associative cultures in brain heart infusion was due to nutrient depletion rather than to the production of bacteriocins, since *C. piscicola* is a microorganism of rapid growth and may produce an insufficiency of a limiting nutrient for the pathogen to grow. Nilsson et al. (56) added cells of *C. piscicola* to control the growth of *L. monocytogenes* on cold-smoked salmon, and selected for strains with fast growth rates since their objective was to enhance the production of bacteriocins at refrigeration temperatures, but they did not take into account the increasing numbers of the LAB. Buncic et al. (12) reported that an effective strain of *Lactobacillus sake* Lb 706 against *L. monocytogenes* on beef samples grew from  $10^3$  CFU/g to  $10^8 - 10^9$  CFU/g within 3 weeks of storage at  $4^\circ\text{C}$ , affecting the quality of the meat. Indeed, these same authors reported that some strains that did not have fast growing rates, but were inoculated at low levels ( $10^3 - 10^4$  CFU/g), were unable to prevent *L. monocytogenes* growth in different media and food systems at refrigeration temperatures. While these approaches inhibited the growth of *L. monocytogenes*, they are unlikely to be applicable in the meat-processing industry because the fast growth rates of the LAB could reduce the shelf life and sensory properties of the products.

In our study, we isolated and selected three strains of LAB that effectively inhibited the growth of *L. monocytogenes* in frankfurters and cooked ham at  $5^\circ\text{C}$  over 28 days of storage. During this time, the numbers of the LAB only increased by approximately 1  $\log_{10}$  cycle in all cases, and no visible signs of spoilage were evident (i.e. detrimental effect on some organoleptic properties related to external appearance such as color changes, undesirable aromas, and stickiness or texture changes on the surface of the products).

Of these selected isolates, strain D3 (*P. acidilactici*) is of particular interest. This strain synthesized a bacteriocin-like compound at refrigeration temperatures, that exhibited effective antilisterial activity for the first 8 hours of incubation at  $37^\circ\text{C}$ , and after that period of time, the

pathogen was able to re-grow. This is important because it demonstrates the difference between using live cells and using only the pure metabolites as antimicrobial agents. In the first case, producer cells may either generate more bacteriocin or continuously excrete bacteriocin so as to replenish the inhibitor over the entire period of storage, whereas when finite amounts of bacteriocin are present, *L. monocytogenes* cells may overcome the bacteriocin levels, either by proteolytic degradation of the inhibitor, or through the outgrowth of a mutant population resistant to the bacteriocin (32, 61). Duffes et al. (22) reported the inhibition of *L. monocytogenes* in co-cultures with bacteriocin-producing strains of *Carnobacterium* spp. in a simulated cold smoked fish system during the first 18 days of storage at 4°C. However, after 21 days of storage, a slight growth of *L. monocytogenes* occurred. This re-growth of the pathogen was explained by a diminution of the inhibitory substance concentration due to production of proteolytic enzymes by *L. monocytogenes*, and not by the appearance of resistant mutants. In a similar study, Pilet et al. (57) reported a decrease in bacteriocin concentration during the stationary growth phase of *C. divergens* and *C. piscicola* in production studies conducted in MRS broth. These authors attributed this phenomenon to a possible proteolytic activity of these strains. Furthermore, Schillinger et al. (61) isolated nisin-resistant mutants of *L. monocytogenes* Scott A after a single exposure to nisin at 100 IU/ml, during incubation for only 3 h at 30°C.

Bacteriocins are known to be active at pH values that fall in the acid range (38, 45). Nisin, for instance, becomes increasingly ineffective in foods that approach pH neutrality, and is completely inactivated under alkaline conditions. Hence, we hypothesized that the use of a combined culture (3 strains) of the selected LAB isolates might have a potential synergistic effect enhancing the activity of the bacteriocin produced by strain D3, favored by the stronger acid production of the other two strains. However, we did not detect significant differences in the antilisterial activity when the strains were studied individually or in combined cultures.

Because the samples inoculated individually with strain D3 (*P. acidilactici*) showed no significant declines in pH and there were no significant effects on the antilisterial activity perceived among the three isolates, a sensory evaluation was conducted to evaluate whether consumers would detect any differences between products treated with this microorganism versus non-treated products. Panelists were not able to identify any significant differences between control and LAB-treated samples in a triangular test over the course of a 56-day evaluation period. Therefore, the use of cells of this strain represents a promising alternative for the control of *L. monocytogenes* in RTE meat products during the refrigerated storage, distribution and retail commercialization, without affecting the sensory quality of the products significantly.

## CONCLUSIONS

From a public health standpoint, it is relevant to evaluate if the antilisterial effect obtained was sufficient to ensure the safety of the products without causing spoilage. According to our results, effective bacteriostatic and bactericidal activities (product-dependent) were observed against *L. monocytogenes* over the course of 28 days of refrigerated storage in different RTE meat systems and no sensory changes were observed over a 56 day storage period. Since the minimum infective dose to cause food-borne listeriosis is relatively low ( $< 10^3$  cells/g or ml of food), it is likely that in a product that has been contaminated with *L. monocytogenes* after the heat treatment, this microorganism reaches those levels after few weeks of storage at refrigeration temperature. Therefore, the approach we propose here is important because the continuous production of antilisterial compounds by the LAB cells during refrigerated storage would prevent growth of *L. monocytogenes* to infective levels, hence ensuring the safety of the products. This type of interventions might be introduced as a CCP in a HACCP plan after cooking steps and before the final refrigerated storage.

**TABLE 1.** Numbers of lactic acid bacteria (LAB) in samples of ready-to-eat (RTE) meat products co-inoculated with *L. monocytogenes* during storage at 5°C for 28 days. “Frankfurters 1” is a commercial brand, “Frankfurters 2” is a special formulation made for this study by the UNL Meat Laboratory (no smoke added), and “Cooked Ham” is a commercial brand.

Days of Storage	Numbers of LAB (Log <sub>10</sub> CFU/ml rinsate) <sup>I</sup>		
	Frankfurters 1	Frankfurters 2	Cooked Ham
0	6.92 <sup>a</sup>	6.88 <sup>a</sup>	7.04 <sup>a</sup>
7	7.51 <sup>b</sup>	7.75 <sup>b</sup>	7.82 <sup>b</sup>
14	7.90 <sup>c</sup>	8.09 <sup>c</sup>	8.04 <sup>b</sup>
21	7.77 <sup>bc</sup>	7.76 <sup>bc</sup>	7.83 <sup>b</sup>
28	8.01 <sup>c</sup>	8.00 <sup>bc</sup>	7.91 <sup>b</sup>

<sup>I</sup> All values are the means of three replications.

<sup>abc</sup> Values with different alphabetical notations within each product differ significantly ( $P < 0.05$ )

**TABLE 2.** Changes of pH in samples of ready-to-eat (RTE) meat products inoculated with *L. monocytogenes* only and co-inoculated with the selected lactic acid bacteria (LAB) strains + *L. monocytogenes* stored at 5°C for 28 days. “Frankfurters 1” is a commercial brand, “Frankfurters 2” is a special formulation made for this study by the UNL Meat Laboratory (no smoke added), and “Cooked Ham” is a commercial brand.

Days of Storage	pH values <sup>1</sup>					
	Frankfurters 1		Frankfurters 2		Cooked Ham	
	Control (no LAB)	Co-inoculated	Control (no LAB)	Co-inoculated	Control (no LAB)	Co-inoculated
0	6.27 <sup>a</sup>	6.20 <sup>a</sup>	6.10 <sup>a</sup>	5.83 <sup>ab</sup>	6.60 <sup>a</sup>	6.50 <sup>a</sup>
7	6.30 <sup>a</sup>	5.77 <sup>b</sup>	6.07 <sup>a</sup>	5.37 <sup>c</sup>	6.50 <sup>a</sup>	5.83 <sup>bc</sup>
14	6.27 <sup>a</sup>	5.03 <sup>cd</sup>	5.67 <sup>b</sup>	4.90 <sup>d</sup>	6.10 <sup>b</sup>	5.73 <sup>cd</sup>
21	5.80 <sup>b</sup>	4.77 <sup>de</sup>	5.37 <sup>c</sup>	4.70 <sup>e</sup>	6.03 <sup>bc</sup>	5.63 <sup>d</sup>
28	5.30 <sup>c</sup>	4.57 <sup>e</sup>	5.27 <sup>c</sup>	4.57 <sup>e</sup>	6.03 <sup>bc</sup>	5.67 <sup>d</sup>

<sup>1</sup> All values are the means of three replications.

<sup>abcde</sup> Values with different alphabetical notations within each product differ significantly ( $P < 0.05$ )

**TABLE 3.** Results of triangle test on frankfurters treated or not treated (control) with the selected strain of lactic acid bacteria (*P. acidilactici*, strain D3) during storage at 5°C for 56 days.

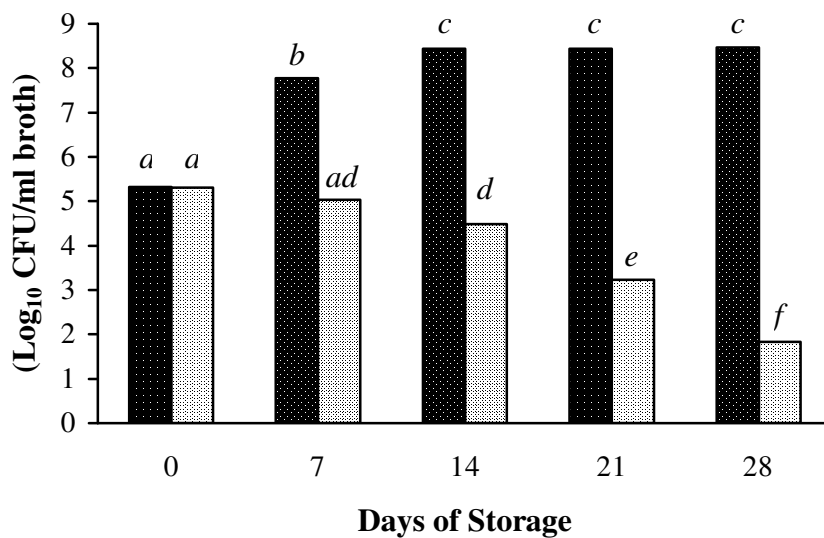
<b>Days of Storage</b>	<b>Total Number of Panelists</b>	<b>Total Correct<sup>1</sup></b>	<b>Number Needed for Significant Difference at 5% (<math>P &lt; 0.05</math>)</b>
0	47	18	23
7	49	16	23
14	54	15	25
21	47	20	23
28	46	17	22
35	54	13	25
42	52	14	25
49	48	21	23
56	46	12	22

<sup>1</sup>Number of panelists who identified the odd sample in the triangle test.

**FIGURE 1.** Quantification of antilisterial activity in associative cultures in MRS broth at 5°C.

Figure shows numbers of *L. monocytogenes* in the control sample with no lactic acid bacteria (LAB) (▀) and in the sample treatment containing LAB (▨).

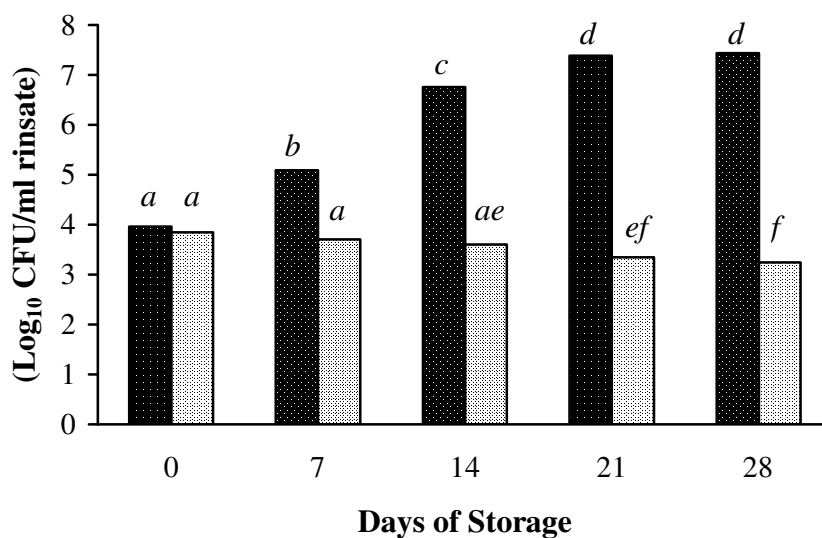
<sup>abcdef</sup> Bars with different alphabetic notations differ significantly ( $P < 0.05$ ). All values are the means of three replications. Standard error = 0.23



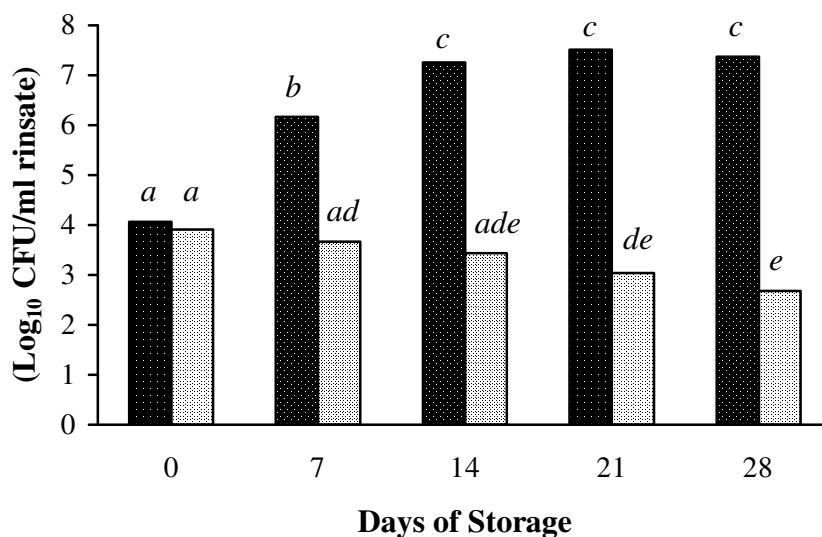
**FIGURE 2.** Inhibition of *L. monocytogenes* by the selected lactic acid bacteria (LAB) strains in RTE meat products during storage at 5°C for 28 days. (A) “Frankfurters 1”, a commercial brand. (B) “Frankfurters 2”, a pork-based formulation prepared at the University of Nebraska Lincoln Meat Laboratory. (C) Cooked ham, a commercial brand. Figure shows numbers of *L. monocytogenes* in the control sample (with no LAB added) and in the sample treatment containing LAB.

<sup>abcdef</sup> Bars with different alphabetic notations differ significantly ( $P < 0.05$ ). All values are the means of three replications. Standard errors: (A) = 0.13, (B) = 0.29, (C) = 0.19

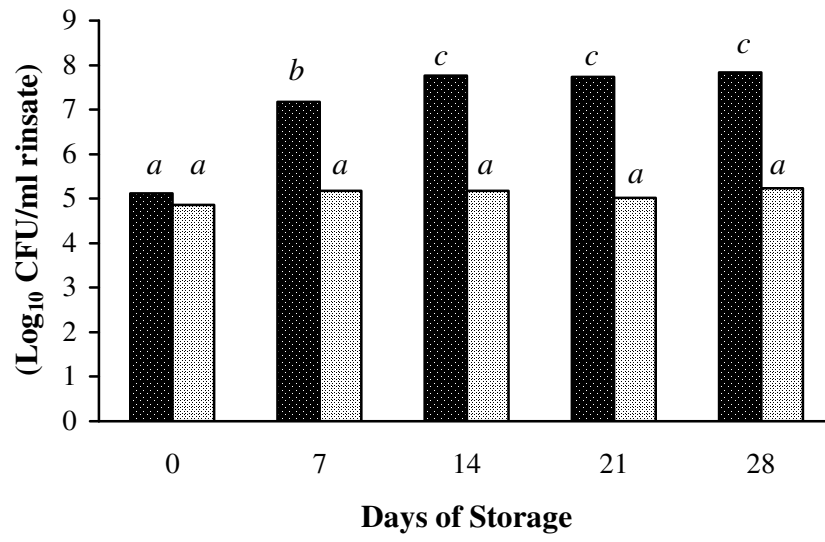
**A.**



**B.**



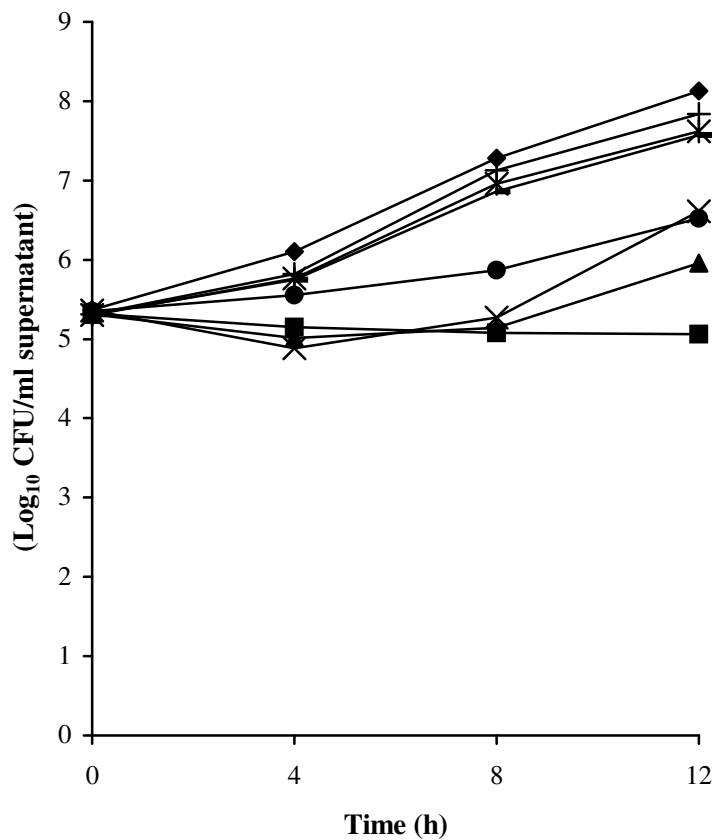
C.

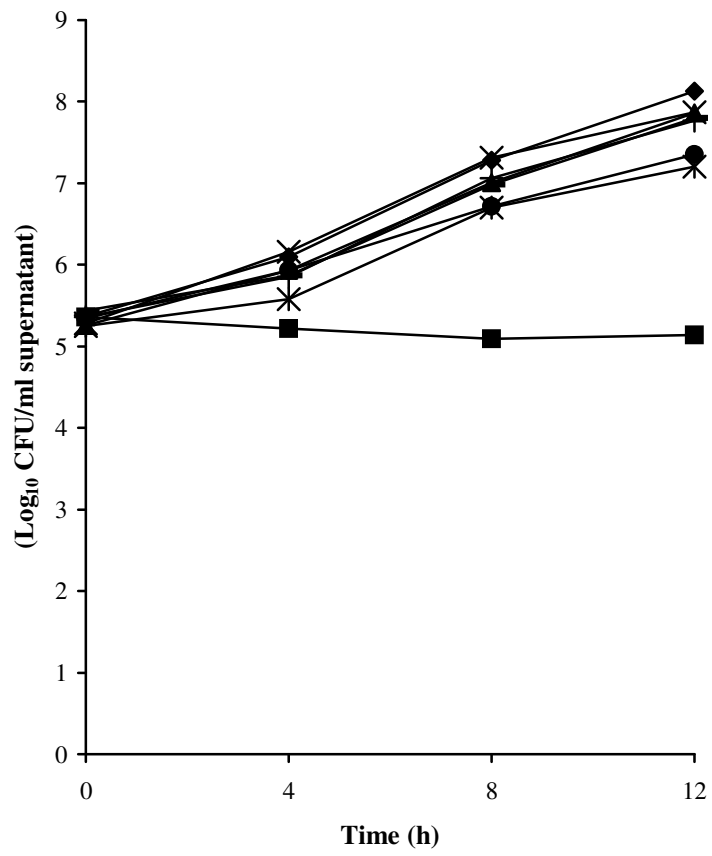




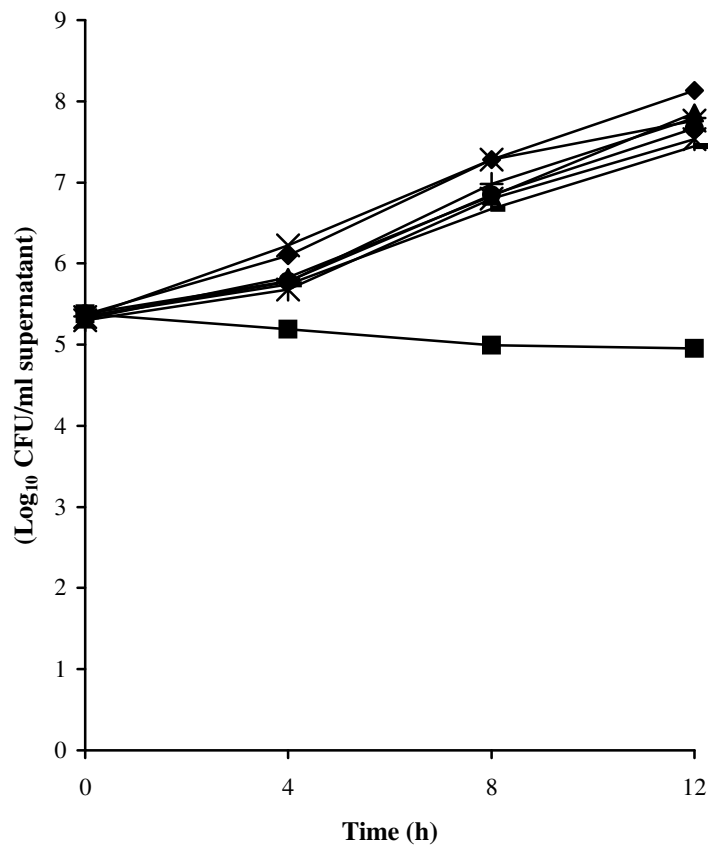
**FIGURE 3.** Characterization of antilisterial activity by the selected lactic acid bacteria (LAB) strains. (A) Strain D3, *Pediococcus acidilactici*. (B) Strain D6, *Lactobacillus casei*. (C) Strain I5, *Lactobacillus paracasei*. Curves show the growth and numbers of *L. monocytogenes* at 37°C for 12 h in the control of MRS broth (○), in untreated cell-free supernatant (□), in cell-free supernatant with pH adjusted to 6.50 (△), and in cell-free supernatants treated with 0.5 mg/ml catalase (X), 1 mg/ml trypsin (■\*), 1 mg/ml pepsin (◇), 1 mg/ml protease type XIV (+), 1 mg/ml proteinase K (●). All values are the mean of three replications. Standard errors: (A) = 0.19, (B) = 0.18, (C) = 0.17

A.

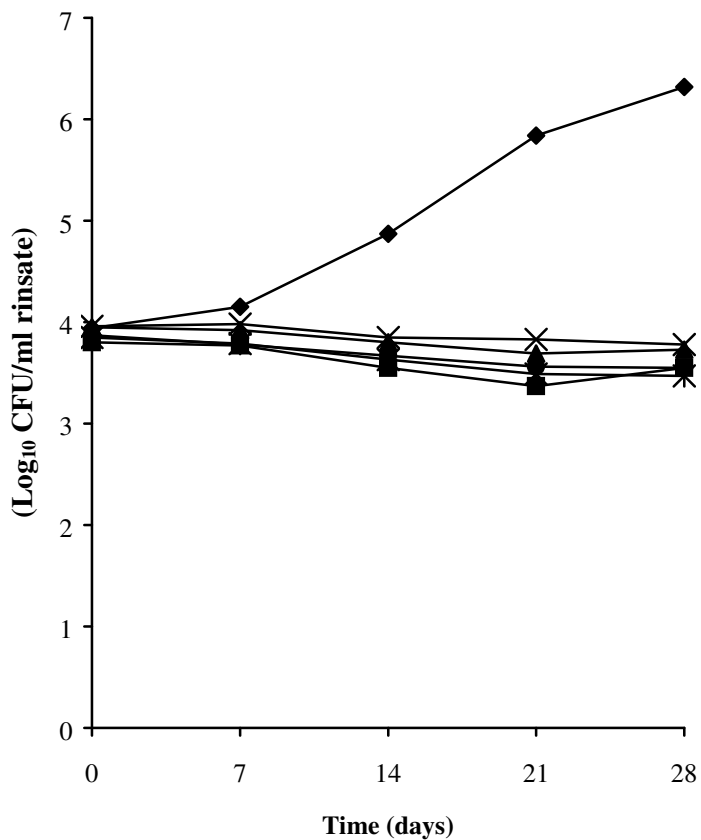


**B.**

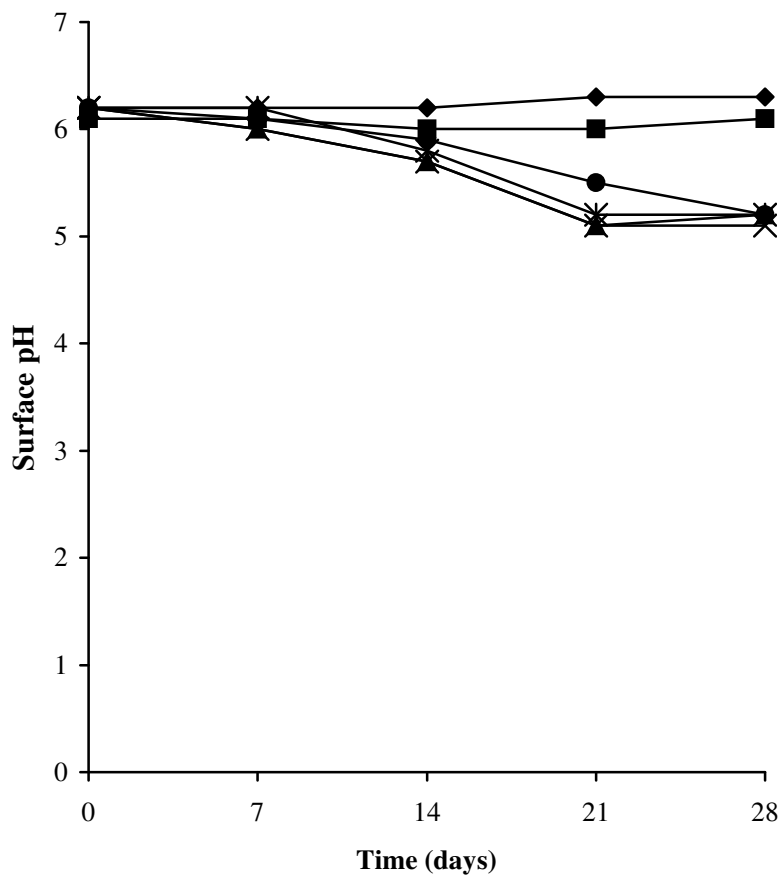
C.



**FIGURE 4.** Synergistic effect of the selected lactic acid bacteria (LAB) strains on the growth of *L. monocytogenes* in frankfurters during storage at 5°C for 28 days. Curves show the growth and numbers of *L. monocytogenes* in the control sample with no LAB added (○), and in the sample treatments containing strain D3 only (■), strain D6 only (▲), strain I5 only (X), strains D3 and D6 combined (\*), and strains D3 and I5 combined (●). All values are the mean of three replications. Standard error = 0.07



**FIGURE 5.** Changes of pH in frankfurters inoculated with *L. monocytogenes* only and co-inoculated with the selected lactic acid bacteria (LAB) strains + *L. monocytogenes* either individually or as combined cultures at 5°C for 28 days. Curves show the growth and numbers of *L. monocytogenes* in the control sample with no LAB added ( ), and in the sample treatments containing strain D ( ), strain D6 only ( ), strain I5 only ( X ), strains D3 and D6 combined ( ● ), and strains D3 and I5 combined ( ). All values are the mean of three replications. Standard error = 0.01



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