RESEARCH REPORT

PORK SAFETY

Title: Competitive Inhibition of *Listeria monocytogenes* in Ready to Eat Pork Products – **NPB #99-216**

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I. Abstract

Lactic acid bacteria (LAB) were isolated from ready-to-eat (RTE) pork products. The inhibitory action towards *Listeria monocytogenes* (LM) was observed in agar spot tests, laboratory media and in RTE pork products. A total of 49 isolates were obtained with 15 showing significant inhibition towards LM in agar spot tests. The six most inhibitory isolates were chosen for quantification of inhibition in laboratory media. API identification of these isolates indicated that 5 were *Lactobacillus plantarum* while one was *Lactobacillus brevis*. In laboratory media there was a 4 log reduction in numbers of LM after a 28 day storage period at 5 C while control samples increased more than 3 log cycles. Ultimately there was more than a 6 log reduction of LM on day 28 compared with the control samples. A mixture of the LAB was added to hot dogs and ham cold cuts along with LM. The LAB had a bacteriostatic effect on the LM completely inhibiting growth of the organism in the RTE products. After 28 days of storage, there was greater than a 4 log difference in the amount of LM in the RTE products containing LM compared to the controls. There were no significant increases in the numbers of LAB so their presence should not have significant impacts on shelf life.

II. Introduction

According to the Centers for Disease Control (CDC), *Listeria monocytogenes* (LM) causes 2,000 reported cases of illness annually of which 25% result in death. One of the most susceptible groups to listeriosis is pregnant women who are 20 times more likely to contract the illness than non-pregnant, healthy individuals. Additionally, the organism can cross the placenta and cause illness in the fetus which can result in miscarriage, stillbirth, or serious medical complications in the newborn. Elderly and immunocompromised individuals are also more susceptible to listeriosis than other healthy individuals.

In late 1998 and early 1999 there was a multi-state outbreak of listeriosis. On March 17, 1999 the CDC reported that 100 illnesses and 21 deaths had occurred in 22 U.S. states (Anonymous, 1999). The company initiated a voluntary recall of ready-to-eat products including hotdogs and various deli meats. LM was isolated from both opened and previously unopened packages of hot dogs produced in this plant. This outbreak

prompted the USDA-FSIS to call for a re-assessment of HACCP plans to ensure that LM was adequately addressed in the process.

LM is usually not addressed in a HACCP plan because it is a sanitation issue. Most processors have implemented aggressive sanitation programs and environmental monitoring to control LM. Additionally, the lack of post-packaging interventions makes it difficult to pinpoint a CCP to control LM in a ready to eat product. It is important to identify methods which will prevent the growth of LM in ready-to-eat foods.

L. monocytogenes typically gets into the food supply from the plant environment after a heat treatment has been applied. After cooking, the casing must be removed from hot dogs and cold cuts must be sliced. Additionally, products must be packaged which is often done by hand. Handling after processing increases the risk of LM contamination from the plant environment or from the employees. Because a heat treatment has already been applied to the product and LM has the potential to grow during refrigerated storage, more interventions to reduce microbial growth after packaging need to be examined.

Lactic acid bacteria (LAB) are inhibitory towards various pathogenic bacteria and spoilage organisms during growth and refrigerated storage in associative cultures (Brashears et al. 1998; Brashears and Gilliland, 1997; Brashears et al. 1996; Brashears and Durre (1999); Dahiya and Speck, 1967; Gilliland and Speck, 1975; Gilliland and Speck, 1977; Martin and Gilliland, 1980; Price and Lee, 1969; Kao and Frazier, 1966; Shahani et al., 1976). Reviews of the various antagonistic actions of LAB towards pathogens, including *L. monocytogenes*, can be found in articles by Hurst (1973) Barefoot and Nettles (1993), Klaenhammer (1988) and Holzapfel et. al (1995). The inhibitory actions were due to the production of acid, hydrogen peroxide, or bacteriocins. These reviews suggest that pathogens and spoilage organisms in foods may be inhibited during growth of LAB or during refrigerated storage. It is possible to select strains of lactic acid bacteria that do not grow at refrigeration temperatures, but produce inhibitory substances. Many species of LAB are food grade and can be safely added to food products. The production of inhibitory compounds by the LAB can continue during storage of the product so there is continuous inhibition of the pathogen (Gilliland and Villegas, 1998; Jaroni and Brashears).

Several scientists (Schillinger et. al., 1990; Sulzer and Busse, 1991; Winkowski and Montville, 1992; Winkowski et. al., 1993) have reported that *L. monocytogenes* was inhibited in various meat products by bacteriocin-producing strains of LAB. Recently, Nilsson et. al. (1999) added cells of *Carnobacterium piscicola* to control the growth of *Listeria monocytogenes* on smoked salmon. They found that the cultures produced bacteriocins that inhibited *L. monocytogenes* in laboratory media. The bacteriocin producing strains of *Carnobacterium* caused a long lag phase of *L. monocytogenes* growth and reduced numbers from 10^3 to <10 cfu/m after 32 days of incubation. Additionally, even strains of *Carnobacterium* that did not produce bacteriocins inhibited the *L. monocytogenes* suggesting that some other inhibitory compound is produced during refrigerated storage that inhibits the pathogen.

III. Objectives

The overall objective of this study is to reduce the numbers *Listeria monocytogenes* in ready to eat (RTE) pork products by competitive inhibition by lactic acid bacteria and thus reduce the number of outbreaks associated with these products.

IV. Procedures

Isolation of Inhibitory Organisms from Ready to Eat Pork Products

Inhibitory organisms were isolated from commercially available pork-based hot dogs and ham cold cuts. Products were obtained from a local supermarket and from the UNL meat laboratory. A 1 gram sample was added to lactobacillus selection (LBS) broth and allowed to

grow at 32°C for 18 h. The resulting culture was streaked onto LBS agar to obtain isolated colonies. Individual colonies were grown in MRS broth and restreaked onto MRS agar until they were pure. The colonies were identified using the API system, Gram stains, and catalase tests.

The agar spot test was used to screen isolated colonies for their ability to inhibit a cocktail mixture of strains of *L. monocytogenes* in laboratory media at refrigeration temperatures. The isolated strains was grown for 18 h at 37°C in MRS broth. Each test culture was spotted onto the surface of MRS agar and incubated for 24 hours at 37°C to allow colonies to develop. Approximately 5 x 10^7 cells of a cocktail mixture of *L. monocytogenes* was added to 15 ml of Nutrient Agar and poured over the spot-inoculated plates. For preliminary results, the plates were incubated at 37°C for 24 hours. The most inhibitory strains were chosen for further testing and the plates were incubated for 5 days at 5°C. The inhibition was measured daily by measuring the inhibitory zone (clear zone) around the colonies. Generally, a diameter of 0.5 mm or larger was considered inhibitory.

The most inhibitory strains were identified and those with acceptable identifications were used for further studies. To quantify the amount of inhibition in broth and to determine if the LAB grew at refrigeration temperatures, we inoculated sterile MRS broth with a cocktail mixture of streptomycin resistant (strep^R) LM to obtain approximately 1 x 10^5 cfu/ml. Strep^R strains were used so the LM could be plated on nonselective media and the recovery of injured cells could be facilitated. The inoculated broth was split into two separate portions and one was inoculated with a concentrated culture of the inhibitory strains of LAB (approximately 1 x 10^7/ml) and the other served as the control. Samples were stored at 7°C and samples were collected on days 0, 7, 21, and 28 to determine the numbers of LAB and LM. The total LM present was determined using the pour plate method onto trypiticase soy agar containing 1000 ug/ml streptomycin. The numbers were compared to the control that contained only added pathogen, no LAB, to determine the amount of inhibition. Samples were plated onto MRS agar to determine if the LAB were growing.

Inhibition of *Listeria monocytogenes* in Pork-based Ready to Eat Products

This study was conducted as a completely randomized design with 3 treatments and 5 sampling intervals over time. The treatments were, 1) products containing both the pathogen and the LAB, 2) products containing only the pathogen, and 3) a background control containing neither the pathogen or the LAB.

Frozen concentrated cultures of the most inhibitory strains selected in phase 1 were prepared as described by Brashears et al. (1998) and combined into a “cocktail” mixture to add to the food products. We examined the inhibition of *L. monocytogenes* in the products by the selected isolates.

Hot dogs and ham cold cuts were inoculated with the pathogen by dipping them into a cocktail mixture of streptomycin resistant (strep^R) *L. monocytogenes* containing approximately 1 x 10^4 cfu/ml. Excess fluid was drained into a sterile container and the products were allowed to dry for 10 minutes. Cells of the inhibitory isolates were added to the hot dogs/cold cuts by dipping them in a diluted mixture of the isolates containing a population of 1 x 10^7 cfu/ml after they have dried. Controls were prepared by dipping the pathogen inoculated samples into sterile water. Background controls containing no isolates or pathogen were prepared by dipping products into sterile water. All samples will be vacuum packaged.

Samples were stored for 28 days at 5°C. Samples were taken on days 0, 7, 14, 21, and 28 and the total number of LM and LAB present were determined as previously described.

V. Results and Discussion

A total of 49 isolates were obtained from 10 commercially available RTE pork products (primarily ham and hot dogs). Fifteen of the isolates showed significant inhibition (inhibitory zone >.05) towards LM on agar spot tests after 24 hours at 37°C (Figure 1). The 6 most

inhibitory isolates were selected for screening at 5°C and 12°C. All six of the isolates completely inhibited the growth of LM in the agar spot test at 5°C after 7, 14, 21, and 28 days of storage. At 12°C all isolates produced zones of inhibition greater than 0.5mm with three of the isolates showing the most inhibition (Figure 2).

The six isolates were all identified using the API system as possibly being lactic acid bacteria. Three of the isolates had doubtful profiles while the other three were positively identified. We had two strains of *Lactobacillus plantarum* and one of *Lactobacillus brevis*. These were chosen for further studies.

The three chosen strains were used to quantify the amount of inhibition of LM during storage at 5°C in MRS broth. The amount of reduction in populations of LM during storage at 5°C by the LAB was significant (Figure 3). After 7 days of storage, the numbers of LM in the control samples increased 3 log cycles from 5.0 $\log_{10}$ to 8.0 $\log_{10}$. Samples containing LAB had no significant increases. Over the 28 day storage period, the amount of LM in the control samples continued to increase to 9.0 $\log_{10}$ cfu/ml while those that contained LAB had no significant increases. The LAB not only inhibited the growth of the LM, they also had lethal action on the LM. The numbers of LM in samples containing LAB decreased from 5.0 $\log_{10}$ cfu/ml on day 0 to 1.5 $\log_{10}$ cfu/ml on day 28. There was less than a 1 log cycle increase in the numbers of lactic acid bacteria in the broth during the storage period (Table 1).

The same three strains were added to commercially available hot dogs. The numbers of LM in hot dogs containing no LAB increased more than 3 log cycles (Figure 4). The amount of LM in hot dogs containing both LM and LAB showed no significant increases and the numbers of LM declined almost one log cycle. Again, there were no significant increases in the numbers of LAB during the 5 day storage period. Similar results were observed in ham cold cuts.

The results of this study indicate that the strains of LAB we have isolated prevent the growth of LM in ready to eat hot dogs during refrigerated storage. Adding LAB to hot dogs after cooking could be an effective intervention that prevents LM growth during distribution and storage prior to consumption. We are currently conducting studies to identify the inhibitory substances produced by the LAB and to determine if the LAB have any impacts on the sensory properties of the product.

Figure 1. Inhibition of *Listeria monocytogenes* by lactic acid bacteria at 5°C. Isolates with zones > 0.5 mm are considered inhibitory.

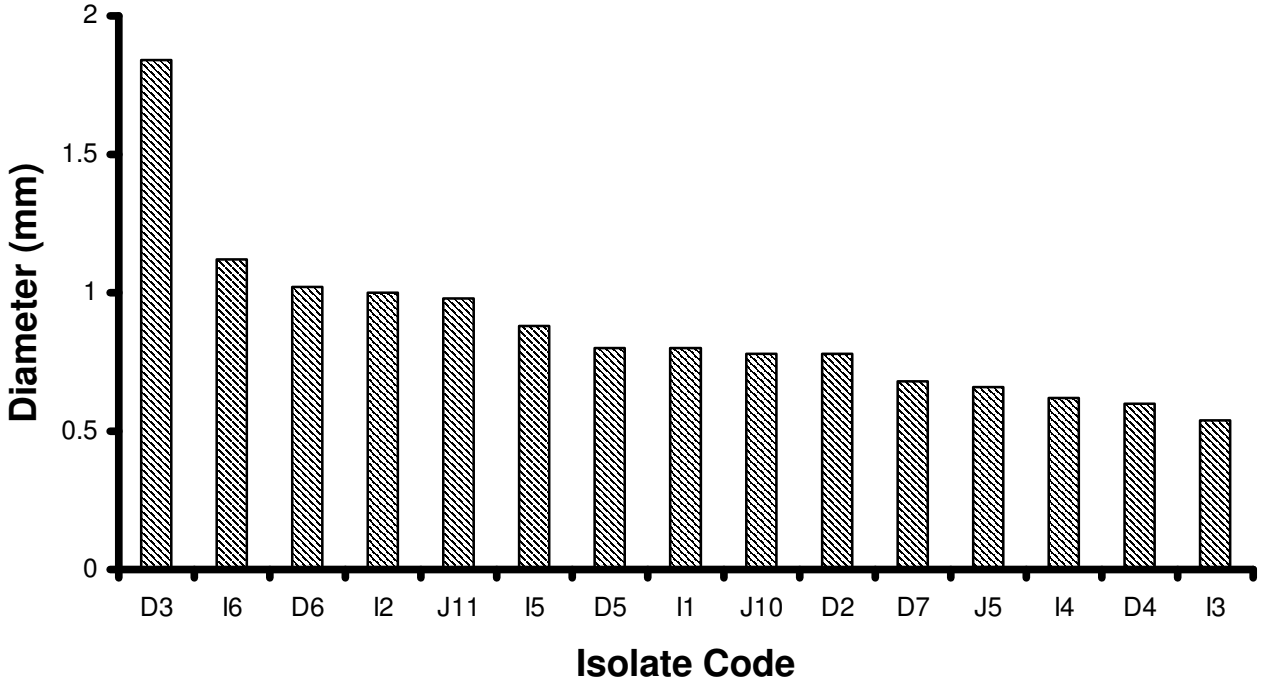

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Figure 2. Inhibition of *Listeria monocytogenes* by lactic acid bacteria at 12 C. Isolates with zones > 0.5 mm are considered inhibitory.

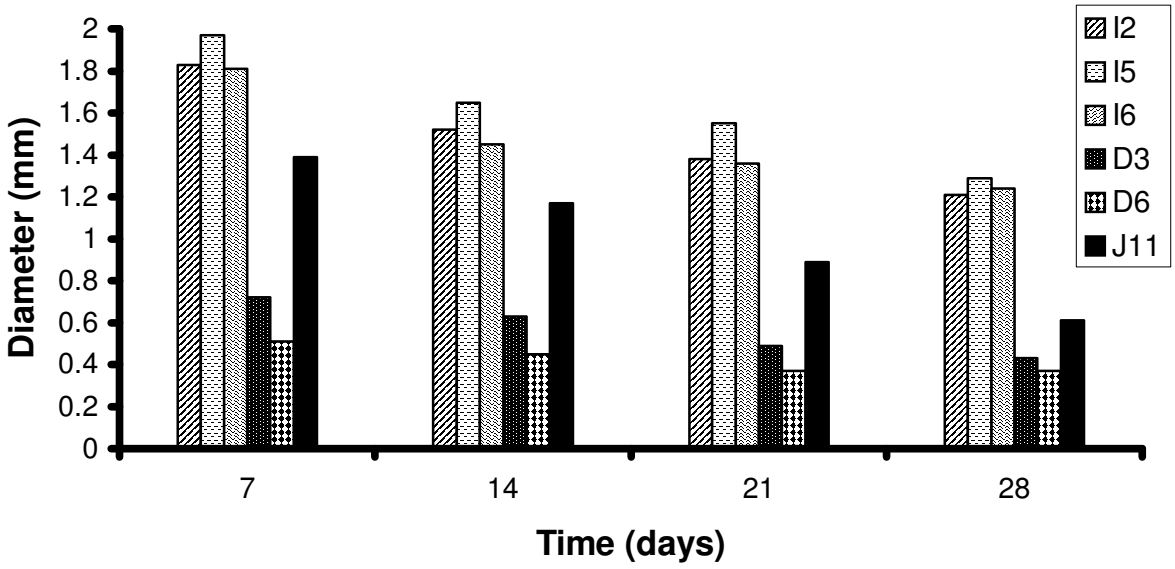


Figure 3. Inhibition of a cocktail mixture of *Listeria monocytogenes* by lactic acid bacteria during storage in MRS broth at 5°C

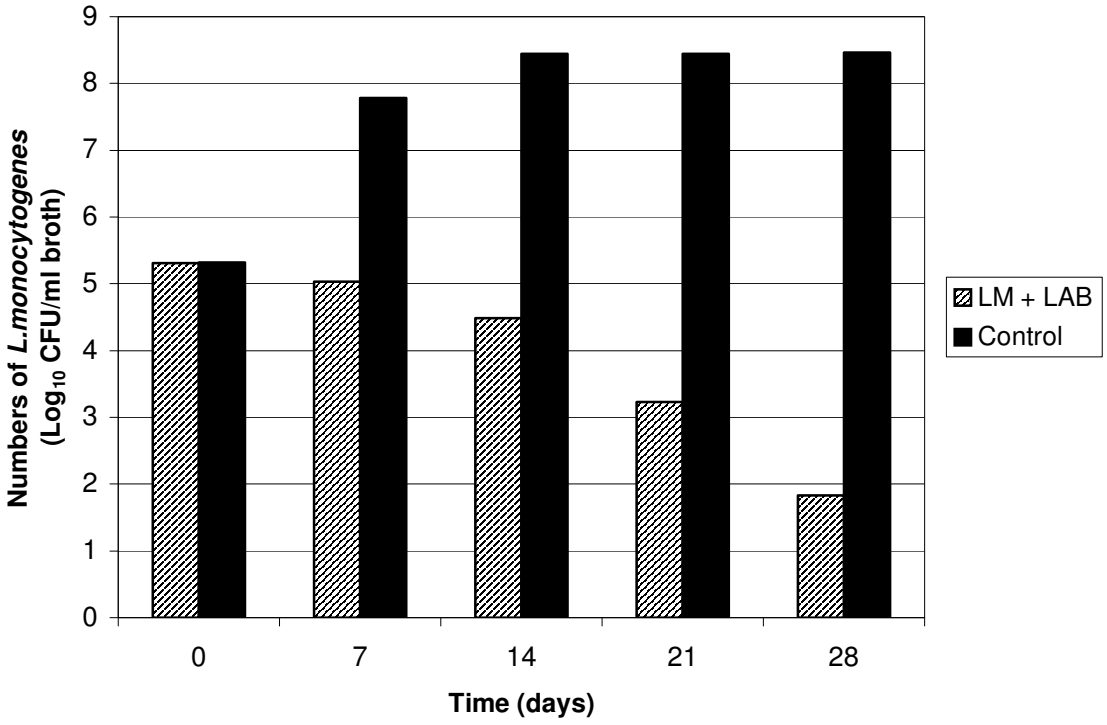
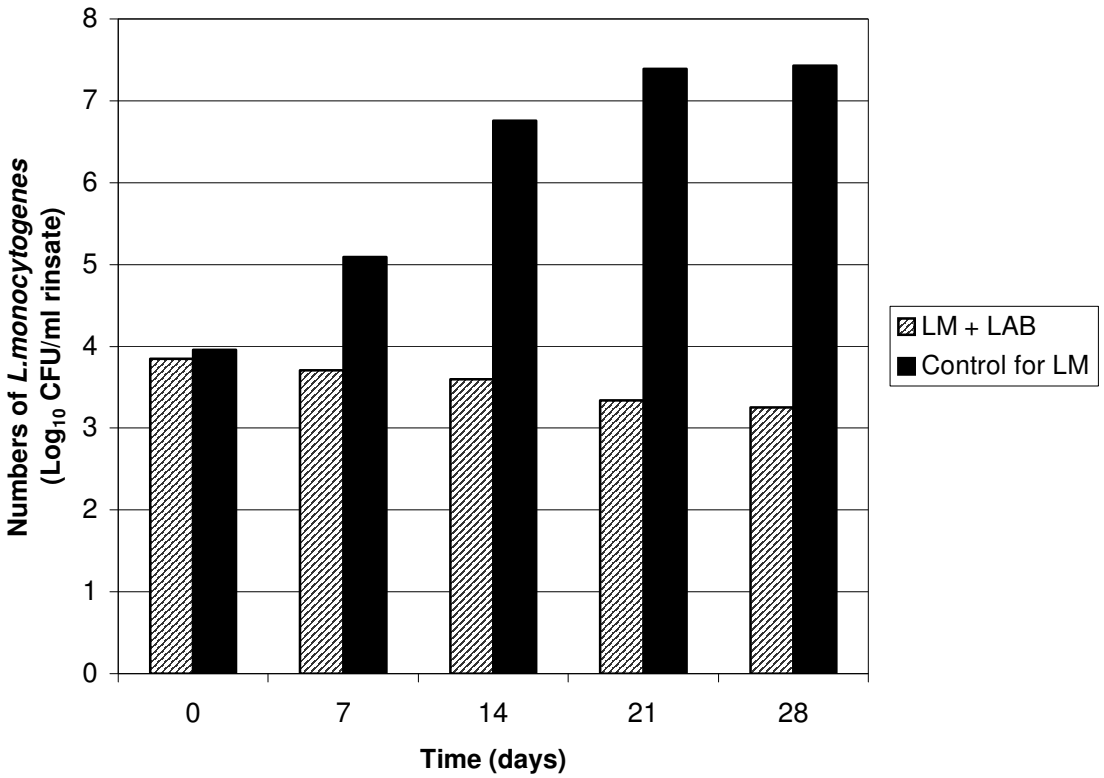


Table 1. Numbers of Lactic Acid Bacteria in MRS broth stored at 5°C for 28 days

Time (days)	Log_{10} CFU/ml of rinsate
0	7.49
7	7.44
14	7.35
21	8.00
28	8.43

Figure 4. Inhibition of *Listeria monocytogenes* in hot dogs by Lactic Acid Bacteria during Storage at 5°C for 28 days (similar results in ham cold cuts).



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