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Inhibition of *Listeria monocytogenes* by Bacteriocin-Producing *Pediococcus* During the Manufacture of Fermented Semidry Sausage[1]

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ABSTRACT

A bacteriocin-producing *Pediococcus* species inhibitory to *Listeria monocytogenes* was used to manufacture fermented semidry sausage. Separate 13.6 kg batches of a commercial summer sausage formulation were inoculated to contain an initial level of 10^6 cells/g of *Listeria monocytogenes* Scott A. In each of two independent studies, an ca. 2 $\log_{10}$ CFU/g reduction of *L. monocytogenes* occurred over the fermentation period, as compared to a less than 1 $\log_{10}$ CFU/g reduction in sausage fermented with a non-inhibitory *Pediococcus* strain. Inactivation of *L. monocytogenes* was also observed in one study where adequate acid production did not occur (pH>5.5), indicating that bacteriocin production occurred independently of carbohydrate fermentation. Following heating to an internal temperature of 64.4°C and storage up to 2 weeks, 9 of 90 sausages sampled were positive for *Listeria*. Recovery was intermittent and did not indicate that the bacteriocin was effective in eliminating *L. monocytogenes* that had survived the heating process.

The presence of *L. monocytogenes* in foods has become a major concern to the food processing industry and government regulatory agencies. A recent report of a case of listeriosis associated with the consumption of turkey franks provides the first direct evidence of the infection linked to a poultry product *(3)*. Studies done in recent years have shown that *L. monocytogenes* occurs commonly in seafoods (5), poultry (*2, 7, 14*), and meats, including cured and fermented meats (*5,6,14,15,16*).

The preservation of cured, fermented sausage from spoilage and pathogenic microorganisms is due to a number of factors, including low water activity, sodium chloride, sodium nitrite, and low pH due to the production of lactic acid by starter culture organisms. Research on the survival of *L. monocytogenes* in dried, fermented sausages has revealed, however, that while the growth is suppressed by the combination of these factors during fermentation and drying processes, the organism may survive in the finished product (*8,10,11*).

A potential means of preserving such fermented meats from *L. monocytogenes* is through the use of bacteriocin-producing lactic acid bacteria. Many pediococci, some species of which are widely used as meat starter cultures, have been shown to produce bacteriocins that are inhibitory to *L. monocytogenes* (*4,9,17*). The objective of this work was to determine the behavior of *L. monocytogenes* in semidry sausage fermented with a bacteriocin-producing strain of *Pediococcus*.

MATERIALS AND METHODS

Cultures

L. monocytogenes Scott A (serotype 4b) was grown aerobically in tryptic soy broth fortified with 0.6% yeast extract (TSB-YE) (Difco Laboratories, Detroit, MI) for 24 h at 37°C. Inoculum was prepared by transferring 1.0 ml of the 24 h culture into 100 ml TSB-YE, which was incubated aerobically for 24 h at 37°C. A sufficient volume of this culture was used to yield 10^6 CFU/g when added to 13.6 kg of meat.

The two meat starter cultures (designated JD1-23 and MP1-08) used were obtained from commercial suppliers as frozen, concentrated cultures, and were of the genus *Pediococcus*. For use in sausage fermentation, the cultures were thawed and added according to manufacturers' directions (ca. 10^7 cells/g).

Sausage manufacture

Five sausage treatments were examined in each of two independent studies (each study consisted of two independent replicate trials): (1) JD—starter culture JD1-23 with no added *L. monocytogenes*; (2) MP—starter culture MP1-08 with no added *L. monocytogenes*; (3) Scott A—no starter culture with added *L. monocytogenes*; (4) JD/Scott A—JD1-23 with added *L. monocytogenes*; and (5) MP/Scott A—MP1-08 with added *L. monocytogenes*. For study 1, a commercial seasoning premix processed from spice extracts was used in the sausage formulation; for study 2, a commercial seasoning premix consisting of natural spices was used.

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Boneless beef trimmings (20% fat) and regular pork trimmings (20% fat) were obtained from the University of Nebraska-Lincoln Meat Science Laboratory. All meat was stored at -20°C before use and was thawed at 4°C for 3 d before sausage manufacture.

Each treatment consisted of 13.6 kg (30 lb.) of meat. Cold pork trim was ground through a 1.27 cm plate and cold beef trim was ground through a 0.48 cm plate, both on a Hobart grinder (model 4732, Hobart Manufacturing Co., Troy, OH). An 8.2 kg portion of ground beef was added to a mixer (model 100DA, Leland Detroit Manufacturing Co., Detroit, MI) with 150 ml tap water, 450 g salt, 33.6 g of a commercial cure mixture containing 2.25 g $NaNO_2$, 300 g dextrose, 8.25 g sodium erythorbate, and 75.2 g of the indicated spice premix and mixed for 3 min. After this initial mixing, a 5.4 kg portion of the ground pork was added and mixed for an additional 2 min. At this time, cultures were added and mixed for an additional 3 min. For treatments inoculated with starter culture or *L. monocytogenes* only, the culture was suspended in 150 ml tap water immediately before addition to the mixer. For treatments inoculated with both starter culture and *L. monocytogenes*, each culture was suspended in 75 ml water before addition to the mixer.

Following mixing, the meat was reground through a 0.48 cm grinder plate. The sausage batter was then stuffed into 6.35 cm diameter fibrous casings with a mechanical stuffer (Model 1000DC, Vemag, Verden, Federal Republic of Germany) and clipped on both ends. The sausage chubs were approximately 20 cm long and weighed approximately 450 g each.

The finished sausages were hung on metal racks and placed in a smokehouse (Alkar, Lodi, WI) maintained at 37.8°C (100°F) and 50% relative humidity (RH). Sausages in study 1 were fermented under these conditions for 12 h; in study 2, the sausages were fermented for 14 h. Following the fermentation period, the temperature was raised to 60°C (140°F) and 55% RH for 1 h, then to 82.2°C (180°F) and 40% RH until sausages were finished to an internal temperature of 64.4°C (148°F). The sausages were then showered for 10 min with cold water and placed in a cooler maintained at 4°C. Samples to be stored were vacuum-packaged in individual, gas-impermeable polyethylene bags using a Kramer-Grebe vacuum packaging machine (model 167/401, Kramer-Grebe, Wallau, Federal Republic of Germany) and held at 4°C until sampled.

Sausage sampling

Two sausages from each treatment were chosen randomly at 0, 3, 6, 9, and 12 h following placement into the smokehouse and after the complete heat treatment. (In study 2, duplicate samples were taken following the same schedule and also at 14 h.) Duplicate storage samples for analysis were taken at 1 and 2 weeks following manufacture.

Sausages were sampled for *L. monocytogenes* by aseptically opening the casing with a sterile scalpel and removing approximately 25 g of meat mix, including the surface and the geometric center of the meat mass in the sample. This sample was mixed, and approximately 11 g was added to 99 ml sterile phosphate-buffered saline (PBS) contained in a sterile stomacher bag (Tekmar Co., Cincinnati, OH). The contents were homogenized for 2 min using a stomacher (model 400, Tekmar), serially diluted (1:10) in PBS, and 0.1 ml portions were spread plated in duplicate onto McBride *Listeria* Agar (MLA) (*13*). Plates were incubated at 37°C for 24 h and enumerated.

In addition to direct plating as described above, 25 g portions of mixed meat samples from sausages exposed to the entire heat process were subjected to enrichment procedures by the method of the Microbiology Division, Food Safety and Inspection Service, U.S. Department of Agriculture, Beltsville, MD.

Two colonies per plate which gave typical appearance for *L. monocytogenes* on MLA or lithium chloride-phenylethanol-moxalactam (LPM) agar (*12*) with 45° transillumination were confirmed by morphological and biochemical tests, and by serology using Bacto-*Listeria* O Antisera Type 4 (Difco).

Analytical methods

The pH of each sausage sample was determined by inserting a combination electrode (Corning Glass Works, Medfield, MA) into the interior of the meat mass and obtaining readings from five different locations within the sausage; the average value was reported. Moisture, fat, Kjeldahl protein, salt, and nitrite determinations were done on duplicate samples of finished sausages from each treatment from each trial (excluding replicate trial 1 of study 1) according to the procedure of the Association of Official Analytical Chemists (*1*).

RESULTS AND DISCUSSION

Initial studies involved the screening of several commercial starter cultures, both meat and dairy, for antimicrobial activity against *L. monocytogenes* Scott A using both direct and deferred methods (unpublished data). Meat starter culture JD1-23 was found to have activity against this organism, and the inhibitory agent has been subsequently characterized as a bacteriocin (*18*). The bacteriocin is inactivated by treatment with several proteolytic enzymes, and is stable to both heating at 91.5°C for 1 h and autoclaving at 121°C for 15 min. Meat starter culture MP1-08, which did not inhibit *L. monocytogenes* in initial screening, was chosen as a control for subsequent sausage experiments.

Fermentation data from studies 1 and 2 are summarized in Tables 1 and 2. In both studies, in those treatments containing both starter culture and *L. monocytogenes*, the number of *L. monocytogenes* was reduced after the end of the fermentation period; however, high numbers still remained in all sausages examined. These results are consistent with those reported by other workers on the survival of *L. monocytogenes* during sausage fermentation (*8,10,11*). The sausage made with JD1-23, however, showed a greater decrease in the numbers of *L. monocytogenes* present after fermentation, as compared to sausage made with MP1-08. This additional decrease, from 1.2 to 1.4 $\log_{10}$ CFU/g, was not due to any additional acid produced by JD1-23, since the final pH of the sausage fermented with JD1-23 in both studies was the same or higher than that fermented with MP1-08.

Evidence of the effectiveness of the bacteriocin produced by JD1-23 against *L. monocytogenes* during sausage fermentation was further demonstrated in study 1, in which the starter cultures failed to produce acid. Due to the lack of adequate acid production and a subsequent drop in pH during the fermentation period, this study was not expected to produce meaningful results. However, subsequent enumeration of *L. monocytogenes* in sausages sampled during

TABLE 1. *Sausage pH and viable* L. monocytogenes *count during fermentation period without adequate acid production (study 1).*[a]

	Treatments									
	MP		JD		Scott A		MP/Scott A		JD/Scott A	
Time (h)	CFU/g	pH	CFU/g	pH	Log_{10} CFU/g	pH	Log_{10} CFU/g	pH	Log_{10} CFU/g	pH
0	<100	5.80(0.01)	<100	5.80(0.01)	6.29(0.07)	5.81(0.01)	6.27(0.09)	5.77(0.01)	6.29(0.20)	5.78(0.01)
3	<100	5.83(0.01)	<100	5.84(0.01)	6.22(0.14)	5.84(0.01)	6.14(0.06)	5.82(0.01)	6.13(0.07)	5.83(0.01)
6	<100	5.82(0.01)	<100	5.86(0.01)	5.97(0.21)	5.87(0.01)	6.03(0.04)	5.79(0.01)	5.66(0.31)	5.86(0.01)
9	<100	5.75(0.01)	<100	5.83(0.02)	5.87(0.13)	5.88(0.01)	6.00(0.04)	5.75(0.01)	4.97(0.53)	5.82(0.02)
12	<100	5.70(0.03)	<100	5.75(0.01)	5.95(0.13)	5.91(0.02)	5.69(0.16)	5.62(0.01)	4.48(0.44)	5.77(0.02)

[a]Values reported are means of duplicate determinations from each of two replicate trials. Values within parentheses represent standard error.

TABLE 2. *Sausage pH and viable* L. monocytogenes *count during fermentation (study 2).*[a]

	Treatments									
	MP		JD		Scott A		MP/Scott A		JD/Scott A	
Time (h)	CFU/g	pH	CFU/g	pH	Log_{10} CFU/g	pH	Log_{10} CFU/g	pH	Log_{10} CFU/g	pH
0	<100	5.83(0.01)	<100	5.83(0.01)	6.27(0.08)	5.81(0.02)	6.29(0.05)	5.79(0.02)	6.28(0.04)	5.80(0.02)
3	<100	5.81(0.02)	<100	5.83(0.02)	6.21(0.02)	5.83(0.01)	6.23(0.03)	5.82(0.02)	6.20(0.02)	5.82(0.02)
6	<100	5.75(0.01)	<100	5.77(0.02)	6.23(0.05)	5.88(0.02)	5.97(0.14)	5.72(0.02)	5.75(0.11)	5.74(0.01)
9	<100	5.53(0.01)	<100	5.54(0.03)	6.08(0.03)	5.87(0.01)	5.69(0.06)	5.51(0.02)	4.37(0.01)	5.50(0.01)
12	<100	5.30(0.02)	<100	5.30(0.01)	5.98(0.03)	5.72(0.03)	5.31(0.10)	5.25(0.02)	4.04(0.06)	5.27(0.02)
14	<100	5.19(0.01)	<100	5.23(0.04)	5.76(0.03)	5.65(0.01)	5.48(0.05)	5.20(0.01)	4.11(0.10)	5.22(0.01)

[a]Values reported are means of duplicate determinations from each of two replicate trials. Values within parentheses represent standard error.

these fermentations showed that the bacterium was inhibited by the bacteriocin-producing culture JD1-23 even in the absence of adequate acid production, indicating that bacteriocin production occurred independently of carbohydrate fermentation. At the end of the fermentation period, numbers of *L. monocytogenes* in sausage fermented with JD1-23 were decreased an additional 1.2 log_{10} CFU/g as compared to numbers of *L. monocytogenes* in sausage fermented with MP1-08, similar to results seen in study 2, where acid production occurred. The pH of sausages in study 1, including those without added *L. monocytogenes*, did not fall below pH 5.5; however, the pH of JD1-23 sausage was slightly higher than that of MP1-08 sausage, so the additional reduction of *L. monocytogenes* in JD/Scott A sausage was not due to a pH effect.

Following the "failed" fermentations of study 1, tests were conducted in beakers to determine the cause of the starter culture inhibition. Testing of the individual sausage ingredients implicated the spice extract premix. The oil-based spice extract had been stored for a long period of time; by-products of hydrolytic or oxidative rancidity may have inhibited the starter cultures. When the spice extract was replaced with natural spices in study 2, sausage pH of ca. 5.2 was achieved within 14 h of fermentation in those treatments containing starter culture.

In beaker sausage prepared without starter culture and held at 32.2°C for 16 h, Glass and Doyle *(8)* observed a 2 log_{10} CFU/g increase in *L. monocytogenes* from an initial level of approximately 5 x 10^3 CFU/g. In our studies, no growth of *L. monocytogenes* from an initial level of 10^6 CFU/g was observed in treatments in which starter culture was omitted; in fact, numbers of *L. monocytogenes* in these treatments decreased slightly during the fermentation period.

The finished sausages had an average of 19.3% protein and 20.3% fat. Moisture content ranged from 50.0 to 55.9%, with an average moisture content of 54.6%. Mean sodium chloride content was 3.8%, with a range of 2.9 to 5.5%; mean sodium nitrite content was 5.5 ppm, with a range of 3.7 to 7.4 ppm.

No *L. monocytogenes* were recovered by direct plating onto MLA of sausages subjected to the entire heat treatment; nine of 90 total post-cook and storage samples (including duplicates) were found to be positive for *L. monocytogenes* only after enrichment. (No post-cook or storage sausages of the first replicate trial of study 1 were examined for the presence of *L. monocytogenes* beyond the fermentation period.) One of the nine positives occurred in one duplicate of JD sausage of study 1, indicating that the meat used to manufacture the sausage may have been contaminated with *L. monocytogenes*. The remaining eight positive sausages occurred among all three treatments inoculated with *L. monocytogenes*, with no association with sausage treatment. However, seven of the nine positives obtained occurred in stored sausages, indicating that the storage time may have allowed for the recovery of heat-injured cells.

The intermittent recovery of *L. monocytogenes* from the cooked sausages suggests that the heating to an inter-

nal temperature of 64.4°C (148°F) may be a borderline treatment for the destruction of the bacterium in this diameter and type of sausage; however, it is recognized that very high numbers of *L. monocytogenes* were present in the sausage following fermentation and before heating. Glass and Doyle (*8*) isolated *L. monocytogenes* sporadically during the drying process of pepperoni that was first heated to an internal temperature of 51.7°C (125°F) for 4 h. The same workers found that treating pepperoni by the same heating schedule following the drying cycle eliminated viable *L. monocytogenes* from the product. Given the wide variety of types and diameters of both fermented and nonfermented sausages, more research on effective heat treatments targeted for *L. monocytogenes* in these products is necessary.

Because *L. monocytogenes* is now known to occur commonly in meats and meat products, more research is needed to develop measures to eliminate the organism from these foods. Results of this work show that the use of bacteriocin-producing starter cultures is a novel means of reducing the populations of *L. monocytogenes* in fermented sausage. In this study, sausage meat was inoculated at high levels of *L. monocytogenes* (10^6 cells/g) in order to evaluate the effect of the bacteriocin on the organism. Had initial inoculum levels been similar to those reported to occur in meats (*5*), it is possible that *L. monocytogenes* may have been eliminated from the sausage during fermentation, although this hypothesis remains to be tested. Use of starter culture organisms that produce bacteriocins have potential applications in fermented vegetable and dairy products as well as meats. In addition to their primary functions of improving the keeping quality and enhancing the flavor and texture of foods, bacteriocin-producing starter cultures could provide a natural and specific preservative effect targeted at particular pathogenic or spoilage organisms of concern.

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