Journal of Food Protection, Vol. 71, No. 8, 2008, Pages 1550–1555

Survival of *Listeria monocytogenes* Strains in a Dry Sausage Model

RIINA TOLVANEN,[1] SANNA HELLSTRÖM,[1]* DIETER ELSSER,[2] HEIKE MORGENSTERN,[2] JOHANNA BJÖRKROTH,[1] AND HANNU KORKEALA[1]

[1]*Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, P.O. Box 66, FIN-00014 University of Helsinki, Finland; and* [2]*Food Protection PD, Danisco A/S, Edwin Rahrs Vej 38, DK-8220 Brabrand, Denmark*

MS 07-273: Received 22 May 2007/Accepted 22 February 2008

ABSTRACT

The survival of five inoculated *Listeria monocytogenes* strains (DCS 31, DCS 184, AT3E, HT4E, and HR5E) was studied in dry fermented sausages prepared using two different starter cultures (starter A and B) with or without a protective *Lactobacillus plantarum* DDEN 2205 strain. *L. monocytogenes* was detected throughout ripening in every sausage sample in which the *L. plantarum* DDEN 2205 strain had not been used. The use of either starter A, with a high concentration of protective culture, or starter B, with a low concentration of protective culture, resulted in *L. monocytogenes*–negative sausages after 17 days of ripening. Differential survival was noted among the *L. monocytogenes* strains during fermentation. Strains AT3E and DCS 31 survived in sausages with protective cultures more often than did the other strains, whereas HT4E and HR5E were inhibited during ripening by all starter and protective cultures used. Protective cultures such as *L. plantarum* may be used as part of a hurdle strategy in dry sausage processing, but variations in susceptibility of different *L. monocytogenes* strains can create problems if other hurdles are not included.

Raw meat and a wide variety of meat products, including fermented sausages, can be contaminated with *Listeria monocytogenes (11, 14, 18). L. monocytogenes* has caused several outbreaks linked to such meat products as rillettes, pâté, and jellied pork tongue *(12, 21, 22).* Clinical listeriosis cases have not been linked to consumption of fermented sausage, but *L. monocytogenes* is known to survive and has frequently been detected in these products *(11, 14, 17).* Unlike many other food pathogens, *L. monocytogenes* infection has a high mortality rate of 20 to 30%, causing concern in the food processing industry *(18, 20).* The infective dose of *L. monocytogenes* to a susceptible population is not known. Therefore, in some countries such as the United States, a zero tolerance of *L. monocytogenes* in ready-to-eat foods has been implemented *(29).* According to European Union regulations, concentration of *L. monocytogenes* must not exceed 100 CFU/g in ready-to-eat foods during their shelf life *(2).* The food industry has been seeking effective control strategies to prevent or decelerate the growth of *L. monocytogenes* in food products to meet the legal regulations, avoid economical losses, and ensure safe products for consumers.

The safety of dry fermented sausage is based on the presence of such factors as nitrite, low water activity (a_w), and low pH, which are often used together as part of the hurdle concept of food safety *(26).* However, these hurdles are insufficient to prevent the survival of *L. monocytogenes* in dry fermented sausages *(14, 18).* The possibility of *L. monocytogenes* survival and growth during the ripening and storage of dry sausage has created the need to develop new processes to enhance the inactivation of *L. monocytogenes.* Bacteriocinogenic lactic acid bacterial strains offer an appealing addition to the antilisterial hurdles.

Lactic acid bacteria can be used as protective cultures in commercial products *(7).* Bacteriocinogenic lactic acid bacteria have been studied as bioprotective agents in food products *(25, 32).* These bioprotective cultures may be used as starter cultures in the food fermentation process or may be added to protect foods without affecting sensory qualities *(33).* Lactic acid bacteria can produce a variety of antilisterial bacteriocins *(6, 8).* Many of these bacteriocins are class IIa bacteriocins, i.e., pediocin-like bacteriocins, which are small ribosomally synthesized peptides that permeabilize the cell membrane *(10).* Pediocin AcH produced by *Pediococcus acidilactici, Pediococcus parvulus, Lactobacillus plantarum,* and *Lactococcus lactis* is one of the bacteriocins that has been effective against *L. monocytogenes (9, 15)* and can reduce the growth of *L. monocytogenes* in dry fermented sausage *(19, 32).*

The inhibitory effect of bacteriocins on the growth of *L. monocytogenes* is considered greater than the effect of reduced pH alone *(6, 25).* In dry and semidry sausages, utilization of bacteriocinogenic strains as fermenting agents has resulted in 1- to 3-log reductions in *L. monocytogenes* counts per gram compared with control sausages *(6, 19, 32).* Temperature and acidity during the fermentation of dry fermented sausage are optimal for the production of some bacteriocins, such as sakacin K *(27),* but may also inhibit bacteriocin production by other strains *(5). L. plantarum* WHE 92 can produce pediocin AcH even at a pH exceeding 5.0 *(15).* Several properties of foods, e.g., ingredients, pH,

* Author for correspondence. Tel: +358-9-191 57134; Fax: +358-9-191 57170; E-mail: sanna.hellstrom@helsinki.fi.

carbohydrate content, temperature, and salt content, can affect the growth of bacteriocinogenic strains and the production of bacteriocins *(5, 35).* The inoculation level and the lactic acid bacteria chosen are critical factors in successful biopreservation *(8).*

L. monocytogenes strains have naturally existing variability in resistance to class IIa bacteriocins, including pediocin AcH *(15, 16, 34),* and thus may pose a problem for use of these bacteriocins in the food industry *(15, 28, 34). L. monocytogenes* strains isolated from sausage or sausage industry environment seem to survive better in fermented sausage than do strains from other sources *(31).* Thus, some strains may be harder to eliminate from dry sausages, and an optimal hurdle combination is needed to ensure safe products. This study was conducted to observe the survival of different meat-derived *L. monocytogenes* strains in dry fermented sausage made with two different starter cultures and with or without a pediocin AcH–producing culture of *L. plantarum* DDEN 2205.

MATERIALS AND METHODS

Bacterial strains and preparation of *L. monocytogenes* inoculant. Two starter cultures were used. Starter A (Imperial Meat Products, Lovendegem, Belgium) contained *Kocuria varians, Lactobacillus sakei, Pediococcus pentosaceus, Staphylococcus carnosus,* and *Staphylococcus xylosus,* and starter B (Danisco, Niebüll, Germany) contained *Lactobacillus curvatus, L. lactis, S. carnosus,* and *S. xylosus.* The protective strain was *L. plantarum* DDEN 2205 (designated previously as WHE 92 *(15)*). Concentrations of starter cultures were 10^6 CFU/g, and the protective strain was used at two different concentrations.

L. monocytogenes strains DCS 31 and DCS 184 (Danisco) and AT3E, HT4E, and HR5E (Department of Food and Environmental Hygiene, University of Helsinki, Helsinki, Finland) were all originally isolated from meat. All *L. monocytogenes* strains were tested for their pediocin resistance by the well diffusion test *(30).* All strains were sensitive to pediocin AcH and had an inhibition zone diameter of 7 to 9 mm.

L. monocytogenes strains were incubated in brain heart infusion (BHI) broth (Difco, Becton Dickinson, Sparks, Md.) for 18 h at 37°C. Concentrations were measured by optical densities, and equal amounts of each strain were inoculated into 10 ml of a 0.1% peptone plus 0.85% saline solution to yield a final *L. monocytogenes* concentration of 10^3 CFU/g in the sausage.

Determination of inhibition potential. The inhibitory activity of *L. plantarum* DDEN 2205 was evaluated using *Listeria innocua* DSMZ 20649 as an indicator strain and nisin (Nisaplin, Danisco Beaminster Ltd., Beaminster, UK) as a reference. A preculture of the indicator strain was prepared by inoculating 50 ml of lactic broth *(13)* with a frozen stock sample of *L. innocua* DSMZ 20649 and incubating overnight at 37°C. For the main culture, 1 ml of the preculture was used to inoculate 50 ml of lactic broth, and the culture was grown overnight at 37°C. The cell count of the main culture was determined by plate counting onto lactic agar (heipha Dr Müller GmbH, Eppelheim, Germany) after incubation for 23 h at 37°C. The main culture was diluted to a cell count of 2.50 to 5.00 CFU/ml with diluting solution (0.0425 mg/liter potassium dihydrogen phosphate), and 1 ml of the diluted culture was added to 50 ml of lactic broth for use in the bioassay. The nisin standard was prepared by adjusting to a concentration of 1.000 IU/ml with diluting solution. The dried culture containing *L. plantarum* DDEN 2205 was diluted 100-fold with diluting solution, and 10 ml was distributed into each sterile tube and heated for 20 min at 80°C in a water bath (Memmert GmbH & Co. KG, Schwabach, Germany) to inactivate living cells. After the samples were cooled to room temperature, the sterility was determined on lactic agar after incubation 23 h at 37°C. A 96-well microplate (Nunc GmbH & Co. KG, Wiesbaden, Germany) was prepared by adding 100 μl of lactic broth to each well. In the first well of the plate, 200 μl of the nisin standard was added to the 100 μl of lactic broth. After mixing, 200 μl was removed and added to the next well. The procedure is repeated until the end of the row, and the 200 μl removed from the last well was discarded. The same procedure was used for the diluted sample of *L. plantarum* DDEN 2205. All samples were tested in duplicate. The indicator strain solution (100 μl) was added to each well and mixed, and the microplate was placed in a reader (Tecan, Crailsheim, Germany) preheated to 37°C. Growth was measured over 23 h at 37°C by determination of the optical density at 492 nm. After incubation, the growth curves of the strain samples were compared with those of the Nisin standard, where no clear inhibition is visible. The curve that was the best match to the Nisin standard curve with no clear inhibition was used to represent the concentration in each well. The unknown concentration of the sample was then calculated back by the dilution factor in the well. A value of 1 IP (inhibition potential) corresponded to 10^7 IU of the nisin standard. The concentration of *L. plantarum* was 10^6 CFU/g and 1 IP corresponded to 10 g of culture.

Preparation of dry sausage. Ingredients in the 15-kg batch of dry sausage were 5 kg of frozen lean pork, 5 kg of frozen pork back fat, 5 kg of lean pork shoulder without rind (not frozen), 2.8% salt, 170 ppm of sodium nitrite, 1.1% milk protein, 0.7% dextrose, 0.35% spice mix (pepper, nutmeg, and garlic), and 0.04% sodium ascorbate.

All ingredients, starters, protective cultures, and *L. monocytogenes* were mixed in a bowl cutter, vacuum packed, and stored in a freezer for about 2 h before being stuffed into fibrous collagen casings (Naturin, Weinheim, Germany). After stuffing, the sausages were fermented and ripened according to the following processing parameters: the sausages were stored at 24°C and 90% relative humidity for 72 h. On the first 2 days (at 24 and 48 h), the sausages were smoked for 1 h. After 72 h, the sausages were dried at 14°C and 75% relative humidity for 25 days to obtain the final product on day 28. The experiment was conducted twice.

Sampling. Sausages were weighed after stuffing (day 0) and on days 3, 7, 10, 14, 17, and 21, and pH was measured at 0, 24, 48, and 63 or 70 h after stuffing. Triplicate samples of each sausage batch with different starter cultures were taken at each sampling time. The experiment was repeated, yielding a total of six samples of each sausage batch at each sampling time. For microbiological analyses, samples were taken after 4 h and on days 2, 7, 17, and 28 of ripening. Samples of control sausages without *L. plantarum* DDEN 2205 were taken immediately after stuffing. Samples for enrichment of *L. monocytogenes* also were taken from raw materials.

Microbiological analyses. *L. monocytogenes* was enumerated with the nine-tube most-probable-number (MPN) method, i.e., for three replicate samples of three tubes in both experiments. For MPN analyses, 10 g of meat from the middle of a dry sausage was mixed with 90 ml of peptone saline and blended with a laboratory blender (Stomacher 400, Seward Medical, London, UK) for 1 min. Further decimal dilutions were made to obtain samples of 1, 0.1, 0.01, 0.001, and 0.0001 g. Samples were enriched by a

TABLE 1. Listeria monocytogenes *populations in dry sausage at different sampling times*

Starter culture[a]	Expt	*L. monocytogenes* population (95% confidence interval) (MPN/g) at:					
		0 h	4 h	2 days	7 days	17 days	28 days
A + 0	1	860 (380–1,900)	520 (220–1,200)	93 (42–210)	93 (42–210)	19 (9.4–40)	12 (5.7–26)
	2	340 (160–740)	430 (190–970)	130 (61–280)	110 (51–240)	55 (23–130)	14 (6.9–30)
A + 2.5	1	NA[b]	19 (9.2–39)	0.5 (0.2–1.3)	0.2 (0.1–0.9)	0.1 (0.01–0.8)	<0.1[c]
	2	NA	23 (11–47)	1.4 (0.7–2.9)	1.6 (0.8–3.3)	0.4 (0.1–1.1)	0.1 (0.01–0.8)
A + 10	1	NA	2.2 (0.6–8.9)	<0.1	0.1 (0.01–0.8)	ND[d]	ND
	2	NA	9.3 (4.2–21)	0.1 (0.01–0.8)	ND	ND	ND
B + 2.5	1	NA	6.9 (2.9–17)	ND	<0.1	ND	ND
	2	NA	1.9 (0.92–3.9)	0.2 (0.06–0.9)	0.1 (0.01–0.8)	ND	ND

[a] Starter cultures A and B were combined with *Lactobacillus plantarum* DDEN 2205 as an inhibitor at inhibitory potentials of 0, 2.5, or 10.

[b] NA, not analyzed.

[c] Values of <0.1 indicate that *L. monocytogenes* was not detected in the MPN analyses but was detected after enrichment of 25 g of sausage sample.

[d] ND, not detected after enrichment of 25 g of sausage sample.

two-step enrichment process. The first enrichment was done in half-Fraser broth (Oxoid, Basingstoke, UK) for 24 h at 30°C, and the secondary enrichment was done in Fraser broth (Oxoid) for 48 h at 37°C. To determine the MPN, three consecutive dilutions were used. Cultures were plated on two *L. monocytogenes*–selective plates, PALCAM (Oxoid) and *L. monocytogenes* blood agar (LMBA; LAB M, Bury, UK), and incubated for 48 h at 37°C. For confirmation of *L. monocytogenes,* five typical colonies from two or three selective plates at each sampling time were streaked onto sheep blood agar plates and incubated for 24 h at 37°C. Catalase-positive, gram-positive rods that produced hemolysis on sheep blood agar were considered to be *L. monocytogenes.*

Qualitative analysis for *L. monocytogenes* was conducted for sausage ingredients (meats and fat) and samples that gave negative results in the MPN analysis. Enrichment was performed according to the ISO standard *(1)* except an LMBA plate was used instead of an Oxford plate. Samples (25 g) of dry sausage were mixed with 225 ml of half-Fraser broth with a stomacher for 1 min and incubated for 24 h at 30°C. Secondary enrichment was performed by transferring 0.1 ml of half-Fraser broth into Fraser broth and incubating for 48 h at 37°C. Cultures were then streaked onto selective agar plates. Confirmation of *L. monocytogenes* was performed as in the MPN analyses. During the first experiment, 10 confirmed isolates from sheep blood agar plates were genotyped for each sampling. In the second experiment, 10 isolates were genotyped from 10 10-g samples from the last samples in which *L. monocytogenes* was detected.

PFGE. Pulsed-field gel electrophoresis (PFGE) was used to identify the *L. monocytogenes* isolates obtained from the sausages. In situ DNA isolation and PFGE were performed as described by Autio et al. *(3, 4)* except using Pronase (Roche Diagnostics GmbH, Mannheim, Germany) instead of proteinase K. *Asc*I (New England Biolabs, Beverly, Mass.) was used for restriction endonuclease digestion. The samples were electrophoresed through a 1.0% (wt/vol) agarose gel (SeaKem Gold, FMC Bioproducts, Rockland, Maine) in 0.5× Tris-borate-EDTA (45 mM Tris, 4.5 mM boric acid, pH 8.3, and 1 mM sodium EDTA) at 200 V and 14°C in a Gene Navigator system with a hexagonal electrode (Pharmacia, Uppsala, Sweden). The pulse times ramped from 1 to 35 s over 18 h. The gels were stained with ethidium bromide and photographed under UV transillumination. A low-range pulsed-field gel marker (New England Biolabs) was used for fragment size determination. PFGE profiles of the strains were compared using BioNumerics software (Applied Maths, Austin, Tex.).

RESULTS

Weight loss and pH changes. Weight losses over 21 days of ripening were 200 to 240 g (27 to 32%). The pH values of the sausages decreased from 5.6 to 5.7 initially to 4.7 to 5.0 after 48 h of fermentation and 4.5 to 4.7 after 63 to 70 h.

Survival of *L. monocytogenes*. *L. monocytogenes* was detected at the end of ripening in sausages without *L. plantarum* DDEN 2205 at 12 to 14 MPN/g and in sausages with starter A plus a low level (2.5 IP) of *L. plantarum* DDEN 2205 at <0.1 to 0.1 MPN/g (Table 1). The other two sausage treatments were *Listeria* free after 17 and 28 days of ripening. All sausages with *L. plantarum* DDEN 2205 contained less than 100 MPN/g at all sampling times.

Differences in survival between *L. monocytogenes* strains. The PFGE pulsotypes of five inoculated *L. monocytogenes* strains and one natural contaminant are presented in Figure 1. Survival of the different *L. monocytogenes* strains during ripening of sausages is presented in Table 2. Strain HT4E was not detected in any of the samples, and HR5E was detected only in samples from sausages without *L. plantarum* DDEN 2205. The other three inoculated strains were detected in samples from several sausages, and strain AT3E was detected in all sausages at some point during ripening. Raw material was contaminated with a *L. monocytogenes* strain before inoculation, and the same strain also was detected in all sausages expect the one with no *L. plantarum* DDEN 2205. PFGE revealed that the natural contaminant was the same genotype in all raw materials and sausages.

DISCUSSION

The *L. monocytogenes* population decreased throughout the ripening and reached <100 MPN/g at 17 days in

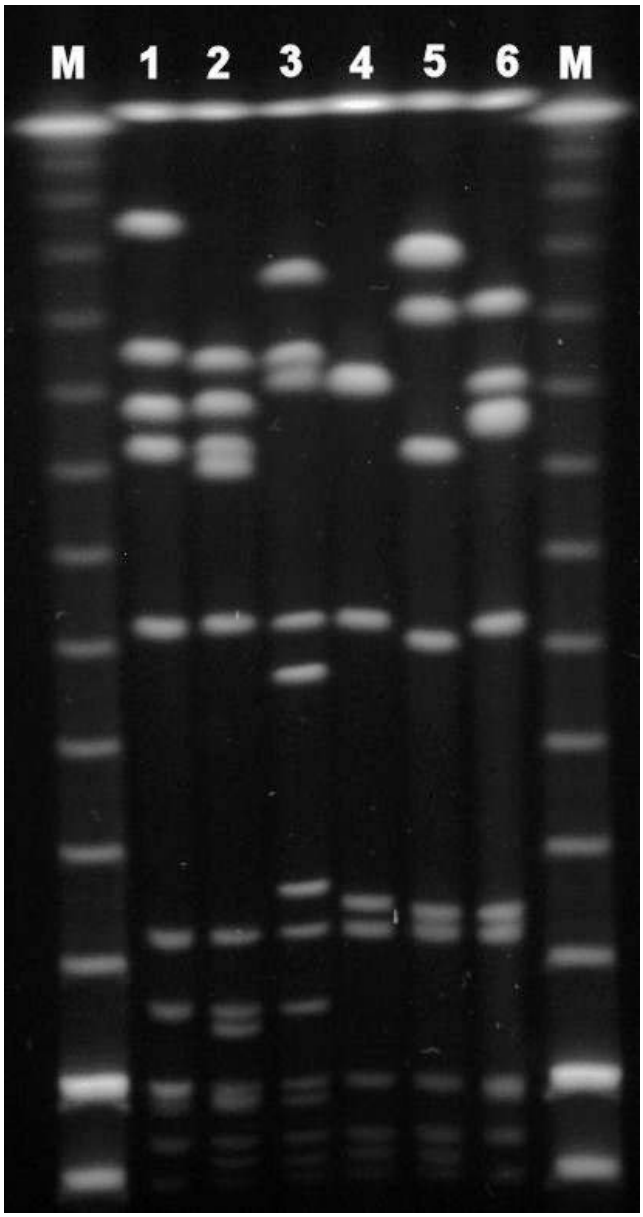

FIGURE 1. *PFGE profiles of five inoculated* Listeria monocytogenes *strains and the natural contaminant. M, molecular weight marker (Low Range PFG Marker, New England Biolabs); lanes 1 through 5, inoculated strains (AT3E, HR5E, HT4E, DCS31, and DCS148, respectively); lane 6, natural contaminant.*

all sausages; this level conforms with the EU regulation for ready-to-eat foods such as dry sausage during their shelf life *(2).* The decrease in pH during ripening was not alone sufficient to eliminate *Listeria*; this bacterium is capable of multiplying in pH values of 4.0 to 9.6 *(24),* but other hurdles, such as low a_w and pediocin production by *L. plantarum* DDEN 2205, inactivated the bacterium. Weight loss during ripening should have increased the salt concentration and reduced the a_w. *L. monocytogenes* was detected in higher levels in sausages without pediocin-producing *L. plantarum* DDEN 2205 than in sausages with this protective strain. In sausages with starter A, *L. monocytogenes* decreased more rapidly when the concentration of the protective culture was higher. Starter A with lower concentrations of protective culture did not produce *L. monocytogenes*–negative sausages after 28 days of ripening, but starter B with the same concentration of protective culture produced *L. monocytogenes*–negative sausages after 2 days of ripening. Starter cultures contained different lactic acid bacteria, possibly explaining the difference in survival of *L. monocytogenes.* Conditions during fermentation and ripening can adversely affect the production of bacteriocin. *L. plantarum* WHE 92 produces pediocin consistently in culture broth between pH 4.0 and 6.0 *(15).* In our study, pH of the sausages was 4.7 to 5.0 after 48 h of fermentation. According to these results, starter A with the high concentration of protective culture DDEN 2205 and starter B with the low concentration of DDEN 2205 seem to be effective combinations for eliminating *L. monocytogenes* from dry sausages during ripening.

The elimination of *L. monocytogenes* during sausage ripening differed among strains. Strains HT4E and HR5E were inhibited in all sausages with the protective *L. plantarum* strain, and strain HT4E was not detected in any of the sausages. Strain AT3E was detected in all sausages at some point of ripening, and strains DCS148 and DCS31 were detected in all sausages other than that with the highest concentration of protective culture. Overall, strains DCS31 and AT3E were detected more often than other strains. These results clearly support the hypothesis that some *L. monocytogenes* strains are harder to eliminate from dry sausages. Thévenot et al. *(31)* found differences in survival of *Listeria* strains on dry sausage, but they compared strains from sausage and sausage-making environments to strains isolated from other sources. In our study, all strains were isolated originally from meats. This strain-specific adaptation to conditions during ripening needs more study. Differences in survival of *L. monocytogenes* strains in dry sausage stress the need to use several strains in inoculation studies. The hurdle concept for dry sausage making should be effective against the most resistant strains of *L. monocytogenes.*

The natural *L. monocytogenes* contaminant was isolated in all sausages expect those without pediocin-producing protective culture. This strain probably was more resistant to pediocin than were the inoculated strains, and it was a better competitor in sausages with *L. plantarum* DDEN 2205 than were the other *L. monocytogenes* strains. *L. monocytogenes* strains differ in their susceptibility to class IIa bacteriocins in vitro *(14, 15, 23).* Observed survival of the natural contaminant indicates that raw material may contain strains, possibly originating from meat-processing environments, that are well adapted to fermentation conditions.

Protective starter cultures such as *L. plantarum* provide an appealing hurdle in dry sausage processing and assist in elimination of *L. monocytogenes* contamination. Possible variations in susceptibility and development of resistance in *L. monocytogenes* strains can create problems when other hurdles are not used. Starter cultures with different bacteriocinogenic strains or competitive strains might prevent the development of resistant strains of *L. monocytogenes.*

TABLE 2. *Survival of different* Listeria monocytogenes *strains during sausage ripening*

Starter culture[a]	Expt	Day of ripening	No. of collected strains	No. of detected strains:					
				DCS148	DCS31	AT3E	HR5E	HT4E	Other[b]
A + 0	1	7	10	5			5		
	1	17	10		3	6	1		
	1	28	10		7	3			
	2	28	10			10			
A + 2.5	1	7	10	5	5				
	1	17	10		10				
	1	28	10						10
	2	28	10	1	1	8			
A + 10	1	7[c]	10			10			
	2	2[c]	1						1
B + 2.5	1	7[c]	10	5	2				3
	2	7[c]	6	2	2	2			

[a] Starter cultures A and B were combined with *Lactobacillus plantarum* DDEN 2205 as an inhibitor at inhibitory potentials of 0, 2.5, or 10.

[b] *L. monocytogenes* strain from raw materials. All the isolates had similar PFGE pulsotypes.

[c] *L. monocytogenes* was not detected at later sampling times.

ACKNOWLEDGMENTS

The authors thank Kirsi Ristkari, Heimo Tasanen, and Hans Van Laerebeke for excellent technical assistance.

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