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Research Note

Antagonistic activity of lactic acid bacteria against *Listeria monocytogenes* in sliced cooked cured pork shoulder stored under vacuum or modified atmosphere at $4 \pm 2^{\circ}C$

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Abstract

Lactic acid bacteria (LAB) strains, isolated from dry fermented sausages were tested as protective cultures in vacuum- or modified atmosphere-packaged sliced cooked cured pork shoulder at 4°C. The inhibitory capacity of the bacteriocins produced by *Leuconostoc mesenteroides* L124 and *Lactobacillus curvatus* L442 against different strains of *Listeria monocytogenes* and *L. innocua* were also studied. In samples inoculated with the LAB, the listeriae population decreased by about $1.5 \log_{10}$ cfu g^{-1}. Addition of bacteriocins in samples reduced the listeriae population below the enumeration limit (10^2 cfu g^{-1}). Similar results were obtained with samples stored in modified atmosphere. Acetate concentration was increased in all samples. Thus, these lactic acid strains may be used as protective cultures to inhibit growth of *L. monocytogenes* or their bacteriocins in meat.

Keywords: Lactic acid bacteria; Protective cultures; Antagonistic activity; *Listeria monocytogenes*; Vacuum; Modified atmosphere; Pork shoulder

1. Introduction

Sliced cooked cured pork shoulder is a meat product sensitive to spoilage. The low salt content (2.0% in water phase), a pH value above 6.0 and a water activity higher than 0.95 are only small hurdles in terms of inhibiting the growth of psychrotrophic pathogens like *Listeria monocytogenes*. After cooking, the normal flora of the product, consisting of LAB, is too low to protect the products against the growth of pathogens (Bredholt et al., 1999). *L. monocytogenes* and other *Listeria* spp., mainly *L. innocua*, contaminate most areas where raw materials are being processed and colonize certain equipment which are not made from stainless steel or are not disinfected properly. Ham and ham-like products are contaminated to a great extent during tumbling since the need to operate tumblers continuously does not enable their proper cleaning and disinfection on a daily basis (Samelis and Metaxopoulos, 1999). Also, recent studies have shown that *L. monocytogenes* can survive the combination of low pH, low water activity, sodium chloride and sodium nitrate associated with meat drying and fermentation (Johnson et al., 1988; Glass and Doyle, 1989). Its ability to proliferate at refrigeration temperatures contributes significantly to its hazard status (Vignolo et al., 1996).

The presence of *L. monocytogenes* in meat products is a particular food safety concern. Novel strategies such as 'biopreservation' have gained increasing attention as a means of 'naturally' controlling the growth of pathogenic and spoilage organisms. Some LAB among those commonly associated with meats produce antimicrobial peptides such as bacteriocins (Garriga et al., 1993; Vignolo et al., 1996). LAB naturally dominate the microflora of many foods including raw meats and meat products that are chill-stored under vacuum or in an environment enriched with CO_2 and their use as protective cultures can increase the safety of food products (Holzapfel et al., 1995; Stiles, 1996).

Consequently, the aim of the present study was to investigate the effect of two bacteriocinogenic LAB strains, *Leuconostoc mesenteroides* L124 and *Lactobacillus curvatus* L442 and their bacteriocins, on

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L. monocytogenes growth, with the intention to use them as protective cultures.

2. Materials and methods

2.1. Antibacterial activity, partial purification and assay

The antibacterial activity of the two bacteriocins was assayed by the Agar Well Diffusion Assay (AWDA) described by Schillinger and Lücke (1989). The two bacteriocins were screened against a range of 15 *Listeria* spp. indicator strains (growth in BHI broth at 30°C for 24 h). A 50 μl of supernatant fluids were spotted on BHI agar (MERCK) plates containing the 'indicator' strains (1% inoculum) and were incubated at 30°C for 24 h. Inhibition was recorded as negative if no zone was observed around the agar well. The supernatant was prepared by centrifugation of a 18 h culture (at 30°C) of *Ln. mesenteroides* L124 and *L. curvatus* L442 in MRS broth (MERCK) at 10,844*g* for 15 min at 4°C (Heraeus Sepatech, Biofuge 22R), adjustment to pH 6.5 and treatment with catalase (300 AU ml^{-1}) (C-3515, Sigma) to exclude the antimicrobial effect of organic acids and hydrogen peroxide and sterilization through a microbiological filter (Acrodisc, 0.22 μm) (Gelman).

For the partial purification the supernatants (500 ml) obtained from the two LAB cultures strains were precipitated with ammonium sulphate (SERVA) [18 h (overnight) at 4°C] (Schobitz et al., 1999). After precipitation a formation of pellet and pellicle was observed and they were collected after precipitation and by centrifugation (10,844*g* for 30 min at 4°C), respectively, and together resuspended in 20 ml of 50 mM sodium phosphate buffer pH 7. The concentrated bacteriocins were diluted with the same buffer to 1:2 and 1:4 for the bacteriocins of *Ln. mesenteroides* L124 and *L. curvatus* L442, respectively, to give the same activity, 1280 AU ml^{-1} and sterilized through a microbiological filter (Acrodisc, 0.22 μm).

To check bacteriocin activity after the partial purification, twofold serial dilutions of the extracts were made in sterile 1/4 strength Ringer's solution. Then 50 μl of the diluted samples were spotted on MRS agar (MERCK) plates containing the indicator strain (*L. curvatus* L267 (1% inoculum). After incubation (30°C for 24 h) the arbitrary units of activity (AU) of the bacteriocin were determined as the reciprocal of the highest dilution showing inhibition of the indicator strain (Barefoot and Klaenhammer, 1983).

2.2. Meat products, bacterial cultures, inoculation and storage.

The product studied was sliced cooked cured pork shoulder. It was produced by an industrial meat company and on the day of its production, transferred to the laboratory. The antagonistic effect between two strains of LAB or their bacteriocins [*Ln. mesenteroides* L124 (ID3985) and *L. curvatus* L442 (ID3986) as they were identified by BCCM™/LMG] and *Listeria* sp., was studied. *Ln. mesenteroides* L153 was used as a negative control due to its inability to produce bacteriocin (bac^-). *Listeria innocua* 7510 S1 was used for the above experiments, instead of *L. monocytogenes* since the two micro-organisms show similar physiological properties with the difference that the former does not belong to the pathogen species of *Listeria* and the results of the sensitivity of some *Listeria* species (see Section 3) against the two bacteriocins, generally, indicated a greater sensitivity of *L. monocytogenes*. All species were isolated from different meat products and kept frozen at –20°C in MRS or BHI broth (MERCK), supplemented with 20% glycerol.

The organisms were subcultured twice (24 h, 30°C) in 10 ml MRS broth or BHI broth, 1% inoculum. The cells were harvested by centrifugation (10,844*g* for 30 min at 4°C), washed twice, resuspended in 10 ml of 50 mM sodium phosphate buffer pH 7 and diluted with sterile 1/4-strength Ringer's solution (OXOID). One millilitre of the bacteriocin solution (1280 AU ml^{-1}) or the bacterial suspension (10^5 and 10^3 cfu ml^{-1} for the LAB and *L. innocua*, respectively) was used on each surface (10 cm × 10 cm, 25 g) of slice with a pipette. After assuring good contact of the inoculum with the meat surface (manually massaging of the exterior of the bags), the samples were vacuum or modified atmosphere (80% CO_2 + 20% N_2) packaged with a packaging machine (Henkovac 1900, The Netherlands) and stored at 4 ± 2°C for 28 d. The packaging material used were Cryovac-type bags with low oxygen permeability (35 $cm^3\,m^{-2}\,d^{-1}$ at 22°C, 65% r.h.). One package from each condition was examined at intervals of 0, 3, 7, 14, 21 and 28 d of storage for microbiological and physicochemical analysis. The experimental conditions were: (1) uninoculated slices of cooked cured pork shoulder as control, (2) *Ln. mesenteroides* L124 + *L. innocua* 7510, (3) *L. curvatus* L442 + *L. innocua* 7510, (4) *Ln. mesenteroides* L153 (bac^-) + *L. innocua* 7510 as negative control, (5) *L. innocua* 7510 as positive control, (6) bacteriocin from *Ln. mesenteroides* L124 + *L. innocua* 7510 and (7) bacteriocin from *L. curvatus* L442 + *L. innocua* 7510.

2.3. Microbiological sampling and analysis

A 25 g of sample was weighted, aseptically, into a sterile Stomacher bag (Seward Stomacher 400 bags, London, UK). Then 225 ml of sterile 0.1% (w/v) peptone-water (OXOID) were added and the sample was homogenized in a Stomacher (Lab Blender, Seward, London, UK) for 2 min at normal speed at room

temperature. Serial decimal dilutions in sterile 1/4-strength Ringer's solution (OXOID) were prepared from this 10^{-1} dilution and 0.1 ml samples of the appropriate dilutions were spread in duplicate on selective agar plates.

LAB were determined on the de Man, Rogosa, Sharpe Agar (MRS, MERCK), incubated at 30°C for 72 h under anaerobic conditions (Gas-Pack, BBL); *L. innocua* on PALCAM Agar (MERCK) and incubated at 30°C for 48 h.

The enumeration limit was at 10^2 cfu g^{-1} for LAB and *L. innocua*. The selectivity of the growth media was checked with the catalase reaction on about 10% of the colonies grown on countable plates.

2.4. pH, a_w and acetate determinations

pH was measured immersing the electrode into the Stomacher bag with the diluted sample with a pH-meter (WTW, pH 526, Germany). The water activity was measured with a calibrated electric hygrometer, Rotronic DT (Rotronic AG, Bassersdorf, Switzerland), according to the manufacturer's instructions. The preparation of the samples and acetate determination were conducted as described by Drosinos and Board (1995).

3. Results

3.1. Antibacterial activity, partial purification and assay

Table 1 shows the results of the antibacterial activity of the two LAB strains. The sensitivity of the *Listeria* 'indicator' strains was estimated based on the diameter (mm) of the inhibition zones. The two bacteriocins produced by *Ln. mesenteroides* L124 and *L. curvatus* L442 were precipitated with ammonium sulphate at 60% and 50% saturation, respectively, and their activity was 2560 and 5120 AU ml^{-1}, respectively.

3.2. Microbiological analysis

Fig. 1a shows that both LAB had an antagonistic effect on *L. innocua* growth in the pork product under vacuum conditions. At the beginning of storage (7th day) the *L. innocua* population increased, from ca. 3.5 to 3.9 $\log_{10}$ cfu g^{-1} and to 4.4 $\log_{10}$ cfu g^{-1} in the presence of *Ln. mesenteroides* L124 and *L. curvatus* L442, respectively. After this initial increase, the number of the listeriae population decreased until the end of storage, with final population numbers of 2.5 and 2.9 $\log_{10}$ cfu g^{-1}, respectively. The reduction of the listeriae population was the same (ca. 1.5 $\log_{10}$ cfu g^{-1}) for both LAB strains.

Table 1
Antimicrobial activity of the crude extract against *Listeria* sp.

Strains	Inhibition zone (mm)	
	L124[a]	L442[b]
L. innocua S1[c]	6	10
L. innocua S2	6	10
L. innocua S3	6	8
L. innocua S4	4	8
L. innocua 7510 S1	6	6
L. innocua 7510 S2	6	10
L. monocytogenes S1	6	8
L. monocytogenes S2	6	6
L. monocytogenes S3	8	10
L. monocytogenes S4	8	10
L. monocytogenes S5	8	8
L. monocytogenes S6	6	8
L. monocytogenes S7	6	6
L. monocytogenes 6510	6	8
L. monocytogenes 5310	6	6

[a] Crude extract from *Ln. mesenteroides* L124 culture.
[b] Crude extract from *L. curvatus* L442 culture.
[c] Different strains isolated from meat products and stored in the culture collection of the laboratory.

In the presence of *Ln. mesenteroides* L153 (bac^-), *L. innocua* increased by ca. 1 $\log_{10}$ cfu g^{-1}, from 3.3 to 4.0 $\log_{10}$ cfu g^{-1} at the end of storage. In the samples inoculated with *L. innocua*, the population reached 7.3, 4 $\log_{10}$ cfu g^{-1} more than the initial number. When the bacteriocins were present, listeriae population reduced by 1 $\log_{10}$ cfu g^{-1} on the 3rd day of storage and on the 14th day the population was below the enumeration limit. In the uninoculated samples, *Listeria* was not detected during the storage, reflecting the good hygiene during the production of meat product.

In the modified atmosphere packaging of pork product, the listeriae population reduced by 1 log in the inoculated samples with the bac^+ LAB strains, from ca. 3.5 to 2.3 $\log_{10}$ cfu g^{-1} at the end of storage. In presence of *Ln. mesenteroides* L153 (bac^-) and *L. innocua* 7510, listeriae population remained constant (3.3–3.6 $\log_{10}$ cfu g^{-1} and 3.1–3.5 $\log_{10}$ cfu g^{-1}, respectively). In presence of the bacteriocins, the bacterial cells of *L. innocua* were below the enumeration limit after 14 d of storage (Fig. 1b).

Both, endogenous and inoculated LAB antagonized the *L. innocua* strain very well and became the dominant flora. Even in the samples inoculated only with *L. innocua*, the endogenous microflora grew rapidly with faster growth rates. Also, the bacteriocins had an effect on the endogenous LAB growth because the growth rate was slower than that in the uninoculated samples (Fig. 2a). The results in the modified atmosphere were similar (Fig. 2b).

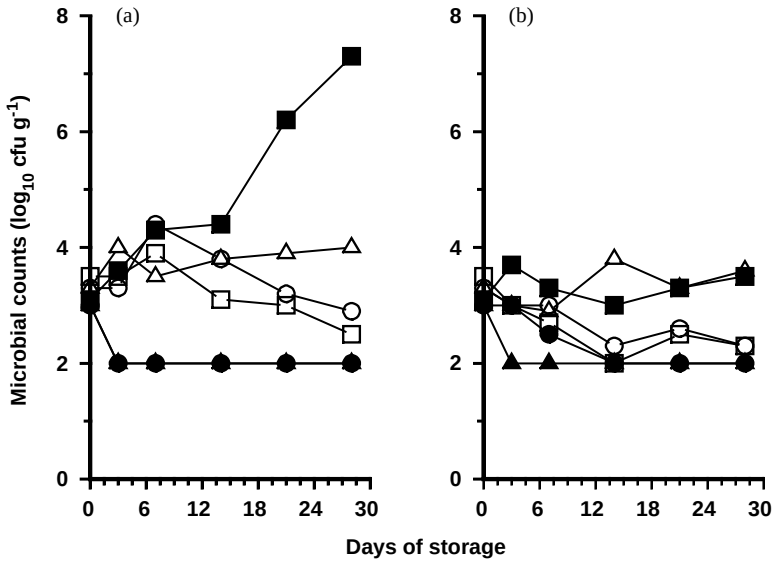

Fig. 1. Growth of listeriae population in the sliced cooked cured pork shoulder, stored under (a) vacuum or (b) MA (80% CO_2 + 20% N_2) conditions at 4±2°C, in the presence of (□) *Ln. mesenteroides* L124 (bac^+), (○) *L. curvatus* L442 (bac^+), (△) *Ln. mesenteroides* L153 (bac^-), (■) *L. innocua* 7510 only, (●) bacteriocin from *Ln. mesenteroides* L124, (▲) bacteriocin from *L. curvatus* L442.

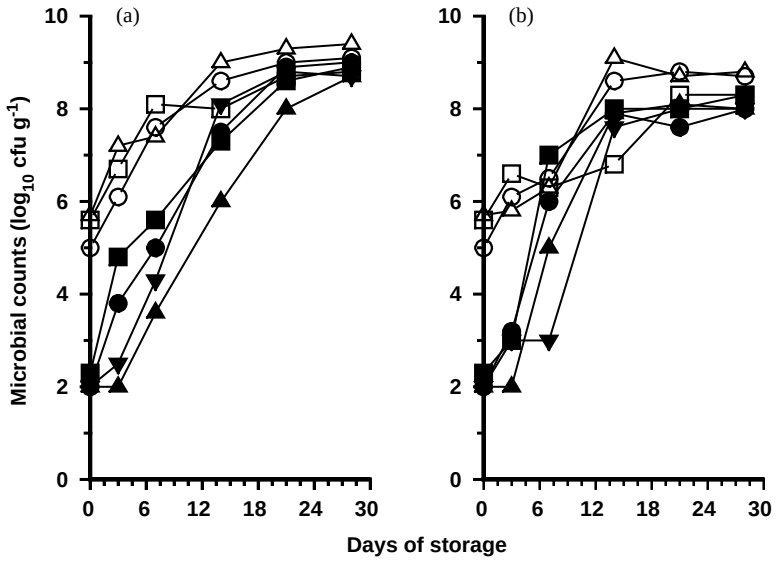

Fig. 2. Growth of endogenous LAB or added bac^+ and bac^- LAB in sliced cooked cured pork shoulder, in the presence of *L. innocua* 7510, under (a) vacuum or (b) MA (80% CO_2 + 20% N_2) conditions at 4±2°C. (□) *Ln. mesenteroides* L124 (bac^+), (○) *L. curvatus* L442 (bac^+), (△) *Ln. mesenteroides* L153 (bac^-), (■) uninoculated, (●) presence of *L. innocua* only, (▲) presence of the bacteriocin from *Ln. mesenteroides* L124, (▼) presence of the bacteriocin from *L. curvatus* L442.

3.3. *pH, a_w and acetate determinations*

Figs. 3a and b show the changes of pH during the storage under vacuum or modified atmosphere. The initial pH value of the pork product was 6.5. The final pH value in the samples inoculated with the bac^+ and bac^- LAB was similar, indicating that the production of lactic acid in the bac^- *Leuconostoc* strain was about the same as in bac^+ LAB strains. But in the samples inoculated with the listeriae strain, the pH value remained at high levels, close to 6.0, until the 21st day of storage and dropped at the end of storage to 5.4. The reduction of pH in the samples with the bacteriocins was similar to that of the uninoculated samples. The pH value remained close to 6.0 until the 14th or 21st day of storage and dropped at the end of the experiment. Similar results were obtained with the modified atmosphere packed pork product, with the exception that the reduction of pH was slower than that in the vacuum packaging. a_w remained constant (0.98) during the storage, therefore did not have a significant effect on the listeriae growth. Under vacuum or

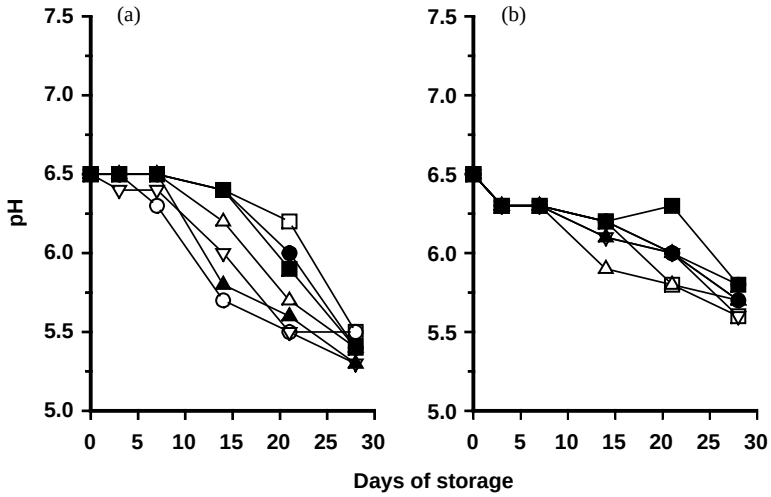

Fig. 3. Changes of pH in the sliced cooked cured pork shoulder, under (a) vacuum or (b) MA (80% CO_2 + 20% N_2) conditions at 4±2°C. (□) Uninoculated, (○) *Ln. mesenteroides* L124 (bac$^+$), (△) *L. curvatus* L442, (▽) *Ln. mesenteroides* L153 (bac$^-$), (■) presence of *L. innocua* only, (●) presence of the bacteriocin from *Ln. mesenteroides* L124, (▲) presence of the bacteriocin from *L. curvatus* L442.

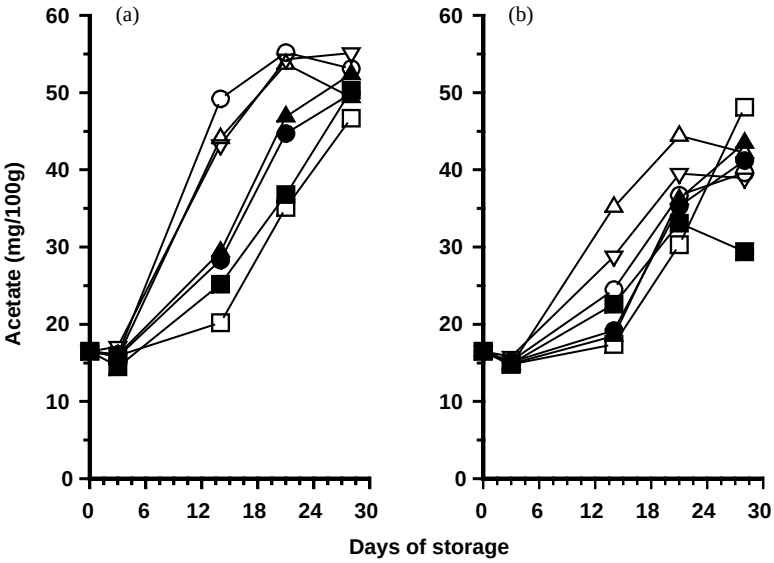

Fig. 4. Changes in acetate concentration in the sliced cooked cured pork shoulder, stored under (a) vacuum or (b) MA (80% CO_2 + 20% N_2) conditions at 4±2°C. (□) Uninoculated, (○) *Ln. mesenteroides* L124 (bac$^+$), (△) *L. curvatus* L442, (▽) *Ln. mesenteroides* L153 (bac$^-$), (■) presence of *L. innocua* only, (●) presence of the bacteriocin from *Ln. mesenteroides* L124, (▲) presence of the bacteriocin from *L. curvatus* L442.

modified atmosphere conditions, the concentration of acetate increased during the storage in all samples. The production of acetate was more pronounced when high levels of LAB counts were achieved. The production of acetate in the samples with *L. innocua* was slower than that in the samples inoculated with the bacteriocin-producing and non-bacteriocinogenic LAB (Fig. 4).

4. Discussion

Factors such as temperature, pH, a_w, nutrients and atmosphere composition constitute hurdles which play a critical role in the growth of the micro-organisms. In the present study, the results showed that *L. innocua* was able to grow at 4°C under vacuum packaging, but with slow growth rates during the first 2 weeks of storage. The minimum growth temperature for *L. monocytogenes* ranges from −0.1°C to −0.4°C and has a long generation time at refrigeration temperatures (Walker et al., 1990), though other researchers found that *L. monocytogenes* was able to grow in roast beef at –1.5°C (Hudson and Mead, 1994). Temperatures likely to be used for refrigerated storage (0–10°C) are unable to inhibit the growth of *L. monocytogenes*, but refrigerated storage will extend the time before growth occurs and reduce the rate of growth (Walker et al., 1990). *L.*

monocytogenes is well adapted to survival on equipment and in production facilities and the occurrence of this micro-organism in cooked meat products is known to be connected with cross contamination after heat treatment (Bredholt et al., 1999). Heat survivors appear to be the main cause of contamination (Samelis and Metaxopoulos, 1999). *Listeria* sp. and *L. monocytogenes* survived in ham and other tumbled meats cooked in boilers at core temperatures below 70°C and in traditional Greek country-style sausages heated to 65–68°C. In contrast, listeriae did not survive in samples heated to 72–75°C (Samelis and Metaxopoulos, 1999).

In our study, CO_2 showed a bacteriostatic effect on the listeriae growth, since the population remained constant until the end of storage. In general, atmospheres in which *L. monocytogenes* multiplication is inhibited are not bactericidal. In cases where a decrease in listeriae count was observed during storage, it was of little relevance. These data suggest that the modification of the atmosphere cannot be considered as the only factor involved in listeriae inhibition since changes in temperature, pH and even possibly competition of other flora also affect the growth (De Fernando et al., 1995).

In the present study, the results showed that the bacteriocins were able to inhibit strains of different *Listeria* species. Also, the bacteriocins or the LAB as protective cultures were able to inhibit *Listeria* sp. in deliberately contaminated pork product, with complete inhibition of the micro-organism after 14 d of storage, indicating that the bacteriocins have a bactericidal effect on the listeriae population. A similar antibacterial spectrum for other bacteriocins produced by LAB has been reported in broth or meat (Nielsen et al., 1990; Berry et al., 1991; Vignolo et al., 1996; Bredholt et al., 1999). For the meat inoculation experiment, the bacteriocins were partially purified to exclude the action of non-specific inhibitors during the storage. A medium activity level (1280 AU ml^{-1}) of the bacteriocins was chosen for the inoculation purposes and was lower than other values found in the literature. It was reported that more than 3000 or 4200 AU ml^{-1} of a bacteriocin have been required to effectively inhibit *L. monocytogenes* in a broth culture (El-Khateib et al., 1993) and on meat (Nielsen et al., 1990; Vignolo et al., 1996). Also, in the samples inoculated with the LAB, the population of listeriae decreased, probably due to the production of bacteriocin. *L. innocua* numbers, in the presence of the LAB strains, firstly increased and then decreased. This may be explained by the fact that the bacteriocins are produced after 24–48 h and they reach the maximum activity after 7 d of incubation at 4 ± 2°C (results not shown). Inhibition of pathogenic micro-organisms by LAB may be due to the effect of one or synergism between several mechanisms, such as competition for nutrients, lowering of pH, production of lactic acid, acetic acid, hydrogen peroxide, gas composition of atmosphere or antimicrobial substances such as bacteriocins (Skytta et al., 1991; Vandenbergh, 1993; Drosinos and Board, 1994). The results showed that endogenous or inoculated LAB grew rapidly within the first days of storage and these faster growth rates and the greater competitiveness for nutrients give to LAB a selective advantage over slower growing competitors. Inhibition was not only due to a decrease in pH because *L. monocytogenes* is able to survive in acid conditions with pH as low as 4.8 in foods (Conner et al., 1986; Glass and Doyle, 1989). Also, the hydrogen peroxide is not formed under anaerobic conditions (Gill, 1982). In addition, the results in our study showed that the production of lactic acid in the samples with the bacteriocin-negative strain was about the same as in the samples inoculated with the bac^+ LAB strains (proportional decrease of pH). Recent studies support the fact that bacteriocinogenic LAB inhibit listeriae regardless of acid production (Skytta et al., 1991). However, the acid production may enhance the antimicrobial action of LAB (Becker et al., 1994; Schillinger et al., 1995). The results showed that acetate concentration was increased during the storage of the samples. Acetate is more effective inhibitor of *L. monocytogenes* than lactate (Ostling and Lindgren, 1993; Drosinos and Board, 1994).

Results from this study showed that the bacteriocins did not inhibit the endogenous LAB, though at the beginning of storage the LAB showed a delay in their growth compared to uninoculated samples. In order to suppress the growth of the naturally growing LAB the inoculation of a lactic starter culture plus the bacteriocin could be an effective way to control undesirable organoleptic changes in vacuum- or modified atmosphere-packaged meat products (Schobitz et al., 1999). Mathieu et al. (1994) and Hanlin et al. (1993) studied the use of a combination of two bacteriocins to provide greater antibacterial activity against *L. monocytogenes* and other Gram-positive bacteria and their spores.

This study demonstrated that *Ln. mesenteroides* L124 and *L. curvatus* L442 have several attributes as potential protective organisms against listeriae. Since the listeriae can grow or survive under vacuum or modified atmosphere, respectively, at low temperatures and the two bac^+ LAB strains grow rapidly because they are well adapted to growth in the meat products and they can reduce the listeriae numbers, can be either employed as biopreservatives or as starter cultures with anti-listerial properties in meat products. Additionally, their bacteriocins have a bactericidal effect on the listeriae growth and could be used to improve meat safety.

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