

EFFECT OF LACTIC ACID BACTERIA ON EXTENTION OF SHELF LIFE AND GROWTH OF *LISTERIA MONOCYTOGENES* IN BEEF STEAKS STORED IN CO₂-RICH ATMOSPHERE

Djamel Djenane^{1,2*}; Luis Martínez²; Domingo Blanco²; Javier Yangüela²; José A. Beltrán²; Pedro Roncalés²

¹Université Mouloud Maameri, Faculté des Sciences Biologiques et des Sciences Agronomiques, Département de Biochimie et de Microbiologie, Complexe Hasnaoua, Tizi-Ouzou, Algérie; ²Department of Animal Production and Food Science, Laboratories of Food Technology and Food Hygiene, Faculty of Veterinary Science, University of Zaragoza, Zaragoza, Spain

Submitted: September 30, 2004; Returned to authors for corrections: August 02, 2005; Approved: November 17, 2005

ABSTRACT

Beef steaks were inoculated with one or other of two protective strains of lactic acid bacteria (bacteriocinogenic *Lactobacillus sakei* CTC 372 or the uncharacterized *Lactobacillus* CTC 711), and stored under modified atmospheres (20-40% CO₂). Inoculation of meat with LAB inhibited growth of the spoilage bacteria. Neither CO₂ in the pack atmosphere or inoculation with protective strains, nor a combination of both, affected formation of metmyoglobin or the development of off-odours. The formation of metmyoglobin in meat pigments and the sensory odour scores were compatible to those of fresh meat which had not undergone either oxidative deterioration or microbial spoilage. *Listeria monocytogenes* was inhibited in broth by meat surface microbiota containing the protective strains. With initial numbers of 5.6 log cfu mL⁻¹, after 7 days incubation at 3°C, *L. monocytogenes* were recovered at log mean numbers of 2.8 log cfu mL⁻¹ when no protective strain was present. At 8°C, the numbers of *L. monocytogenes* were reduced by about 2.5 or 1.5 log mL⁻¹ in the presence of *Lb. sakei* CTC 372 or *Lb. CTC 711*, respectively. At 25°C, the numbers of *L. monocytogenes* recovered from broth containing either protective strain were about 5 log lower than the numbers recovered from broth containing *L. monocytogenes* only.

Key words: fresh meat, modified atmosphere, preservation, lactic acid bacteria, spoilage, *Listeria monocytogenes*

INTRODUCTION

Meat is a very perishable food. Both oxidative and microbial processes are involved in meat deterioration. The potential for psychrotrophic spoilage microorganisms to grow during the extended refrigerated storage period and decrease organoleptic quality or spoil the food product is also a concern. With sufficient time at refrigeration temperatures, several types of psychrotrophic bacteria may grow to levels that may cause meat spoilage. These microorganisms include *Brochothrix thermosphacta* (*B. thermosphacta*), lactic acid bacteria (LAB), and *Pseudomonas* spp. (38). *B. thermosphacta*, which is aerobic to facultatively anaerobic, has been recovered from packaged beef, pork and lamb. Spoilage may involve development of

sliminess and production of off-odors and off-flavors conferred by short chain fatty acids (32).

Pseudomonas spp., which are aerobic, are among the most common spoilage agents of refrigerated foods (19). Growth of *Pseudomonas* spp. and other Gram-negative psychrotrophs is affected by oxygen tension and other factors. During growth, pseudomonads produce proteases and lipases that can catalyze reactions causing degradation of protein and fat. The consequence of these reactions is formation of peptides and fatty acids of undesirable flavor and odor. Sometimes these bacteria also produce unsightly green pigments (38).

Oxygen (O₂) concentrations of 60-80% are used in modified atmosphere packaging (MAP) to maintain myoglobin (Mb) in its oxygenated form (MbO₂). High O₂ concentration retards

*Corresponding Author. Mailing address: Université Mouloud Maameri. Faculté des Sciences Biologiques et des Sciences Agronomiques. Département de Biochimie et de Microbiologie. Complexe Hasnaoua, Tizi-Ouzou, 15000. Algérie. E-mail: djenane@unizar.es

metmyoglobin (MetMb) formation at meat surfaces and does not accelerate growth of aerobic organisms (13,37,43). If pathogens are present they may grow and the meat may become hazardous for consumers. A major concern associated with packaging fresh meat under modified atmospheres (MA) is the risk of growth of psychrotrophic pathogens such as *Listeria monocytogenes* (18,27). *L. monocytogenes* is a mesophilic foodborne pathogen, which shows psychrotrophic behaviour. It is widely distributed in nature and its control in food is difficult due to the relatively high tolerance to inhibitory conditions when compared to other food borne pathogens (16). Growth of *L. monocytogenes* in meat packaged under MA has been the focus of many studies, but the effect of carbon dioxide (CO₂) on the growth of *L. monocytogenes* is not clear, while the effect of including O₂ in the atmosphere is also uncertain (51). Inhibition of *L. monocytogenes* in meats cannot be achieved by MA alone (27).

Some strains of LAB are antagonistic against many microorganisms, including spoilage and pathogenic bacteria, because they produce bacteriocins (1,3,31). It has been suggested that bacteriocin-producing LAB might be useful as natural preservatives to enhance meat shelf life and safety by inhibiting spoilage and pathogenic bacteria (5,6). Inhibition of spoilage of fresh meat by bacteriocinogenic *Lactobacillus sakei* (*Lb. sakei*) CTC 494 (sakacin K-producing) together with MAP has been reported (26). Aymerich *et al.* (4) characterized *Lb. sakei* CTC 372 as a sakacin T producing strain; they also demonstrated that it can strongly inhibit *L. monocytogenes* and *Staphylococcus aureus* (*S. aureus*). LAB CTC 711 has not been characterized for bacteriocin production. However, according to Hugas (personal communication) it inhibited not only *L. monocytogenes* and *S. aureus* but also Gram-negative bacteria, such as *Salmonella* and *Escherichia coli*. The effects of bacteriocin-producing LAB on pathogens in meat may be difficult to evaluate because intrinsic factors of the product can influence the activity of bacteriocins (11,28,49). The inhibitory effects of LAB on *L. monocytogenes* might then be more reality discussed in broth cultures than with meat (8,41,45).

The aims of this study were to examine the effects of two protective LAB inoculated onto meat on the preservation of the quality characteristics of beef steaks stored under CO₂-rich atmospheres and to test the effects of the LAB against *L. monocytogenes* in broth at various temperatures.

MATERIALS AND METHODS

Meat samples

The *Longissimus dorsi* (LD) muscle from a single beef carcass was obtained at 48 h *post-mortem* (pH 5.6-5.7), and trimmed of external fat. Forty-five steaks, each 1.5 cm thick and weighting about 150 g, were aseptically cut and divided into halves. The steak portions were exposed to air for about 1 h at 1°C to allow for blooming.

Bacterial cultivation and media

The strains of LAB used, bacteriocinogenic *Lb. sakei* CTC 372 and the uncharacterized *Lb.* CTC 711, were isolated from meat or meat products (4,25) and kindly provided by Dr. M. Hugas (Centro de Tecnología de la Carne, IRTA, Monells, Spain). They were grown on Man, Rogosa and Sharpe (MRS) agar (Merck; Darmstadt, Germany) at 30°C.

The indicator strain of *L. monocytogenes* used in this study was from the Spanish Type Culture Collection (STCC 4031, corresponding to ATCC 15313; Valencia, Spain) and was kindly provided by Prof. Dr. Sala (University of Zaragoza, Spain). *L. monocytogenes* was grown in Tryptic Soy Broth (TSB; Biolife, S.r.l. Milano, Italy) supplemented with 0.6% yeast extract (YE; Biolife) and maintained on slants of Tryptic Soy Agar (TSA; Biolife) supplemented with 0.6% of yeast extract. The inoculum was prepared by transferring one colony of *L. monocytogenes* from a plate to a test tube containing 5 mL of sterile supplemented TSB (TSB-YE). After inoculation, the tube was incubated at 36 ± 1°C for 24 h. Erlenmeyer flasks (250 mL) containing 50 mL of TSB-YE were inoculated with this culture to obtain approximately 10⁶ cells mL⁻¹.

To prepare inocula of LAB, an isolated colony of each strain was transferred from MRS agar into a test tube containing 10 mL of sterile MRS broth (Merck; Darmstadt, Germany), which were incubated overnight at 30°C to obtain a culture containing approximately 10⁶ cfu mL⁻¹. When necessary, the culture was diluted with MRS broth to obtain the required cell numbers. Stock cultures of LAB strains were maintained as frozen stocks at -80°C in 20% (vol/vol) sterile glycerol (Panreac, Barcelona, Spain).

Inoculation of meat with LAB strains

After blooming, the 90 portions of meat were divided into three groups of 30. One group was sprayed with a culture of *Lb. sakei* CTC 372 to achieve 10⁴-10⁵ cm⁻². The second group was similarly sprayed with a culture of LAB CTC 711. The final group of steaks was sprayed with sterile 0.1% peptone water. Uniform spraying of the surface of beef steaks was achieved using a spray gun. To ensure that the inoculum was evenly distributed on meat surfaces, steaks were selected at random for determination of numbers of LAB.

Packaging and storage

Each meat portion was placed on an expanded polystyrene tray (15.5 x 21.5 x 2.5 cm). Each tray was placed into a polyethylene and polyamide (PE/PA, 80/20 µm thickness) laminate pouch (Sidlaw Packaging-Soplalril, Barcelona, Spain) with a water vapour permeability of 5-7 g m⁻² 24 h⁻¹ at 23°C and oxygen permeability of 40-50 mL m⁻² 24 h⁻¹ atm⁻¹ at 23°C. For pouches containing steaks subjected to the same treatment, 15 were filled with 1.5 L of 70% O₂/20% CO₂/10% N₂ (Abelló Linde S.A.; Barcelona, Spain), and the other 15 with 1.5 L of 60% O₂/40% CO₂ (Abelló Linde S.A.). The pouches were heat sealed, and stored in the dark at 1±1°C.

On days 7, 12, 17, 22 and 28 of storage, 3 packs from each treatment group with each atmosphere were opened. One steak from each set of three was used for microbial sampling, while the other two were used for sensory analysis and for instrumental and chemical analyses.

Inhibitory effect on *L. monocytogenes*

An overnight culture of *L. monocytogenes* was suspended in TSB-YE, prepared with 50 mM sodium phosphate buffer, pH 5.6-5.7, to simulate the normal pH of meat. Cultures of *Lb. sakei* CTC 372 and *Lb. CTC 711* were obtained from inoculated meat on day 7 of storage by swabbing 10 cm² of meat surfaces with a sterile cottonwool swab. Swabs were stirred in 10 mL of 0.1% peptone water. One mL of each suspension was added to each of 4 test tubes containing 10 mL of the suspension of *L. monocytogenes*. The broth containing LAB and *L. monocytogenes* was incubated for up to 10 days at 3, 8 or 25°C. To prepare the control samples, uninoculated beef steaks were swabbed on day 7 of storage and cultures containing LAB and *L. monocytogenes* were prepared and incubated as before.

pH measurements

The meat pH was measured after homogenisation of 5 g of meat in distilled water, using a Micro pH 2001 pH meter (CRISON mod.) with an INGOLD type U 402 electrode. Three readings were obtained for each steak portion.

Microbiological analysis

Two sterile cottonwool swabs moistened with 0.1% peptone water were used to swab 10 cm² of meat surface delimited by a sterile, stainless steel template. Swabs were stirred in 10 mL of 0.1% peptone water. Serial ten-fold dilutions were prepared by diluting 1 mL in 9 mL of 0.1% peptone water. Three plates were prepared from each dilution by pouring 1 mL into the fluid agar appropriate for each microbial species. LAB were enumerated in plates of MRS agar, which were incubated anaerobically at 30°C for 48-72 h. *B. thermosphacta* were enumerated in plates of streptomycin thallos acetate actidione (STAA) agar (Biolife s.r.l; Milano, Italy), which were incubated aerobically at 25°C for 72 h. *Pseudomonas* spp. were enumerated in plates of cephaloridine fucidin cetrinide (CFC) agar (Oxoid; Basingstoke, England), incubated at 25°C for 48-72 h (30). *L. monocytogenes* was enumerated in plates of PALCAM agar (Merck; Darmstadt, Germany), incubated aerobically at 36±1°C for 24 to 48 h. The logs of mean values for the counts from plates were recorded.

Colour determination

Meat surface colour was measured using a reflectance spectrophotometer (CM-2002, Minolta, Osaka, Japan) 30 min after package opening, to allow for colour stabilisation after exposure to air. CIE L*, a*, b* values (9) were recorded. Hue-

angle (h) and Chroma (C*) were calculated using the formulae: $h = \tan^{-1}(b^*/a^*)$ and $C^* = (a^{*2} + b^{*2})^{1/2}$, respectively.

Metmyoglobin analysis

The MetMb percentage of the total myoglobin perceptible at the steak surface was estimated spectrophotometrically, by the method of Stewart *et al.* (48), with measurement of steak surface reflectance at 525 and 572 nm (Minolta CM-2002; Osaka, Japan). The maximum value of the ratios of (K/S)_{572 nm} to (K/S)_{525 nm} at the beginning of the experiment was fixed as 0% MetMb; K and S were the absorption and the scattering coefficients, respectively, and K/S ratios were calculated from reflectivity (R_∞) values using the Kubelka-Munk equation [$K/S = (1 - R_{\infty})^2 / 2R_{\infty}$]. The value of 100% MetMb was obtained following the same procedure after oxidising a sample of meat in a 1% (w/v) solution of potassium ferricyanide (35). The average value for each steak was the mean of 20-25 determinations.

Lipid oxidation

Lipid oxidation was assessed in triplicate by the 2-thiobarbituric acid (TBA) method of Pfalzgraf *et al.* (40). Thiobarbituric acid reactive substances (TBARS) values were calculated from a standard curve of malonaldehyde (MA) and expressed as mg MA kg⁻¹ meat.

Sensory evaluation

Meat samples were evaluated for off-odour by a six-member panel, trained according to the method of Cross *et al.* (10). For rating odour, meat samples presenting different off-odour characteristics within the range of the evaluation scale were used. Samples used for rating included packaged beef steaks that were either fresh or stored at 4°C for up to 3 weeks.

In all assessments, beef steaks were evaluated 20 min after pack opening. Two samples for each treatment and time were taken, identified with 3-digit random numbers and placed in polystyrene trays of 15.5 cm x 21.5 cm. Each panelist received two half steak portions from each treatment, randomly numbered and served. The samples for evaluation were presented at room temperature of about 25°C.

The attribute off-odour was rated using a 5-point descriptive scale, in which: 1 = no off-odour, 2 = slight off-odour, 3 = little off-odour, 4 = moderate off-odour, and 5 = strong off-odour (12).

Analysis of data

The significance of differences amongst treatments after each day of storage was determined by analysis of variance using the Least Square Difference (LSD) method of the General Linear Model procedure of Statistical Package for Social Sciences (SPSS) program for Windows, version 6.1.2 (47). All other calculations were performed using Microsoft Excel, version 5, statistical functions (Microsoft Corp., Redmond, WA, USA). Differences were considered significant at the P<0.05 level.

RESULTS

Few presumptive *Pseudomonas* spp. were recovered from steaks after 12 days of storage (Fig. 1). The numbers of *Pseudomonas* spp. recovered from uninoculated steaks packaged in the 40% CO₂ atmosphere were 0.8 log unit lower than the numbers recovered from uninoculated steaks packaged in 20% CO₂ atmosphere after 17 days of storage. However, after 22 days of storage, the numbers recovered from uninoculated steaks packaged under either atmosphere were not significantly different ($P>0.05$).

Initial numbers of LAB were less than 10 cfu cm⁻² on uninoculated steaks (result not shown). The inoculated steaks all carried LAB at 4 to 5 log cfu cm⁻². After inoculation with *Lb. sakei* CTC 372, the numbers of *Pseudomonas* spp. recovered from steaks were about 1 log unit lower than the numbers recovered from uninoculated steaks after 28 days of storage under both CO₂ atmospheres. However, the numbers recovered from steaks inoculated with *Lb. CTC 711* were about 1 log unit lower than the numbers recovered from uninoculated steaks throughout storage, when steaks were packaged under 20% CO₂. Presumptive *B. thermosphacta* reached maximum values of about 5 log cfu cm⁻² at the end of storage (Fig. 2). The log numbers recovered from uninoculated steaks were similar during 22 days of storage; but the numbers recovered from steaks packaged under 40% CO₂ were about 1 log unit lower than the numbers recovered from steaks packaged under 20% CO₂ after 28 days of storage. The numbers of *B. thermosphacta* recovered from steaks inoculated with *Lb. sakei* CTC 372 were about 2 log units lower than the numbers recovered from uninoculated steaks after 28 days of storage under either CO₂ atmosphere. The numbers recovered from steaks inoculated with *Lb. CTC 711* were about 1 log unit lower than the numbers recovered from uninoculated steaks throughout storage when steaks were packaged under 20% CO₂.

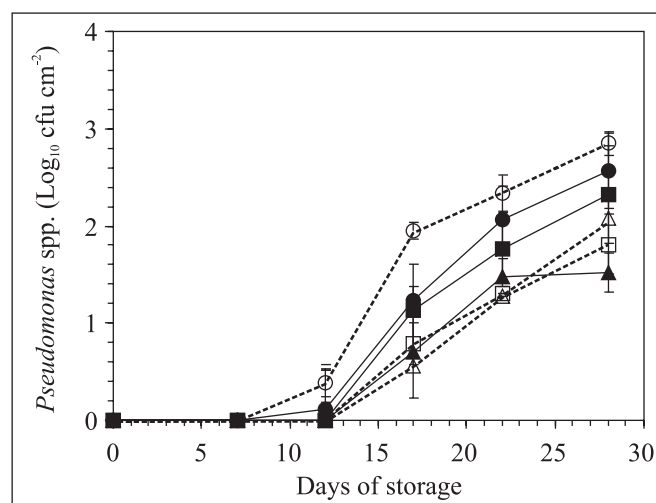


Figure 1. Numbers of *Pseudomonas* spp. recovered from beef steaks stored at 1±1°C under atmospheres of 70% O₂/20% CO₂/10% N₂ (open symbols) or 60% O₂/40% CO₂ (closed symbols) without being inoculated (circles) or after being inoculated with *Lactobacillus sakei* CTC 372 (triangles) or *Lactobacillus* CTC 711 (squares).

Neither high CO₂ concentrations, nor inoculation with LAB protective strains appeared to affect MetMb formation (Fig. 3). The results of the CIE indices of redness (CIE a*, hue and Chroma; data not shown) agreed with those for MetMb formation. The pH values of steaks did not differ significantly ($P>0.05$) during storage (data not shown).

Table 1 shows the sensory off-odour scores for beef steaks during storage. Neither variation in CO₂ concentration, nor inoculation with the protective LAB strains affected the odour of the meat.

Table 1. Sensory panel scores (mean±SD) for off odours of beef steaks packaged under atmospheres containing 20 or 40% CO₂ without or after being inoculated with lactic acid bacteria, after storage at 1±1°C for various times.

Off-odour*	Treatments		Days of storage					
	CO ₂ %	Inoculum	0	7	12	17	22	28
	20	None	1±0.0	1±0.0	1.3±0.4 ^x	2.5±0.3 ^x	3.6±0.2 ^x	4.8±0.3 ^y
	20	LAB 1 ^a	1±0.0	1±0.0	1.0±0.0 ^x	2.3±0.6 ^x	3.3±0.4 ^x	4.3±0.2 ^x
	20	LAB 2 ^b	1±0.0	1±0.0	1.0±0.0 ^x	2.4±0.5 ^x	3.4±0.5 ^x	4.2±0.3 ^x
	40	None	1±0.0	1±0.0	1.2±0.3 ^x	2.3±0.6 ^x	3.2±0.4 ^x	4.2±0.4 ^x
	40	LAB 1 ^a	1±0.0	1±0.0	1.0±0.0 ^x	2.2±0.4 ^x	3.2±0.4 ^x	4.2±0.3 ^x
	40	LAB 2 ^b	1±0.0	1±0.0	1.2±0.3 ^x	2.4±0.5 ^x	3.3±0.4 ^x	4.2±0.2 ^x

^aLAB 1, *Lactobacillus sakei* CTC 372; ^bLAB 2, uncharacterized *Lactobacillus* CTC 711; *1=No off-odour, 2=Slight off-odour, 3=Little off-odour, 4=Moderate off-odour, 5=Strong off-odour; ^{xy}Mean values in the same column are significantly different when accompanied by different superscripts ($P<0.05$).

After 7 or 5 days of incubation at 3 or 8°C, respectively, and at latter times, the numbers of *L. monocytogenes* recovered from broths containing *Lb. sakei* CTC 372 or *Lb. CTC 711* were substantially lower than the numbers recovered from broth

containing *L. monocytogenes* only (Fig. 4). The presence of either protective strains resulted in a severe inactivation of *L. monocytogenes* after 5 days at 25°C. The pH values of broths did not differ significantly ($P>0.05$) during incubation (data not shown).

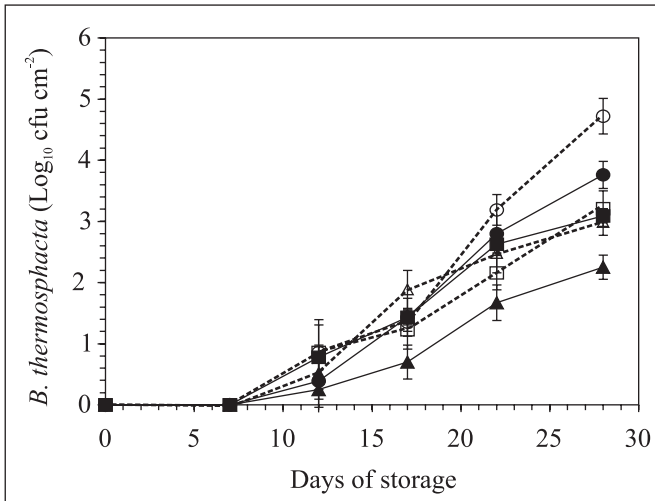


Figure 2. Numbers of *Brochothrix thermosphacta* recovered from beef steaks stored at $1\pm 1^\circ\text{C}$ under atmospheres of 70% O_2 /20% CO_2 /10% N_2 (open symbols) or 60% O_2 /40% CO_2 (closed symbols) without being inoculated (circles) or after being inoculated with *Lactobacillus sakei* CTC 372 (triangles) or *Lactobacillus* CTC 711 (squares).

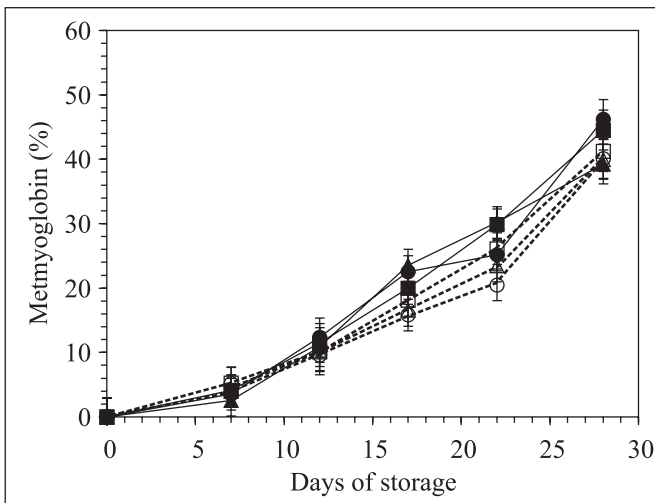


Figure 3. Metmyoglobin percentage on the surface of beef steaks stored at $1\pm 1^\circ\text{C}$ under atmospheres of 70% O_2 /20% CO_2 /10% N_2 (open symbols) or 60% O_2 /40% CO_2 (closed symbols) without being inoculated (circles) or after being inoculated with *Lactobacillus sakei* CTC 372 (triangles) or *Lactobacillus* CTC 711 (squares).

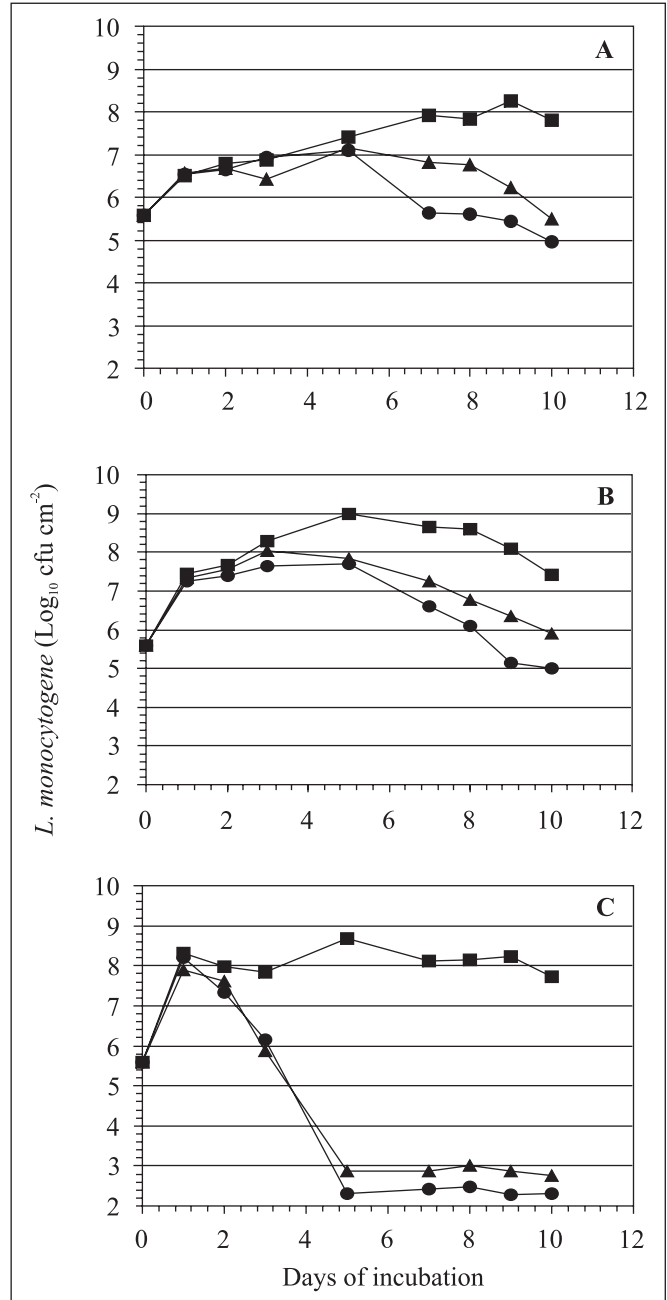


Figure 4. Effect of meat surface microbial flora without (■) or with *Lactobacillus sakei* CTC 372 (●) or *Lactobacillus* CTC 711 (▲) on the numbers of *Listeria monocytogenes* in broths incubated at (A) 3°C , (B) 8°C , or (C) 25°C .

DISCUSSION

Due to consumer demands and convenience at retail stores, there is great interest in developing MAP of consumer-ready cuts of meats. Spoilage bacteria tolerate high concentrations of O₂ and their growth rate can be reduced by including CO₂ in the gas mixture. As to the microbiological effects of CO₂ atmospheres, our findings indicated that numbers of bacteria recovered from steaks packaged under 40% CO₂ atmosphere were lower than those packaged under 20% CO₂. Similar results were observed in packed steaks for 28 days of storage at 1°C (14). An increase of shelf-life of meat packaged in high CO₂ atmospheres was expected and previously reported (2,21), as was synergistic effect between CO₂ and low temperature (20,21).

Studies of bacteriocin-producing strains have mainly focused on the inhibition of food pathogens, but the effects of these bacteria against specific spoilage organisms is unknown. The inoculation of steaks with *Lb. sakei* CTC 372 reduced the numbers of *Pseudomonas* spp. and *B. thermosphacta* on steaks packed under either CO₂ atmosphere. After 28 days of storage, *Lb. sakei* CTC 372 delayed microbial growth by about 10 days for steaks packed under either CO₂ atmosphere, as compared with uninoculated steaks. However, the numbers of bacteria were apparently reduced by inoculation of steaks with *Lb.* CTC 711 only when steaks were packaged under 20% CO₂, with bacterial growth being delayed approximately 9 days in this case. Further studies are required to explain the ineffectivity of *Lb.* CTC 711 to inhibit spoilage bacteria in steaks packed under 40% CO₂. Inhibition of spoilage bacteria on inoculated steaks can be attributed to the production of bacteriocins by either LAB strains. The combination of meat packing under MA and inoculation with bacteriocins has been shown to reduce the numbers of *B. thermosphacta*, which reached undetectable levels after 25 days of storage (50).

In this study, *Pseudomonas* spp. seemed to be the most vulnerable group to both protective strains of LAB. In beef packed under lower or higher concentration of CO₂, *Pseudomonas* spp. were always minor fractions of the flora, while *B. thermosphacta* was predominant. If it is accepted that a number of 7 log cfu cm⁻² is the approximate point at which spoilage becomes apparent (29), the numbers of the spoilage flora recovered from uninoculated and inoculated steaks were always lower than the numbers required for spoilage.

The bacteriocinogenic LAB strains examined in this study grew and produced antimicrobial substances at refrigeration temperatures. Thus, *Lb. sakei* CTC 372 or *Lb.* CTC 711, originally isolated from meat products, could be useful for control of microorganisms in refrigerated meats.

With respect to meat quality characteristics, the CIE a* values, MetMb percentage and sensory odour scores during 22 days of storage can be considered similar to those of fresh meat. It has been suggested that £ 40% MetMb on meat surface

might be considered as satisfactory (23,24,42), therefore, by this criterion, all the steaks should be considered satisfactory up to 22 days of storage.

With respect to off-odours, Greene and Cumuze (22) found that a TBARS value of at least 2.0 mg malonaldehyde/kg is required for perception of rancid odours. Results not shown of the present study showed that TBARS values were < 2 mg malonaldehyde /kg in steaks inoculated by either protective strain; therefore, perceptible off-odours would not be present during 3 weeks of storage, which is in accordance with the sensory analysis results.

Steaks inoculated with either protective strains had lower numbers of total psychrotrophic aerobes throughout storage (results not shown). However, the reduction in numbers of total psychrotrophic aerobes did not result in any extension of the shelf life of steaks. Those findings suggest that changes in the colour and odour of steaks packed in MA were due to oxidative processes rather than the growth of spoilage microorganisms.

The ubiquity of *L. monocytogenes* is well known. In food and in the environment, as *in vivo* infection, *L. monocytogenes* is exposed to many stress signals that can alter its virulence. According to Kathariou (33), stress caused by heating, freezing, dehydration, refrigeration, acids and salts, as well the exposure to disinfectants and other antimicrobial substances are of special relevance for the physiological status and virulence of this pathogen in foods.

Several studies have been performed using LAB strains to inhibit *L. monocytogenes* in dairy, fish and meat products (1,15,27,39). Unfortunately, individual foods may also be inhibitory towards bacteriocins and thus reduce or eliminate their efficiency (17). It has been shown that the use of LAB protective strains was less efficient in foods compared to broths (7). Thus, other studies have examined their effect only in broth cultures and extrapolated results to a wider range of uses. This may be in part explained by a more thorough contact between *L. monocytogenes* and bacteriocin in broth than in meat, which seems to be crucial for the efficacy of the protective culture. An even distribution of LAB on the surface of product was also found to be essential for the antilisterial activity.

In the present study, we used a high inoculum in broth of *L. monocytogenes* in order to assure that the presence of high numbers of other bacteria would not give rise to inhibition because of microbial competition. The effect of the inoculated LAB on *L. monocytogenes* was by far higher at 25°C than at lower temperature. *Lb. sakei* CTC 372 or *Lb.* CTC 711 inhibited *L. monocytogenes* in broth by 70.24 or 64.30% respectively, after 10 days incubation. Inactivation of *L. monocytogenes* at 25°C can be attributed to the rapid growth and early production of bacteriocins by the bacteriocinogenic strains of LAB at this temperature. But inhibition of *L. monocytogenes* was also substantial at refrigeration temperatures. At 8°C, inhibitions by *Lb. sakei* CTC 372 or *Lb.* CTC 711 were 32.70 or 20.72%

respectively, after 10 days incubation. But at 3°C, *Lb. sakei* CTC 372 or *Lb. CTC 711* inhibited *L. monocytogenes* by 36.5 or 29.5% respectively. The enhancement of the antilisterial efficacy of bacteriocinogenic protective culture had already been shown in broth in previous reports (45,46). Experiments were conducted at higher temperatures in order to simulate possible temperature abuse throughout the meat commercial chain. Inhibition of *L. monocytogenes* by LAB at low temperatures has been reported in broth or on meat or salmon (8,34,44). That contrast with reports of the higher production of bacteriocin by LAB at low temperature than at temperatures optimal for growth (27). In accordance with the findings of this study, Luchansky *et al.* (36) reported that on meat inoculated with *L. monocytogenes* and LAB, *L. monocytogenes* was inhibited at 25°C but not at 4°C. Inoculation with protective strains did not significantly alter the pH of either steaks or broths (results not shown); so pH was not a factor in the inhibition of bacterial growth.

The 40% CO₂ atmosphere exerted a significant inhibitory effect on the growth of spoilage bacteria on steaks; and LAB protective strains *Lb. sakei* CTC 372 or CTC 711 might be useful as natural preservatives for further controlling spoilage bacteria, as well as providing a hurdle to the growth of *L. monocytogenes* at either refrigeration or abusive temperatures. Neither the increase of CO₂ in the packaging atmosphere, inoculation with LAB protective strains, nor a combination of both, resulted in any modification of meat quality characteristics.

ACKNOWLEDGEMENTS

This research was supported by project grants ALI 96-0587 and AGL 2000-1407 from the Comisión Interministerial de Ciencia y Tecnología (CICYT). D. Djenane was the recipient of a fellowship from the Agencia Española de Cooperación Internacional (A.E.C.I.). L. Martínez was the recipient of a fellowship from the Fondo Social Europeo and the Gobierno de Aragón (Grant B072/2001). The authors wish to thank Prof. Dr. F.J. Sala and Dr. M. Hugas, who kindly provided the strains of *L. monocytogenes* and lactic acid bacteria, respectively and Abelló Linde S.A. (Spain), which provided the packaging equipment and gas mixtures. The assistance of Mrs. Olga Isla Corral is greatly acknowledged.

RESUMO

Efeito de bactérias lácticas na extensão da vida de parateleira e multiplicação de *Listeria monocytogenes* em fatias de carne bovina armazenadas em atmosfera rica em CO₂

Fatias de carne bovina foram inoculadas com uma de duas bactérias lácticas protetoras (*Lactobacillus sakei* CTC 372

bacteriocinogênica e *Lactobacillus* CTC 711 não caracterizado) e armazenadas em atmosfera modificada (20-40% CO₂). A inoculação da carne com bactérias lácticas protetoras inibiu a multiplicação de bactérias deteriorantes. O CO₂ da atmosfera e/ou a presença de bactérias lácticas não causou a formação de metmioglobina ou de odores indesejáveis. A formação de metmioglobina e as características sensoriais foram equivalentes às apresentadas por carne fresca não deteriorada e não submetida a tratamento. A multiplicação de *Listeria monocytogenes* em caldo foi inibida por bactérias isoladas da superfície das carnes inoculadas com as bactérias lácticas. Após 7 dias a 3°C, a contagem inicial de *L. monocytogenes* de 5.6 log UFC.mL⁻¹ caiu para 2.8 log UFC.mL⁻¹ na ausência de bactéria láctica protetora. A 8°C, as contagens de *L. monocytogenes* foram reduzidas em 2.5 ou 1.5 log na presença de *Lb. sakei* CTC 372 ou *Lb. CTC 711*, respectivamente. A 25°C, as contagens de *L. monocytogenes* no caldo contendo uma das bactérias lácticas protetoras foram 5 log mais baixas do que nos caldos contendo *L. monocytogenes* somente.

Palavras-chave: carne bovina, atmosfera modificada, conservação, bactérias lácticas, deterioração, *Listeria monocytogenes*

REFERENCES

1. Ammor, S.; Tauveron, G.; Dufour, E.; Chevallier, I. Antibacterial activity of lactic acid bacteria against spoilage and pathogenic bacteria isolated from the same meat small-scale facility: Behaviour of pathogenic and spoilage bacteria in dual species biofilms including a bacteriocin-like-producing lactic acid bacteria. *Food Control*, 17, 454-461, 2006.
2. Asensio, M.A.; Ordóñez, J.A.; Sanz, B. Effect of carbon dioxide and oxygen enriched atmospheres on the shelf-life of refrigerated pork packed in plastic bags. *J. Food Prot.*, 51, 356-360, 1988.
3. Aymerich, M.T.; Hugas, M.; Monfort, J.M. Review: Bacteriocinogenic lactic acid bacteria associated with meat products. *Food Sci. Technol. Int.*, 4, 141-158, 1998.
4. Aymerich, M.T.; Garriga, M.; Monfort, J.M.; Nes, I.; Hugas, M. Bacteriocin-producing *lactobacilli* in spanish-style fermented sausages: characterization of bacteriocins. *Food Microbiol.*, 17, 33-45, 2000.
5. Barnby-Smith, F.M. Bacteriocins: applications in food preservation. *Tren. Food Sci. Technol.*, 3, 133-137, 1992.
6. Bredholt, S.; Nesbakken, T.; Holck, A. Industrial application of an antilisterial of *Lactobacillus sakei* as a protective culture and its effect on the sensory acceptability of cooked, sliced, vacuum-packaged meats. *Int. J. Food Microbiol.*, 66, 191-196, 2001.
7. Budde, B.B.; Hornbaek, T.; Jacobsen, T.; Barkholt, V.; Koch, A. G. *Leuconostoc carnosum* 4010 has the potential as a new protective culture for vacuum-packed meats: culture isolation, bacteriocin identification, and meat application. *Int. J. Food Microbiol.*, 83, 171-184, 2003.
8. Castellano, P.H.; Holzappel, W.H.; Vignolo, G.M. The control of *Listeria innocua* and *Lactobacillus sakei* in broth and meat slurry with the bacteriocinogenic strain *Lactobacillus casei* CRL 705. *Food Microbiol.*, 21, 291-298, 2004.
9. CIE. Recommendations on uniform color spaces-color difference equations psychometric color terms. *Commission International de l'Eclairage*, Paris, 1978. Supplement N° 2 to CIE Publication N° 15 (E-1.3.1) 1971/(TC-1.3.).

10. Cross, H.R.; Moen, R.; Stanfield, M.S. Training and testing of judges for sensory analysis of meat quality. *Food Technol.*, 32(7), 48-54, 1978.
11. De Martinis, E.C.P.; Franco, B.D.G.M. Inhibition of foodborne pathogens by bacteriocin producing *Leuconostoc* spp. and *Lactobacillus sake* isolated from linguça frescal. *Rev. Microbiol.*, 28, 284-287, 1997.
12. Djenane, D.; Sánchez-Escalante, A.; Beltrán, J.A.; Roncalés, P. Extension of the retail display life of fresh beef packaged in modified atmosphere by varying lighting conditions. *J. Food Sci.*, 66, 181-186, 2001.
13. Djenane, D.; Sánchez-Escalante, A.; Beltrán, J.A.; Roncalés, P. Extension of the shelf life of beef steaks packaged in a modified atmosphere by treatment with rosemary and displayed under UV-free lighting. *Meat Sci.*, 64, 417-426, 2003a.
14. Djenane, D.; Sánchez-Escalante, A.; Beltrán, J.A.; Roncalés, P. The shelf life of beef steaks treated with DL-lactic acid and antioxidants and stored under modified atmospheres. *Food Microbiol.*, 20, 1-7, 2003b.
15. Duffes, F.; Leroi, F.; Boyaval, P.; Dousset, X. Inhibition of *Listeria monocytogenes* by *Carnobacterium* spp. strains in a simulated cold smoked fish system stored at 4°C. *Int. J. Food Microbiol.*, 47, 33-42, 1999.
16. Farber, T.M.; Peterkin, P.I. *Listeria monocytogenes*: a food-borne pathogen. *Microbiol. Rev.*, 55, 476-511, 1991.
17. Ganzle, M.G.; Weber, S.; Hammes, W.P. Effect of ecological factors on the inhibitory spectrum and activity of bacteriocins. *Int. J. Food Microbiol.*, 46, 207-217, 1999.
18. García, De Fernando, G.D.; Nychas, G.J.E.; Peck, M.W.; Ordóñez, J.A. Growth/survival of psychrotrophic pathogens on meat packaged under modified atmospheres. *Int. J. Food Microbiol.*, 28, 221-231, 1995.
19. Gill, C.O. The control of microbial spoilage in fresh meats. In "Advances in meat research", Vol. 2, "Meat and Poultry Microbiology", eds Pearson, A.M.; Dutson, T.R. p. 49, AVI Publishing Co., Westport, Conn, 1986.
20. Gill, C.O. The solubility of carbon dioxide in meat. *Meat Sci.* 22, 65-71, 1988.
21. Gill, C.O.; Tan, K.H. Effect of carbon dioxide on growth of meat spoilage bacteria. *Appl. Environ. Microbiol.*, 39, 317-319, 1980.
22. Greene, B.E.; Cumuze, T.H. Relationship between TBA-numbers and inexperienced panelists assessments of oxidized flavor in cooked beef. *J. Food Sci.*, 47, 52-58, 1981.
23. Greene, B.E.; Hsin, I.M.; Zipser, M.W. Retardation of oxidative color changes in raw ground beef. *J. Food Sci.*, 36, 940-942, 1971.
24. Hood, D.E.; Riordan, E.B. Discoloration in pre-packaged beef: measurement by reflectance spectrophotometry and shopper discrimination. *J. Food Technol.*, 8, 333-343, 1973.
25. Hugas, M.; Garriga, M.; Aymerich, T.; Monfort, J.M. Biochemical characterization of *Lactobacilli* from dry fermented sausages. *Int. J. Food Microbiol.*, 18, 107-113, 1993.
26. Hugas, M.; Garriga, M.; Aymerich, T.; Monfort, J.M. Inhibition of *L. monocytogenes* in dry fermented sausages by the bacteriocinogenic *Lactobacillus sake* CTC 494. *J. Appl. Bacteriol.*, 79, 322-330, 1995.
27. Hugas, M.; Pagés, F.; Garriga, M.; Monfort, J.M. Application of the bacteriocinogenic *Lactobacillus sakei* CTC 494 to prevent growth of *L. monocytogenes* in fresh and cooked meat products packed with different atmospheres. *Food Microbiol.*, 15, 639-650, 1998.
28. Hugas, M.; Garriga, M.; Pascual, M.; Aymerich, M.T.; Monfort, J.M. Enhancement of sakacin K activity against *Listeria monocytogenes* in fermented sausages with pepper or manganese as ingredients. *Food Microbiol.*, 19, 519-528, 2002.
29. Hunt, M.C.; Mancini, R.A.; Hachmeister, K.A.; Kropf, D.H.; Merriman, M.; Delduca, G.; Milliken, G. Carbon monoxide in modified atmosphere packaging affects color, shelf-life, and microorganisms of beef steaks and ground beef. *J. Food Sci.*, 69, 45-52, 2004.
30. ICMSF. International Commission of Microbiological Specifications for Foods. Microorganisms in foods. Vol. 1. Their significance and methods of enumeration. (Elliott, R.P.; Clark, D.S.; Lewis, K.H.; Lundbeck, H.; Olson, J.C.Jr.; Simonsen, B. (eds). Toronto, 1983. University of Toronto Press.
31. Jamuna, M.; Babusha, S.T.; Jeevaratnam, K. Inhibitory efficacy of nisin and bacteriocins from *Lactobacillus* isolates against food spoilage and pathogenic organisms in model and food systems. *Food Microbiol.*, 22, 449-454, 2005.
32. Jay, J.M. "Modern Food Microbiology." 4th eds. Chapman & Hall, New York, 1992.
33. Kathariou, S. *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. *J. Food Prot.*, 65, 1811-1829, 2002.
34. Katla, T.; Møretø, T.; Aasen, I.M.; Holck, A.; Axelsson, L.; Naterstad, K. Inhibition of *L. monocytogenes* in cold smoked salmon by addition of sakacin P and/or live *Lactobacillus sakei* cultures. *Food Microbiol.*, 18, 431-439, 2001.
35. Ledward, D.A. Metmyoglobin formation in beef stored in carbon dioxide enriched and oxygen depleted atmospheres. *J. Food Sci.*, 35, 33-37, 1970.
36. Luchansky, J.B.; Glass, K.A.; Harsono, K.D.; Degnan, A.; Faith, N.G.; Cauvin, B. Genomic analysis of *Pediococcus* starter cultures to control *L. monocytogenes* in turkey summer sausage. *Appl. Environ. Microbiol.*, 58, 3053-3059, 1992.
37. Martínez, L.; Djenane, D.; Cilla, I.; Beltrán, J.A.; Roncalés, P. Effect of different concentrations of carbon dioxide and low concentration of carbon monoxide on the shelf-life of fresh pork sausages packaged in modified atmosphere. *Meat Sci.*, 71, 563-570, 2005.
38. Marth, E.H. Extended shelf life refrigerated foods. *Food Technol.*, 52(2), 57-62, 1998.
39. Mcauliffe, O.; Hill, C.; Ross, R.P. Inhibition of *Listeria monocytogenes* in cottage cheese manufactured with a lacticin 3147-producing starter culture. *J. Appl. Microbiol.*, 86, 251-256, 1999.
40. Pfalzgraf, A.; Frigg M.; Steinhart, H. Alpha tocopherol contents and lipid oxidation in pork muscle and adipose tissue during storage. *J. Agric. Food Chem.*, 43, 1339-1342, 1995.
41. Pleasants, A.B.; Soboleva, T.K.; Dykes, A.; Jones, R.J.; Filippov, A.E. Modelling of the growth of populations of *Listeria monocytogenes* and a bacteriocin-producing strain of *Lactobacillus* in pure and mixed cultures. *Food Microbiol.*, 18, 605-615, 2001.
42. Renerre, M.; Mazuel, J.P. Relation entre méthodes de mesure instrumentales et sensorielles de la couleur de la viande. *Sci. Alim.*, 5, 541-557, 1985.
43. Sanchez-Escalante, A.; Djenane, D.; Torrecano, G.; Beltrán, J.A.; Roncalés, P. Antioxidant action of borage, rosemary, oregano and ascorbic acid in beef patties packaged in modified atmosphere. *J. Food Sci.*, 68(1), 339-344, 2003.
44. Schillinger, U.; Lücke, F.K. Lactic acid bacteria as protective cultures in meat products. *Fleischwirtsch.*, 70, 1296-1299, 1990.
45. Schillinger, U.; Lücke, F.K. Behaviour of *L. monocytogenes* in meat and its control by a bacteriocin-producing strain of *Lactobacillus sake*. *J. Appl. Bacteriol.*, 70, 473-478, 1991.
46. Schillinger, U.; Chung, H.S.; Keppler, K.; Holzapfel, W.H. Use of bacteriocinogenic lactic acid bacteria to inhibit spontaneous nisin-resistant mutants of *Listeria monocytogenes* Scott A. *J. Appl. Microbiol.*, 85, 657-663, 1998.
47. SPSS. SPSS for Windows, 6.1.2. Chicago. SPSS Inc. 1995.
48. Stewart, M.R.; Zipser, M.W.; Watts, B.M. The use of reflectance spectrophotometry for the assay of raw meat pigments. *J. Food Sci.*, 30, 464-469, 1965.
49. Stiles, M.E. Biopreservation by lactic acid bacteria. *Ant. van Leeuwen.*, 70, 331-345, 1996.
50. Tu, L.; Mustapha, A. Reduction of *Brochothrix thermosphacta* and *Salmonella* serotype *Typhimurium* on vacuum packaged fresh beef treated with nisin and nisin combined with EDTA. *J. Food Sci.*, 67, 302-306, 2002.
51. Wimpfheimer, L.; Altman, N.S.; Hotchkiss, J.H. Growth of *L. monocytogenes* Scott A, serotype 4 and competitive spoilage organisms in raw chicken packaged under modified atmospheres and in air. *Int. J. Food Microbiol.*, 11, 205-214, 1990.