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ORIGINAL ARTICLE

Inhibition of *Listeria innocua* and *Brochothrix thermosphacta* in vacuum-packaged meat by addition of bacteriocinogenic *Lactobacillus curvatus* CRL705 and its bacteriocins

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bacteriocins, biopreservation, *Lactobacillus curvatus*, vacuum-packaged meat.

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Abstract

Aims: To evaluate the inhibition effectiveness of *Lactobacillus curvatus* CRL705 used as a bioprotective culture and of its bacteriocins, lactocin 705 and lactocin AL705, against *Listeria innocua*, *Brochothrix thermosphacta* and indigenous lactic acid bacteria (LAB) in vacuum-packaged meat stored at 2°C.

Methods and Results: The live culture of *Lact. curvatus* CRL705 as well as synthetic lactocin 705 and purified lactocin AL705 were shown to be similarly effective in preventing the growth of *B. thermosphacta* and *L. innocua* in meat discs in contrast to control samples in which these micro-organisms grew rapidly, their numbers increasing by 3·0- and 2·1-log cycles respectively. In addition, indigenous LAB population showed a lower growth rate in the presence of lactocin 705. Bacteriocin activity was detected in the meat discs during 36 days at 2°C irrespective of the biopreservation strategy applied. Changes in pH were not significantly different in meat discs treated with the protective culture when compared with control samples.

Conclusions: *Lactobacillus curvatus* CRL705 and the produced bacteriocins, lactocin 705 and lactocin AL 705, were effective in inhibiting *L. innocua* and *B. thermosphacta*. The use of the bioprotective culture in refrigerated vacuum-packaged fresh meat would be more feasible from an economic and legal point of view.

Significance and Impact of the Study: Establishment of biopreservation as a method to ensure the microbiological safety of vacuum-packaged fresh meat at 2°C.

Introduction

Antagonistic cultures added to food to inhibit pathogens and/or extend shelf life while changing the sensory properties of the product as little as possible are termed protective cultures. Their use or that of their metabolic products (organic acids, hydrogen peroxide, enzymes and bacteriocins) is often referred to as biopreservation (Lücke 2000). Some lactic acid bacteria (LAB) commonly associated with meats demonstrate antagonism towards pathogenic and spoilage organisms. Biopreservation systems such as bacteriocinogenic LAB cultures and/or their bacteriocins have received increasing attention and new approaches to control pathogenic and spoilage micro-organisms have been developed. Many studies have demonstrated bacteriocins antagonism towards *Listeria monocytogenes* in cooked meat products (Hugas *et al.* 1998; Bredholt *et al.* 2001; Jacobsen *et al.* 2003; Mataragas *et al.* 2003; Vermeiren *et al.* 2004). However, little is known about the inhibitory activity of protective cultures against specific spoilage organisms in refrigerated vacuum-packaged raw meat. Meat storage in gas-impermeable packs restricts the growth of *Pseudomonas* species so that LAB, *Brochothrix thermosphacta* and Enterobacteriaceae become the major

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components of the spoilage microbiota (Hansen and Bautista 2000; Sakala *et al.* 2002; Vermeiren *et al.* 2004). LAB and *B. thermosphacta* significantly influence the quality of meat and meat products, both micro-organisms being associated with spoilage appearing as sour off-flavours and off-odours, slimy, pack swelling and/or greening (Samelis *et al.* 2000). Cutter and Siragusa (1996) reported the efficacy of the nisin spray treatment in reducing *Listeria innocua* and *B. thermosphacta* populations in refrigerated vacuum-packaged beef. Metaxopoulos *et al.* (2002), who inoculated the bacteriocin producing *Leuconostoc mesenteroides* L124 and *Lactobacillus curvatus* L422 on sliced cooked cured pork shoulder stored under vacuum packaging, also showed that the number of *B. thermosphacta* and enterococci was decreased during storage at 4°C. Despite the promising information and laboratory studies, bacteriocinogenic strains often suffer from a limited effectiveness in foods due to factors such as a narrow spectrum, spontaneous loss of bacteriocinogenecity, inactivation through proteolytic enzymes or binding to foods ingredients and poor adaptation to food environment (Holzapfel *et al.* 1995).

In previous works (Vignolo *et al.* 1996a, 1996b), we observed an inhibitory effect on *L. monocytogenes* and some LAB exerted by an antimicrobial substance present in the supernatant of *Lactobacillus casei* CRL705 and named lactocin 705. However, after its purification, lactocin 705 was found to be only active against closely related LAB and *B. thermosphacta*. This substance was classified as belonging to class IIb, two-peptide bacteriocins, whose activity relies upon the complementation of two peptides (Cuozzo *et al.* 2000; Castellano *et al.* 2003). As the supernatant of *Lact. casei* CRL705 also inhibited the growth of *L. monocytogenes*, the production of more than one bacteriocin was assumed. In fact, genetic studies (S. Cuozzo, F. Sesma and R. Raya, unpublished data) as well as broth and meat slurry assays (Castellano *et al.* 2004) demonstrated the existence of a second inhibitory substance, an anti-*Listeria* bacteriocin called AL705, which was found to be inactive against indigenous LAB and *B. thermosphacta*.

With the aim of increasing the range of effectiveness of biopreservatives available to food technologists, the inhibitory ability of bacteriocins (lactocin 705 and AL705) and of a live culture of *Lact. curvatus* CRL705 (formerly identified as *Lact. casei*) against *L. innocua*, *B. thermosphacta* and indigenous LAB was studied in vacuum-packaged meat discs stored at 2°C.

Materials and methods

Bacterial strains and culture conditions

Lactobacillus curvatus CRL705 (formerly identified as *Lact. casei* CRL705), a lactocin 705 and lactocin AL705 producer and *Lactobacillus plantarum* CRL691, used as an indicator of lactocin 705, were isolated from dry-cured sausages (Vignolo *et al.* 1993). *Lactobacillus curvatus* CRL1579, which produces only lactocin AL705, is a derivative of CRL705 that carries a plasmid pBlueScript with the 7·6 kb *Hin*dIII–*Eco*RI fragment from pRC18; this fragment contains the immunity protein, ABC transporter and accessory (Acc) protein of lactocin 705. Both ABC and Acc protein are required for the processing and secretion of the chromosomically encoded lactocin AL705 (S. Cuozzo, F. Sesma and R. Raya, unpublished data). All three lactobacilli strains were grown in de Man, Rogosa, Sharpe (MRS) broth (Britania, Buenos Aires, Argentina) at 30°C. *Listeria innocua* 7, used as an indicator of lactocin AL705, was obtained from the Unité de Recherches Laitieres et Genetique Appliqué, INRA (France) and was grown in trypticase soy broth (TSB; BBL, Cockeysville, MD, USA) with 0·5% added yeast extract at 30°C. *Brochothrix thermosphacta* ATCC11509, obtained from the Unité de Recherches sur la Viande, INRA was grown in APT broth at 25°C.

Lactocin 705 and lactocin AL705 preparation

Purification and amino acid sequencing of lactocin 705 have been reported by Palacios *et al.* (1999) and Cuozzo *et al.* (2000). The synthesis of the 33-amino acid lactocin 705α peptide and of the 33-amino acid 705β peptide was performed in Geminys Biotech (Alachua, FL, USA) and Bio-synthesis (Lewisville, TX, USA) respectively. Purified lactocin AL705 was obtained from the supernatant of *Lact. curvatus* CRL1579 using the desorption pH-dependent method. The sample was then loaded onto a C_8 cartridge (Supelclean LC-8; Supelco, Bellefonte, PA, USA) and lactocin AL705 was eluted in the 40% acetonitrile fraction. After concentration, the active sample was injected on a C_8 Vydac 208 TP RP-HPLC analytical column (10 μm to 4·6 mm × 250 mm; Alltech Associates, Deerfield, IL, USA) and eluted with a 30 min linear gradient of 5–100% isopropanol in aqueous 0·1% (v/v) trifluoroacetic acid. The active fractions from RP-HPLC were collected and concentrated in a N_2 concentrator (Turbo Vap LV; Zymark, Holkienton, MA, USA) and stored at −40°C.

Preparation and inoculation of vacuum-packaged meat discs

Meat discs (3-cm diameter × 0·5-cm thick) were aseptically obtained from bovine M. semimembranosus. *Lactobacillus curvatus* CRL705 was sprayed onto the surface of the meat using a hand-operated spraying bottle to obtain 10^6 CFU cm^{-2} while lactocin 705 and

lactocin AL705 were sprayed to reach a concentration of 2·8 μmol l^{-1} and 6400 AU ml^{-1} respectively. Meat discs with and without *Lact. curvatus* or its bacteriocins were inoculated with *L. innocua* and *B. thermosphacta* to obtain *c.* 10^3 CFU cm^{-2}. Samples were vacuum-packaged using a film (Cryovac, Buenos Aires, Argentina) with a diffusion coefficient of 6/14 cm^3 m^{-2} atm^{-1} 24 h^{-1} to oxygen at 25°C and 75% relative humidity. The packages were sealed at a final vacuum of 99% using a Turbovac 320 ST vacuum packaging machine (HFE Vacuum Systems, Hertogenbosh, the Netherlands). All samples were stored at 2°C for 36 days.

Microbiological analysis

Immediately after inoculation and after 7, 14, 21, 28 and 36 days of storage, duplicate discs for each treatment and sampling interval were counted for *L. innocua*, *B. thermosphacta*, LAB and *Lact. curvatus* CRL705. Discs from each treatment were mixed (1 : 10) with a dilution medium (0·85% NaCl) and placed in a stomacher bag for 1 min. *Listeria innocua* and *B. thermosphacta* populations were counted after serial decimal dilutions using sterile peptone water (0·1% w/v) and plated on PALCAM selective media (Difco Laboratories, Inc., Detroit, MI, USA) and selective Streptomycin Thallous Acetate Agar base (Gardner 1966) incubated at 30 and 25°C for 48 h respectively. Differential count of indigenous LAB and *Lact. curvatus* CRL705 was performed in MRS agar with and without spectinomycin (200 μg ml^{-1}) and incubated for 48 h at 30°C. Growth and selection of antibiotic-resistant *Lact. curvatus* were performed using 300 μg ml^{-1} of spectinomycin in MRS. The antibiotic was purchased from Sigma Chemical Co (St Louis, MO, USA).

Antimicrobial activity and pH determination

A semiquantitative modified well-diffusion assay (Vignolo *et al.* 1993) was used to determine bacteriocin activity (lactocin 705 and lactocin AL705). Samples were stomached for 8 min (Stomacher Lab-Blender 400; A.J. Seward Laboratory, London, UK) in a stomacher bag and 0·85% NaCl (one disc plus dilution medium, 1 : 2) was added. The homogenates were centrifuged at 12 000 ***g*** for 10 min and 30 μl of serial twofold dilutions of neutralized supernatant was placed on each well cut in a semisolid MRS and TSB overlay inoculated with *Lact. plantarum* CRL691 and *L. innocua* 7, respectively, as indicator organisms. Positive bacteriocin activity was evidenced as a zone of inhibition on the indicator organism lawn. The measurement of pH was performed using a Metrohn 692 pH/Ion Meter (Metrohn SA, Herisau, Switzerland) on the first homogenized dilution of the meat samples.

Statistical analysis

Each experiment was repeated on two separate replications. One-way analysis of variance (ANOVA) was used (Minitab Statistic Program, release 8.21; Minitab Inc., Philadelphia, PA, USA).

Results

The bacteriocinogenic strain *Lact. curvatus* CRL705 inoculated on meat discs was able to grow from $1·2 \times 10^6$ to $3·2 \times 10^8$ CFU cm^{-2} after 36 days of incubation at 2°C (Fig. 1a). The same viable counts on MRS agar plates with and without spectinomycin were obtained, indicating that the bioprotective culture was the dominant population after 36 days. The growth of the psychrophilic microbiota naturally present in the meat reached in the absence of lactocin 705 a final cell count of $5·0 \times 10^6$ CFU cm^{-2} after 36 days while the population was 2-log cycles lower ($5·0 \times 10^4$ CFU cm^{-2}) in meat samples with the bacteriocin added. Figure 1b shows a listeriostatic effect when the protective culture or lactocin AL705 was added to meat discs while *L. innocua* grew rapidly, increasing its number by 3·0 logs from days 7 to 28 in the control samples. On the other hand, cell counts of *B. thermosphacta* after the addition of either the protective culture or lactocin 705 decreased from $6·4 \times 10^2$ to $2·1 \times 10^1$ CFU cm^{-2} after incubation for 14 days and remained at this low level until the end of the incubation period (Fig. 1c). In untreated samples this micro-organism was able to increase its population by 2-log cycles.

The presence of inhibitory activity in meat discs during the 36 days of storage at 2°C was determined (Table 1). After 14 days of incubation, the same antibacterial activity level was detected in the meat discs when *Lact. curvatus* CRL705, lactocin 705 or AL705 were added and remained stable throughout the storage period. The low bacteriocin activity detected during the first 2 weeks in the presence of the biopreservative culture was possibly due to the adaptation of *Lact. curvatus* CRL705 growth to the meat substrate. Homogenates from the control series did not exhibit any inhibitory activity in the assay, thus indicating that bacteriocins were responsible for the inhibitory activity in treated meat discs (data not shown). The final pH values as a result of the bacteriocinogenic *Lact. curvatus* CRL705 growth in meat discs stored at 2°C are shown in Table 2. The analysis of the effect of different treatments demonstrated that the pH value of meat control discs (5·50) was not significantly different from that found in samples inoculated with the protective culture (5·48) and challenged with *L. innocua* (5·42) or *B. thermosphacta* (5·40).

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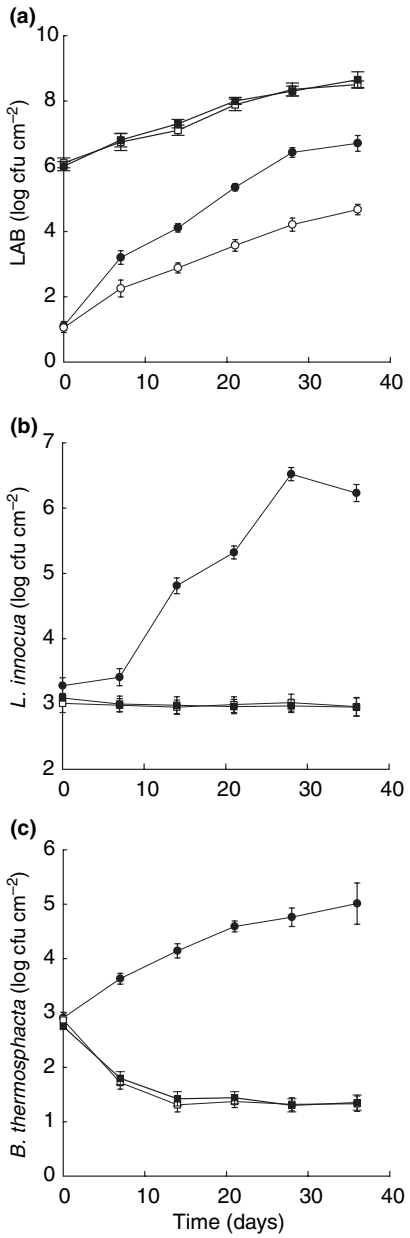

Figure 1 Growth of LAB, *Listeria innocua* and *Brochothrix thermosphacta* on meat discs incubated at 2°C for 36 days. (a) Control (●); lactocin 705 addition (○); *Lactobacillus curvatus* CRL705 growth in the presence/absence of spectinomycin (□)/(■). (b) Control (●); addition of *Lact. curvatus* CRL705 (■) and lactocin AL705 (□). (c) Control (●); addition of *Lact. curvatus* CRL705 (■) and lactocin 705 (□). The numbers represent duplicates of treated and control samples.

Discussion

In this study, the potential of lactocin 705 and lactocin AL705 as well as the live culture of *Lact. curvatus* CRL705 used as a bioprotective culture to control the growth of *L. innocua*, *B. thermosphacta* and indigenous LAB in fresh meat discs incubated at 2°C was evaluated. In a previous study, Castellano *et al.* (2004) demonstrated that *L. innocua* had a similar growth rate and sensitivity to lactocin AL705 at refrigeration temperatures when compared with *L. monocytogenes*, suggesting that this pathogen might be reduced by similar amounts of bacteriocin. *Lactobacillus curvatus* CRL705 used as a bioprotective culture as well as lactocin AL705 were shown to be similarly effective in preventing the growth of *L. innocua* in meat discs throughout the storage period (Fig. 1b). A similar bacteriostatic effect was reported by Katla *et al.* (2002) when comparing the anti-*Listeria* effect of sakacin P and sakacin P-producing *Lact. sakei* strain on chicken cold cuts. Moreover, Hugas *et al.* (1998) working on vacuum-packaged fresh meat products, also found that *Lact. sakei* CTC494 and the bacteriocin produced, sakacin K, exerted a bacteriostatic effect on *Listeria*. On the other hand, Jacobsen *et al.* (2003) showed that the live cells of *Leuconostoc carnosum* 4010 were more effective than leucocins alone for the growth inhibition of *L. monocytogenes* in sliced meat products. In contrast, the addition of *Enterococcus faecium* CTC492 in fermented sausages and hamburgers did not exert any positive antilisterial effect when compared with the batches treated with enterocins A and B, this being attributed to a highly inhibition of the producer strain by refrigeration temperatures and by sausage ingredients (Aymerich *et al.* 2000).

Table 1 Bacteriocin activity in vacuum-packaged meat discs treated with *Lactobacillus curvatus* CRL705 or its bacteriocins, lactocin 705 and lactocin AL705, incubated at 2°C for 36 days

	Days of storage					
Treatment	0	7	14	21	28	36
Lact. curvatus CRL705	–	+	++	++	++	++
Lactocin 705	++	++	++	++	++	++
Lactocin AL705	++	++	++	++	++	++

–, no inhibition; +, inhibition halos <10 mm; ++, inhibition halos >10 mm.

The use of *Lact. curvatus* CRL705 as a bioprotective culture as well as lactocin 705 also showed an effective inhibition of *B. thermosphacta* in chill stored meat discs. This is in agreement with results from Metaxopoulos *et al.* (2002), who demonstrated that *Leuc. mesenteroides* L124 and *Lact. curvatus* L442 or their bacteriocins reduced *B. thermosphacta* population in cooked cured meat products under vacuum or modified atmospheres at 4°C. In addition, Cutter and Siragusa (1996) also demonstrated that nisin spray treatments followed by refrigerated vacuum packaging could increase the shelf life of fresh beef by inhibiting the growth of *Listeria* and *B. thermosphacta*. On the other hand, the control of indigenous LAB in meat would provide a shelf-life extension without

Table 2 Effect of *Lactobacillus curvatus* CRL705 on the pH values (±SE) of meat discs incubated at 2°C for 36 days

Treatment	Days of storage					
	0	7	14	21	28	36
Lact. curvatus CRL705	5·40 ± 0·15	5·34 ± 0·19	5·27 ± 0·22	5·37 ± 0,19	5·36 ± 0·15	5·48 ± 0·17
Listeria innocua + CRL705	5·34 ± 0·18	5·33 ± 0·20	5·30 ± 0·18	5·42 ± 0·22	5·31 ± 0·19	5·42 ± 0·19
Brochothrix thermosphacta + CRL705	5·37 ± 0·20	5·32 ± 0·17	5·33 ± 0·21	5·40 ± 0·18	5·34 ± 0·23	5·40 ± 0·23
Control	5·41 ± 0·16	5·41 ± 0·16	5·36 ± 0·22	5·39 ± 0·16	5·40 ± 0·20	5·50 ± 0·21

compromising the microbiological safety of the product. In the present study, the psychrophilic microbiota grew at a higher rate in the absence of the bacteriocin when compared to meat discs treated with lactocin 705, in which the final population was 2-log cycles lower after 36 days of incubation at 2°C. As part of the naturally growing LAB was suppressed, the addition of the bacteriocin could be an effective way of controlling undesirable organoleptic changes in vacuum-packaged meat. In addition, the bioprotective culture *Lact. curvatus* CRL705 has been successfully used to control beef spoilage *Lact. sakei* CRL1424 in meat slurry as was demonstrated by Castellano *et al.* (2004). Others bacteriocinogenic strains were also reported to control natural LAB microbiota such as *Lactococcus lactis*, producer of lacticin 3147 in fresh pork sausages (Scannell *et al.* 2000) and *Lact. sakei* TH1 during the commercial production of cooked meat products (Bredholt *et al.* 2001).

The biopreservative culture *Lact. curvatus* CRL705 was able to produce bacteriocins on the chilled meat discs during the 36 days of incubation at 2°C. Even when a low antimicrobial activity was detected during the first two weeks in the meat discs co-inoculated with *Lact. curvatus* CRL705, a decrease in *B. thermosphacta* population by more than 1-log cycle was produced. This low amount of bacteriocins that would be correlated with the adaptation period of the bioprotective culture to the meat substrate would be enough to inhibit bacterial growth. The antimicrobial activity detected throughout the experiment in meat discs inoculated with *Lact. curvatus* CRL705 or with lactocin 705 or AL705 would be responsible for bacterial suppression. This result agrees with those obtained in a previous study (Castellano *et al.* 2004), in which the co-inoculation of a meat slurry with the Bac^- variant of the bacteriocins producer *Lact. curvatus* CRL705 was unable to decrease the viable cell counts of *L. innocua* and *Lact. sakei* strains to the same extent as the bioprotective culture. On the other hand, changes in pH were not significantly different after incubation for 36 days at 2°C in meat discs treated with the protective culture when compared with control samples, which indicates that the presence of the bacteriocinogenic *Lact. curvatus* CRL705 will not produce organoleptic defects related to pH decrease.

As a result of this study it can be concluded that in addition to the hurdle represented by low temperature and vacuum packaging, the use of the bioprotective culture *Lact. curvatus* CRL705 as well as its bacteriocins lactocin 705 and AL705 in fresh meat will ensure its microbiological safety. Moreover, the use of live cells of *Lact. curvatus* CRL705 would be more feasible from an economic point of view – and without legal restrictions – compared with the addition of purified bacteriocins.

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