Research Article

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Characterization of a multilayer film activated with *Lactobacillus curvatus* CRL705 bacteriocins

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Abstract

BACKGROUND: Bacteriocins produced by lactic acid bacteria offer enormous promise for food safety preservation. In this study an active multilayer film obtained by the incorporation of lactocin 705 and lactocin AL705, two bacteriocins produced by *Lactobacillus curvatus* CRL705 with antimicrobial activity against *Lactobacillus plantarum* CRL691 and *Listeria innocua* 7, respectively, was characterized for its potential application in active packaging technology. Film activity performance at different storage conditions, bacteriocins transfer into water and sunflower oil, and film surface properties were evaluated.

RESULTS: Film activity against *L. innocua* 7 was maintained during 2, 4 and 6 weeks at 30, 10 and 5 °C respectively. At 30 and 10 °C, activity loss against *L. plantarum* CRL691 was observed on the second week of storage and after the fourth week at 5 °C. Results showed no significant difference for active multilayer film contact angle and seal properties compared to the control (without bacteriocins). A decrease in lactocin 705 inhibitory activity after sunflower oil contact was observed, while lactocin AL705 remained unaffected. After water contact, film activity was retained for both bacteriocins.

CONCLUSIONS: As demonstrated by antimicrobial activity and physico-mechanical properties retention, lactocin 705 and AL705 active multilayer film present potential for application in active packaging technology.

Keywords: active packaging technology; bacteriocins; *Listeria*; multilayer film

INTRODUCTION

Food packaging has undergone a tremendous development over the last 30 years. Packaging has been considered as a socio-scientific discipline ensuring that goods are delivered to the consumer in the best conditions intended for their use, performing one or more of the following functions: containment, protection, preservation, communication, utility and performance.[1,2] In particular, for the meat industry, the increased demands for greater stringency in relation to hygiene and safety, as well as the need to meet consumer expectations for convenience and quality, led to huge progress in food packaging materials and systems. Packaging of fresh meat is carried out to avoid contamination, delay spoilage, reduce weight loss, allow enzymatic activity to improve tenderness, and to ensure the presence of oxymyoglobin in red meats at the retail level,[3] while avoiding dehydration, lipid oxidation, discoloration and loss of aroma in processed meat products.[4] Even when meat products have been processed and packed under good manufacturing and hygienic practices, their shelf life is determined by the conditions applied during storage and distribution.

From a microbiological standpoint, meat and meat products are good supports for bacterial growth that will cause spoilage unless they are controlled or destroyed. The range of microbial taxa found in meat and meat products under various storage conditions include aerobes such us *Pseudomonas*,[5] cold-tolerant *Enterobacteriaceae, Brochothrix thermosphacta*, and lactic acid bacteria (LAB) occurring in chilled meat stored both aerobically and anaerobically.[6,7] The major threat to food safety are the so-called emergent pathogens, among which *Listeria monocytogenes, Escherichia coli* O157:H7, *Campylobacter jejuni, Yersinia enterocolitica* and *Vibrio parahemolyticus* have been related to the increase in outbreaks when compared to traditional food pathogens.[8,9]

In order to prevent meat from spoilage and the growth of pathogens, a series of selective preservative barriers must be applied. As, nowadays, meat trade throughout the world is carried out in vacuum-packed controlled atmosphere packs, low temperature and modified packaging constitute the most important hurdles.[7] Many meat packaging systems currently exist, from which active and intelligent technologies represent the major approaches.[10] Packaging may be termed 'active' when it performs some desired role in food preservation other

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than providing an inert barrier to external conditions.[11] Active packaging refers to the incorporation of certain additives into packaging systems with the aim of maintaining or extending product quality and shelf life. Several types of active substances can be incorporated into the packaging material to improve its functionality. For meat products in particular, and considering that microbial contamination occurs mainly on the surface due to post-processing handling, the use of packaging films containing bactericidal or bacteriostatic substances could be highly efficient. Slow migration from the packaging material, could help to maintain high antimicrobial concentrations where they are needed.[12–14] Many studies have been conducted using both, plastic (low/high density polyethylene, polypropylene, ethylene–vinyl acetate, ethylene–methacrylic co-polymers) and edible and/or biodegradable materials (vegetable and animal proteins films) as carriers for bioactive agents.[15]

Bacteriocins produced by LAB offer enormous promise for food safety improvements. Numerous antimicrobial peptides have been characterized and their antimicrobial potential has been well documented.[16,17] Nisin was used in most of the studies as antimicrobial for films activation, probably due to availability, regulatory concerns, and well known effectiveness against Gram-positive pathogens and spoilage agents. However, the application of bacteriocins other than nisin as antimicrobials for films has also been reported.[18–20] The efficacy of antimicrobial-incorporated material in food preservation is an important issue, in which the release rate and the extent of migration of the active agent from the packaging material to the food surface are the main factors. A dependence of active compounds migration on food simulants with polymer type, degree of cross-linking, simulant, and nature of the active compound was reported.[21,22] Since in a previous study bacteriocins produced by *Lactobacillus curvatus* CRL705 active against *Listeria, Brochothrix thermosphacta* and other LAB have been incorporated in a multilayer film with a linear low-density polyethylene food contact layer,[23] the functional characteristics of the active multilayer film are evaluated here, looking into its potential for use in active packaging technology.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Lactobacillus curvatus CRL705 (a producer of the bacteriocins lactocin 705 and lactocin AL705) and *Lactobacillus plantarum* CRL691 (which is sensitive to the activity of lactocin 705) were isolated from dry-fermented sausages[24] and grown in man rogosa sharpe (MRS) broth (Britania, Buenos Aires, Argentina) at 30 °C for 16 h. *Listeria innocua* 7 (sensitive to the activity of lactocin AL705) was obtained from the Unité de Recherches Laitières et Génétique Appliquée, INRA (Paris, France) and grown in trypticase soy broth (Britania) with 5 mg cm^{-3} of added yeast extract (Britania, Argentina) at 30 °C for 16 h. All strains were maintained and stored at −20 °C in 0.15 g cm^{-3} of glycerol until use.

Preparation of bacteriocins from *Lactobacillus curvatus* CRL705

The active solution containing lactocin 705 and lactocin AL705 was obtained by ammonium sulfate precipitation as described earlier.[23] An overnight culture of the bacteriocins producer *L. curvatus* CRL705 was centrifuged (2500 × *g*; 15 min); the supernatant was precipitated using 0.44 g cm^{-3} ammonium sulfate (Biopack, Buenos Aries, Argentina), centrifuged (20 000 × *g*; 20 min) and freeze dried. The active powder obtained was re-suspended in water (1 mg cm^{-3}, 267 AU cm^{-3} and 2133 AU cm^{-3} for lactocin 705 and lactocin AL705, respectively) and was subsequently used as a partially purified aqueous bacteriocins (PPAB) solution for the activation of the multilayer film (see below).

Activity of the bacteriocins and determination of stability

Bacteriocins antimicrobial activity in the PPABs solution was determined by a modification of the agar well diffusion assay.[25] Fifteen microliters of serial two-fold dilutions of the bacteriocins solution were added to 5 mm diameter wells cut in semi-solid MRS agar plates seeded with *L. plantarum* CRL691 for lactocin 705. The same experiment was performed with trypticase soy agar + yeast extract seeded with *L. innocua* 7 for lactocin AL705 quantification. The agar plates were stored at 4 °C for 24 h to allow pre-diffusion, incubated for 16–18 h at 30 °C and examined for zones of inhibition. Bacteriocin titer, expressed in arbitrary units (AU cm^{-3}) was defined as the reciprocal of the highest dilution, yielding a visible uniform zone of inhibition on the sensitive strain. Each determination was performed in duplicate. Antimicrobial stability of 1 mg cm^{-3} PPAB solution stored at −20 °C was determined every 6 months during 2 years by using the agar well diffusion assay.

Preparation and stability of the active multilayer film

A 100 µm multilayer film composed of an external polypropylene layer, an internal polyamide–polyethylene structure, a barrier layer of ethylene vinyl alcohol co-polymer, and a linear low density polyethylene (LLDPE) food contact layer (Cryovac; Sealed Air Co., Buenos Aires, Argentina), was used in this study. The multilayer film (19.6 cm^2) was contacted by its food contact face (LLDPE) with the PPAB solution (4.6 cm^3 of 1 mg cm^{-3}) during 1 h at 30 °C to incorporate the two antimicrobial agents (lactocin 705 and AL705). After treatment, the multilayer film was removed from the solution, rinsed with sterile distilled water and dried for 10 min at 50 °C.[23] Film antimicrobial activity was assayed by placing 1.0 cm diameter punched circles directly on two separate agar plates seeded with the corresponding sensitive strain for each bacteriocin. After incubation, film bioactivity was revealed as an inhibition zone of the sensitive organism beneath and around the multilayer film and was expressed as relative inhibition area (RIA) according to Equation 1, where *I* is the inhibition zone around the multilayer film and *A* is the film area. To study the utilization of the PPAB solution to perform more than one film activations, three multilayer films were contacted with 4.6 cm^3 of the same PPAB solution (1 mg cm^{-3}) and the relative inhibition area beneath and around the film was evaluated. Temperature and time influence on the film antimicrobial activity was evaluated by relative inhibition area determination after 7, 14, 32 and 45 days of storage at 30, 10 and 5 °C. Activity was expressed as the % inhibition, according to Equation 2, where RIA_0 and RIA_t are the relative inhibition areas at the beginning of the experiment and at time *t*, respectively.

$$RIA = \frac{I}{A} \quad (1)$$

$$\text{Inhibition (\%)} = \frac{RIA_t}{RIA_0} \times 100 \quad (2)$$

Contact angles and seal properties

Water contact angles on the LLDPE face of the active multilayer and control films (without bacteriocins), were measured using a contact angle goniometer (Rame-Hart 500; Netcong, NJ, USA)

under ambient conditions (50% relative humidity and 23 °C); tests were performed in quintuplicate. Seal strength of treated and non-treated films was compared using an Instron model 1125 (Instron, Norwood, MA, USA) at 50% relative humidity and 23 °C according to ASTM F 88/F88M-09.[26] Initial grip separation was set at 25 mm and cross-head speed at 200 mm min^{-1}. Seven replicates of thermo-sealed films were obtained by using a thermo-sealing machine (TP-701S Heat Seal Tester; Tester Sangyo Co., Tokyo, Japan) at 150 °C for 1 s and 2.5 Pa.

Evaluation of lactocin 705 and lactocin AL705 residual antimicrobial activity on the activated film after water and sunflower oil contact

The active multilayer film (0.95 cm^2) was contacted with water and sunflower oil (260 µL) representing hydrophilic and hydrophobic media, respectively. The test was carried out at 5 °C. After 10 days of contact, films were removed and evaluated for residual antimicrobial activity by relative inhibition areas determination, as described above. An active multilayer film stored at 5 °C for 10 days was used as bacteriocins positive control. Bacteriocins activity in water and sunflower oil, after the films were removed, was determined by the agar well diffusion assay. To determine the influence of hydrophilic and hydrophobic media on bacteriocins activity, the active freeze-dried powder containing *L. curvatus* CRL705 bacteriocins was re-suspended in each medium (lactocin 705 and lactocin AL705 concentration 67 and 533 AU cm^{-3}, respectively), stored 10 days at 5 °C and tested for activity every two days. All tests were performed in quadruplicates.

Statistical analysis

Experimental data were subjected to general linear model analysis of variance (GLM ANOVA), and the Tukey test was applied with a level of significance of 95%. All statistical analyses were performed using Minitab Statistic Program, release 12 (Pennsylvania, USA).

RESULTS

Antimicrobial activity of the PPAB solution as a function of time was evaluated. After 2 years of storage at −20 °C, full activity retention was observed (267 and 2133 AU cm^{-3} for lactocin 705 and lactocin AL705, respectively). PPAB solution re-utilization was determined after three consecutive films were contacted with the same bacteriocins solution. Activity evaluation of the treated films against *L. plantarum* CRL691 and *L. innocua* 7 showed that only the first contacted film was able to inhibit the growth of both sensitive organisms (Table 1).

Table 1. Anitimicrobial activity of three LDPE films after direct contact with the same activation solution (PPABs)

Bacteriocin	Relative inhibition area*		
	Film 1 (first activation)	Film 2 (second activation)	Film 3 (third activation)
Lactocin 705**	1 ± 0.3	ND	ND
Lactocin AL705***	1.7 ± 0.2	ND	ND

Results are the average of four replicates.
* Dimensionless quantity.
** Affected microorganism, *L. plantarum* CRL691.
*** Affected microorganism, *L. innocua* 7.
ND, not detected.

The active multilayer film exhibited different inhibitory activities when stored at 5, 10, and 30 °C during 45 days (Fig. 1). For both bacteriocins, the multilayer film antimicrobial activity was influenced by time and temperature ($P < 0.05$, GLM ANOVA). When lactocin 705 activity on the multilayer film surface was analyzed using *L. plantarum* CRL691 as sensitive strain, inhibitions of 32, 66 and 100% after 7 days of storage at 30, 10 and 5 °C, respectively, were observed (Fig. 1a) After 14 days, no antimicrobial activity on the multilayer film stored at 30 and 10 °C was detected. During the same period, the film stored at 5 °C retained 100% activity, then decreasing to 18% at day 32. On the other hand, anti-listerial lactocin AL705 adsorbed on the multilayer film showed inhibitory activity during longer periods of time for the three assayed temperatures (Fig. 1b). At 30 °C, the active multilayer film inhibition decreased up to 33% (day 14); while at 10 °C, an inhibition of 60% on the day 32 was observed. Active

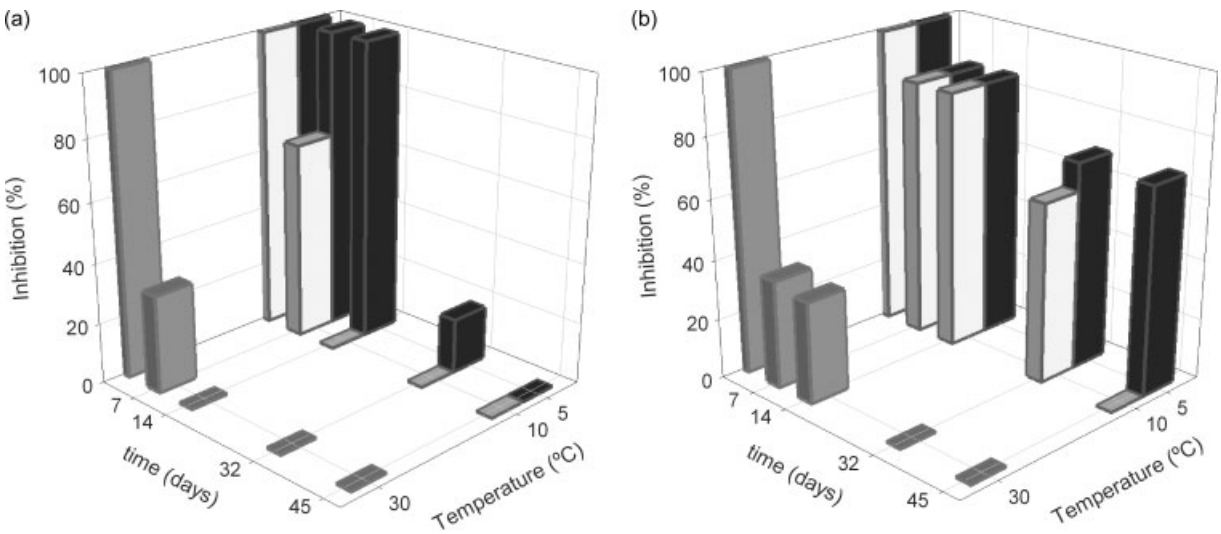

Figure 1. Antimicrobial stability of (a) lactocin 705 (antagonistic effect against *L. plantarum* CRL691), and (b) lactocin AL705 (antagonistic effect against *L. innocua* 7), on the active multilayer films stored at different temperatures, expressed as percent of inhibition.

Table 2. Residual antimicrobial activity of the active multilayer film after direct contact with water and sunflower oil

Control/activity	Bacteriocin	
	Lactocin 705*	Lactocin AL705**
Bacteriocin positive control***	$0.7^{a} \pm 0.4$	$1.8^{a} \pm 0.4$
Residual activity of the activated film***		
After water contact	$0.6^{a} \pm 0.2$	$1.5^{a} \pm 0.5$
After sunflower oil contact	$0.1^{b} \pm 0.1$	$1.1^{a} \pm 0.7^{a,b}$

[a,b] In each column, means with different superscripts are significantly different ($P < 0.05$).
* Affected microorganism, *L. plantarum* CRL691.
** Affected microorganism, *L. innocua* 7.
*** Residual antimicrobial activity is expressed as relative inhibition area from four replicates (see Materials and methods).

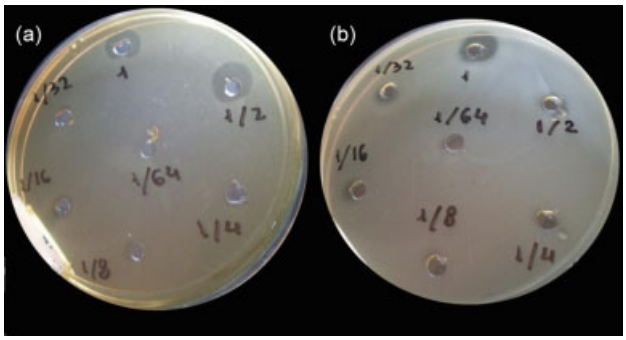

Figure 2. Agar well diffusion assay for (a) lactocin 705 and (b) lactocin AL705 quantification when re-suspended in sunflower oil.

multilayer films stored at 5 °C exhibited 69% inhibitory activity retention at the end of the test (45 days). Even when lactocin AL705 proved to be more stable than lactocin 705, lower storage temperatures helped to keep longer antimicrobial activities on the active multilayer film.

Contact angle and seal strength values after activation treatment were measured. Results of five replicates demonstrated that contact angle of the control ($92 \pm 2^{\circ}$) and active film ($90 \pm 2^{\circ}$) were not significantly different ($P \geq 0.05$). Films seal strength studies showed material elongation with delamination and seal break, and remote material break failures. As remote material break is due to the material itself, it was not taken into account to calculate material seal strength.[26] No significant difference ($P \geq 0.05$) was observed for the control (2420 ± 221 N m^{-1}) and the activated film (2477 ± 142 N m^{-1}) seal strength.

Results of residual antimicrobial activity of the multilayer film after contact with water and sunflower oil are shown in Table 2. In the presence of water, lactocin 705 activity on the film surface was not significantly different ($P \geq 0.05$) compared with bacteriocins positive control film (see materials and methods). After contact with sunflower oil, the activated film showed a decrease in lactocin 705 inhibitory activity compared to control film. As for lactocin AL705, the activated film remained active after contact with water and sunflower oil (Table 2). After 10 days of active multilayer film contact with water and sunflower oil, no antimicrobial activity was detected by the well agar diffusion assay in both studied media. In addition, the active powder containing lactocin 705 and AL705 (see materials and methods) re-suspended in water showed bacteriocins activity retention over 10 days at 5 °C (67 and 533 AU cm^{-3} for lactocin 705 and lactocin AL705, respectively). As the active powder was not soluble in sunflower oil, activity evaluation by the well agar diffusion assay showed no correlation between inhibition areas and two-fold dilution (Fig. 2), consequently titer evaluation in this medium did not lead to reliable results.

DISCUSSION

The development of efficient delivery systems becomes important in order to maximize the biopreservative potential of bacteriocins. In view of its potential application in packaging technologies, functional characterization of a multilayer antimicrobial film containing two bacteriocins produced by *L. curvatus* CRL705 was carried out in this study. It was shown that the PPAB solution used to obtain the active multilayer film retained antimicrobial activity and remained fully stable after storage at −20 °C for 2 years. This result is in accordance with other authors who also found that cold temperature seems to be the most appropriate preservation condition for long-term storage of bacteriocins.[27–29] Although the PPAB solution remained active after the first multilayer film activation, direct contact of second and third films with the same PPAB solution resulted in inactive films (Table 1). This lack of activation ability made it impossible to re-use bacteriocins solution for more than one film adsorption process. A decrease in bacteriocin concentration in the PPAB solution, below the minimum necessary to reach the adsorption equilibrium of lactocin 705 and AL705 onto the film surface, could explain this result.[23]

Before its application in food products, it is important to first evaluate the shelf life of the bioactive film. Previous studies have shown that LAB bacteriocins retain their activity when incorporated in various materials under different storage conditions.[30–32]

Immobilized nisin on different plastic materials showed antimicrobial activity retention after 3 months at chill and room temperatures.[18,33] Also, the anti-listerial bacteriocin from *Lactobacillus curvatus* 32Y adsorbed on polythene-oriented polyamide (PE-OPA) films was able to maintain its activity for 4 months at room temperature.[19] In this study, the antimicrobial stability of a multilayer film decreased when storage temperature increased. It was shown that the optimal time–temperature storage conditions to assure activity for both bacteriocins on the multilayer film were 2 weeks and 5 °C. Therefore, while the active solution (PPAB) can be stored without loss of activity for 2 years at −20 °C, active multilayer films containing lactocin 705 and AL705 must be prepared a few days before its application. In the active film, antilisterial bacteriocin lactocin AL705 maintained its activity during 45 days at chill temperatures. However, in the same conditions lactocin 705 antimicrobial activity was retained after 2 weeks. These results encourage promising applications of lactocin 705 and AL705 active multilayer films in ready-to-eat meat products, such as Bologna and Vienna sausages,[34] susceptible to LAB spoilage, and *Listeria* post-process contamination.

According to Han,[35] several factors must be considered in antimicrobial film design for packaging applications. The physicochemical nature of the polymer, the characteristics of antimicrobial substances and foods, the interaction between active compounds and the polymer matrix, and migration from the active material, are among the most important.[35–37] Antimicrobials adsorbed or immobilized onto polymer surfaces may alter heat sealing strength, adhesion and printing properties of plastics.[38] The

contact angle of the film surface serves as a measure of the wettability which, in turn, is indicative of the adherability of the film surface.[39] In this study, the active multilayer film wettability, determined by contact angle, and the seal strength values remained unaffected after the activation treatment, compared to control films. These results suggest that multilayer film activated with lactocin705 and AL705 did not change surface hydrophobic pattern (contact angles 90–96°).[40,41] Similarly, Mauriello *et al*.[19] found no significant differences in solderability after PE-OPA films activation with the antilisterial bacteriocin produced by *Lactobacillus curvatus* 32Y. Unaffected multilayer film sealing properties would assure active packaging integrity during food products storage.

As a general observation, proteinaceous substances adsorb on hydrophobic surfaces in greater amounts as opposed to hydrophilic surfaces.[42] However, Bower *et al*.[30] and Daeschel *et al*.[42] found higher nisin adsorption to hydrophilic modified silicon surfaces, thus lower contact angle surfaces showed higher nisin activities. In our work, the LLDPE hydrophobic (higher contact angle) film surface presented lactocin 705 and AL705 antimicrobial activity that remained active even after water contact. This result could suggest that hydrophobic forces would be responsible for adsorption of bacteriocins onto the multilayer film surface (LLDPE layer). Studies on bacteriocins and film surface interactions are currently being carried out.

Research in food technology reveals that chemical composition and treatment of foods may play an important role in the antimicrobial activity of bacteriocins.[43–45] In this study, water and sunflower oil, selected as hydrophilic and hydrophobic media, were chosen in order to determine bacteriocin inactivation and/or release from the film. Our results showed that the physico-chemical nature of the media in contact with the active multilayer film could affect residual activity of adsorbed lactocin 705 and AL705. Retention of antimicrobial activity was observed when bacteriocins were in contact with water (from active powder or the multilayer film). When the active multilayer film was in contact with sunflower oil, a decrease in lactocin 705 residual activity compared to control film, was observed. Reduction of lactocin 705 activity in the presence of sunflower oil may be related to its mode of action on target cells. Lactocin 705 activity depends upon the complementation of two peptides that interact with sensitive organism membrane at different bilayer levels. Lac705α peptide interacts with the interfacial region inducing dehydration, while lac705β peptide interacts with the hydrophobic core to form transmembrane oligomers involved in membrane permeabilization.[46] In contact with sunflower oil, a reduced ability of lac705β peptide to interact with *L. plantarum* CRL691 sensitive cell membrane may be suggested. Similar results have been reported for nisin and sakacin P anti-listerial activity in presence of high fat content in different foods.[36,43–45]

Active polymeric materials, developed by the incorporation of new bacteriocins isolated from LAB, may be used in food packaging technology in order to improve food quality and safety. In this study, it was demonstrated that a multilayer film activated with lactocin 705 and lactocin AL705 shows promising functional properties for applications to extend the shelf life of meat products. The LLDPE food contact layer from the multilayer film served as a bacteriocin carrier, showing effective antimicrobial activity without affecting film sealing properties. Multilayer film anti-listerial activity was retained for at least 6 weeks at chill temperatures and after contact with water and sunflower oil. The active solution containing the bacteriocins stored at −20 °C retained its activity for a period of 2 years. The application of lactocin 705 and lactocin AL705 for the control of spoilage and pathogen microorganisms may contribute to maintaining the stability of perishable foods, thus avoiding economic losses.

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