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Natural and artificial casings as bacteriocin carriers for the biopreservation of meats products

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Abstract

The aim of the study was to determine if natural and artificial casings (ovine, porcine, bovine, collagen, and cellulose casings), serve as carriers for the application of the bacteriocin sakacin G produced by *Lactobacillus curvatus* ACU-1. Mode of action of the cell-free supernatant (CFS) containing the bacteriocin was also studied to determine whether *Listeria monocytogenes* inhibition could be tantamount to *Listeria innocua* one. Cylindrical vessels were filled with sterile meat emulsion and were wrapped with the different casings. Half the systems were treated with the CFS containing sakacin G, while the other half was taken as control systems. All systems were inoculated with *L. innocua* ATCC 33090. *L. innocua* was able to grow on all the studied casings. Collagen casing treated with CFS inhibited *Listeria* growth. A bacteriostatic effect was observed in the systems with porcine, ovine, bovine and cellulosic casings treated with CFS. The bacteriocin maintained its activity until the end of the trial. All casings were widely promising as antimicrobial application supports, being effective carriers to be used in a wide range of meat products.

Practical applications

Based on its advantageous characteristics, bacteriocins attracted considerable interest as natural food preservatives to extend shelf life and safety of meat and meat products. The application of sakacin G produced by *Lactobacillus curvatus* ACU-1 on different natural and artificial casings would allow the preservation of a wide range of meat products that are stuffed into them, such as several types of sausages, wiener, salami, and mortadella, among others. The major advantage relies on its application, since it is not necessary to modify the production process or add extra wrappings to support the antimicrobial substance.

1 | INTRODUCTION

Different types of sausages, stuffed into natural or artificial casings, are regarded as one of the oldest types of processed meat products (Feng, Drummond, Zhang, & Sun, 2014). Natural casings are tender and have high permeability to both moisture and smoke (Savic & Savic, 2002) and are often considered the golden standard of sausage casings. These casings are made by cleaning and stripping away both the mucosa and muscle layers from the submucosa layer of the animal's small intestine (Bakker, Houben, Koolmees, Bindrich, & Sprehe, 1999; Koolmees, Tersteeg, Keizer, van den Broek, & Bradley, 2004). The ready-to-use casing consists of the submucosa which is made up of cylindrical sheets of collagen as well as elastic components, being structurally the most important layer of the intestinal tract. Collagen is the main factor responsible for the natural casing's tensile strength (Harper, Barbut, Lim, & Marcone, 2012; Savic & Savic, 2002). Artificial casings were created at the beginning of the 20th century, when the requirements of the meat industry—which was rapidly developing—overcame the supply of natural casings. Artificial casings have gained increasing interest for sausage manufacture, due to their uniform size, shape and strength, flexibility, hygienic quality, because microbiological contamination is negligible, storage at low temperatures unnecessary, and there is no problem with product spoilage during storage and transport. Artificial casings are usually not as permeable, elastic, and tension resistant as natural casings, especially after cooking (Djordjevic et al., 2015; Feng et al., 2014; Pecanac et al., 2015).

Several studies have shown that the addition of bacteriocins to the surface of meat products by spraying, immersion or packaging systems

is more efficient than its addition to the meat mass (Barros, Kunigk, & Jurkiewicz, 2010; Kouakou et al., 2008; Rivas, Castro, Vallejo, Marguet, & Campos, 2014). In this sense, the use of a packaging system containing bacteriocins could be highly efficient, by continuous release of bacteriocins from the packaging to the surface of the products, thus helping maintain its effective concentrations (Quintavalla & Vicini, 2002). The application of bacteriocins in food-contact materials requires lower amounts of bacteriocins compared to the direct addition into the whole meat volume, thus reducing the use of the preservative and decreasing the processing costs (Balciunas et al., 2013). Lowering the costs of biopreservation processes may be highly attractive, especially for small economies and developing countries, where food safety may be seriously compromised (Holzapfel, 2002).

The two major methods for bacteriocin incorporation into meat packaging usually used are: (1) direct incorporation of bacteriocins into film matrix, and (2) indirect incorporation by coating bacteriocins onto the film surface (Woraprayote et al., 2016). Natural and artificial casings that are currently used in the elaboration of meat products would be highly advantageous as carriers for the application of bacteriocins since: (1) the biopreservation process would not increase the cost of production, that is, being functional as a support for the bacteriocin, the casings would be used as they are currently used; (2) the bacteriocin application would not involve major changes in the production process, yet only a previous stage of immersion in a bulk containing the antimicrobial substance would be added. The carrier acts as a reservoir and diffuser of the bacteriocin molecules to the food, guaranteeing a gradient-dependent continuous supply of bacteriocin. The carrier may also protect the bacteriocin from inactivation by interaction with food components and enzymatic inactivation (Ercolini, La Storia, Villani, & Mauriello, 2006).

Bacteriocin-producing *Lactobacillus curvatus* ACU-1 was isolated from dried sausages made in Chaco, Argentina (Castro, Palavecino, Herman, Garro, & Campos, 2011). The cell-free supernatant (CFS), containing sakacin G (Mechoud et al., 2017), showed to be effective for the control of *Listeria innocua* on the surface of pork without losing effectiveness by adsorption to the fatty or meat tissues (Rivas et al., 2014). These results suggest it as a potential natural preservative for application in various meat products.

The aim of the study was to determine if natural casings, that is, ovine, porcine and bovine, and artificial wrappers, that is, collagen and cellulose, serve as support for the application of sakacin G, contained in the CFS of the strain *Lb. curvatus* ACU-1, using a model system composed of a sterile meat emulsion contained in vessels embedded in the different treated casings. Mode of action of the CFS containing the bacteriocin was also studied to determine whether *Listeria monocytogenes* inhibition could be tantamount to *L. innocua* one.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains, culture conditions, and CFS

Strains used in this study were: (1) *Lb. curvatus* ACU-1, isolated from artisanal dry sausages manufactured in Chaco, Argentina (GenBank accession number JX979220) (Castro et al., 2011; Rivas et al., 2014), (2) *L. innocua* ATCC 33090 and *Listeria monocytogenes* 01/155 (Applied Bacteriology Laboratory, INIQUI-CONICET, Salta, Argentina). Bacteria were maintained as frozen stocks at −30 °C in the suitable medium and were propagated twice in the appropriate culture media at 30 °C before use. *Lb. curvatus* was recovered in de Man, Rogosa, and Sharpe broth (MRS, Biokar Diagnostics, Allonne, France), while both strains of *Listeria* were recovered in Brain Heart Infusion (BHI, Biokar Diagnostics).

The CFS of *Lb. curvatus* ACU-1, that contained the bacteriocin sakacin G, was obtained from a culture of 36 hr at 30 °C in MRS broth. The culture was centrifuged at 4000 × g for 20 min, then the supernatant was carefully collected, and sterile-filtered using a 0.22 μm pore size cellulose acetate filter (Sartorius, Goettingen, Germany). Afterwards, the CFS was adjusted to pH 7.0 with sterile 1 M NaOH (Rivas et al., 2014). Antimicrobial activity of the CFS was expressed in arbitrary units (AU), being 800 AU/ml.

2.2 | Mode of action

Sakacin G mode of action in liquid medium was determined for both strains of *Listeria*. Overnight cultures of *L. innocua* ATCC 33090 and *L. monocytogenes* 01/155 were treated with the CFS at a ratio of 1:10 to yield an initial bacterial count of about 10^8 cfu/ml. Control samples consisted of bacteriocin-free BHI broth inoculated with the corresponding indicator bacterium. Samples were incubated at 30 °C. For determination of viability loss of sensitive cells, samples were withdrawn at 1 hr intervals for OD measurements (600 nm). Bacterial enumeration was also carried out along the trial by plating samples on BHI agar. Log10 colony forming units (cfu/ml) against time were used to determine the kinetics of indicator viability loss. A reduction in cfu/ml after the treatment is an indication of cell viability loss (Bhunia, Johnson, & Ray, 1988). The trial was carried out in duplicate and shown data are the means of the replicates.

2.3 | Evaluation of the antimicrobial effectiveness of the CFS applied onto different casings

Casings tested in this study were the ones currently used by meat processing plants in Argentina; they were supplied by a local small-scale facility. Natural casings used were: ovine, porcine and bovine; and artificial wrappings: collagen and cellulosic. To simulate the industrial process prior to the stuffing, the natural casings were washed for 3 hr in sterile distilled water, while the artificial ones were only moistened.

Sterile cylindrical vessels (2.4 cm long and 1.8 cm diameter) were manually filled with sterile meat emulsion. This filling emulsion was obtained from Viennese sausages (provided by a local small-scale facility) processed in a cutter and sterilized by autoclave. Each studied system was set up filling the vessels with the meat paste and wrapping them with each one of the studied casings. These coverings were adjusted by attaching them to the opposite end of the vessel with a cotton thread (Figure 1). They were then placed in water at 85 °C for 10 min, simulating the conditions to which sausages are subjected during the manufacturing process. Half of systems wrapped with each

FIGURE 1 Receptacle filled with the meat paste, and wrapped with the casing which was embedded with the CFS. Casing was tied at the opposite end with a cotton thread

type of casing was treated with the CFS by 5 min immersion. While the other half—not treated with CFS—was immersed in sterile MRS broth and taken as control systems. Then, they were inoculated with a suspension of *L. innocua* ATCC 33090 (10^5 cfu/ml) by spraying, simulating postproduction surface contamination. The different systems thus obtained were vacuum packed individually in polyethylene bags (oxygen permeability: 70 [cm^3/m^2/24 h/atm] at 25 °C and 75% RH) and stored at 5 °C for 28 days. Samples were withdrawn along storage at 0, 2, 5, 8, 11, 14, 21, 28, and 35 days. The extreme of the vessel where the casing was in contact with the meat mass was swabbed to sample *L. innocua* for bacterial count determination on PALCAM agar. Bacterial counts were expressed in log cfu per square centimeter, considering the circular surface of the cylindrical vessel that was swabbed (area = πr^2).

2.4 | Statistical analyses

A randomized block design was adopted and the entire experiment was replicated three times on three different days. An analysis of variance (ANOVA) was carried out to analyze the microbial counts, which considered the storage time for each casing as a fixed effect, and the replications of the experiments as a random term (n = 3). The level of significance was set at $p \leq .05$ for all analyses and Tukey test was performed to separate means. All the tests were performed with the software Statgraphics Plus 4.0 (Manugistics, Inc., Rockville, MD).

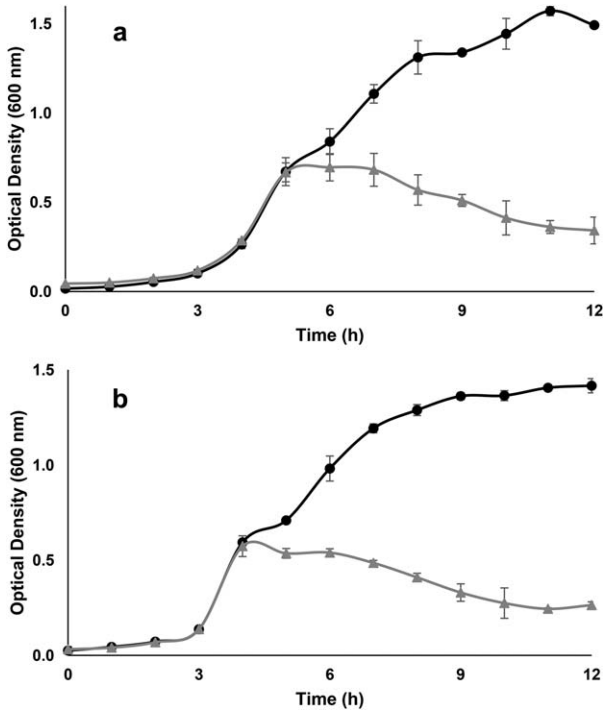

FIGURE 2 Effect of *Lb. curvatus* ACU-1 cell-free supernatant (CFS) on the growing cells of (a) *L. innocua* ATCC 33090 and (b) *L. monocytogenes* 01/155. (●) Indicator strain culture without CFS; (▲) indicator strains cultures with CFS was added

3 | RESULTS AND DISCUSSION

3.1 | Mode of action

Autochthonous strain *Lb. curvatus* ACU-1 has been investigated due to its antimicrobial effect, which can be mainly attributed to the production of bacteriocins. Although three bacteriocins were found to be genetically expressed, that is, sakacin Q, sakacin P, and sakacin G (Rivas et al., 2014), the latter is the only one effectively produced and released to the extracellular environment (Mechoud et al., 2017). Mode of action determination comprises an important primary data for the prospective application of the bacteriocin-producing strain and/or its metabolites as part of a hurdle system in the biopreservation of food. In this sense, two *Listeria* strains were chosen as target microorganisms. The addition of *Lb. curvatus* ACU-1 CFS at the exponential phase of growth of *L. innocua* ATCC 33090 and *L. monocytogenes* 01/155 cell suspensions resulted in a decrease in optical density following a similar behavior (Figure 2). These results are in keeping with bacterial counts of both indicator microorganisms which showed a decrease in viable cells from 10.25 ± 0.14 (Log10 cfu/ml)—at the moment of adding the CFS—to 9.31 ± 0.01 (Log10 cfu/ml) at the end of the trial, thus confirming the inhibitory effect of the CFS (Table 1). Moreover, as no statistical differences were observed between them, neither at the beginning of the trial, nor at the moment of bacteriocin addition, or at

TABLE 1 Bacterial counts (Log10 cfu/ml) of indicator strains in the presence and absence of CFS

	Time (hr)			
	0	4	8	12
L. innocua ATCC33090	$8.12^{a} \pm 0.78$	$10.33^{a} \pm 0.02$	$11.89^{a} \pm 0.05$	$11.88^{a} \pm 0.03$
L. innocua ATCC33090 + CFS	$8.38^{a} \pm 0.43$	$10.38^{a} \pm 0.20$	$9.58^{b} \pm 0.07$	$9.31^{b} \pm 0.01$
L. monocytogenes 01/155	$8.42^{a} \pm 0.33$	$10.23^{a} \pm 0.08$	$11.76^{a} \pm 0.05$	$11.83^{a} \pm 0.05$
L. monocytogenes 01/155 + CFS	$8.53^{a} \pm 0.16$	$10.10^{a} \pm 0.04$	$9.43^{b} \pm 0.06$	$9.32^{b} \pm 0.00$

[a–b]Means within the same column with different superscript letters are different ($p < .05$—Tukey Test).

the end of the experiment, it can be assumed that sakacin G from *Lb. curvatus* ACU-1 exerted the same inhibition pattern toward *L. innocua* ATCC 33090 and *L. monocytogenes* 01/155. In view of this finding, and the fact that *L. monocytogenes* is a well-known pathogen, *L. innocua* was chosen as the indicator bacteria for further experiments.

3.2 | Antimicrobial effectiveness of sakacin G applied onto different casings

About 10% of all foodborne fatalities were caused by listeriosis (FDA-SFSAN, USDA-FIS, 2003). Facilities that produce ready-to-eat (RTE) meats must control this hazard through their HACCP program since products are post-lethality expose to possible *L. monocytogenes* contamination. Introduction of bacterial metabolites with antilisterial activity into the hurdle technology concept has been exploited in the past 30 years. Nevertheless, scarce references can be found when searching for natural and artificial casings as carriers for these metabolites (Barbut, 2010; Djordjevic et al., 2015; Harper et al., 2012). Consequently, five different types of casings were studied to find out which could be the best suited for superficial bacteriocin application in RTE meat products.

L. innocua—a surrogate of *L. monocytogenes* that shares physiological characteristics with the pathogen—was able to colonize and grow on the different studied systems (Table 2). Significant increase in *Listeria* counts can be observed in all control systems until the end of the trial, reaching counts in the range of 6.10–9.04 Log cycles. It has to be mentioned that, among the control systems, the bovine casing showed to slow down *Listeria* growth. This fact could be attributed to histological differences between this particular type of natural casing among the others (Wijnker, Tersteeg, Berends, Vernooij, & Koolmees, 2008). Massani et al. (2014) reported that all control systems filled into artificial casings (without bacteriocins) inoculated with *L. innocua*, showed the typical growth of the indicator microorganism reaching maximum level of 7–8 Log cycles at Day 45 of storage at 5 °C.

Regarding the systems treated with the CFS containing sakacin G, differences in antimicrobial activity were found depending on the casing used as carrier. The porcine and collagen casings had a statistically significant reduction of *Listeria* from second and fifth storage days, respectively. At end of the test, the collagen casing had a reduction close to 2 Log cycles. Since differences of one order of magnitude are generally regarded as being of microbial significance (Gill & Holley, 2000), collagen casing might have a promissory use as *Lb. curvatus* ACU-1 CFS carrier. Conversely, a bacteriostatic effect was observed in the systems with porcine, ovine, bovine, and cellulosic casing until the end of the trial; there were not significant differences in the count of the indicator microorganism throughout whole essay. Similar trends to the ones registered for ovine, bovine and cellulosic casing were reported by Massani et al. (2014). In their study, lactocins produced by *L. curvatus* CRL705 exerted a bactericidal effect, against *L. innocua* 7 in laboratory media, whereas when applied to sausages using an active packaging system only bacteriostatic effect was observed; stating the importance of studying the real food, since interactions with food components (such as enzymes and lipids) can affect the activity of the antimicrobial substance.

When comparing each system with its own control, different inhibition rates of *Listeria* were observed depending on the treated casing. Thus, from the second day of the trial, significant differences were registered in the counting of the indicator microorganism between the CFS treated cellulose casing system and its respective control. After 5 days, significant differences were detected in the ovine, porcine, and collagen treated systems compared to their untreated controls. From 8 days ahead, all the systems exhibited significant differences with respect to their controls. Several studies had reported the effectiveness of the contact method in providing active films incorporating antimicrobial substances. Nisaplin was incorporated into cellophane-based packaging by contact, which was effective for the control of microbial growth in chopped meat (Guerra, Macías, Agrasar, & Castro, 2005). In addition, Daeschel, McGuire, and Al-Makhlafi (1992) reported the antimicrobial activity of nisin after adsorption onto hydrophobic and hydrophilic silicon surfaces using the contact method. Bacteriocins other than nisin were also applied by various methods to develop active plastic materials for *L. monocytogenes* inhibition in agar media or food systems, such as the bacteriocin from *L. curvatus* 32Y and CWBI-B28 (Ercolini et al., 2006; Ghalfi, Allaoui, Destain, Benkerroum, & Thonart, 2006; Mauriello, Ercolini, La Storia, Casaburi, & Villani, 2004), lacticin 3147 produced by *Lactococcus lactis* subsp. lactis (Scannell et al., 2000)—and pediocin from *Pediococcus acidilactici* (Massani, Fernandez, Ariosti, Eisenberg, & Vignolo, 2008; Ming, Weber, Ayres, & Sandine, 1997).

Preservatives must have low diffusivity in the film where they are applied to remain at the surface of the food, since diffusion into the food center results in a preservative concentration reduction at the surface (Scannell et al., 2000). Throughout this study the bacteriocin maintained its activity in the different treated systems, suggesting that the

TABLE 2 Counts of *Listeria innocua* ATCC 33090 inoculated onto the different studied casings

Time (days)	Casings									
	O	O + CFS	P	P + CFS	B	B + CFS	Co	Co + CFS	Ce	Ce + CFS
0	2.58 ± 0.31a	2.22 ± 0.07a	2.61 ± 0.27a	2.40 ± 0.35a	2.62 ± 0.22a	2.27 ± 0.17a	2.88 ± 0.06a	2.31 ± 0.47b	2.26 ± 0.17a	2.05 ± 0.11a
2	2.84 ± 0.07b	1.99 ± 0.41a	2.81 ± 0.21b	2.31 ± 0.11b	2.73 ± 0.09b	2.13 ± 0.32a	2.92 ± 0.10b	2.34 ± 0.14a	2.34 ± 0.20b	1.47 ± 0.12a*
5	3.46 ± 0.03c	1.79 ± 0.48a*	3.06 ± 0.30c	2.20 ± 0.13e*	2.99 ± 0.27c	2.28 ± 0.36a	3.60 ± 0.03c	1.53 ± 0.25d*	3.46 ± 0.38c	1.17 ± 0.02a*
8	3.89 ± 0.56d	1.66 ± 0.42a*	3.72 ± 0.37d	2.05 ± 0.29f*	3.25 ± 0.42d	1.64 ± 0.18a*	4.41 ± 0.79d	1.31 ± 0.51e*	3.85 ± 0.16d	0.77 ± 0.10a*
11	5.05 ± 0.92e	1.79 ± 0.39a*	4.20 ± 0.97e	1.54 ± 0.51g*	3.27 ± 0.14e	2.08 ± 0.25a*	5.55 ± 0.10e	1.91 ± 0.13c*	4.56 ± 0.63e	1.41 ± 0.56a*
14	5.85 ± 0.55f	1.66 ± 0.36a*	5.04 ± 0.44f	1.14 ± 0.72i*	3.63 ± 0.48f	1.58 ± 0.42a*	6.02 ± 0.50f	0.89 ± 0.36f*	5.78 ± 0.66f	1.75 ± 0.84a*
21	6.34 ± 0.60g	1.72 ± 0.32a*	6.88 ± 0.54g	1.37 ± 0.78h*	4.67 ± 0.82g	1.67 ± 0.08a*	6.03 ± 1.10g	0.66 ± 0.31h*	6.64 ± 0.18g	0.87 ± 0.14a*
28	7.77 ± 0.51h	2.11 ± 1.47a*	6.65 ± 1.28h	2.20 ± 0.50d*	5.81 ± 2.19h	1.88 ± 1.03a*	7.70 ± 0.65h	0.80 ± 0.19g*	7.54 ± 0.63h	1.61 ± 1.72a*
35	9.04 ± 0.00i	1.69 ± 0.29a*	8.44 ± 0.70i	2.22 ± 1.15c*	6.10 ± 1.85i	1.90 ± 1.13a*	8.99 ± 0.62i	0.54 ± 0.11i*	8.08 ± 0.67i	1.44 ± 0.32a*

Values are expressed as the mean ± SD from three independent experiments. Mean values in the same column with the same letter are not significantly different. The mean values of system + CFS marked with * have significant differences compared to the respective control system at each sampling time ($p \leq .05$).
O = ovine; P = porcine; B = bovine; Co = collagen; Ce = cellulosic; + CFS = are the respective systems treated with CFS.

diffusivity of the antimicrobial from all studied carriers to the food was sufficiently low during the 35 days of the trial. At the end of storage at 5 °C (35 days), *L. innocua* counts were 6.99 Log cycles lower in ovine casing; 6.00 in porcine casing; 3.85 in bovine casing; 7.89 in the collagen cover and 6.43 in the cellulose cover, regarding their respective control, which demonstrates the effectiveness of bacteriocin to prevent *Listeria* from growing steeply. On the contrary, Iseppi et al. (2008) reported a decrease in *Listeria* inhibition as a function of time when inoculated frankfurter samples which were packed with an enterocin-doped LDPE film, suggesting that bacteriocin diffusion out of the coating was fast. Barros et al. (2010) disclosed a decrease in nisin activity in sausages superficially treated with nisin in acid solution over storage period, they suggested that the diminished activity was due to an adsorption of nisin onto meat proteins or lipid particles.

Many studies have reported the development of coatings that would be used as a support in the application of bacteriocins in different food products (Daeschel et al., 1992; Ercolini et al., 2006; Ghalfi et al., 2006; Iseppi et al., 2008; Massani et al., 2008; Mauriello et al., 2004; Scannell et al., 2000; Woraprayote et al., 2013). A marked diminution of the antimicrobial activity was observed throughout these studies when bacteriocins were included as part of the core of the packaging. In this regard, the advantage of the application proposed in this study is the use of the same packaging—casings—used in the manufacture of various meat products. The antimicrobial substance is directly incorporated into the casing by immersion, being simpler, and lower cost compared to other proceedings, since bacteriocin addition does not modify the production process. Furthermore, it can protect bacteriocin from inactivation (Iseppi et al., 2008).

Based on the presented results, it is concluded that the collagen casing was more effective as carrier of the bacteriocin sakacin G, contained in the CFS, since there was a statistically significant reduction of *Listeria* in these systems. While in the porcine, ovine, cellulosic and bovine casings the CFS only had a bacteriostatic effect. Despite this important difference, all casings were widely promising as antimicrobial application carriers in a wide range of meat products where these casings are used.

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