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Occurrence of antilisterial structural bacteriocins genes in meat borne lactic acid bacteria

Cecilia Fontana [a], Pier S. Cocconcelli [a], Graciela Vignolo [b], Lucila Saavedra [b, *]

[a] Istituto di Microbiologia, Centro Ricerche Biotecnologiche, Università Cattolica del Sacro Cuore, Piacenza-Cremona, Italy
[b] Centro de Referencia para Lactobacilos (CERELA), CONICET, Chacabuco 145, Tucumán, Argentina

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ABSTRACT

The ability to inhibit the growth of *Listeria* cells and the presence of bacteriocin encoding genes was examined in 115 LAB strains isolated from Argentinean vacuum-packaged beef and different traditional fermented sausages. *Lactobacillus* (*L*) *sakei*, *Lactobacillus* (*L*) *curvatus* and *Enterococcus* (*E*) *faecium* showed a great inhibition of all *Listeria* strains evaluated while *Pediococcus* (*P*) *acidilactici* and *Lactobacillus* (*L*) *plantarum* demonstrated a limited or absent antilisterial activity. Both *L. curvatus* and *L. sakei* carried the *sppA*, *sppQ* and *sapA* structural genes, encoding for sakacin P, sakacin Q and curvacin A bacteriocins, respectively. Whilst *L. curvatus* exhibited a higher occurrence of these genes, *L. sakei* strains were more effective at inhibiting *Listeria* (*L*) strains, *Listeria monocytogenes* UC8159 and *Listeria innocua* 7 being the most sensitive to these bacteriocins. Among analyzed *E. faecium* strains, the wide distribution of *entA*, *entB* and *entP* genes accounted for the high antilisterial activity particularly observed against *L. monocytogenes* FBUNT. The structural gene *plantEF* was mostly present in *Lactobacillus plantarum* strains and no *pedA* gene was found in *P. acidilactici* evaluated strains. The antilisterial potential of *L. sakei* and *E. faecium* offers great possibilities for the meat industry as biopreservative cultures, although more studies are needed in order to conclude about this issue.

1. Introduction

Processing technologies as well as nutrients availability in meat are known to limit bacterial growth to those specially featured organisms, determining the type and number of bacteria present in the different meat ecosystems (Nieto-Lozano, Reguera-Useros, Pelaez-Martinez Mdel, & Hardisson de la Torre, 2006; Nychas, Skandamis, Tassou, & Koutsoumanis, 2008). Despite the progress in food biotechnology, meat industry is still under scrutiny due to the frequent outbreaks of foodborne illness. *Listeria monocytogenes* is a food-borne pathogen capable of surviving unfavorable environmental conditions, such as low pH and high sodium chloride levels, contaminating the end-products. It is also able to grow at low temperatures. Ready-to-eat foods, including deli-meats are considered as high risk products and this microorganism cannot be completely eliminated even when many risk assessment strategies have been developed for its control (Klontz et al., 2008; Ross, Rasmussen, Fazil, Paoli, & Sumner, 2009).

Biopreservation by using bacteriocinogenic LAB has gained increased attention as a means of naturally controlling the shelf life and safety of meat products (Castellano, Belfiore, Fadda, & Vignolo, 2008; Vignolo, Saavedra, Sesma, & Raya, 2012). Bacteriocins are bacterially produced antimicrobial peptides with narrow or broad host ranges (Cotter, Hill, & Ross, 2005). The production of these low-molecular-weight peptides seems to be a common phenotype among LAB, since numerous bacteriocins have been isolated (Cotter et al., 2005) (Rea et al., 2011). Based on their physico-chemical properties these small antimicrobial peptides have been classified into two major classes: class I (lantibiotics) consists of bacteriocins containing intramolecular thioether ring structures and modified amino acids resulting from post-translational modifications. Nisin is a well-known member from this family (McAuliffe, Ross, & Hill, 2001) and is currently used in many countries as food preservative (Delves-Broughton, Blackburn, Evans, & Hugenholtz, 1996). Class II includes the bacteriocins with non-modified peptides except for bacteriocins with thioester bridges and circular bacteriocins. This class has been divided into subgroups: class IIa, characterized by a conserved sequence (YGNGVXCXK/NXXC) at their N-terminus and known for their strong anti-listerial activity, IIb (two-component peptides), and IIc (thiol-activated peptides) requiring

* Corresponding author. Chacabuco 145, (T4000ILC) San Miguel de Tucumán, Tucumán, Argentina. Tel.: +54 381 4311720x169; fax: +54 381 4005600.
E-mail addresses: lucila@cerela.org.ar, lulusaav@gmail.com (L. Saavedra).

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reduced cysteine residues for activity (Nes, Yoon, & Diep, 2007; Nishie, Nagao, & Sonomoto, 2012).

During fermentation and ripening of traditional sausages *Lactobacillus sakei*, *Lactobacillus curvatus* and to a lesser extent *Lactobacillus plantarum* and species from *Pediococcus* and *Enterococcus* genera are by far the most often isolated LAB species which must have adapted to the existing stringent conditions (Cocconcelli & Fontana, 2010; Fontana, Cocconcelli, & Vignolo, 2005; Fontana, Vignolo, & Cocconcelli, 2005; Vignolo, Fontana, & Cocconcelli, 2010). Several antilisterial bacteriocins are known to be produced by meat borne *L. sakei* strains such as sakacin A produced by *Lactobacillus sake* Lb 706 isolated from meat products (Holck, Axelsson, Birkeland, Aukrust, & Blom, 1992), sakacin P produced by *L. sake* LTH 673 and Lb674 strains isolated from fermented dry sausages (Holck, Axelsson, Hühne, & Kröckel, 1994; Tichaczek, Vogel, & Hammes, 1994); from *L. sakei* strain I151 isolated from naturally fermented Italian sausages Urso, R., Rantsiou, K., Cantoni, C., Comi, G., & Cocolin, L. (2006). and sakacin Q produced by *L. sakei* Lb674 and LTH673 (Mathiesen, Huehne, Kroeckel, Axelsson, & Eijsink, 2005) among others. On the other hand, antilisterial bacteriocins produced by *L. curvatus*, curvacin A produced by *L.curvatus* LTH1174 isolated from fermented sausages (Tichaczek, Vogel, & Hammes, 1993), curvaticin L442 produced by *L. curvatus* L442 isolated from Greek traditional fermented sausage (Xiraphi et al., 2006) and lactocin AL705 produced by *Lb. curvatus* CRL705 isolated from fermented sausages (Castellano & Vignolo, 2006) were reported. Other antilisterial compounds produced by meat borne LAB are pediocin PA-1/AcH produced by *Pediococcus acidilactici* (Pucci, Vedamuthu, Kunka, & Vandenbergh, 1988), plantaricins (A, EF and JK) produced by *L. plantarum* (Anderssen, Diep, Nes, Eijsink, & Nissen-Meyer, 1998) as well as enterocins A, B and P produced by enterococci (Aymerich et al., 2000; Cintas, Casaus, Havarstein, Hernandez, & Nes, 1997).

The aim of the present study is to analyze the occurrence of bacteriocin structural genes and various gene combinations in a target of 115 LAB isolated from different Argentinean raw meat and meat fermented products as well as its correlation with antilisterial activity.

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

A total of 115 LAB strains isolated from Argentinean raw meat and meat fermented products were used in this study. The ability to produce antilisterial bacteriocins was evaluated in *L. sakei* (46 strains) and *L. curvatus* (14 strains) from pork/beef fermented sausages and vacuum-packaged beef (Castro, Palavecino, Herman, Garro, & Campos, 2011; Fontana, Cocconcelli, et al., 2005; Fontana, Vignolo, et al., 2005); *L. sakei* (25 strains) from llama fermented sausages (Lopez et al., 2012); *L. plantarum* (7 strains) from pork fermented sausages (Fontana, Cocconcelli, et al., 2005; Fontana, Vignolo, et al., 2005); *E. faecium* (16 strains) from pork/llama fermented sausages (Lopez et al., 2012), and *P. acidilactici* (7 strains) from pork/beef fermented sausages (Fontana, Cocconcelli, et al., 2005; Fontana, Vignolo, et al., 2005). *L. curvatus* CRL705, isolated from traditional fermented sausages (Vignolo, Suriani, Pesce de Ruiz Holgado, & Oliver, 1993) and *Enterococcus mundtii* CRL35 from artisanal cheeses (Saavedra, Minahk, de Ruiz Holgado, & Sesma, 2004) were used as controls for bacteriocin production. *Lactobacillus* and *Pediococcus* strains were cultured in MRS broth (Difco, BD, Buenos Aires, Argentina) and incubated at 30 °C for 18 h while *Enterococcus* strains were cultured in BHI (Brain heart infusion, Britania, Buenos Aires, Argentina) broth (Oxoid, Bioartis S.R.L., Buenos Aires, Argentina) at 37 °C for 18 h. *Listeria innocua* 7 [kindly gifted by Dr. Jean Richard del Laboratoire d' Ecologie Microbienne de la Unité de Recherches Laitieres et Gentique Appliqueé, Institut National de la Recherche Agronomique, Centre de Recherche de Jouy- en-Josas (INRA)] was grown in BHI at 30 °C; *L. monocytogenes* UC8158; UC8159; UC8160 (Università Cattolica del Sacro Cuore Culture Collection, Cremona, Italy) and FBUNT (Cátedra de Bacteriología, Facultad de Bioquímica, Química y Farmacia, UNT, Tucumán, Argentina) were grown in BHI at 37 °C for 18 h. All *Listeria* strains were used as indicator strains.

2.2. Detection of antilisterial activity

The anti-listerial activity was analyzed in all LAB strains by an agar spot test using all *L. monocytogenes* strains and *L. innocua* 7 as sensitive microorganism. Cell-free supernatants (CFS) were obtained by centrifugation at 15.600 g for 10 min; the cell-free supernatant fluid was then adjusted to pH 7.0 with 1 N NaOH. 5 μl of neutralized CFS was spotted in plates containing 10 ml of BHI 1.5% agar plus 10 ml of BHI soft agar (0.7%) inoculated with 10^7 CFU/ml^{-1} of an overnight culture of the indicator strains. Positive antimicrobial activity was evidenced after 24–48 h at 30 °C (*L. innocua* 7) or 37 °C (*L. monocytogenes*) as a clear inhibition zone on indicator organism's lawn. All experiments, unless stated otherwise, were performed in duplicate. Inhibitory activity was expressed in halo diameter as −/+ (≤0.5 mm), + (0.5–2 mm), ++ (2–4 mm), +++ (>4 mm) or − (no halos) around the spot.

2.3. PCR analysis for bacteriocin-encoding genes

All strains were tested by PCR to search the presence of different bacteriocin encoding genes. DNA was isolated from LAB colonies using Microlysis (Labogen, Milan, Italy) or following the protocol by Pospiech and Neumann (Pospiech & Neumann, 1995). Primers used and conditions for each of structural genes amplified are listed in Table 1. PCR reactions were performed using a GeneAmp® PCR Instrument System 9700 (Applied Biosystems, Italy) and My Cycler TM Thermal cycler (BIORAD, Tecnolab, Buenos Aires Argentina). The following conditions were used for PCR reactions: an initial denaturation step of 94 °C for 1 min, followed by 35 cycles of 1 min at 94 °C, 30 s at different annealing temperatures according to the primers used (Table 1) and 1 min at 72 °C, and final extension at 72 °C for 5 min. The amplified product was visualized in a 1.5% (w/v) agarose gel stained with SYBER Safe (Invitrogen, Italy) or GelRel (Biotium-Genbiotech, Argentina). All PCR runs included a blank control consisting of PCR-grade water and a non-template control (no DNA). All PCR products were purified and subject to sequencing at CERELA Sequencing Service and BMR Genomics (Padova, Italy). The resulting sequences were analyzed with the Blast program (Altschul et al., 1997).

2.4. Statistical analysis

Relations between meat borne LAB antilisterial activity and bacteriocin encoding genes presence were analyzed by means of a Multiple Correspondence Analysis (MCA) method (Abdi & Valentin, 2007). MCA is the generalization of simple correspondence analysis to several categorical variables. It is a geometric technique for displaying the rows and columns of a multi-way contingency table as points in a low-dimensional Euclidean space, such that the positions of the row and column points are consistent with their associations in the table. A matrix was created in which the rows of the data corresponded to bacteriocinogenic meat borne LAB strains and the columns to the analyzed bacteriocin genes and inhibitory activity level against the different *Listeria* strains used as indicators. The goal is to have a global view of the data that is useful for