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Improving safety of salami by application of bacteriocins produced by an autochthonous *Lactobacillus curvatus* isolate

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ABSTRACT

The aims of this study were to isolate LAB with anti-*Listeria* activity from salami samples, characterize the bacteriocin/s produced by selected isolates, semi-purify them and evaluate their effectiveness for the control of *Listeria monocytogenes* during manufacturing of salami in a pilot scale. Two isolates (differentiated by RAPD-PCR) presented activity against 22 out of 23 *L. monocytogenes* strains for bacteriocin MBSa2, while the bacteriocin MBSa3 inhibited all 23 strains in addition to several other Gram-positive bacteria for both antimicrobials and were identified as *Lactobacillus curvatus* based on 16S rRNA sequencing. A three-step purification procedure indicated that both strains produced the same two active peptides (4457.9 Da and 4360.1 Da), homologous to sakacins P and X, respectively. Addition of the semi-purified bacteriocins produced by *Lb. curvatus* MBSa2 to the batter for production of salami, experimentally contaminated with *L. monocytogenes* (10^4–10^5 CFU/g), caused 2 log and 1.5 log reductions in the counts of the pathogen in the product after 10 and 20 days respectively, highlighting the interest for application of these bacteriocins to improve safety of salami during its manufacture.

1. Introduction

Lactic acid bacteria (LAB), especially *Lactobacillus sakei* and *Lactobacillus curvatus*, are part of the microbiota of many types of fermented meat products. These two species of LAB are well adapted to the meat environment, conveying the improved flavor and accelerated maturation of fermented meat products (Chaillou et al., 2005; Lahtinen et al., 2011). LAB are also essential in fermented food products, preventing growth of spoilage and pathogenic microorganisms by acidification and production of antimicrobial compounds, contributing to improved safety and quality (Fadda et al., 2010; Balciunas et al., 2013; Mangia et al., 2013).

Bacteriocins produced by LAB are antimicrobial proteinaceous compounds synthesized via the ribosomes, presenting variable spectrum of activities. Most bacteriocins are small molecules with amphipathic characteristics and high isoelectric points. The producer cells are resistant to the bacteriocins they produce due to synthesis of specific immunity proteins (Deegan et al., 2006; Mills et al., 2011; Dobson et al., 2012; Nishie et al., 2012). Numerous bacteriocins produced by different LAB species have been already described (Balciunas et al., 2013). According to Cotter et al. (2005), between 30 and 99% of the prokaryotes (Bacteria and Archaea) produce at least one bacteriocin.

Bacteriocins produced by LAB are well known for their activity against *Listeria monocytogenes*, a ubiquitous Gram-positive pathogen that has caused several food related outbreaks in the last decades (Kumar, 2011; Scallan et al., 2011). In the most recent classification of bacteriocins (Heng et al., 2007), there is a special class dedicated to bacteriocins with anti-*Listeria* activity. Control of *L. monocytogenes* in processed foods is a serious problem, due to the capability to survive the hurdles usually encountered during manufacture of dry fermented products, such as low pH, salt and presence of nitrites (Vogel et al., 2010). Due to this anti-*Listeria* activity, bacteriocinogenic LAB and their bacteriocins are beneficial as preservation agents in fermented products, and can be used as technological alternatives to chemical preservatives, fitting the increased demand for foods with less or no additives (Dickson-Spillmann et al., 2011).

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Many surveys in Brazil indicate that *L. monocytogenes* is a frequent contaminant in fermented meat products consumed in the country, such as sausages and salamis (Borges et al., 1999; Sakate et al., 2003; Martins and Germano, 2011). In our study, we describe the isolation of LAB with anti-*Listeria* activity from Italian type salami produced in Brazil, characterization and purification of the bacteriocins produced by selected LAB and evaluation of their effectiveness in the control of *L. monocytogenes* during the fermentation step of salami manufacture.

2. Material and methods

2.1. Isolation and identification of bacteriocinogenic LAB from salami

Italian type salami samples were purchased in retail markets in the city of Sao Paulo (Brazil), and 50 g of each sample were submitted to isolation of bacteriocinogenic LAB as described by Todorov et al. (2010). Identification of the strains was done using recommended morphological and biochemical tests and 16S rRNA sequence analysis, using primers 8f (5′-CAC GGA TCC AGA CTT TGA T(C/T)(A/C) TGG CTC AG-3′) and 1512r (5′- GTG AAG CTT ACG G(C/T) T AGC TTG TTA CGA CTT-3′) as described by Felske et al. (1997). Purified amplified PCR products were sequenced at the Center for Human Genome Studies, Institute of Biomedical Sciences, University of Sao Paulo, Brazil and sequences were compared to known sequences in GenBank using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). The genetic similarity of the bacteriocinogenic isolates was tested by Random Amplification of Polymorphic DNA (RAPD) with primers OPL-01 (5′- GGC ATG ACC T-3′), OPL-02 (5′- TGG GCG TCA A-3′), OPL-04 (5′- GAC TGC ACA C-3′), OPL-14 (5′- GTG ACA GGC T-3′) and OPL-20 (5′- TGG TGG ACC A-3′) as described by Todorov et al. (2010).

2.2. Determination of bacteriocin activity

The amount of bacteriocin produced by two selected bacteriocinogenic isolates (*Lb. curvatus* MBSa2 and *Lb. curvatus* MBSa3) was determined testing two-fold dilutions of cell free supernatants (CFS) for antimicrobial activity according to Todorov et al. (2010), using *L. monocytogenes* Scott A as indicator strain. For preparation of the CFS, bacteriocin producer strains were grown in MRS broth (Difco, Detroit, MI, USA) for 24 h at 30 °C and cells were removed by centrifugation at 4000 × *g* for 15 min at 4 °C (Hettich Zentrifugen, model Mikro 22R, Tuttlingen, Germany). The pH of CFS was adjusted to 6.0–6.5 with 1 M NaOH (Synth, Sao Paulo, Brazil), heated for 30 min at 70 °C and filter-sterilized (Millex GV 0.22 μm, Millipore, Billerica, MA, USA). One arbitrary unit (AU) was defined as the reciprocal of the highest dilution that resulted in production of a clear zone of inhibition of *L. monocytogenes*. Results were expressed in AU/mL (Todorov et al., 2010).

2.3. Characterization of the bacteriocinogenic strains

2.3.1. Growth and bacteriocin production in MRS broth

The bacteriocinogenic strains *Lb. curvatus* MBSa2 and *Lb. curvatus* MBSa3 were tested for growth and bacteriocin production in MRS broth at 25 °C, 30 °C and 37 °C. Growth was monitored measuring absorbance at 600 nm (Ultrospec 2000; Pharmacia Biotech, Little Chalfont, UK) every 2 h up to 24 h. Changes in pH of the cultures were recorded. Presence of bacteriocins in the CFS was monitored every 2 h up to 24 h, using the spot-on-the-lawn method and *L. monocytogenes* Scott A as indicator of activity (Todorov et al. (2010)).

2.3.2. Influence of NaCl content and pH of MRS broth on bacterial growth

Bacteriocinogenic strains *Lb. curvatus* MBSa2 and *Lb. curvatus* MBSa3 were tested for growth in MRS broth containing increasing NaCl contents and at acid pH, simulating conditions occuring during the manufacture of salami. Strains were grown in MRS broth for 24 h at 30 °C and then aliquots (10^6–10^7 CFU/mL) were transfered to MRS broth containing 1%–10% NaCl, with pH adjusted to 4 or to 6 with 1 M lactic acid, and incubated at 30 °C. Growth was monitored every 2 h up to 24 h, measuring changes in absorbance as described in 2.3.1.

Table 1
Spectrum of activity of the bacteriocins produced by *Lactobacillus curvatus* MBSa2 and MBSa3.

Target microorganism	Source	Diameter of the inhibition zone (mm)	
		MBSa2	MBSa3
Listeria ivanovii subsp. *ivanovii*	ATCC 19119	15	16
Listeria innocua	ATCC 33090	18	21
Listeria innocua 225/07 serovar 6a	FIOCRUZ[a]	15	16
Listeria innocua 224/07 serovar 6a	FIOCRUZ	11	15
Listeria innocua 047/07 serovar 6a	FIOCRUZ	15	14
Listeria innocua 588/08 serovar 6a	FIOCRUZ	14	11
Listeria monocytogenes Scott A	USP[b]	13	13
Listeria monocytogenes 602/08 serovar 1/2a	FIOCRUZ	13	13
Listeria monocytogenes 046/07 serovar 1/2c	FIOCRUZ	11	14
Listeria monocytogenes 103 serovar 1/2a	USP	0	15
Listeria monocytogenes 106 serovar 1/2a	USP	13	14
Listeria monocytogenes 104 serovar 1/2a	USP	14	15
Listeria monocytogenes 409 serovar 1/2a	USP	12	14
Listeria monocytogenes 506 serovar 1/2a	USP	14	14
Listeria monocytogenes 709 serovar 1/2a	USP	11	12
Listeria monocytogenes 607 serovar 1/2b	USP	18	17
Listeria monocytogenes 603 serovar 1/2b	USP	10	20
Listeria monocytogenes 426 serovar 1/2b	USP	10	14
Listeria monocytogenes 637 serovar 1/2c	USP	10	14
Listeria monocytogenes 422 serovar 1/2c	USP	12	15
Listeria monocytogenes 712 serovar 1/2c	USP	13	15
Listeria monocytogenes 408 serovar 1/2c	USP	14	15
Listeria monocytogenes 211 serovar 4b	USP	15	16
Listeria monocytogenes 724 serovar 4b	USP	19	16
Listeria monocytogenes 101 serovar 4b	USP	18	18
Listeria monocytogenes 703 serovar 4b	USP	18	20
Listeria monocytogenes 620 serovar 4b	USP	20	20
Listeria monocytogenes 302 serovar 4b	USP	15	14
Enterococcus hirae D105	USP	10	13
Enterococcus faecium S105	AGRIS[c]	10	15
Enterococcus faecium S154	AGRIS	0	11
Enterococcus faecium ST62BZ	USP	10	10
Lactobacillus fermentum ET35	UCV[d]	10	10
Lactobacillus curvatus ET31	UCV	0	9
Lactobacillus sakei	ATCC 15521	10	11

No activity have been recorded against: *Bacillus cereus* ATCC 1178, *Staphylococcus aureus* ATCC 29213, ATCC 25923 and ATCC 6538, *Listeria welshimeri* USP, *Listeria seeligeri* USP, *Escherichia coli* ATCC 8739, *Escherichia coli* O157:H7 ATCC 35150, *Enterobacter aerogenes* ATCC 13048, *Salmonella typhimurium* ATCC 14028, *Salmonella enteritidis* ATCC 13076, *Enterococcus faecalis* ATCC 12755, *Enterococcus faecium* S100 AGRIS, ST211Ch USP, ET12 UCV[d], ET88 UCV and ET05 UCV, *Lactococcus lactis* V94 USP, V69 USP and B16 USP, *Pediococcus pentosaceus* ET34 UCV, *Lactobacillus curvatus* ET06 UCV and ET30 UCV, *Lactobacillus sakei* subsp. *sakei* 2a USP, *Lactobacillus delbrueckii* B5 USP and ET31 UCV, *Lactobacillus acidophilus* La14 Rhodia, Lac4 Rhodia and La5 Rhodia, *Lactococcus lactis* subsp. *lactis* MK02R USP, D2 USP, D3 USP, D4 USP, D5 USP, B1 USP, B2 USP, B15 USP, B17 USP and R704 Chr. Hansen.

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