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Partial characterization of bacteriocins produced by three strains of *Lactobacillus sakei*, isolated from *salpicao*, a fermented meat product from North-West of Portugal

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ABSTRACT

Lactobacillus sakei ST22Ch, ST153Ch and ST154Ch were isolated from traditional pork product from Northwest of Portugal, and identified based on API50CHL, PCR with specific primers and 16s rDNA sequencing. RAPD-PCR analysis showed significant differences between isolates. The selected isolates inhibited the growth of *Enterococcus* spp., *Listeria* spp., *Escherichia coli*, *Klebsiella* spp., *Pseudomonas* spp., *Staphylococcus* spp., and *Streptococcus* spp. The mode of action of the bacteriocins was bactericidal, as observed against *Enterococcus faecium*. A reduction in antimicrobial activity was recorded after treatment of the bacteriocins with proteolytic enzymes, but not when they were exposed in presence of α-amylase, suggesting that they are not glycosylated. Maximal activity of bacteriocins was recorded during the early stationary phase and remained stable only for a short period, followed by a decrease. According to tricine/SDS-PAGE, the size of bacteriocins ST22Ch, ST153Ch and ST154Ch are approximately 3.0 kDa, 10.0 kDa and 3.0 kDa, respectively. Bacteriocins were heat tolerant and remained active after 2 h at 100 °C. Activity of bacteriocins was not affected by treatment with 1% Triton X-100, Tween 20, Tween 80, SDS, NaCl, urea and EDTA. In presence of 1% Triton X-114 bacteriocins were inactivated. PCR reactions targeting genes for enterocin A, enterocin P, sakacin P, sakacin G1 and sakacin G2 in the total DNA of *L. sakei* ST22Ch, ST153Ch and ST154Ch, generated positive results. Curvacin A gene was detected only in *L. sakei* ST154Ch DNA.

1. Introduction

Consumption of fermented meat products is essential part of the diet in the Iberian Peninsula in Europe. However, besides contributing to the gastronomic characteristics of these products, fermentation is one of the oldest ways to preserve meat and meat products. *Salpicao* (in Portuguese "*salpicão*") is the traditional fermented cured/smoked sausage prepared from pork filet using salt, white or red wine, garlic and chili pepper produced in Portugal. Generally fermentation takes 8 days.

Lactic acid bacteria (LAB) are known for their production of antimicrobial compounds, including bacteriocins or bacteriocin-like peptides (Todorov, 2009). Bacteriocins of LAB are defined as ribosomally synthesized proteins or protein complexes usually antagonistic to genetically closely related organisms (Nes & Johnsborg, 2004). They are generally low molecular weight proteins that gain entry into target cells by binding to cell surface receptors. Their bactericidal mechanism varies and may include pore formation, degradation of cellular DNA, disruption through specific cleavage of 16S rDNA, and inhibition of peptidoglycan syntesis (Heu et al., 2001).

Recently, according to a complete genome analysis, it was proposed that strains of *Lactobacillus sakei* can be used to control pathogens in meat because the species' metabolism is particularly well adapted to a meat medium. *Lactobacillus* species represent the dominant LAB strains currently found in meat starter cultures

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(Chaillou et al., 2005). Moreover, *L. sakei* and *Lactobacillus curvatus* isolates from meat are often bacteriocinogenic. Up to now, several bacteriocins are known to be produced by *L. sakei* strains and their potential application in meat preservation has been studied. Examples are: curvacin A (Tichaczek, Nisen-Meyer, Nes, Vogel, & Hammes, 1992) identical to sakacin A (Shillinger & Lücke, 1989); sakacin P (Aasen et al., 2003; Tichaczek, Vogel, & Hammes, 1994; Urso, Rantsiou, Cantoni, Comi, & Cocolin, 2006) identical to bavaricin A (Larsen, Vogensen, & Josephsen, 1993); sakacin 674 (Holck, Axelsson, Hühne, & Kröckel, 1994), sakacin B (Samelis, Roller, & Metaxopolos, 1994), sakacin K (Hugas, Pages, Garriga, & Monfort, 1998), sakacin V18 (Cintas, Casaus, Fernandez, & Hernandez, 1998) and sakacin M (Sobrino et al., 1992) identical to lactocin S (Mortvedt, Nissen-Meyer, Sletten, & Nes, 1991); bavaricin MN (Kaiser & Montville, 1996), sakacin T (Aymerich et al., 1996), sakacin G (Simon, Fremaux, Cenatiempo, & Berjeaud, 2002), sakacin X (Vaughan, Eijsink, & Van Sinderen, 2003), sakacin Q (Mathiesen, Huehne, Kroeckel, Axelsson, & Eijsink, 2005) and sakacin 1 (Alves, Martinez, Lavrador, & De Martinis, 2006). All sakacins possess strong antilisterial activity and most of them belong to the Class IIa bacteriocins (Klaenhammer, 1988).

Recent approaches in the preservation of meat products are increasingly directed towards biocontrol using bacteriocinogenic *Lactobacillus* strains as protective microbiota to inhibit growth of *Listeria monocytogenes* and other undesired microorganisms (Castellano, Holzapfel, & Vignolo, 2004; Hugas et al., 1998; Mataragas, Metaxopoulos, Galiotou, & Drosinos, 2003).

Bacteriocin production does not always correlate with the increase in cell mass or growth rate of the producer strain (Bogovic-Matijasic & Rogelj, 1998). Higher bacteriocin levels are often recorded in the absence of growth stimulating nutrients, or at temperatures and pH conditions lower than required for optimal growth (Aasen, Moreto, Katla, Axelsson, & Storro, 2000; Krier, Revol-Junelles, & Germain, 1998; Matsusaki, Endo, Sonomoto, & Ishizaki, 1996; Todorov, Gotcheva, Dousset, Onno, & Ivanova, 2000). Optimal bacteriocin production is often recorded in medium with limiting concentrations of sugars, nitrogen sources, vitamins and potassium-phosphate, or when the medium pH is regulated (Vignolo, Dekairuz, Holgado, & Oliver, 1995).

Studies conducted on bacteriocins from different other LAB, e.g. pediocin PD-1 (Nel, Bauer, Vandamme, & Dicks, 2001), sakacin P (Aasen et al., 2000), and bacteriocins produced by *Leuconostoc mesenteroides* L124 (Mataragas et al., 2003) have suggested that production is often regulated by growth pH and temperature. In some cases, higher bacteriocin activity has been recorded at sub-optimal growth conditions (Aasen et al., 2000; Bogovic-Matijasic & Rogelj, 1998; Krier et al., 1998; Matsusaki et al., 1996; Todorov et al., 2000).

Little is known about the microbial population of *Salpicao*. In our knowledge we are the first reporting on the isolation of bacteriocinogenic *L. sakei* strains from *Salpicao*. In this study, we report on the isolation of bacteriocin-producing strains from different samples of *Salpicao* produced by "Fumeiro do Laboreiro", North-West of Portugal. The strains have been identified to species level, the bacteriocins have been characterized, the mode of activity has been studied and levels of production have been determined in model media and different growth media.

The objective of this study was to characterize bacteriocins ST22Ch, ST153Ch and ST154Ch, produced by *L. sakei* strains ST22Ch, ST153Ch and ST154Ch isolated from *Salpicao*, with the aim of using the strain as co-starter bioprotective cultures in meat fermentation.

2. Materials and methods

2.1. Isolation of lactic acid bacteria and screening for bacteriocin activity

Samples of 50 g *Salpicao* obtained from the "Fumeiro do Laboreiro" (Portugal), were macerated in a Stomacher (BagMixer, Interscience, Weymouth, USA) for 10 min at 20 °C. Serial dilutions of the sample were made with sterile saline (0.85%, w/v NaCl), plated onto MRS agar (Difco) and incubated at 30 °C for 24 h.

Screening for bacteriocin-producing isolates was carried out according to the triple-agar-layer method described by Todorov and Dicks (2005b). The second layer of agar (1.7%, w/v) was supplemented with 50.0 mg/l Actidion (Sigma) to prevent fungal growth. All plates were incubated at 30 °C for 24 h. Colonies were then overlaid with a third layer of 1% (w/v) Brain Heart Infusion (BHI) agar (Difco), seeded with 10^6 CFU/ml *Enterococcus faecium* ATCC 19443, *L. sakei* ATCC 15521 and *Listeria ivanovii* subsp. *ivanovii* ATCC 19119, respectively. The plates were incubated at 37 °C for 24 h. Colonies with the largest zones of growth inhibition were isolated, inoculated into MRS broth (Difco) and incubated for 24 h at 30 °C. Pure cultures were obtained by streaking onto MRS agar.

Antimicrobial activity was confirmed by using the agar-spot-test method (Todorov, 2008). Activity was expressed as arbitrary units (AU) per ml, with one AU defined as the highest dilution showing a clear zone of inhibition (Todorov, 2008). *E. faecium* ATCC 19443 was used as a sensitive test strain.

2.2. Identification of strains ST22Ch, ST153Ch and ST154Ch

Morphology of strains ST22Ch, ST153Ch and ST154Ch was determined by using an AFM – EasyScan II from Nanosurf (Switzerland) according to Meincken and Todorov (2009).

Identification was conducted by physiological and biochemical tests (Stiles & Holzapfel, 1997). Carbohydrate fermentation reactions were recorded by using API50CHL (Biomérieux, Marcy-l'Etiole, France). Identification to species level was by PCR with primers specific for *L. sakei* (Ls: 5′-ATG AAA CTA TTA AAT TGG TAC-3′ and 16: 5′-GCT GGA TCA CCT CCT TTC-3′) as described by Berthier and Ehrlich (1998). Confirmation of identification was obtained by amplifying the genomic DNA with primers F8 (5′-CAC GGA TCC AGA CTT TGA TYM TGG CTC AG-3′) and R1512 (5′-GTG AAG CTT ACG GYT AGC TTG TTA CGA CTT-3′), as described by Felske, Rheims, Wolterink, Stackebrandt, and Akkermans (1997). The amplified fragments were cleaned using SigmaSpin™ Post-Reaction Clean-Up Columns (Sigma, St Louis, MO, USA), sequenced, and compared to sequences in GenBank using BLAST (Basic Local Alignment Search Tool).

Differentiation of the strains ST22Ch, ST153Ch and ST154Ch was performed by random amplification of polymorphic DNA (RAPD) PCR. DNA was isolated according to the manufacture's protocol using ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA). Primers OPL-02 and OPL-08 were used (Kit L of the RAPD® lomer kits, Operon Biotechnologies, Cologne, Germany). Amplification reactions were performed according to Todorov, Ho, Vaz-Velho, and Dicks (2010). The amplified products were separated by electrophoresis in 1.4% (w/v) agarose gels in 1× TAE buffer at 100 V for 2 h. Gels were stained in TAE buffer containing 0.5 μg/ml ethidium bromide (Sigma Diagnostics, St. Louis, Mo., USA). Banding patterns were analysed using Gel Compare, Version 4.1 (Applied Maths, Kortrijk, Belgium).