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BIOENERGY/BIOFUELS/BIOCHEMICALS

Cloning, production, and functional expression of the bacteriocin sakacin A (SakA) and two SakA-derived chimeras in lactic acid bacteria (LAB) and the yeasts *Pichia pastoris* and *Kluyveromyces lactis*

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Abstract Mature sakacin A (SakA, encoded by *sapA*) and its cognate immunity protein (SakI, encoded by *sapiA*), and two SakA-derived chimeras mimicking the N-terminal end of mature enterocin P (EntP/SakA) and mature enterocin A (EntA/SakA) together with SakI, were fused to different signal peptides (SP) and cloned into the protein expression vectors pNZ8048 and pMG36c for evaluation of their production and functional expression by different lactic acid bacteria. The amount, antimicrobial activity, and specific antimicrobial activity of SakA and its chimeras produced by *Lactococcus lactis* subsp. *cremoris* NZ9000 depended on the SP and the expression vector. Only *L. lactis* NZ9000 (pNUPS), producing EntP/SakA, showed higher bacteriocin production and antimicrobial activity than the natural SakA-producer *Lactobacillus sakei* Lb706. The lower antimicrobial activity of the SakA-producer *L. lactis* NZ9000 (pNUS) and that of the EntA/SakA-producer *L. lactis* NZ9000 (pNUAS) could be ascribed to secretion of truncated bacteriocins. On the other hand, of the *Lb. sakei* Lb706 cultures transformed with the pMG36c-derived vectors only *Lb. sakei* Lb706 (pGUS) overproducing SakA showed a higher antimicrobial activity than *Lb. sakei* Lb706. Finally, cloning of SakA and EntP/SakA into pPICZαA and pKLAC2 permitted the production of SakA and EntP/SakA by recombinant *Pichia pastoris* X-33 and *Kluyveromyces lactis* GG799 derivatives although their antimicrobial activity was lower than expected from their production.

Keywords Bacteriocins · Sakacin A · Sakacin A-derived chimeras · Lactic acid bacteria (LAB) · *Pichia pastoris* · *Kluyveromyces lactis* · Heterologous bacteriocin production

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Introduction

Lactic acid bacteria (LAB) secrete small, cationic, heat-stable antimicrobial peptides, commonly known as bacteriocins and many are more active than conventional antibiotics against pathogenic and drug-resistant Gram-positive bacteria, yet display no toxicity towards eukaryotic cells [16, 35]. Accordingly, bacteriocins produced by LAB may find use as natural antimicrobial peptides in food, medical, veterinary, and animal production applications, whereas bacteriocin-producing LAB could be evaluated for potential use as probiotics [8, 9, 23, 41]. However, the low bacteriocin yields obtained after their purification from natural producers and the high cost of synthetic bacteriocin synthesis drive the exploration of recombinant microbial systems for the biotechnological production of bacteriocins.

Bacteriocins produced by LAB may be classified into the class I lantibiotics, containing post-translationally modified amino acids, and the class II nonlantibiotics, containing non-modified amino acids. Class II bacteriocins may be further subdivided into the (1) pediocin-like (class IIa) bacteriocins, (2) two-peptide (class IIb) bacteriocins,

(3) cyclic (class IIc) bacteriocins, and (4) nonpediocin one-peptide linear (class IId) bacteriocins [16, 43]. All pediocin-like bacteriocins have similar amino acid sequences, especially in their rather hydrophilic N-terminal half, which contains a disulfide bridge and a common YGNGVxC "pediocin box" motif, followed by a hinge and a somewhat more hydrophobic and diverse C-terminal half [31]. The target receptor for class IIa bacteriocins on sensitive cells has been identified as proteins of the sugar transporter mannose phosphotransferase system (Man-PTS) [21]. It has been suggested that the conserved N-terminal half of these bacteriocins initially interacts with the receptor to enable the C-terminal half to engage in more profound and active interactions with the receptor rendering the permeases open as pores, thereby causing leakage of solutes, disruption of membrane integrity, and eventually cell death [35].

Most class IIa bacteriocins are synthesized as biologically inactive precursors or prepeptides containing an N-terminal extension of the so-called double-glycine type (leader sequence) that is cleaved concomitantly with export across the cytoplasmic membrane by dedicated ATP-binding cassette transporters (ABC transporters) and their accessory proteins [32]. However, many secreted prokaryotic proteins and a few bacteriocins such as enterocin P [14] and hiracin JM79 [44] contain N-terminal extensions of the so-called Sec-type (signal peptide) which are proteolytically cleaved concomitantly with peptide externalization by the general secretory pathway (GSP) or Sec-dependent pathway. Secretory proteins are equipped with an N-terminal signal peptide (SP) that functions as a target and recognition signal for signal peptidases that remove the SP from the translocated protein, resulting in the extracellular release of the mature protein or peptide [24, 42]. Accordingly, the SP of secretory proteins may drive the access of fused mature proteins or peptides to the GSP for their secretion by the heterologous producer cells.

Sakacin A (SakA) is a class IIa, pediocin-like, antilisterial bacteriocin produced by *Lactobacillus sakei* Lb706 [33]. The *sap* locus responsible for SakA production in *Lb. sakei* Lb706 is located in a 60-kb pLSA60 plasmid harboring the *sapAIPhKRTE* operon. The genes *sapA* and *saiA* encode the 59 amino acid SakA precursor, consisting of a 18 amino acid leader sequence (LS_{sapA}) and the 41 amino acid mature bacteriocin (SakA), and the 90 amino acid immunity protein (SakI), respectively. The synthesis of SakA in *Lb. sakei* Lb706 is a temperature-sensitive process regulated by the peptide pheromone through a three-component regulatory system. In this respect, *sap*-Ph, *sapK*, and *sapR* encode the pheromone induction factor, the histidine protein kinase, and the response regulator, respectively. Finally, *sapT* and *sapE* encode an ABC transporter and its cognate accessory protein [20].

The production of SakA and immunity to the bacteriocin has been obtained in the recombinant non-bacteriocin-producing host *Lb. sakei* Lb790 but not in other LAB, probably because signal-transducing systems were not functioning properly [1]. Accordingly, as a further attempt to improve the production and functional expression of this tightly regulated bacteriocin by other microbial hosts we have evaluated the fusion of mature sakacin A (SakA, encoded by *sapA*) and its cognate immunity protein (SakI, encoded by *sapiA*), and of two SakA derived chimeras mimicking the N-terminal end of mature enterocin P (EntP/SakA) and mature enterocin A (EntA/SakA) to different signal peptides and their expression by *Lactococcus lactis* subsp. *cremoris* NZ9000 and *Lb. sakei* Lb706. Moreover, the cloning of SakA and the EntP/SakA-derived chimera in different yeast protein expression vectors has also permitted the evaluation of their functional expression by the yeasts *Pichia pastoris* X-33 and *Kluyveromyces lactis* GG799.

Materials and methods

Microbial strains, plasmids, and growth conditions

The microbial strains and plasmids used in this study are listed in Table 1. *Lactobacillus sakei* Lb706 was used as the source of *sapA* (SakA) and *saiA* (SakI), whereas *L. lactis* MG1363, *Enterococcus faecium* P13, and *E. hirae* DCH5 were used as the source of the signal peptides Usp45 (SP_{usp45}), EntP (SP_{entP}), and HirJM79 ($SP_{hirJM79}$), respectively. The primary amino acid and nucleotide sequence of the SP_{usp45}, SP_{entP}, and $SP_{hirJM79}$ are given by the GenBank accession numbers M60178, AF005278, and DQ664500, respectively. The GenBank accession number for the structural sakacin A (*sapA*) and immunity (*saiA*) genes of *Lb. sakei Lb706* is Z46867. The lactococcal strains were propagated at 32 °C in M17 broth (Oxoid Ltd., Basingstoke, UK) supplemented with 0.5 % (w/v) glucose (GM17). The enterococcal strains and the lactobacilli were grown in MRS broth (Oxoid) at 32 °C. *Pichia pastoris* X-33 (Invitrogen Life Technologies, Barcelona, Spain) and *K. lactis* GG799 (New England Biolabs, Ipswich, MA, USA) were cultured in YPD medium (Sigma-Aldrich Inc., St. Louis, MO, USA) at 30 °C with shaking. *Escherichia coli* JM109 (Invitrogen) was grown in LB broth (Sigma) at 37 °C with shaking. *Listeria* spp. strains were cultured in BHI broth (Oxoid) at 32 °C. Agar plates were made by addition of 1.5 % (w/v) agar (Oxoid) to the liquid media. When necessary, chloramphenicol (Sigma), Zeocin (Invitrogen), and ampicillin (Sigma) were used at a concentration of 5, 25, and 50 μg/ml, respectively. Cell dry weights of late exponential phase bacterial cultures, expressed as cell dry mass, were determined gravimetrically.

Table 1 Microbial strains and plasmids used in this study

Strain or plasmid	Description	Source and/or reference[a]
Strains		
E. coli JM109	Selection of recombinant plasmids	Invitrogen Life Technologies
E. faecium		
T136	Enterocin A and B producer; MPA indicator	DNBTA, Casaus et al. [11]
P13	Enterocin P producer	DNBTA, Cintas et al. [14]
E. hirae		
DCH5	Hiracin JM79 producer	DNBTA, Sánchez et al. [44]
Lb. sakei		
Lb706	Sakacin A producer	LMG, Axelsson and Holck [1]
L. lactis subsp. *lactis*		
BB24	Nisin A producer	DNBTA, Cintas [13]
L. lactis subsp. *cremoris*		
NZ9000	MG1363 pepN::*nisRK*	NIZO, Kuipers et al. [36]
MG1363	Plasmid-free derivative of NCDO712; source of *usp45*	IFR, Gasson [27]
P. pastoris X-33	Yeast laboratory strain	Invitrogen Life Technologies
K. lactis GG799	Yeast laboratory strain	New England Biolabs
Plasmids		
pMG36c	Cm^r, pMG36e derivative	RUG-MG, van de Guchte et al. [49]
pNZ8048	Cm^r; inducible expression vector carrying the *nisA* promoter	NIZO, Kuipers et al. [36]
pPICZαA	Zeo^r; integrative plasmid carrying the secretion signal sequence from the *Saccharomyces cerevisiae* α-factor prepropetide and functional sites for the integration at the 5′AOX1 locus of *P. pastoris* X-33	Invitrogen Life Technologies
pKLAC2	Amp^r; integrative plasmid carrying the *Aspergillus nidulans* acetamidase (*amdS*) gene, the secretion signal sequence from the *S. cerevisiae* α-factor prepropetide and functional sites for the integration at the LAC4 locus of *K. lactis* GG799	New England Biolabs
pNUS	Cm^r; pNZ8048 derivative carrying the PCR product NUS (SP_{usp45} fused to mature *sapA* and *saiA* genes)	This work
pNPS	Cm^r; pNZ8048 derivative carrying the PCR product NPS (SP_{entP} fused to mature *sapA* and *saiA* genes)	This work
pNHS	Cm^r; pNZ8048 derivative carrying the PCR product NHS ($SP_{hirJM79}$ fused to mature *sapA* and *saiA* genes)	This work
pNUAS	Cm^r; pNZ8048 derivative carrying the PCR product NUAS (SP_{usp45} fused to mature *entA/sapA* and *saiA* genes)	This work
pNUPS	Cm^r, pNZ8048 derivative carrying the PCR product NUPS (SP_{usp45} fused to mature *entP/sapA* and *saiA* genes)	This work
pGUS	Cm^r; pMG36c derivative carrying the PCR product GUS (P_{32} ribosome binding site and the SP_{usp45} fused to mature *sapA and saiA* genes)	This work
pGUAS	Cm^r; pMG36c derivative carrying the PCR product GUAS (P_{32} ribosome binding site and the SP_{usp45} fused to mature *entA/sapA* and *saiA* genes)	This work
pGUPS	Cm^r; pMG36c derivative carrying the PCR product GUPS (P_{32} ribosome binding site and the SP_{usp45} fused to mature *entP/sapA* and *saiA* genes)	This work
pPSA	pPICZαA derivative carrying the PCR product KR-SA	This work
pPPSA	pPICZαA derivative carrying the PCR product KR-PSA	This work
pKSA	pKLAC2 derivative carrying the PCR product KR-SA	This work
pKPSA	pKLAC2 derivative carrying the PCR product KR-PSA	This work

MPA microtitre plate asay, Cm^r chloramphenicol resistance, Zeo^r Zeocin resistance, Amp^r ampicillin resistance

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Basic genetic techniques and enzymes

Total genomic DNA from *Lb. sakei* Lb706, *L. lactis* MG1363, *E. faecium* P13, and *E. hirae* DCH5 was isolated using the Wizard® DNA Purification Kit (Promega, Madison, WI, USA). Plasmid DNA isolation was carried out using the QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany), as suggested by the manufacturer, but cells were suspended with lysozyme (40 mg ml^{-1}) and mutanolysin (500 U ml^{-1}) and incubated at 37 °C for 10 min to weaken the cell wall before following the kit instructions. DNA restriction enzymes were supplied by New England Biolabs. Ligations were performed with the T4 DNA ligase (Roche Molecular Biochemicals, Mannheim, Germany). *E. coli* JM109 and *K. lactis* GG799 competent cells were transformed as described by the supplier (Invitrogen and New England Biolabs, respectively). Competent *P. pastoris* X-33 cells were obtained as recommended by the supplier (Invitrogen) and transformed by electroporation with a Gene Pulser™ and Pulse Controller apparatus (Bio-Rad Laboratories, Hercules, CA, USA), as previously described [29].

PCR amplification and nucleotide sequencing

Oligonucleotide primers were purchased from Sigma-Genosys Ltd. (Cambridge, UK). Conditions for PCR amplifications were performed as previously described [8]. The PCR-generated fragments were purified by a NucleoSpin® Extract II Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) for cloning and nucleotide sequencing. Nucleotide sequencing of purified PCR products was done using the ABI PRISM® BigDye™ Terminator cycle sequence reaction kit and the automatic DNA sequencer ABI PRISM, model 377 (Applied Biosystems, Foster City, CA, USA), at the Unidad de Genómica (Facultad de Ciencias Biológicas, Universidad Complutense de Madrid, Spain).

Recombinant plasmids derived from pNZ8048

The primers and inserts used for the construction of the recombinant plasmids are listed in Table 2. Plasmid derivatives were constructed as follows: the primer pairs USPBS-F/USPSKA-R, EPBS-F/EPSA-R, and HNZ-F/SAPSH-R were used for PCR amplification from total genomic DNA of *L. lactis* MG1363, *E. faecium* P13, and *E. hirae* DCH5 of the NU, NP, and NH fragments encoding the SP_{usp45}, SP_{entP}, and $SP_{hirJM79}$, respectively, with a tail complementary to the N-terminal sequence of SakA. Primers USPBS-F/USEA-R were used for PCR amplification from total genomic DNA of *L. lactis* MG1363 of a 137-bp *Bsp*HI fragment (NUA) containing the SP_{usp45} and the N-terminal nucleotide sequence *ACCACTCATAGTGG TAAATAT::sapA* mature (*entA/sapA*), encoding the first 7 amino acids (TTHSGKY) from EntA fused to mature *sapA*. Primers USPBS-F/USEP-R were used for PCR amplification from *L. lactis* MG1363 of a 117-bp *Bsp*HI fragment (NUP) containing the SP_{usp45} and the N-terminal nucleotide sequence of the *GCTACG::sapA* mature (*entP/sapA*) encoding the first 2 amino acids (AT) from EntP fused to mature *sapA*. Primers SKA-F/SKAJEI-R were used for PCR amplification from total genomic DNA of *Lb. sakei* Lb706 of a 428-bp *Hin*dIII fragment (A) containing mature *sapA* and *saiA*. Primers SEA-F/SKAJEI-R were used for PCR amplification from *Lb. sakei* Lb706 of a 440-bp *Hin*dIII fragment (B) containing the *entA/sapA* chimera and *saiA*. Primers SEP-F/SKAJEI-R were used for PCR amplification from *Lb. sakei* Lb706 of a 431-bp *Hin*dIII fragment (C) containing the *entP/sapA* chimera and *saiA*. Mixtures of fragments NU and A, of fragments NP and A, and of fragments NH and A were used as templates to amplify the PCR products NUS, NPS, and NHS encoding mature *sapA* fused to the SP_{usp45}, SP_{entP}, and $SP_{hirJM79}$, respectively, and *saiA*. Similarly, mixtures of fragments NUA and B, and NUP and C were used as templates to amplify the 532-bp *Bsp*HI-*Hin*dIII fragment NUAS, and the 523-pb *Bsp*HI-*Hin*dIII fragment NUPS, encoding the EntA/SakA and the EntP/SakA chimeras, respectively, fused to the SP_{usp45}, and SakI. Fragments NUS, NPS, NHS, NUAS, and NUPS were digested with the corresponding restriction enzymes and inserted into pNZ8048, with transcription of the cloned bacteriocins under control of the inducible P_{nisA} promoter, which was also digested with *Nco*I/*Hin*dIII. The ligation mixtures were used to transform *L. lactis* NZ9000 competent cells. The resulting plasmid derivatives pNUS, pNPS, pNHS, pNUAS, and pNUPS, respectively, were confirmed by bacteriocinogenicity tests, PCR, and sequencing of the inserts.

Recombinant plasmids derived from pMG36c

To construct recombinant plasmids derived from pMG36c the purified pNUS, pNUAS, and pNUPS plasmids were used as templates by using the primer pair USP-F/SKAJEI-R for generation of *Sac*I-*Hin*dIII 538-bp (GUS), 550-bp (GUAS), and 541-bp (GUPS) fragments, respectively, containing the P_{32} promoter and ribosome binding site (RBS) of pMG36c fused in frame with the genes of interest. The purified inserts were digested with the corresponding restriction enzymes and inserted into plasmid pMG36c digested with the same enzymes. The ligation mixtures were used to transform *L. lactis* NZ9000 and *Lb. sakei* Lb706 competent cells. The plasmid derivatives

Table 2 Primers, PCR products, and bacteriocins used in this study

Primer or PCR product	Nucleotide sequence (5′–3′) or description	Amplification
Primers		
USPBS-F	ATAAACTCATCATGAAAAAAAAGATTATCTCAGCTATTTTAATGTCTAC	NU, NUA, NUP, NUS, NUAS, NUPS
USP-F	CATAGAGCTCTGTAAGGAGGATTTTTGAAATGAAAAAAAAGATTATCTCAGCTAT	GUS, GUAS, GUPS
USPSKA-R	GTAAACACCGTTGCCATATGATCTAGCAGCGTAAACACCTGACAACGGG	NU
EPBS-F	ATAAACTCATCATGAGAAAAAAATTATTTAGTTTAGCTCTTATTGG	NP, NPS
EPSA-R	GTAAACACCGTTGCCATATGATCTAGCTGCATCAACTTTTGTACCAAAATTTGTC	NP
HNZ-F	AAACTCATCATGAAAAAGAAAGTATTAAAACATTGTGTTATTCTAGG	NH, NHS
SAPSH-R	GTAAACACCGTTGCCATATGATCTTGCTGCATCAACTTTTATTCCTGTACC	NH
SKA-F	GCTAGATCATATGGCAACGGTGTTTACTGTAATAAT	A
SKAJEI-R	ATAAGTTAAGCTTGTACTAACTAAATCCAGACCACGCCTTAGG	A, B, C, NUS, NPS, NHS, NUAS, NUPS, GUS, GUAS, GUPS
SEP-F	GCTACGAGATCATATGGCAACGGTGTTTAC	C
USEP-R	CACCGTTGCCATATGATCTCGTAGCAGCGTAAACACCTGACAACGGG	NUP, NUPS
SEA-F	ACCACTCATAGTGGTAAATATTATGGCAACGGTGTTTACTGTAATAAT	B
USEA-R	GCCATAATATTTACCACTATGAGTGGTAGCGTAAACACCTGACAACGGG	NUA, NUAS
JJSA-F	GAATTCTCGAGAAAAGAGCTAGATCATATGGCAACGGTGTTTAC	KR-SA
JJSA-R	ATAAGTTGGCGGCCGCTATTTACATTTCAGCTAAACCACTAGCCCAG	KR-SA, KR-PSA
JJPSA-F	GAATTCTCGAGAAAAGAGCTACGAGATCATATGGCAACGGTGTTTAC	KR-PSA
PCR products		
A	428-bp *Hin*dIII fragment containing the mature sakacin A (*sapA*) and immunity (*saiA*) genes	
B	440-bp *Hin*dIII fragment containing the chimera *entA/sapA* and *saiA* genes	
C	431-bp *Hin*dIII fragment containing the chimera *entP/sapA* and *saiA* genes	
NU	110-bp *Bsp*HI fragment containing the SP_{usp45} and the N-terminal sequence of *sapA*	
NP	110-bp *Bsp*HI fragment containing the SP_{entP} and the N-terminal sequence of *sapA*	
NH	129-bp *Bsp*HI fragment containing the $SP_{hirJM79}$ and the N-terminal sequence of *sapA*	
NUA	137-bp *Bsp*HI fragment containing the SP_{usp45} and the N-terminal sequence of *entA/sapA*	
NUP	117-bp *Bsp*HI fragment containing the SP_{usp45} and the N-terminal sequence of *entP/sapA*	
NUS	520-bp *Bsp*HI/*Hin*dIII fragment containing the SP_{usp45} fused to mature *sapA* and *saiA* genes	
NPS	520-bp *Bsp*HI/*Hin*dIII fragment containing the SP_{entP} fused to mature *sapA* and *saiA* genes	
NHS	526-bp *Bsp*HI/*Hin*dIII fragment containing the $SP_{hirJM79}$ fused to mature *sapA* and *saiA* genes	
NUAS	532-bp *Bsp*HI/*Hin*dIII fragment containing the SP_{usp45} fused to *entA*/*sapA* and *saiA* genes	
NUPS	523-bp *Bsp*HI/*Hin*dIII fragment containing the SP_{usp45} fused to *entP*/*sapA* and *saiA* genes	
GUS	538-bp *Sac*I/*Hin*dIII fragment containing the P_{32} ribosome binding site and the SP_{usp45} fused to mature *sapA* and *saiA* genes	
GUAS	550-bp *Sac*I/*Hin*dIII fragment containing the P_{32} ribosome binding site and the SP_{usp45} fused to *entA*/*sapA* and *saiA* genes	
GUPS	541-bp *Sac*I/*Hin*dIII fragment containing the P_{32} ribosome binding site and the SP_{usp45} fused to *entP*/*sapA* and *saiA* genes	
KR-SA	158-bp *Xho*I/*Not*I fragment containing the α-factor Kex2 signal cleavage fused to mature *sapA*	
KR-PSA	161-bp *Xho*I/*Not*I fragment containing the α-factor Kex2 signal cleavage fused to mature *entP*/*sapA*	
Bacteriocins		
SakA	ARSYGNGVYCNNKKCWVNRGEATQSIIGGMISGWASGLAGM	
EntP/SakA	ATRSYGNGVYCNNKKCWVNRGEATQSIIGGMISGWASGLAGM	
EntA/SakA	TTHSGKYYGNGVYCNNKKCWVNRGEATQSIIGGMISGWASGLAGM	

pGUS, pGUAS, and pGUPS were checked by bacteriogenicity tests, PCR, and sequencing of the inserts.

Antimicrobial activity of the recombinant bacterial strains

The antimicrobial activity of individual LAB colonies was examined by the stab-on-agar test (SOAT), as previously described [14]. Recombinant cultures of *L. lactis* NZ9000 were induced for production of SakA or the EntA/SakA and EntP/SakA chimeras when they reached an optical density at 600 nm (OD_{600}) of 0.5, using as inducer crude nisin A (NisA) from the supernatant of *L. lactis* BB24 (NisA producer), at a final concentration of 10 ng ml^{-1}. Cell-free culture supernatants from all transformants were obtained by centrifugation of cultures at 12,000*g* at 4 °C for 10 min, adjusted to pH 6.2 with 1 M NaOH, filtered through 0.2-μm-pore-size filters (Whatman Int. Ltd., Maidstone, UK), and stored at −20 °C until use. The antimicrobial activity of the supernatants was examined by a microtiter plate assay (MPA) as described (Gutiérrez et al. [30]), using *E. faecium* T136 and *Pediococcus damnosus* CECT4797 as indicator microorganisms. In the MPA, growth inhibition of the sensitive culture was measured spectrophotometrically at 620 nm with a microtitre Labsystems iEMS plate reader (Labsystems, Helsinki, Finland). One bacteriocin unit (BU) was defined as the reciprocal of the highest dilution of the bacteriocin causing 50 % growth inhibition (50 % of the turbidity of the control culture without bacteriocin). The antimicrobial activity of most recombinant LAB was also tested against *Listeria* spp. obtained from the CECT (Colección Española de Cultivos Tipo, Valencia, Spain), using the MPA.

Cloning of the sakacin A structural gene (*sapA*) and its EntP/SakA (*GCTACG::sapA*) chimera in *P. pastoris* X-33 and *K. lactis* GG799, and determination of their antimicrobial activity

The primers and inserts used for the construction of the recombinant plasmids are listed in Table 2. Derivatives of plasmids pPICZαA and pKLAC2 were constructed as follows: primers JJSA-F/JJSA-R and JJPSA-F/JJSA-R were used for PCR amplification from total genomic DNA of *Lb. sakei* Lb706 DNA of the 158-bp and 161-bp *Xho*I–*Not*I fragments KR-SA and KR-PSA, respectively, carrying the α-factor Kex2 signal-protease cleavage site without the Glu-Ala spacer fused to mature SakA and to the EntP/SakA chimera. The KR-SA and KR-PSA fragments were digested with the restriction enzymes mentioned above and the resulting 138-bp and 141-bp fragments were ligated into the pPICZαA and pKLAC2 vectors at appropriate sites to generate plasmids pPSA and pKSA, and pPPSA and pKPSA, respectively. Competent *E. coli* JM109 cells were transformed with these plasmids and the resulting transformants were confirmed by DNA sequencing. Subsequently, *Sac*I-linearized pPSA and pPPSA and *Sac*II-linearized pKSA and pKPSA were used to transform *P. pastoris* X-33 and *K. lactis* GG799, respectively. The *P. pastoris* X-33SA and *P. pastoris* X-33PSA transformants were selected from YPD agar supplemented with Zeocin (100 and 1,000 μg ml^{-1}) and sorbitol (1 M). The *K. lactis* GG799SA and *K. lactis* GG799PSA transformants were selected on YCB (New England Biolabs) agar supplemented with Tris–HCl Buffer (30 mM) and acetamide (5 mM). Plates were incubated at 30 °C for 3–5 days. The presence of the integrated pPSA, pKSA, pPPSA, and pKPSA linearized plasmids in the genome of the transformed yeasts was confirmed by bacteriocinogenicity tests and by PCR and sequencing of the inserts.

The antimicrobial activity of individual *P. pastoris* X33SA, *P. pastoris* X33PSA, *K. lactis* GG799SA, and *K. lactis* GG799PSA transformants was screened by a streak-on-agar test (STOAT). Briefly, the *P. pastoris* X33SA and *P. pastoris* X33PSA transformants were streaked onto BMMY buffered methanol complex medium [1 % yeast extract, 2 % peptone, 100 mM potassium phosphate (pH 6), 1.34 % yeast nitrogen base (YNB) without amino acids, 4×10^{-5} % biotin, and 0.5 % methanol] agar and grown at 30 °C to induce production of the bacteriocins. During the incubation period, methanol was added daily to the plates at 0.5 % (v/v) final concentration to maintain the induction. The *K. lactis* GG799SA and *K. lactis* GG799PSA transformants were streaked onto YPGal (1 % yeast extract, 2 % peptone and 2 % galactose) agar. After incubation of the plates at 30 °C for 48 h, 40 ml of MRS soft agar containing about 1×10^5 cfu ml^{-1} of the indicator microorganism *P. damnosus* CECT4797 was added, and the plates were further incubated at 30 °C overnight for development of inhibition zones.

To determine the growth of the recombinant yeasts and the antimicrobial activity of their supernatants, the *P. pastoris* X33SA and *P. pastoris* X33PSA bacteriocin producers were grown in the buffered glycerol complex medium BMGY [1 % yeast extract, 2 % peptone, 100 mM potassium phosphate (pH 6), 1.34 % YNB without amino acids, 4×10^{-5} % biotin, and 1 % glycerol] at 30 °C until an OD_{600} of approximately 2–6 was reached. Cells were then harvested by centrifugation (5,000×*g* at 4 °C for 10 min), and resuspended to an OD_{600} of 1 in BMMY medium. Similarly, the *K. lactis* GG799SA and *K. lactis* GG799PSA cultures were grown in the YPD medium at 30 °C until an OD_{600} of approximately 2–6 was reached. Cells were then harvested by centrifugation (5,000×*g* at 4 °C for 10 min), washed with YPGal, and resuspended to an OD_{600} of 1 in fresh YPGal medium. The *P. pastoris* and *K. lactis* cultures

were incubated at 30 °C for 36 h with shaking (250 rpm). During growth, samples were collected periodically for determination of their OD_{600}, bacteriocin production, and the antimicrobial activity of their supernatants by the MPA.

Production of specific anti-SakA polyclonal antibodies and ELISA

The peptide fragment SAJJ (NH_2-CSGWASGLAGM-COOH), deduced from the C-terminal amino acid sequence of SakA, was selected as the antigen for the generation of antibodies of predetermined specificity against SakA. The synthetic peptide SAJJ was synthesized by Invitrogen Ltd. (Paisley, Scotland, UK), with a peptide purity of greater than 95 %. The peptide SAJJ was conjugated to the key-hole limpet hemocyanin (KLH) carrier protein as a SAJJ-KLH conjugate, 1:2 (w/w), using the components of the inject maleimide-activated mariculture KLH kit (Perbio Science, Rockford, IL, USA) for use as the immunogen. Rabbits (New Zealand White Females) were immunized with SAJJ-KLH, as described previously [9]. Serum was obtained from blood samples incubated overnight at 4 °C, centrifuged at $1{,}000\times g$ at room temperature for 15 min, and stored at −20 °C until use. The enzyme-linked immunosorbent assay (ELISA) procedures for determination of antiserum specificity and sensitivity were performed as described previously [9]. A noncompetitive indirect ELISA (NCI-ELISA) was developed to detect and quantify SakA, EntP/SakA, and EntA/SakA in the supernatants of the recombinant bacterial and yeast producer cells. Briefly, wells of flat-bottomed polystyrene microtitre plates (Maxisorp, Nunc, Roskilde, Denmark) were coated overnight (4 °C) with supernatants from *Lb. sakei* Lb706 or the recombinant LAB and yeast hosts. After addition of the anti-JJSA-KLH antibodies and the goat anti-rabbit immunoglobulin G peroxidase conjugate (Cappel Laboratories, West Chester, PA, USA), bound peroxidase was determined with ABTS (2,2′-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid]) (Sigma) as the substrate by measuring the absorbance of the wells at 405 nm with a Labsystems iEMS reader (Labsystems) with a built-in software package for data analysis.

Purification of SakA, EntP/SakA, and EntA/SakA, mass spectrometry analysis, and N-terminal amino acid sequencing

SakA, EntP/SakA, and EntA/SakA were purified from *Lb. sakei* Lb706 and *L. lactis* NZ9000 (pNUS), *L. lactis* NZ9000 (pNUPS), and *L. lactis* NZ9000 (pNUAS), respectively. SakA and EntP/SakA were also purified from *K. lactis* GG799SA and *P. pastoris* X-33PSA and *K. lactis* GG799PSA, respectively, as previously described [8, 45]. Briefly, supernatants from early stationary phase cultures of recombinant LAB strains and 0.5 l of the recombinant yeasts were precipitated with ammonium sulfate, desalted by gel filtration, and subjected to cationic-exchange and hydrophobic-interaction chromatography, followed by reversed-phase chromatography in a fast-protein liquid chromatography system (RP-FPLC) (GE Healthcare, Barcelona, Spain). Purified fractions were subjected to matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) mass spectrometry, as previously described [30]. For N-terminal sequencing, the purified bacteriocins were subjected to automatic Edman degradation and sequence on polyvinylidene difluoride membranes (PVDF) in a Procise 494 HT Sequencing System (Applied Biosystems Inc., Foster City, CA, USA), according to the manufacturer's standard methods, at the Centro de Investigaciones Biológicas (CIB, Madrid, Spain).

Results

Heterologous production and functional expression of SakA and two SakA-derived chimeras, EntP/SakA and EntA/SakA, by different LAB strains

The mature bacteriocin SakA and two SakA-derived chimeras, EntP/SakA and EntA/SakA, fused to different signal peptides (SP_{usp45}, SP_{entP}, and $SP_{hirJM79}$) and cloned into the protein expression vectors pNZ8048, under control of the inducible P_{nisA} promoter, and into pMG36c, carrying the P_{32} constitutive promoter, have been evaluated for their production and functional expression by *L. lactis* subsp. *cremoris* NZ9000 and *Lb. sakei* Lb706. Cloning of SP_{usp45}, SP_{entP}, and $SP_{hirJM79}$ fused to mature *sapA* (SakA) and *saiA* (SakI) into pNZ8048 afforded the NisA-induced derived vectors pNUS, pNPS, and pNHS, respectively, whereas cloning of SP_{usp45} fused to mature *sapA* (SakA) and *saiA* (SakI) into pMG36c afforded the constitutive pGUS. Similarly, cloning of the SP_{usp45} fused to *entP/sapA* (EntP/SakA) and *saiA* (SakI) and fused to *entA/sapA* (EntA/SakA) and *saiA* (SakI) into pNZ8048 and pMG36c afforded the NisA-induced derived vectors pNUPS and pNUAS, and the constitutive pGUPS and PGUAS, respectively. Transformation of competent *L. lactis* subsp. *cremoris* NZ9000 and *Lb. sakei* Lb706 with the pNZ8048- and pMG36c-derived vectors yielded recombinant strains which were further checked for bacteriocin production.

The production and functional expression of the SakA, EntP/SakA, and EntA/SakA in the supernatants of the recombinant LAB strains were quantified by specific anti-SakA antibodies in an NCI-ELISA and by a microtitre plate assay (MPA), respectively. None of the control strains transformed with the empty vectors, except the natural *Lb.*

Table 3 Bacteriocin production and antimicrobial activity of supernatants from recombinant LAB strains producing SakA and the EntP/SakA and EntA/SakA chimeras

Strain	Bacteriocin production (μg mg^{-1} cell dry weight)[a]	Antimicrobial activity (BU mg^{-1} cell dry weight)[b]		Specific antimicrobial activity (BU μg^{-1} SakA)[c]	
		Enterococcus faecium T136	*Pediococcus damnosus* CECT4797	*Enterococcus faecium* T136	*Pediococcus damnosus* CECT4797
Lactococcus lactis subsp. *cremoris*					
NZ9000 (pNZ8048)	NP	NA	NA	NE	NE
NZ9000 (pNUS, SakA)	3.4	124	300	37	89
NZ9000 (pNPS, SakA)	NP	NA	NA	NA	NA
NZ9000 (pNHS, SakA)	2.1	115	247	55	118
NZ9000 (pNUPS, EntP/SakA)	3.2	1,613	14,429	538	4,810
NZ9000 (pNUAS, EntA/SakA)	2.7	34	77	13	29
Lactococcus lactis subsp. *cremoris*					
NZ9000 (pMG36c)	NP	NA	NA	NE	NE
NZ9000 (pGUS, SakA)	4.9	174	31	35	12
NZ9000 (pGUPS, EntP/SakA)	2.5	NA	434	NE	174
NZ9000 (pGUAS, EntA/SakA)	1.9	NA	NA	NE	NE
Lactobacillus sakei					
Lb706 (pMG36c)	2.0	956	2,095	478	1,047
Lb706 (pGUS, SakA)	4.7	1,943	6,538	413	1,391
Lb706 (pGUPS, EntP/SakA)	3.2	419	1,385	130	432
Lb706 (pGUAS, EntA/SakA)	2.4	306	858	128	357
Lactobacillus sakei					
Lb706[d]	2.1	849	2,243	404	1,068

Most of the data are means from two independent determinations in triplicate

BU bacteriocin units, *NA* no activity, *NE* not evaluable, *NP* no production

[a] Production of SakA was calculated by an NCI-ELISA with antibodies specific for the C-terminal amino acid fragment of SakA

[b] Antimicrobial activity was calculated against *E. faecium* T136 ($SakA^s$) and *P. damnosus* CECT4797 ($SakA^s$)

[c] Specific antimicrobial activity refers to the antimicrobial activity against *E. faecium* T136 and *P. damnosus* CECT4797 divided by the SakA produced

[d] *Lb. sakei* Lb706 was used as control for the production and antimicrobial activity of SakA

sakei Lb706 SakA producer, showed production of SakA. The production of SakA by *L. lactis* NZ9000 transformed with the pNZ8048 derivatives was 1.6-fold higher for *L. lactis* NZ9000 (pNUS) and identical for *L. lactis* NZ9000 (pNHS), as compared to production of SakA by *Lb. sakei* Lb706, whereas it was non-detectable for *L. lactis* NZ9000 (pNPS). The production of EntP/SakA by *L. lactis* NZ9000 (pNUPS) and of EntA/SakA by *L. lactis* NZ9000 (pNUAS) was 1.5- and 1.3-fold higher, respectively, than that of SakA by *Lb. sakei* Lb706 (Table 3). The production of SakA and its chimeras by *L. lactis* NZ9000, transformed with the pMG36c-derivatives, was from 0.9-fold lower to 2.3-fold higher than production of SakA by *Lb. sakei* Lb706 (Table 3). Finally, the production of SakA, EntP/SakA, and EntA/SakA by *Lb. sakei* Lb706 transformed with the pMG36c-derivatives pGUS, pGUPS, and pGUAS, respectively, was from 1.1- to 2.2-fold higher than production of SakA by *Lb. sakei* Lb706 (Table 3).

The evaluation of the antimicrobial activity of the recombinant LAB revealed that most of the *L. lactis* NZ9000 transformed hosts showed a much lower (from no activity to 0.14-fold lower) antimicrobial activity than that of *Lb. sakei* Lb706, except for *L. lactis* NZ9000 (pNUPS), with a 1.9- and a 6.4-fold higher antimicrobial activity, depending on the indicator strain, than that of *Lb. sakei* Lb706. On the other hand, supernatants of *Lb. sakei* Lb706 (pGUS) showed a 2.3- and a 2.9-fold higher antimicrobial activity, whereas those of *Lb. sakei* Lb706 (pGUPS) and *Lb. sakei* Lb706 (pGUAS) showed a lower antimicrobial activity (0.36- to 0.62-fold lower) than *Lb. sakei* Lb706. It should be noted that, although most of the recombinant *L. lactis* NZ9000 and *Lb. sakei* Lb706 hosts produced a higher

Table 4 Antimicrobial activity of supernatants from recombinant LAB strains against *Listeria* spp[a]

Strain	*L. ivanovii*	*L. welshimeri*	*L. seeligeri*	*L. innocua*	*L. monocytogenes*				
	913	919	917	910	911	935	936	939	4032
Lactococcus lactis subsp. *cremoris*									
NZ9000 (pNUS, SakA)	61	45	56	46	57	25	58	56	50
NZ9000 (pNUPS, EntP/SakA)	1,012	348	1,178	335	392	453	1,423	1,312	1,758
NZ9000 (pNUAS, EntA/SakA)	32	26	28	NA	25	24	39	27	37
Lactococcus lactis subsp. *cremoris*									
NZ9000 (pGUS, SakA)	25	4	25	NA	23	24	30	27	34
NZ9000 (pGUPS, EntP/SakA)	98	60	88	45	93	101	187	89	184
NZ9000 (pGUAS, EntA/SakA)	NA	NA	NA	NA	NA	NA	NA	25	NA
Lactobacillus sakei									
Lb706 (pMG36c)	203	204	412	149	75	346	815	691	781
Lb706 (pGUS, SakA)	624	1,257	1,541	749	387	584	2,286	1,729	2,743
Lb706 (pGUPS, EntP/SakA)	148	315	594	254	121	249	516	560	691
Lb706 (pGUAS, EntA/SakA)	100	216	369	176	110	180	418	272	428
Lactobacillus sakei									
Lb706[b]	185	272	443	175	90	370	655	1,266	543

Most of the data are mean from two independent determinations in triplicate

NA no activity

[a] Antimicrobial activity expressed in bacteriocin units per milligrams of cell dry weight

[b] *Lb. sakei* Lb706 was used as control for the antimicrobial activity of SakA

extracellular amount of SakA, EntP/SakA, and EntA/SakA than *Lb. sakei* Lb706, only *L. lactis* NZ9000 (pNUPS) and *Lb. sakei* Lb706 (pGUS) showed a 1.3- and 4.5-fold, and a 1.0- and 1.3-fold higher, respectively, specific antimicrobial activity than the SakA produced by *Lb. sakei* Lb706 (Table 3).

Supernatants of the recombinant LAB strains also showed antagonistic activity against *Listeria* spp. by the MPA (Table 4). The pattern of the antagonistic activity of their supernatants was similar to that observed against the indicator bacteria *E. faecium* T136 and *P. damnosus* CECT4797 (Table 3). Supernatants of *L. lactis* NZ9000 (pNUS) showed a 0.04- to 0.63-fold lower antagonistic activity, those of *L. lactis* NZ9000 (pNUPS) a 1.0- to 5.4-fold higher antagonistic activity, and supernatants of *L. lactis* NZ9000 (pNUAS) from no activity to a 0.25-fold lower antagonistic activity, as compared to that of *Lb. sakei* Lb706 against the evaluated *Listeria* strains. A low antilisterial activity was also observed for *L. lactis* NZ9000 transformed with plasmids pGUS, pGUPS, and pGUAS (from no activity to a 1.0-fold antimicrobial activity as compared to that of *Lb. sakei* Lb706). However, *Lb. sakei* Lb706 (pGUS) showed a 1.3- to 5.0-fold higher antilisterial activity than *Lb. sakei* Lb706, whereas *Lb. sakei* Lb706 (pGUPS) and *Lb. sakei* Lb706 (pGUAS) showed a lower antilisterial activity than *Lb. sakei* Lb706 against *Listeria* spp. (Table 4).

Purification of SakA, EntP/SakA, and EntA/SakA, mass spectrometry analysis, and N-terminal amino acid sequencing

The SakA produced by *Lb. sakei* Lb706 and *L. lactis* (pNUS), the EntP/SakA produced by *L. lactis* (pNUPS), and the EntA/SakA produced by *L. lactis* (pNUAS) were purified to homogeneity following a previously used chromatographic procedure (results not shown). MALDI–TOF MS analysis of the purified SakA from *Lb. sakei* Lb706 showed a major peak of 4,306.9 Da close to its predicted molecular mass of 4,308.8 Da (Fig. 1a), whereas the molecular mass of the SakA produced by *L. lactis* (pNUS) showed a much lower molecular mass of 3,805.3 Da (Fig. 1b). Similarly, the molecular mass of the EntP/SakA produced by *L. lactis* (pNUPS) was 4,410.5 Da (Fig. 1c), whereas that of the EntA/SakA produced by *L. lactis* (pNUAS) showed a molecular mass of 3,805.8 Da (Fig. 1d). Given that the obtained molecular masses of SakA and EntA/SakA produced by *L. lactis* NZ9000 are almost identical to each other but lower than their predicted theoretical molecular masses of 4,308.8 and 4,769.3 Da, respectively, it could be that both occur via abnormal processing. Accordingly, determination of the N-terminal amino acid sequence of both bacteriocins by Edman degradation demonstrated that they started with the amino acid sequence NGVY-, meaning that both have lost their first

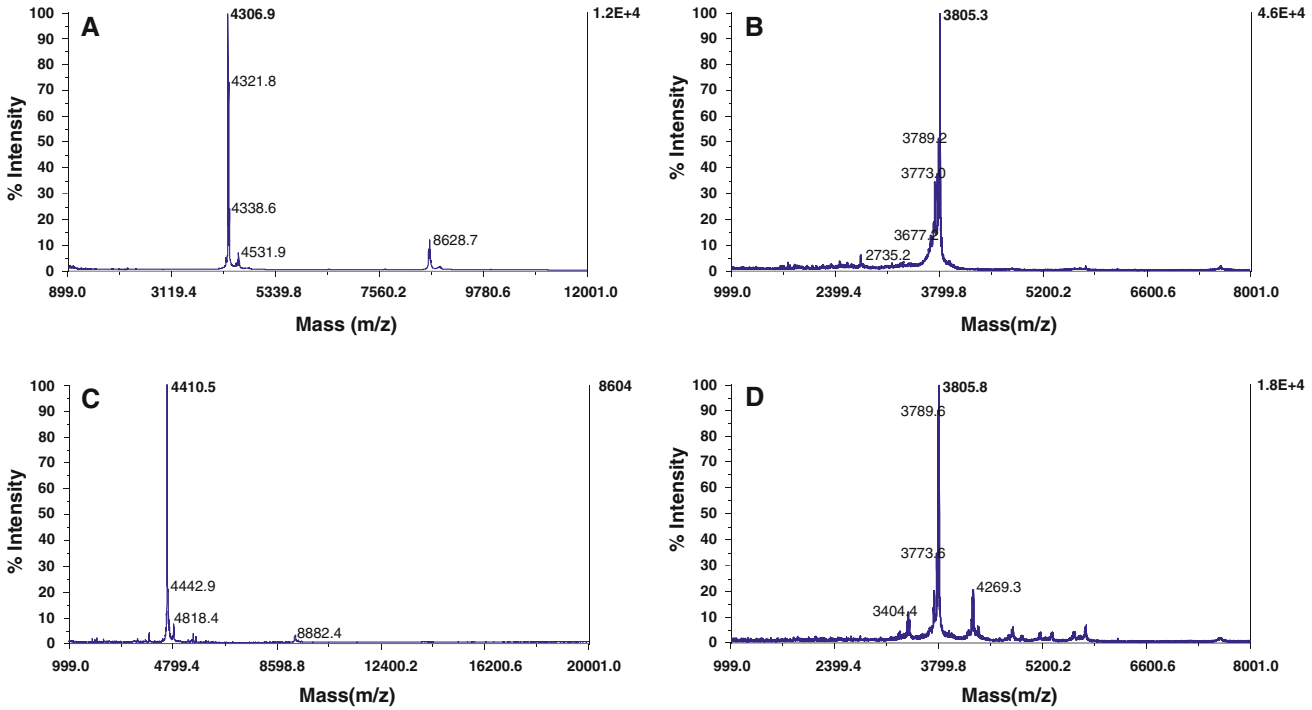

Fig. 1 Mass spectrometry analysis of purified sakacin A from *Lb. sakei* Lb706 (**a**), purified sakacin A from *L. lactis* NZ9000 (pNUS) (**b**), purified enterocin P/sakacin A from *L. lactis* NZ9000 (pNUPS) (**c**), and purified enterocin A/sakacin A from *L. lactis* NZ9000 (pNUAS) (**d**). Numbers indicate the molecular mass in daltons of most of the observed peptide fragments

five (ARSYG-) and nine (TTHSGKYYG-) N-terminal amino acids, respectively, during processing and secretion out of the *L. lactis* NZ9000 producer hosts.

Heterologous production and functional expression of SakA and EntP/SakA by *P. pastoris* and *K. lactis*

Cloning of the PCR fragments containing the α-factor Kex2 signal cleavage fused to mature *sapA* (SakA) or to mature *entP/sapA* (EntP/SakA) into the expression vectors pPICZαA and pKLAC2 afforded the *P. pastoris* X-33 and *K. lactis* GG799 derivatives, respectively, from which to determine the production and functional expression of SakA and EntP/SakA. Since the *P. pastoris* X-33SA, producer of SakA, and *P. pastoris* X-33PSA, producer of EntP/SakA, did not show a defined direct antimicrobial activity when streaked in selective agar plates with the SakA-sensitive indicator *P. damnosus* CECT4797, they were selected on the basis of their high Zeocin resistance (1,000 μg ml^{-1}). However, *K. lactis* GG799SA, producer of SakA, and *K. lactis* GG799PSA, producer of EntP/SakA, showed a low but defined direct antimicrobial activity against *P. damnosus* CECT4797. Colonies of *P. pastoris* X-33 and *K. lactis* GG799, transformed with the linearized control plasmids, were used as bacteriocin-negative controls to discard the possibility that the antimicrobial activity exerted by the recombinant hosts was due to metabolites other than bacteriocins.

The heterologous production and functional expression of the SakA and EntP/SakA produced by the recombinant yeasts were determined by an NCI-ELISA and an MPA, respectively. The growth and production of SakA by *P. pastoris* X-33SA were lower than those of EntP/SakA by *P. pastoris* X-33PSA. The largest production of SakA and EntP/SakA by the recombinant *P. pastoris* producers was 1.8- and 24.2-fold higher, respectively, than production of SakA by *Lb. sakei* Lb706. However, neither the SakA nor the EntP/SakA produced by the *P. pastoris* producers showed a measurable antimicrobial activity (Table 5). On the other hand, the growth of *K. lactis* GG799SA and *K. lactis* GG799PSA was similar, whereas the production of SakA was 11.4-fold higher and that of the EntP/SakA 55.2-fold higher than production of SakA by *Lb. sakei* Lb706. However, the antimicrobial activity and the specific antimicrobial activity of either the SakA or the EntP/SakA produced by the *K. lactis* derivatives were much lower, ranging from 0.01- to 0.06-fold lower for both, than those of the SakA produced by *Lb. sakei* Lb706 (Table 5).

Purification and mass spectrometry analysis of the SakA and EntP/SakA produced by *P. pastoris* and *K. lactis*

Although not a measurable or a very low antimicrobial activity was observed in the supernatants of the

Table 5 Production and antimicrobial activity of SakA and EntP/SakA from supernatants of *P. pastoris* X-33SA, *P. pastoris* X-33PSA, *K. lactis* GG799SA, and *K. lactis* GG799PSA

Strain	Incubation time (h)	OD_{600}	Bacteriocin production (μg SakA ml^{-1})[a]	Antimicrobial activity (BU ml^{-1})[b]	Specific antimicrobial activity (BU μg^{-1} SakA)[c]
P. pastoris X-33SA	0	0.1	ND	NA	NE
	2	0.1	ND	NA	NE
	4	0.2	ND	NA	NE
	6	0.3	1.9	NA	NE
	8	0.4	2.5	NA	NE
	10	0.4	2.7	NA	NE
	12	0.4	2.8	NA	NE
	24	0.4	3.6	NA	NE
	28	0.5	3.0	NA	NE
	36	1.6	0.9	NA	NE
P. pastoris X-33PSA	0	1.0	ND	NA	NE
	2	2.9	ND	NA	NE
	4	5.1	ND	NA	NE
	6	6.5	2.6	NA	NE
	8	7.3	6.4	NA	NE
	10	7.7	9.4	NA	NE
	12	8.8	11.7	NA	NE
	24	13.6	44.3	NA	NE
	28	11.5	48.5	NA	NE
	36	12.2	48.0	NA	NE
K. lactis GG799SA	0	1.0	ND	NA	NE
	2	2.4	ND	NA	NE
	4	4.4	ND	NA	NE
	6	7.2	7.5	20.1	2.7
	8	9.5	11.5	26.0	2.3
	10	14.3	22.9	32.5	1.4
	12	16.6	22.7	NA	NE
	24	17.1	ND	NA	NE
	28	18.4	ND	NA	NE
	36	19.2	ND	NA	NE
K. lactis GG799PSA	0	1.0	ND	NA	NE
	2	3.9	ND	NA	NE
	4	6.3	17.2	NA	NE
	6	8.8	40.6	22.7	0.6
	8	10.8	89.0	23.2	0.3
	10	14.2	110.4	24.9	0.2
	12	15.4	97.9	NA	NE
	24	18.6	90.0	NA	NE
	28	19.0	90.0	NA	NE
	36	20.1	87.2	NA	NE
Lb. sakei Lb706[d]	15	1.2	2.0	830	415

Most of the data are means from two independent determinations in triplicate

BU bacteriocin units, *NA* no activity, *ND* no detection, *NE* not evaluable, OD_{600} optical density of cultures at 600 nm

[a] Production of SakA and EntP/SakA were calculated by using an NCI-ELISA with antibodies specific for the C-terminal amino acid fragment of SakA

[b] Antimicrobial activity against *E. faecium* T136 ($SakA^s$)

[c] Specific antimicrobial activity refers to the antimicrobial activity against *E. faecium* T136 divided by the SakA and EntP/SakA produced

[d] *Lb. sakei* Lb706 was used as control for the production and antimicrobial activity of SakA

recombinant yeasts, their supernatants were subjected to protein purification following the procedure used for the recombinant LAB producers. Interestingly, a high antimicrobial activity was observed after the gel filtration, cationic exchange, hydrophobic interaction, and reverse-phase chromatography steps of all evaluated supernatants, except in those from *P. pastoris* X-33SA (results not shown). The theoretical molecular mass of SakA is 4,308.8 Da and that of the EntP/SakA is 4,410.0 Da. However, MALDI–TOF MS analysis of the purified EntP/SakA produced by *P. pastoris* X-33PSA showed a minor peptide fragment of 4,439.9 Da and major fragments of higher molecular mass (5.4–5.8 kDa). Similarly, the purified SakA from *K. lactis* GG799SA showed a fragment of 4,351.6 Da as well as fragments of higher molecular mass (>4.4 kDa), whereas the EntP/SakA produced by *K. lactis* G799PSA also showed a minor peak of 4,285.3 Da and numerous fragments of higher molecular mass (5.5–6.1 kDa) (Fig. 2).

Discussion

If the use of bacteriocins as natural antimicrobial agents in food, veterinary, and medical applications is ever to meet the high expectations of the research community, a high level production of active bacteriocins in homologous and heterologous microbial hosts is essential. Production of bacteriocins by LAB is essentially based on the expression of native biosynthetic genes, by exchanging or replacing leader peptides and/or dedicated processing and secretion systems (ABC transporters) or by fusion of mature bacteriocins to signal peptides that act as secretion signals [8, 9, 30]. Accordingly, optimization of bacteriocin gene expression and protein production would help the development of LAB as microbial cell factories for production and delivery of bacteriocins of biotechnological interest.

For the heterologous production of SakA and two SakA-derived chimeras, EntP/SakA and EntA/SakA, by *L. lactis* subsp. *cremoris* NZ9000 and *Lb. sakei* Lb706, the mature SakA and its chimeras were fused to different SPs. The SP_{usp45}, SP_{entP}, and $SP_{hirJM79}$ encode the 27 amino acid SP of the major extracellular Usp45 protein from *L. lactis* MG1363 [48], the 27 amino acid SP of the bacteriocin EntP [14], and the 30 amino acid SP of the bacteriocin hiracin JM79 [44], respectively. SakA holds the N-terminal ARS amino acid sequence before the YGNGVxC consensus sequence of the class IIa bacteriocins. However, since positively charged amino acids immediately following the SP cleavage site may interfere with the secretion machinery or passage of the protein through the bacterial membrane [37], the EntP/SakA chimera was constructed by displacement of the arginine (R) residue from position +2 to position +3 of the mature bacteriocin, thus mimicking the N-terminal amino acid sequence of EntP (ATR), a bacteriocin homologous to SakA [14] which is known to be overproduced and adequately processed by recombinant *L. lactis* and *P. pastoris* [8, 29]. The EntA/SakA chimera was also constructed to hold the TTHSGKY amino acid sequence before the YGNGVxC consensus sequence of the class IIa bacteriocins, thus mimicking the N-terminal amino acid sequence of EntA [2], which is also overproduced and adequately processed by recombinant *L. lactis* [9] and the yeasts *P. pastoris*, *K. lactis*, *Hansenula polymorpha*, and *Arxula adeninivorans* [10].

In this work, the development of specific anti-SakA antibodies and an NCI-ELISA has permitted the detection and quantification of SakA and its derived chimeras in the supernatants of the recombinant LAB strains, as well as the determination of their specific antimicrobial activity. Apparently, the production and antimicrobial activity of the SakA, EntP/SakA, and EntA/SakA produced by the recombinant LAB strains depend on the SP, the expression vector, and the host strain (Table 3). The production of SakA and its derivatives by the recombinant LAB strains may also rely on the expression of the SakI immunity (*saiA*) gene. It has been demonstrated that bacteriocin producers are protected from their own bacteriocins by the concomitant expression of a cognate immunity protein, that the expression of the immunity protein may increase bacteriocin production, and that the immunity proteins form a strong complex with the receptor proteins, thereby preventing producer cells from being killed [8, 35]. The production and secretion of SakA by the recombinant *L. lactis* NZ9000 derivatives was observed when *sapA* was fused to SP_{usp45} and $SP_{hirJM79}$. However, the SP_{entP} was unable to drive the production of SakA by *L. lactis* NZ9000. This was an unexpected result because the SP_{entP} fused to mature pediocin PA-1 (PedA-1) [38] and EntA [9, 39] permitted the production and secretion of both bacteriocins by different recombinant *L. lactis* hosts. In this context, secretion levels in Gram-positive bacteria may become not only affected by variations in the SPs but also by differences in the N-terminal part of the mature peptide or protein that may become only evident after fusion with some SP. It is possible that the N-terminal amino acid sequence of mature SakA may affect its secretion because positive charges at the N-terminus may affect export of the peptide by the Sec-dependent pathway [37]. Mature SakA may also remain N-terminally associated to the cell membrane via a Sec-type signal peptide that is not cleaved off during secretion [7]. It has been also hypothesized that variations in secretion capacities can be governed by post-transcriptional factors such as secondary structure of mRNA, codon usage, and translation efficiency [22]. The molecular folding of SakA inside *L. lactis* may also maintain the prepeptide in a secretion-incompetent conformation [40].

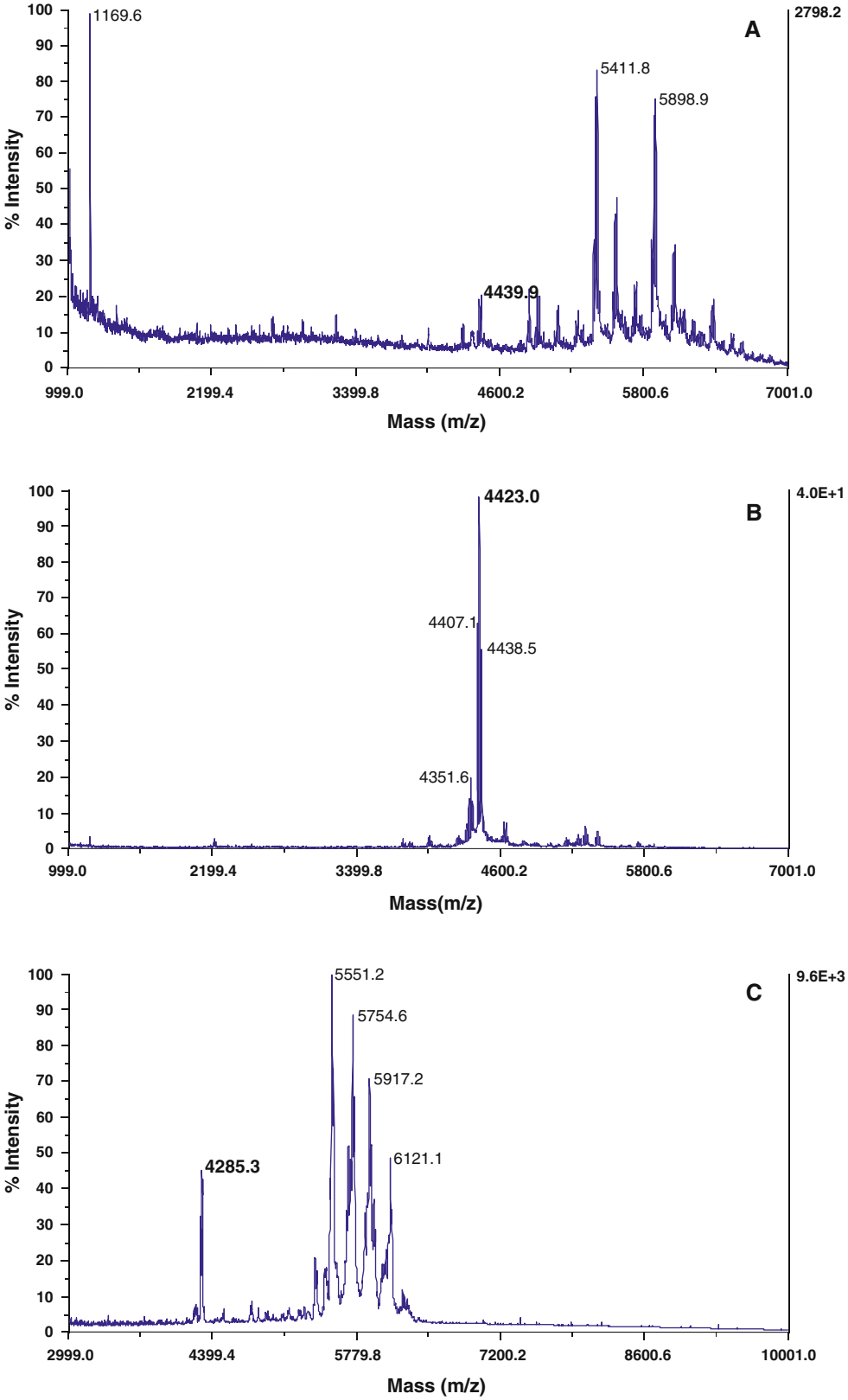

Fig. 2 Mass spectrometry analysis of purified enterocin P/sakacin A from *P. pastoris* X-33PSA (**a**), purified sakacin A from *K. lactis* GG799SA (**b**), and purified enterocin P/sakacin A from *K. lactis* GG799PSA (**c**). Numbers indicate the molecular mass in daltons of most of the observed peptide fragments

In any case, protein secretion is a preferred means of protein expression in the development of LAB as cell factories for production of biologically active bacteriocins, and SP_{usp45} fused to mature *sapA* and its chimeras adequately drive their production and secretion by the recombinant *L. lactis* and *Lb. sakei* hosts (Table 3).

The role of the expression vectors in terms of production and antimicrobial activity of the target bacteriocins seems to depend on the host strain, genes of interest, promoter, and vector copy number (Table 3). Plasmid pNZ8048 contains the high copy number heterogramic replicon of the lactococcal plasmid pSH71 with a unique *Nco*I cleavage site, downstream of the *nisA* ribosomal binding sequence (RBS), used for translational fusions inducible by NisA [19, 36]. The expression vector pMG36c contains the low copy replication origin of the lactococcal plasmid pWV01 and the strong P_{32} promoter to drive the constitutive transcription of inserted genes into the multicloning site (MCS) of pUC18 [49]. In this work, production of SakA and its chimeras by recombinant *L. lactis* and *Lb. sakei* hosts is not closely associated with the protein expression vector used, and this observation is in contrast to results in which production of bacteriocins is higher by pNZ8048-derived LAB transformants than by the pMG36c-derived ones [8, 9, 30, 38, 39, 45]. For optimization of protein expression, inducible systems are often considered superior to constitutive expression systems, because the former enable achievement of sufficient biomass prior to initiation of target protein expression and consequent metabolic burden of the cell. However, other factors such as mRNA stability and secondary structure may steer protein production from the recombinant *L. lactis* and *Lb. sakei* hosts [22].

Of interest is also the observation that supernatants of most recombinant *L. lactis* NZ9000, transformed with the pNZ8048-derived expression vectors, show a lower antimicrobial activity than that expected from the production of SakA, EntP/SakA, and EntA/SakA, except for *L. lactis* NZ9000 (pNUPS), with an antimicrobial activity in the range of that deduced from its production of EntP/SakA. On the other hand, all recombinant *L. lactis* NZ9000 hosts transformed with pMG36c-derived vectors show non-measurable or very poor antimicrobial activity (Table 3). It may occur that the short induction time for bacteriocin production from nisin-inducible systems most probably prevents bacteriocins from attaching to cell walls, forming aggregates, and/or undergoing protease degradation [8, 30]. A high bacteriocin production does not always correspond to a high antimicrobial activity. The low antimicrobial activity of the SakA and its chimeras produced by the recombinant LAB hosts may depend on many factors which are difficult to determine. It is possible that regulatory responses to secretion stress activate quality control networks involving folding factors and housekeeping proteases [18]. Differences in the Sec-dependent translocation and Sec machinery in the different LAB strains, differences in protein folding, and conformational modifications of the bacteriocin to a less extracellular active form may also account for a low antagonistic activity of the secreted bacteriocins [46]. In this respect, the four cysteine residues present in SakA and presumably involved in the formation of two disulfide bonds (DSB) may also play a role in the folding, structural integrity, and antimicrobial activity of the produced bacteriocins [25]. Bacteriocin self-aggregation may also decrease the antagonistic activity of bacteriocins [9].

MALDI–TOF MS analysis of the bacteriocins purified from recombinant *L. lactis* NZ9000 hosts revealed that SakA purified from *L. lactis* (pNUS) had a lower molecular mass than SakA purified from *Lb. sakei* Lb706. The purified EntP/SakA showed a major fragment of a molecular mass of 4,410.5 Da, corresponding to its expected size, whereas the EntA/SakA showed a major fragment of a molecular mass of 3,805.8 Da (Fig. 1). Accordingly, since the theoretical molecular mass of SakA is 4,308.8 Da, that of EntP/SakA is 4,410.0 Da, and that of EntA/SakA is 4,769.3 Da, it seems that the SakA and the EntA/SakA produced by recombinant *L. lactis* NZ9000 are truncated bacteriocin fragments. Furthermore, all the analyzed bacteriocins manifest the presence of major bacteriocin fragments with presumably none, one (+16 Da), and two (+32 Da) methionine residues (Met^{30}, Met^{41}) oxidized to MetSO (Fig. 1). The oxidation of methionine residues during bacteriocin purification and recombinant production by LAB is common [9, 39]. Determination of the N-terminal amino acid sequence of SakA and EntA/SakA by Edman degradation revealed that both bacteriocins started with the amino acid sequence NGVY-, demonstrating that both are truncated forms of the bacteriocins cloned in *L. lactis* NZ9000. As far as we know, this is the first report of N-terminal truncated bacteriocins produced by recombinant LAB. However, the truncated SakA and EntA/SakA produced by *L. lactis* NZ9000 still maintain a lower, but measurable antimicrobial activity, perhaps owing to the nonspecific binding of the pediocin-like N-terminal sequences or their truncated forms to target cells through electrostatic interactions [12].

Transformation of *Lb. sakei* Lb706, natural producer of SakA, with pMG36c-derived vectors permitted a slightly higher production of SakA, EntP/SakA, and EntA/SakA by all transformants. Furthermore, *Lb. sakei* Lb706 (pGUS), producer of SakA, showed higher antimicrobial activity than supernatants of *Lb. sakei* Lb706 (Table 3), confirming the higher production and antimicrobial activity of bacteriocins produced by homologous LAB hosts [9]. In *Lb. sakei* Lb706 modification of the N-terminal sequence of SakA resulted in lower antimicrobial activity in the supernatants of *Lb. sakei* Lb706 (pGUPS) and *Lb. sakei* Lb706 (pGUAS), further supporting that different amino acid sequences for the signal and mature peptides may be required for optimal production and secretion depending on the bacterial host.

Supernatants of *L. lactis* NZ9000 (pNUPS), producers of EntP/SakA, and those of *Lb. sakei* Lb706 (pGUS), producers of SakA, showed up to a 5.4-fold higher antimicrobial activity against several *Listeria* spp. than any other recombinant LAB host (Table 4). These recombinant LAB strains as overproducers of SakA and EntP/SakA, with higher antimicrobial activity than the supernatants of *Lb. sakei* Lb706, may be considered as appropriate cellular factories and an alternative to *Lb. sakei* Lb706 for production and recovery of the SakA and EntP/SakA antilisterial bacteriocins. Moreover, the use as bacteriocin producers of *Lactococcus* spp. and *Lactobacillus* spp. strains, generally recognized as safe (GRAS) and with a qualified presumption of safety (QPS), may also provide means by which the potential benefits of antimicrobial compounds can be exploited in food.

Although heterologous expression systems for production of bacteriocins are being developed in bacteria, yeasts have not been yet fully exploited as alternative hosts for their production [10]. However, a number of yeast platforms have been developed for the successful heterologous production of peptides and proteins [6, 28]. In this work, linearized pPICZαA and pKLAC2 protein expression vectors [15, 17] containing SakA and SakA-derived chimeras have permitted the production of SakA and EntP/SakA by *P. pastoris* X-33SA and *P. pastoris* X-33PSA and the production and expression of these peptides by *K. lactis* GG799SA and *K. lactis* GG799PSA, although both expressed peptides are significantly less active than the SakA and EntP/SakA produced by *Lb. sakei* Lb706. All recombinant yeasts secreted SakA and EntP/SakA, although production of SakA was lower than that of EntP/SakA. Whereas positively charged amino acids immediately following the SP cleavage site do not seem to interfere with the yeasts secretion machinery, displacement of the arginine (R) residue from position +2 to position +3 of the bacteriocin improves EntP/SakA secretion in *P. pastoris* X-33PSA and *K. lactis* GG799PSA. It should be noted that growth of *P. pastoris* X-33SA was severely impaired as compared to that of *P. pastoris* X-33PSA (Table 5). Since one of the main bottlenecks in recombinant protein production is the inability of foreign peptides to reach their native conformation in heterologous yeast hosts, it may happen that incorrectly folded SakA is accumulated in the endoplasmic reticulum (ER), activating the unfolded protein response (UPR) and the ER-associated degradation with generation of reactive oxygen species (ROS), leading to persistent ER stress conditions causing apoptosis and yeast death [26]. In this context, recent studies show that synthetic antimicrobial peptides induce the accumulation of ROS and hydroxyl radicals known to be important regulators of apoptosis and cell death in *Candida albicans* [34].

The SakA and the EntP/SakA produced by the recombinant *P. pastoris* and *K. lactis* hosts showed no activity or a much lower antimicrobial activity than that deduced from their production (Table 5). This observation was not unexpected because bacteriocins cloned into *S. cerevisiae* [3, 47, 50], *P. pastoris* [4, 5, 10, 29, 45], and *K. lactis, H. polymorpha*, and *A. adeninivorans* [10] have been produced with variable success regarding their secretion and functional expression. In this work, MALDI–TOF MS analysis of the purified bacteriocins produced by the recombinant yeasts showed that, besides the presence of tentative oxidized forms of SakA and EntP/SakA, other fragments of high molecular mass were present (Fig. 2). In this respect, these peptides could be associated with unknown biological compounds, as suggested for the inactive pediocin PA-1 (PedPA-1) produced by *P. pastoris* [5]. However, more likely both bacteriocins may have been subjected to post-translational modifications (PTMs) (Fig. 2). Some common PTMs events in peptides and proteins are phosphorylation, acetylation, methylation, oxidation, formylation, disulfide bond formation, and N-linked and O-linked glycosylation [51].

The presence of cysteine and methionine residues in SakA and EntP/SakA (Table 2) may lead to the formation of correct disulfide bridges but also to oxidation of these residues and the covalent attachment of different compounds to cysteine. Cysteine is susceptible to chemical modifications such as glutathionylation and cysteinylation. Similarly, the oxidation of methionine residues to MetSO is common during production of bacteriocins by yeasts [4, 10]. Glycosylation is also a common PTM in eukaryotes involving linkage via the Asn-X-Ser/Thr sequence (N-glycosylation) or the side chain of serine and threonine (O-glycosylation). The absence in SakA and EntP/SakA of attachment sites for N-linkages precludes their N-glycosylation, but the presence of three serines and two threonines makes them suitable for O-glycosylation. However, the correct processing, secretion, and functional expression of the bacteriocins EntP [29], HirJM79 [45], and EntA [10] produced by recombinant yeasts contrast with the low biological activity of the SakA and EntP/SakA produced by the *P. pastoris* and *K. lactis* producers. Misfolding of SakA and EntP/SakA and induction of the yeasts' UPR may be responsible for apoptosis in *P. pastoris* X-33SA and for extensive PTMs in *P. pastoris* X-33PSA, *K. lactis* G799SA, and *K. lactis* G799PSA.

The use of synthetic hybrid bacteriocins and the synthesis of bacteriocins containing modified amino acid sequences by site-directed mutagenesis, error-prone PCR, and gene shuffling techniques have permitted the design of more active bacteriocins [31]. The production of bacteriocins in heterologous LAB and yeasts has also permitted the use of safer microbial hosts, to increase their

production, antimicrobial activity, and specific antimicrobial activity as compared to that of the natural producers, to provide antimicrobial capabilities to LAB that may be useful as starters or protective cultures, or to design potential cell factories for production and delivery of bacteriocins of interest in food, medical, veterinary, and animal production applications [10]. However, further efforts should be made to clarify those critical factors involved in the production and functional expression of different bacteriocins or their chimeras by recombinant LAB and yeasts.

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