Sakacin G is the main responsible bacteriocin for the anti-listerial activity of meat-borne Lactobacillus curvatus *ACU-1*

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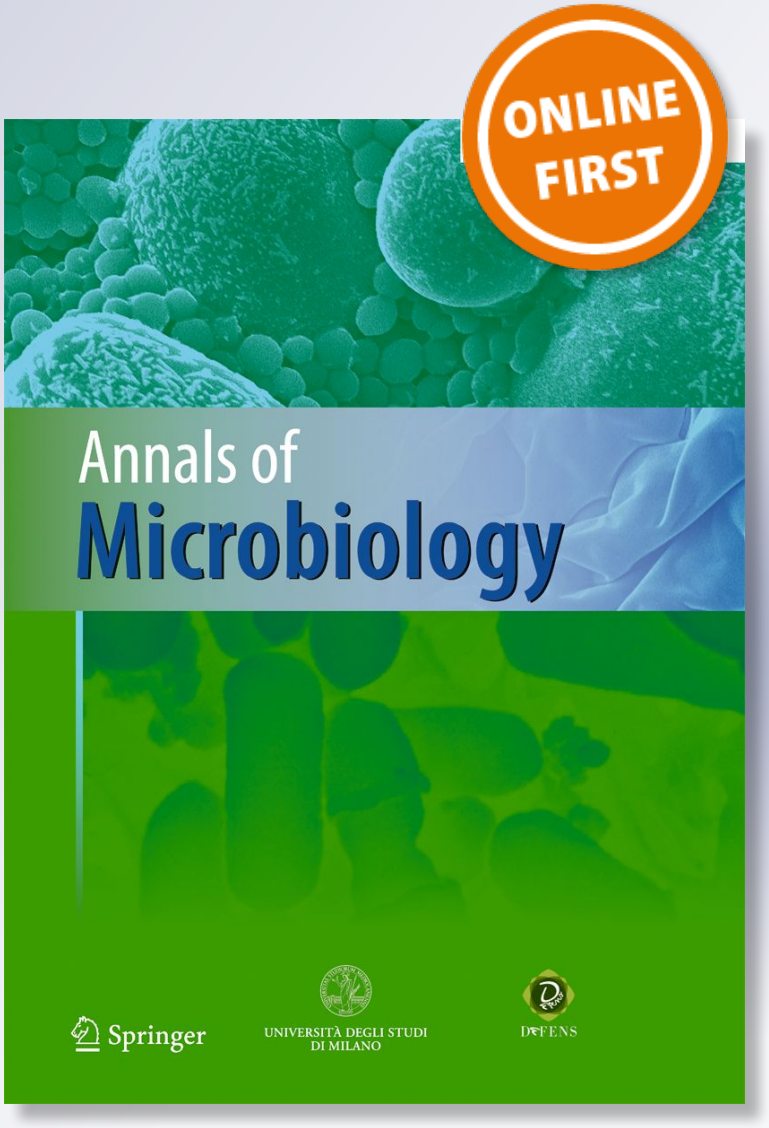

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ORIGINAL ARTICLE

Sakacin G is the main responsible bacteriocin for the anti-listerial activity of meat-borne *Lactobacillus curvatus* ACU-1

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Abstract The present study was conducted to quantify the expression of the sakacins produced by *Lactobacillus curvatus* ACU-1, a strain isolated from artisanal dry fermented sausages of Argentina. Polymerase chain reaction (PCR) screening indicated the presence of sakacin G, P, and Q genes in *L. curvatus* ACU-1. Purification and activity assays determined that anti-*Listeria* activity was mainly associated to sakacin G, as mass spectrometry analysis revealed a single peak of 3832.60 Da. Further characterization by quantitative PCR demonstrated that *L. curvatus* ACU-1 transcription of the sakacin G structural gene was three orders of magnitude higher than the others. Interestingly, *L. curvatus* ACU-1 had *skgA1*/*skgA2* as well as *sppQ* genes encoded in a plasmid, while the *sppA* gene that encodes for sakacin P was present in the bacterial chromosome. These results point out that sakacin G is the main peptide responsible for the anti-listerial activity of *L. curvatus* ACU-1, with little or no contribution of sakacin P and sakacin Q. The high level of expression of sakacin G demonstrated in the present work would facilitate its potential use in food preservation, improving the food quality, safety, and shelf life. In addition, the sakacin G promoter may serve as an interesting tool for the expression of other bacteriocins at higher levels.

Keywords *Lactobacillus* · Bacteriocins · Gene expression · *Listeria* · Artisanal dry sausages

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Introduction

The deep understanding of lactic acid bacteria (LAB) physiology, together with the study of the biochemistry and genetics of bacteriocins, have been the basis for novel industrial applications, such as the production of food biopreservatives, of which nisin is the paradigm (Jones et al. 2005; Casaburi et al. 2016). LAB applications have clearly moved beyond the classic food fermentations; the use of these microorganisms as delivery vehicles and as biochemical cell factories is expected to increase (Gaspar et al. 2013). In this sense, the use of protective cultures, namely bacteriocin-producing bacteria, has become important for food biotechnology.

Bacteriocins are cationic antimicrobial peptides produced by a large number of bacteria, including LAB. These peptides normally act against closely related microorganisms and some spoilage- and disease-related Gram-positive pathogens (Balciunas et al. 2013). Bacteriocin-producing LAB strains have been identified among all genera that comprise this group, including *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Leuconostoc*, *Pediococcus*, and *Carnobacterium*, as well as several *Enterococcus* spp. (Alvarez-Sieiro et al. 2016). Lactobacilli from a wide range of sources have been comprehensively studied as bacteriocin producers. Particularly, *L. curvatus* and *L. sakei* strains isolated from meat have been reported to produce different bacteriocins, mainly curvacins

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and sakacins, i.e., curvacin A produced by *L. curvatus* LTH1174 isolated from fermented sausages (Tichaczek et al. 1993), curvaticin L442 produced by *L. curvatus* L442 isolated from Greek traditional fermented sausage (Xiraphi et al. 2006), sakacin Q produced by *L. sakei* Lb674 and LTH673 (Mathiesen et al. 2005), sakacin P and sakacin X produced by *L. curvatus* MBSa2 and MBSa3 isolated from Italian type salami (de Souza Barbosa et al. 2015), among others. Most of these peptides belong to the class IIa, which are known as pediocin-like bacteriocins and exhibit strong anti-listerial activity (Eijsink et al. 1998; Guyonnet et al. 2000; Katla et al. 2003).

The strain *L. curvatus* ACU-1, isolated from artisanal dry fermented sausages, has a remarkable antimicrobial activity against several strains of *Listeria monocytogenes*, *L. innocua*, and *Staphylococcus aureus* (Castro et al. 2011). This activity was related to a thermostable bacteriocin-like inhibitory substance (BLIS) (Castro et al. 2012). The BLIS from *L. curvatus* ACU-1 retained high activity after prolonged refrigerated storage and several freeze/thaw cycles. Moreover, the bacteriocin was produced at concentrations of NaCl as high as 3% (w/w) combined with refrigeration temperatures (Castro et al. 2011). Consequently, its application in meat products as a protective culture and/or as part of a starter culture seems to be very promising. In an attempt to elucidate the nature of these substances, Rivas et al. (2014) conducted polymerase chain reaction (PCR) amplifications on *L. curvatus* ACU-1 total DNA using specific primers for known *Lactobacillus* bacteriocins. A PCR product was obtained when specific primers designed for the sakacin Q structural gene (*sppQ*) were used, suggesting the production of this bacteriocin by *L. curvatus* ACU-1.

Finding structural genes of determined bacteriocins by molecular tools is sufficient proof for identifying the presence of those bacteriocins in the bacterium under study. However, this result is not sufficient to explain whether these peptides are being effectively expressed and transported across the cell. Hence, the aim of the present work was to quantify the expression of sakacin Q and elucidate whether this was the only bacteriocin synthesized by *L. curvatus* ACU-1 or whether other peptides could be co-purified from the culture supernatant.

Materials and methods

Bacterial strains and culture conditions

The strains used in this study were: (i) the bacteriocin-producing strain *L. curvatus* ACU-1, isolated from artisanal dry sausages manufactured in Chaco (Argentina) (Castro et al. 2011) and (ii) the indicator microorganism *Listeria innocua* 7, because it proved to be an excellent model of the pathogenic *L. monocytogenes* (Friedly et al. 2008). Both bacteria were maintained as frozen stocks at −20 °C in the suitable medium and were propagated twice in the appropriate culture media at 30 °C before use. *Lactobacillus curvatus* ACU-1 was recovered in de Man, Rogosa and Sharpe broth (MRS, Biokar Diagnostics, France), while *L. innocua* was recovered in Tryptic Soy medium (Britania, Argentina).

Bacteriocin(s) purification

Lactobacillus curvatus ACU-1 was grown in 1 L of MRS till stationary phase, since it was previously described that the highest anti-*Listeria* activity was found at this phase (Rivas et al. 2014). Ammonium sulfate fractionation was carried out from 40% up to 80% saturation, stirring the sample for 1 h at 4 °C. Afterwards, the sample was centrifuged at 4000 × g for 30 min at 4 °C. The pellet was dissolved in 500 mL of 20 mM acetate buffer, pH 5.0. Then, a cation-exchange chromatography was carried out using SP Sepharose Fast Flow (GE Healthcare Life Sciences, USA). For this purpose, a plastic 60-mL syringe was filled with SP Sepharose slurry in order to obtain a 10-mL column, which was carefully equilibrated with 20 mM acetate buffer, pH 5.0. Afterwards, the sample was slowly passed through the SP Sepharose column and different fractions were eluted with increasing concentrations of NaCl. The active fraction was re-precipitated with ammonium sulfate and dissolved in a solution of 20% acetonitrile + 0.1% trifluoroacetic acid (TFA). Protein concentration was estimated by the method of Bradford (1976).

Active sample was then further purified by reversed-phase high-performance liquid chromatography (HPLC) in a C18 μBondapak column (10 μm 300 × 4.6 mm Waters) associated to a Gilson HPLC system. A non-linear gradient of acetonitrile was used for the elution at a flow rate of 0.8 $mL \cdot min^{-1}$. Elution was followed by monitoring absorbance at 220 nm. Once active fractions were identified, they were pooled and dried under vacuum with a Savant SpeedVac (Thermo Fisher Scientific). Finally, active sample was submitted for peptide mass determination by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis at the Lanais-Pro facility (IQUIFIB, CONICET-UBA, Argentina).

Antibacterial activity

Anti-*Listeria* activity was assessed by the spot-on-lawn assay, as described by Lewus et al. (1991). Briefly, 10 μL of each fraction was spotted onto sterile Tryptic Soy agar plates (Britania, Argentina). After complete absorption of the samples, 5 mL of soft agar inoculated with the sensitive strain *L. innocua* 7 was poured on top of the plates and they were incubated for 16 h at 30 °C. Clear halos of growth inhibition were indicative of antimicrobial activity.

Table 1 Primers used in this study

Primers	Sequence (5′-3′)	Reference
curA_F	GTAAAAGAATTAAGTATGACA	Remiger et al. (1996)
curA_R	TTACATTCCAGCTAAACCACT	Remiger et al. (1996)
SakG_F	GTAAAAATTATTTAACAGGAGG	Dortu et al. (2008)
SakG_R	TTAGTGCTTTTTTATCTGGTA	Dortu et al. (2008)
SakA_F	GAA(T/A)T(A/G)(C/A)(A/C)ANCAATTA(C/T)(A/C)GGTGG	Dortu et al. (2008)
SakA_R	CAGCCGCTAATCATACCACC	Dortu et al. (2008)
SakG F RT	TGGCGTTAGCTGTAACTCTCAC	This study
SakG R RT	AACTCCATGACCGCCATTAG	This study
SakP F RT	TAGACTGGGGAACAGCTATTGG	This study
SakP R RT	TTTATTCCAGCCAGCGTTTC	This study
SakQ F RT	AGCTGGTGCAGCTGGTTTAG	This study
SakQ R RT	TAATTCTCCGCCGATACCAC	This study

DNA isolation and PCR

Total DNA from the *L. curvatus* ACU-1 strain was obtained as described by Pospiech and Neumann (1995), while plasmids were purified according to Anderson and McKay (1983).

PCR screening of bacteriocin structural genes in *L. curvatus* ACU-1 was performed using both total and plasmid DNA as templates. For this purpose, new sets of primers were designed and synthesized (Genbiotech, Argentina), including specific as well as degenerate primers that were recently tested in our lab (Table 1). PCR reactions were programmed as follows: 10 min heating at 94 °C, followed by 30 cycles consisting of 1 min denaturation at 94 °C, 1 min annealing at 51 °C, and 1 min extension at 72 °C, and a final extension step of 10 min at 72 °C. DNA quantification was performed by using the Qubit® Fluorometer (Invitrogen, USA).

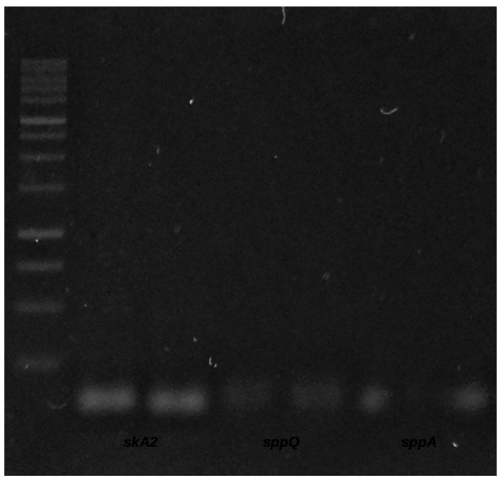

Fig. 1 Polymerase chain reaction (PCR) screening of bacteriocins in *Lactobacillus curvatus* ACU-1 total DNA. *skgA2*, *sppA*, and *sppQ* genes were present in *L. curvatus* ACU-1, as confirmed by PCR amplification. Each sample was tested in triplicate. A kilobase DNA ladder was used in lane 1

Expression of bacteriocin structural genes

RNA isolation

Total RNA was obtained from *L. curvatus* ACU-1 according to Raya et al. (1998). Briefly, cells from either 6-h or 16-h cultures were centrifuged, washed with buffer TE (10 mM Tris, 1 mM EDTA, pH 8), and resuspended in 500 μL of cold TE buffer. Afterwards, 0.6 g of glass beads, 170 μL of 2% Macaloid slurry, 500 μL of TE saturated phenol with chloroform:isoamyl alcohol (1:1), and 50 μL of 10% SDS were added to the cell suspension and bacterial disruption was achieved in a Mini-Beadbeater cell disrupter (Model 607EUR, BioSpec Products) with 10 cycles of 1 min. After 15 min of centrifugation at 12,000 × g, the aqueous supernatant containing the RNA was extracted with 1 volume of phenol-chloroform-isoamyl alcohol and precipitated with 3 M sodium acetate and 3 volumes of 70% ethanol. Then, RNA was recovered by centrifugation, washed with 70% ethanol, and resuspended in RNAse-free water. Isolated RNA was treated with the TURBO DNA-free™ kit (Ambion®, Thermo Fisher Scientific) prior to cDNA synthesis. Finally, DNA-free RNA (1 μg) was used to synthesize cDNA using SuperScript® III First-Strand Synthesis SuperMix (Invitrogen, Thermo Fisher Scientific). RNA quantification was performed by using the Qubit® Fluorometer (Invitrogen, USA).

Real-time qPCR

qRT-PCR reactions were carried out in duplicate using 1 μL of cDNA, 0.3 μM of each primer, and 10 μL of PerfeCTa® SYBR® Green SuperMix for iQ™ (Quanta Biosciences™,

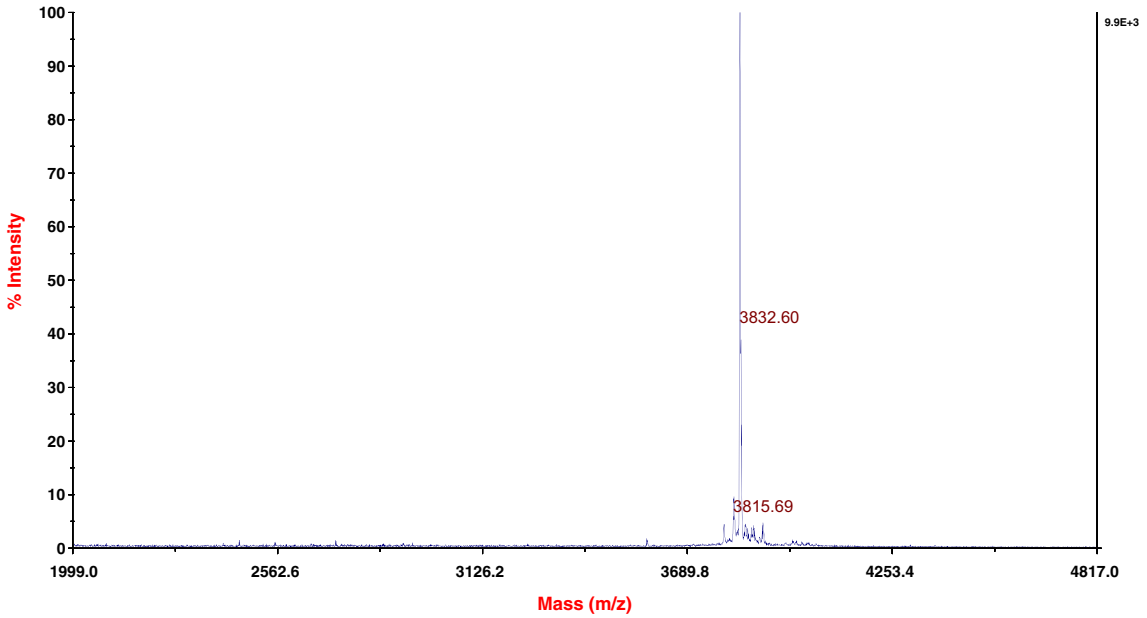

Fig. 2 Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis of antimicrobial peptide secreted by *L. curvatus* ACU-1. The high-performance liquid chromatography (HPLC) sample from the peak at a retention time of 28 min was associated to a 3832.60-Da signal

Genbiotech) for a final reaction volume of 20 μL. The 16S rRNA gene was used as the housekeeping gene, since its expression was invariable under the tested conditions. qPCR primers were designed with the PrimerQuest® Tool from IDT (http://www.idtdna.com/primerquest/Home/Index), in order to obtain amplicons of ~100 bp for each gene, including the housekeeping gene. The copy number of each transcript was calculated by using a standard curve (Suárez et al. 2015). For this purpose, each structural gene was amplified by PCR from total DNA, purified with the AccuPrep PCR Purification Kit (Bioneer, Genbiotech). The concentration of the amplified product was measured with a UV-visible spectrophotometer (Varian, 50MPR Microplate Reader, Palo Alto, CA, USA) at 260 nm using a standard calibration curve, and converted to number of copies per unit volume using the following equation: DNA (copy) = (6.023 × 1023 [copies mol^{-1}] × DNA amount [g])/ (DNA length [bp] × 660 [g mol^{-1} bp^{-1}]) (Whelan et al. 2003). Purified DNA were adjusted to 10^{10} copies per μL and then serial dilutions were prepared in order to obtain standards from 10^{10} to 10^4 copies per μL. The copy transcripts (CT) values of *skgA2*, *sppA*, and *sppQ* for each dilution were plotted against the logarithm of their initial template concentrations and standard curves were obtained by linear regression. Each dilution was analyzed in duplicate. Reactions were performed in an iQ™5 Multicolor Real-Time PCR Detection System (BioRad, USA) under the following conditions: 3 min at 95 °C, 40 cycles of 10 s at 95 °C, 20 s at 51 °C, and 20 s at 72 °C, followed by melting curve cycles. For each reaction, non-template controls (NTC) were included (without cDNA) as well as positive controls (genomic DNA as template).

Results

Previous analysis on *L. curvatus* ACU-1 total DNA demonstrated the presence of the sakacin Q structural gene (*sppQ*) (Rivas et al. 2014). Since the scope of the present paper was to obtain a deeper insight into sakacin production by the *L. curvatus* ACU-1 strain, new PCR amplifications were performed with a broader list of specific sakacins and degenerate primers. Interestingly, it was found that *L. curvatus* ACU-1

Table 2 Copy number of sakacin mRNA in *Lactobacillus curvatus* ACU-1. Sakacin expression was quantified by qPCR as described in the Materials and methods section

Samples	*sakG*		*sakP*		*sakQ*	
	CT	Log	CT	Log	CT	Log
ACU-1, 6 h	23.59 ± 0.8	9.93×10^6	38.64 ± 1.4	3.22×10^2	35.30 ± 0.8	3.94×10^4
ACU-1, 16 h	19.10 ± 1.2	6.13×10^7	37.97 ± 1.3	7.26×10^2	34.42 ± 1.1	7.52×10^4

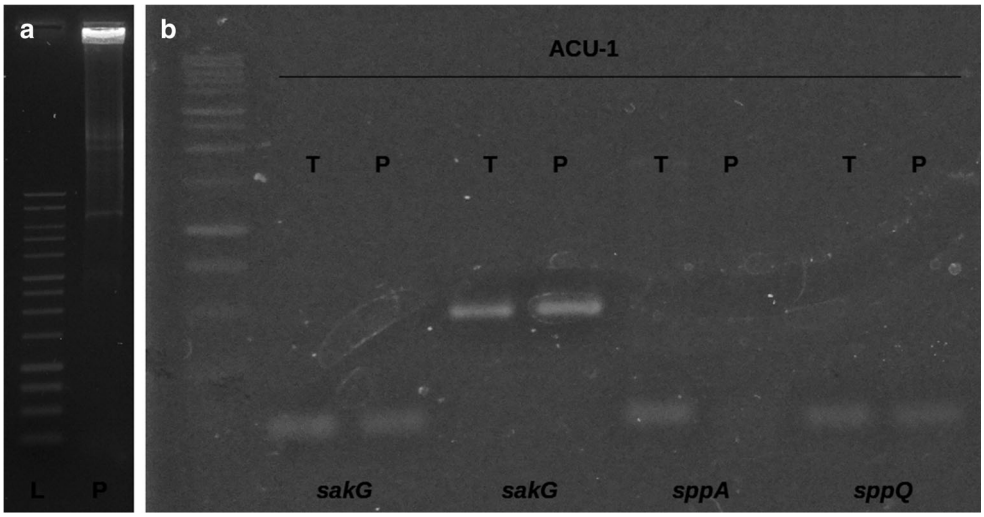

Fig. 3 Plasmid isolation and bacteriocin structural genes localization analysis. **a** Plasmid was observed in the agarose gel (lane denoted with P). A kilobase DNA ladder was used in lane L. **b** *skgA2*, *sppA*, and *sppQ* gene localization in *L. curvatus* ACU-1 was analyzed by carrying out PCR amplifications using total (T) and plasmid (P) DNA as templates. Two different sets of *sakG* primers were tested, as described in the Results section. A kilobase DNA ladder is shown in lane 1

would potentially express not only the previously identified sakacin Q, but also sakacin G and sakacin P (Fig. 1).

Based on these results, bacteriocins purification from cell-free supernatant was carried out by HPLC. Four main peaks were obtained from the C18 column elution (data not shown). The peak eluted from the C18 column with a retention time of 28 min turned out to have a strong antimicrobial activity; hence, it was further characterized by mass spectrometry. The MALDI-TOF analysis undoubtedly indicated that bacteriocin with anti-*Listeria* activity had a mass of 3832.60 Da (Fig. 2), which did not match the expected mass of sakacin Q (4486 Da) but, rather, resembled the mass of sakacin G. In fact, Simon et al. (2002), who described sakacin G for the very first time, reported a 3834-Da peptide.

Taken together, our results would suggest that a differential expression pattern might take place, with sakacin G being the bacteriocin expressed at a higher degree. To analyze the expression profile of the sakacins in *L. curvatus* ACU-1, qPCR was performed upon RNA isolation from exponential and stationary cultures. *Lactobacillus curvatus* ACU-1 showed expression of *skgA2* from early stages, since it was found at almost 10^7 copies per μL after 6 h of culture. Furthermore, the copies increased up to 6.13×10^7 at 16 h (Table 2). Regarding the *sppQ* gene, it was expressed at a much lower proportion in this strain. Actually, $4–8 \times 10^4$ copies of *sppQ* were found, while *sppA* expression was found to be almost negligible, with less than 10^3 copies per μL in stationary phase (Table 2). After qPCR measurements, samples of each condition were taken from the microplate qPCR wells, electrophoresed, and visualized by GelRed staining (Genbiotech, Argentina). Bands were of the expected size, confirming the specificity of the primers used (data not shown).

Lactobacillus curvatus ACU-1 contained at least one plasmid, as it can be observed in Fig. 3a. Therefore, the possible presence of *skgA2*, *sppA*, and *sppQ* genes in that plasmid was analyzed by performing PCR amplifications using not only total DNA but also plasmidic DNA as templates. The PCR results demonstrated that *skgA2* and *sppQ* were located in the plasmid of *L. curvatus* ACU-1, whereas *sppA* was present only in the chromosome (Fig. 3b). Two different sets of primers were used for *skgA2*, with similar results, i.e., (i) primers designed by Dortu et al. (2008) that allow the amplification of a 492-bp fragment and (ii) primers designed in this work, which can amplify a fragment of 94 bp by using the *skgA2* gene as template.

Discussion

In the present work, we found that *L. curvatus* ACU-1 was able to express at least three different bacteriocins, i.e., sakacin P, sakacin Q, and sakacin G. The fact that this strain is able to potentially produce more than one peptide is not surprising, since it is already known that certain *Lactobacillus* strains produce more than one antimicrobial peptide (Cintas et al. 2000). Specifically, the presence of structural genes encoding for sakacin P and sakacin Q in meat-borne *L. curvatus* was described for *L. curvatus* LTH1174 and L442 isolated from meat fermentations (Cocolin and Rantsiou 2007) and several strains of *L. curvatus* isolated from vacuum-packaged beef (Fontana et al. 2015). Sakacin P and sakacin G are class IIa bacteriocins, i.e., they are low-molecular-weight and heat-stable peptides that contain the YGNGV consensus sequence in their N-terminal domain. On the other hand, the expression of sakacin Q was confirmed. This peptide, unlike the other two, is a class IIc bacteriocin, which has no clear structural features, as other members of this subgroup.

In our previous report, we were not able to demonstrate the presence of *sppA*, which encodes for sakacin P (Rivas et al.

2014). In this work, total DNA was extracted by the method of Pospiech and Neumann (1995) instead of using a commercial kit. In addition, a new set of primers was used. Besides, it was demonstrated that *sppA* was only present in the chromosome, while *sppQ* and *skgA2* were located in a plasmid. These results may explain why *sppA* was found in this work, while it could not be detected previously. Furthermore, it could also explain the different degree of expression between *sppQ–skgA2* and *sppA* because the latter gene might have a single copy in the chromosome and the other two genes would have more copies since they are encoded in a plasmid. As a matter of fact, GelRed-stained *sppA* bands always gave weaker signals in agarose gels as compared to the other genes. Gene *sppA* was already described as a gene linked to chromosomes, although it is generally associated to plasmids (Cocolin and Rantsiou 2007).

There is convincing evidence that the structural sakacin G gene is duplicated in *L. curvatus* ACU-1 plasmid. On one hand, primers designed by Dortu et al. (2008) did amplify a nearly 500-bp fragment, as shown in *L. sakei* 2512 and in *L. sakei* CWBI-B1365. On the other hand, in spite of several attempts, sequencing of the region upstream of *skgA2* gave no single peaks in each cycle, which also suggests a duplication of these genes.

However, this finding cannot explain the impressive difference between the expression of *skgA2* and *sppQ*. Further studies focused on the *skgA2* promoter would be necessary. It is tempting to speculate that sakacin G promoter may be stronger than sakacin Q or sakacin P promoters; hence, it could be exploited in the future for bacteriocin expression. Actually, ongoing experiments in our lab were designed to clone the three sakacins under the sakacin G promoter in order to evaluate if all peptides can be expressed at the same level.

There is scarce information on the quantification of mRNA copies of bacteriocins. Recently, Suárez et al. (2015) described an *Enterococcus faecium* able to produce at least six different bacteriocins, with mRNA of enterocin P being the only one to reach 2×10^7 copies·μL^{-1}, while the other mRNAs were found to be in the range 10^4–10^6 copies·μL^{-1}. Interestingly, sakacin G mRNA was found to be at concentrations as high as 6×10^7 copies·μL^{-1} in *L. curvatus* ACU-1, i.e., almost three times more copies than enterocin P in *E. faecium* CRL1879 (Suárez et al. 2015).

Noteworthy, besides having the highest expression, sakacin G has two disulfide bonds, which may suggest that its antimicrobial activity would be intrinsically higher than the other two bacteriocins that have just one disulfide bond (Eijsink et al. 1998; Guyonnet et al. 2000). Therefore, the anti-*Listeria* activity observed in *L. curvatus* ACU-1 supernatant could be ascribed only to sakacin G, with almost no contribution of the other two peptides.

A general conclusion that can be drawn from this work is that, every time PCR screening assays are used for finding new bacteriocins, they must be complemented with classical purification protocols in order to obtain conclusive results.

The high expression level of sakacin G demonstrated in the present work would facilitate the purification of sufficient quantities of this bacteriocin for biochemical and biophysical studies, as well as for its possible use in food preservation, either alone or as part of a combination in a multibarrier design (Leistner 2000). Actually, the strong anti-*Listeria* activity of sakacin G would promote its use as an antimicrobial peptide, to reduce or eliminate pathogenic bacteria growth, improving the food quality, safety, and shelf life. In addition, the sakacin G promoter may serve as an interesting tool for the expression of other bacteriocins at higher levels.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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