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Research Letter

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Purification and characterization of two new cell-bound bioactive compounds produced by wild *Lactococcus lactis* strain

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One sentence summary: The paper describes the purification of novel antimicrobial substances produced by a lactic acid bacteria strain isolated from fermented naturally salami.
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ABSTRACT

Novel compounds and innovative methods are required considering that antibiotic resistance has reached a crisis point. In the study, two cell-bound antimicrobial compounds produced by *Lactococcus lactis* ID1.5 were isolated and partially characterized. Following purification by cationic exchange and a solid-phase C18 column, antimicrobial activity was recovered after three runs of RPC using 60% (v/v) and 100% (v/v) of 2-propanol for elution, suggesting that more than one antimicrobial compound were produced by *L. lactis* ID1.5, which were in this study called compounds AI and AII. The mass spectrum of AI and AII showed major intensity ions at m/z 1070.05 and 955.9 Da, respectively. The compound AI showed a spectrum of antimicrobial activity mainly against *L. lactis* species, while the organisms most sensitive to compound AII were *Bacillus subtilis*, *Listeria innocua*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*. The antimicrobial activity of both compounds was suppressed by treatment with Tween 80. Nevertheless, both compounds showed high stability to heat and proteases treatments. The isolated compounds, AI and AII, showed distinct properties from other antimicrobial substances already reported as produced by *L. lactis*, and have a significant inhibitory effect against two clinically important respiratory pathogens.

Keywords: antimicrobial compounds; cell extract; lactic acid bacteria; purification

INTRODUCTION

The antimicrobial activity of bacteria has long been attributed to the production of small metabolites such as organic acids, hydrogen peroxide, ethanol and diacetyl (Leroy and De Vuyst 2004). It has gradually become clear that additional metabolites often contribute to the antimicrobial ability of bacteria and they have received wide attention because they can be future drug

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therapeutic (Field *et al.* 2015). One such category of metabolites are the ribosomally synthesized peptides, called bacteriocins (Nes and Johnsborg 2004). Antimicrobial peptides (AMPs) are highly diverse molecules that are able to inhibit a broad spectrum of microorganisms, usually cationic, which have an amphipathic character and a high content of hydrophobic residues (Baltzer and Brown 2011). Bacteriocins constitute a heterogeneous group of peptides, and although their classification has been continuously revised, they are divided into two major groups. Lantibiotics (class I) are small peptides of 19–38 residues containing post-translational modifications, which are characterized by thiother-based internal ring structures (lanthionine or β-methyllanthionine). The other group is non-lantibiotic (class II), which are small heat-stable peptides with no modified amino acids, except formation of disulphide bridges and circularization of cyclic peptides (Cotter, Hill and Ross 2005, Nishie, Nagao and Sonomoto 2012). Nisin, Pediocin PA-1, Lacticin 3147 and Enterocin AS-48 are the best known bacteriocins (Egan *et al.* 2016). In addition, it has also become evident that certain bacteria have produced other newly defined antimicrobial compounds, such as lipopeptides (LiPs). They are produced non-ribosomally and consist of a short linear or cyclic peptide sequence, with positive or negative charge, to which a fatty acid moiety is covalently attached to the N-terminus, such as surfactins or lichenysins, iturins and fengycins or plispastatins (Mangoni and Shai 2011). Bacteriocin-like inhibitory substance (BLIS) is a new term applied to antagonistic substances, which are incompletely defined or do not fit the typical criteria defining bacteriocins (Atanassova *et al.* 2003; Gálvez *et al.* 2010). They tend to have a broader spectrum of activity than currently known bacteriocins. A number of these BLIS were reported to be produced by lactobacilli, inhibiting a wide range of both Gram-positive and Gram-negative bacteria as well as fungi (Atanassova *et al.* 2003; Gálvez *et al.* 2010; Gao *et al.* 2011; Ruíz *et al.* 2012). Several strains of *Lactococcus lactis* have been shown to be interesting biosurfactants producers, which inhibit the growth of bacteria and fungi (Rodrigues *et al.* 2006; Moldes *et al.* 2007; Rodríguez *et al.* 2010).

Among lactic acid bacteria, *L. lactis* is quite desirable for industrial application, because it is homofermentative, highly productive and generally recognized as safe (Guinane *et al.* 2005; Neves *et al.* 2005). *Lactococcus lactis* is used in food production, and constitutes a significant part of the indigenous flora of mammals, including humans (Espeche *et al.* 2009; Gao *et al.* 2011). Thus, their antimicrobial metabolites may be considered safe agents for preventing growth of undesirable microorganisms.

In previous work, our research group has isolated the nisin Z-producing strain *L. lactis* ID1.5 from an artisanal fermented sausage (Saraiva 2012). In this work, we describe the purification and partial characterization of two rather different antimicrobial substances produced by *L. lactis* ID1.5, with inhibitory properties against Gram-positive and Gram-negative bacteria.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Lactococcus lactis ID1.5 was grown in LAPTg broth (Juárez, Ocaña and Nader-Macías 2004) at 30°C for 16 h. Indicator strains for determination of antimicrobial spectra were propagated at appropriate temperatures (30°C or 37°C), as recommended by culture collections, for 18 h. *Lactococcus lactis* IL1403, LMGT 2115 and LMGT 2122 were grown in M17 medium (Oxoid, Thermo Fisher Scientific, MA, USA) supplemented with 0.4% (w/v) glucose (GM17). *Lactobacillus sakei* NCDO 2714 was grown in MRS broth (Oxoid). *Bacillus subtilis* DSMZ 347, *Enterococcus faecalis* V583, *Listeria innocua* BL86/26B, *Micrococcus luteus* ATCC 10 240, ATCC 4698, *Staphylococcus aureus* 2002–05-ME 8245–3, 2002–60-8452, *Pseudomonas aeruginosa*, *Pseudomonas* sp., *Salmonella* Typhimurium SL 1344 and *Shigella soneii* ATCC 11 060 were grown in BHI medium (Oxoid). *Streptococcus pneumoniae* TIGR4 was grown in Todd-Hewitt broth (Becton Dickinson, Nova Jersey, USA) supplemented with 0.8% (w/v) glucose, and *Escherichia coli* ATCC 14 763 was grown in LB medium (Bertani 2004). Routinely, fresh bacterial cultures were obtained from frozen stocks for each experiment.

Antimicrobial activity assay

Antimicrobial activity was detected using the agar well diffusion assay described by Ryan *et al.* (1996). GM17 agar at 48°C was seeded with the indicator strains *L. lactis* LMGT 2115, LMGT 2122 and IL1403 (20 μL of an overnight culture per 20 mL agar), dispensed into sterile plates and allowed to solidify. Wells of ~4–6 mm in diameter were made. Aliquots of 30 μL of cell-bound antimicrobial extract (CE), which was obtained with 70% (v/v) 2-propanol and 0.1% (v/v) trifluoroacetic acid (TFA) followed by rotatory evaporation (Buchi, Flawil, Switzerland) (Field *et al.* 2008), and cell-free supernatant (CFS) of *L. lactis* ID1.5, both CE and CFS adjusted to pH 7.0, were separately dispensed into the wells. After incubation overnight at 30°C, antimicrobial activity was observed by the formation of an inhibition halo.

Antimicrobial activity was quantified by using a microtiter assay method (Holo, Nilssen and Nes 1991). A 2-fold serial dilution of 100 μL antimicrobial compounds samples were prepared in a microtiter plate well containing 50 μL of the culture medium added of 150 μL of a diluted overnight culture of the indicator strain. The microtiter plate cultures were incubated for 12 h, after which growth inhibition was measured spectrophotometrically at 620 nm with a microtiter plate reader (Labsystems iEMS reader MF; Labsystems, Helsinki, Finland). One arbitrary unit was defined as the amount of antimicrobial compounds, which inhibited growth of the indicator strains by 50%.

In order to compare the new cell-bound bioactive compounds and nisin Z, the inhibitory activity was assayed by the agar spot test using ammonium sulfate precipitation from CFS (CFE) and CE. CFE was obtained by precipitation of supernatant with ammonium sulfate (40% saturation) and agitation for 30 min at 4°C. The bacteriocin was precipitated from the supernatant by centrifugation (11 490 $\times$ *g* for 30 min at 4°C) and dissolved in 20 mL sterile distilled water. Five microliter of CFE and CE of *L. lactis* ID1.5 were spotted onto GM17 medium plates with the indicator strains. One microliter of trypsin (Sigma-Aldrich, Missouri, USA) and proteinase K (Finnzymes, Thermo Fisher Scientific, MA, USA) solutions (1 mg mL^{-1}) was added separately near of the spots. The antimicrobial activity was observed after incubation overnight at 30°C.

Extraction and purification of the cell-bound active compounds

The extraction of the cell-bound antimicrobial compounds was performed by a bacteriocin extraction protocol (Field *et al.* 2008) with some modifications. *Lactococcus lactis* ID1.5 was grown in 2 L of LAPTg broth at 30°C overnight. The cells were collected by centrifugation at 11 490 $\times$ *g* for 20 min. The cell pellets were resuspended in 300 mL of 70% (v/v) 2-propanol (Arcus, Kemetyl

Norge AS, Oslo, Norway) containing 0.1% (v/v) TFA (Merck, Darmstadt, Germany), pH 2.0 and stirred at room temperature for 3 h. After centrifugation at 11 490 × *g* for 20 min, the supernatant (CE) was retained and cell pellets were discarded.

The purification procedure of antimicrobial compounds involves three steps: ion exchange chromatography, solid-phase extraction (SPE) and reversed-phase chromatography (RPC). In the first step, the sample of CE (adjusted to pH 3.5) was applied to 5 mL SP Sepharose Fast Flow (GE Healthcare Biosciences, Uppsala, Sweden) equilibrated with 10 mM acetic acid. The antimicrobial activity compounds were detected in the unbound fraction. This fraction was then diluted (adjusted to pH 4.0) and subjected to SPE in a 10g C18 column (Resteck Corporation, Svalbard, Norway) pre-equilibrated with methanol 100% (v/v) and one wash with MilliQ water. The fraction eluted in 140 mL of 70% (v/v) 2-propanol with 0.1% (v/v) TFA was further submitted to RPC. In the three steps, the purification was followed by using Äkta Purifier (Pharmacia Biotechnology, Lillestrom, Norway) in a 3 mL column Resource 15 RPC (Pharmacia Biotechnology) equilibrated with 0.1% TFA. The antimicrobial compounds were eluted in the following manner with a gradient of 2-propanol (Merck, Darmstadt, Germany) containing 0.1% TFA at a flow rate of 1 mL min^{-1}: 0–5 min, 0%–30%; 5–15 min, 30%; 15–20 min, 30%–60%, 20–30 min, 60%; 30–35 min, 60%–100%; 35–45 min, 100% of 2-propanol and collected in 1 mL fractions. Fractions of each peak showing inhibitory activity were called RPC I and were re-chromatographed separately twice to improve the purification degree of the antimicrobial compounds producing the fractions RPC II and RPC III. Antimicrobial activity was monitored throughout the purification procedure by the assay above described using *L. lactis* LMGT 2122 as the indicator strain. For evaluating the inhibition spectrum of the enriched fractions of each antimicrobial compound against several bacteria species, the 2-propanol was evaporated before assays.

Molecular mass analysis

The molecular mass of the purified fractions was analyzed by electrospray ionization—time of flight mass spectrometry (ESI-TOF/MS) with a Xevo G2 QTof Schematic mass spectrometer performed at Waters Technologies do Brasil Ltda., Barueri, São Paulo, Brazil.

Effect of heat, proteolytic enzymes and Tween 80 on stability of the antimicrobial compounds

For evaluation of the heat stability, 30 μL of purified samples were dispensed in microtubes, heated at 100°C for 15 min and for 30 min. To test the sensitivity to proteases, aliquots of purified samples were treated with the enzymes trypsin (prepared in 67 mM sodium phosphate buffer, pH 7.6) and proteinase K (prepared in 20 mM Tris-HCl buffer, pH 7.4, 1 mM $CaCl_2$) followed by incubation at 37°C for 5 h. After 2-propanol evaporation, the samples were resuspended in water and then the enzymes were added at a final concentration of 1 mg mL^{-1}. Then, samples were cooled and filtered through 0.22 μm pore-size filters (Merck Millipore, Darmstadt, Germany) and the residual activity was tested against *L. lactis* LMGT 2122 by the microtiter plate assay.

Samples (30 μL) of purified samples were treated with Tween 80 (Sigma-Aldrich, Missouri, USA) at different final concentrations, 0.01%, 0.02%, 0.05%, 0.1%, 0.5% and 1.0% (v/v). Samples were further filtered, and the antimicrobial activity was tested as described above.

Table 1. Antimicrobial activity of CE and CFS from *L. lactis* ID1.5.

Indicator strain	Description	CFS	CE
Lactococcus lactis ID1.5	Nisin producer	–	+
Lactococcus lactis IL1403	Nisin sensitive	+	+
Lactococcus lactis LMGT 2115	Nisin producer	–	+
Lactococcus lactis LMGT 2122	Nisin producer	–	+

CFS, cell-free supernatant; CE, cell extract; (+), presence of antimicrobial activity; (–), absence of antimicrobial activity.

RESULTS AND DISCUSSION

In this study, we reported the detection, isolation and partial characterization of new compounds inhibiting microbial growth, which showed to be different from other antimicrobial peptides or bioactive compounds already reported as produced by *Lactococcus lactis* strains.

In order to confirm the presence of novel antimicrobial compounds produced by *L. lactis* ID1.5, the activity of CE was compared to those obtained in the CFS using nisin producer *L. lactis* strains and a nisin-sensitive strain as indicators (Table 1). Only one lactococcal strain, *L. lactis* IL1403, was sensitive to CFS (Table 1) as well as to CFS subjected to ammonium sulfate precipitation (CFE) (Fig. S1, Supporting Information). All other *L. lactis* strains were not sensitive to CFS and were sensitive to cell extract (Table 1). These strains are also nisin producing (Table 1) and present immunity against this bacteriocin.

Therefore, our results indicated that there are other inhibitory substances present in CE, and these antimicrobial substances seem to be different from the described bacteriocins produced by lactic acid bacteria. These antimicrobial substances were not found in CFS and CFE, since they were not able to inhibit *L. lactis* LMGT 2122 used as indicator when assessed by agar well diffusion and by spot plate methods (Table 1 and Fig. S1). These compounds showed to be cell associated, while bacteriocins from the lactic acid bacteria can be naturally cell associated; however, they could be dislodged from the cell surface to supernatant by reduction in the pH values due to the production of organic acids (Yang, Johnson and Ray 1992, Mantovani *et al.* 2002).

Extraction and purification of the cell-bound bioactive compounds

To identify the presence of these antimicrobial compounds not yet reported to *L. lactis*, and to study their properties, the CE was subjected to sequential purification steps, showing an increase in specific activity and degree of purification at each step (Table 2). The antimicrobial substances did not bind to the cationic exchange resin, indicating the anionic nature of the compounds. The unbound fraction, enriched in the antimicrobial compounds, was then separated on a C18 solid-phase column, when specific activity was increased about two and half times (Table 2).

Upon the first reversed-phase chromatography (RPC I), two distinct fractions with antimicrobial activity, called AI and AII, were obtained (Fig. S2, Supporting Information). Compound AI was eluted as the peak at 60% (v/v) 2-propanol and compound AII was eluted in 100% (v/v) 2-propanol, which indicated high hydrophobicity of both bioactive agents (Fig. 1). These characteristics indicate that these compounds differ from bacteriocins produced by lactic acid bacteria previously described, which are cationic peptides and most often they elute in the 30%–40%

Table 2. Purification of cell-bound antimicrobial compounds produced by *L. lactis* ID1.5.

Purification step[a]	Volume (mL)	Protein concentration (mg mL^{-1})[b]	Antimicrobial activity ($\times 10^3$) (AU mL^{-1})[c]	Specific Activity (AU mg^{-1})	Increase in specific activity (fold)
CE	300	3.88	1.28	330	1.00
IEX	300	2.90	1.28	441	1.33
SPE	140	1.58	1.28	810	2.45
Compound AI					
RPC I	3	0.61	1.28	2.09×10^3	6.33
RPC II	2	0.32	1.28	4.00×10^3	12.12
RPC III	2	0.18	2.56	1.42×10^4	43.03
Compound AII					
RPC I	3	0.90	2.56	2.84×10^3	8.60
RPC II	2	0.30	5.12	1.70×10^4	51.51
RPC III	1	0.14	5.12	3.65×10^4	110.60

[a]CE, cell extract; IEX, ion-exchange chromatography; RPC, reversed-phase chromatography; SPE, solid phase extraction in a Resprep-C18 column.
[b]The protein concentration was determined either by determining the optical density at 280 nm.
[c]*Lactococcus lactis* LMGT 2122 was used as indicator strain.

fractions using 2-propanol as solvent in RPC (Lubelski *et al.* 2008; Nissen-Meyer *et al.* 2009). Pulusani, Rao and Sunki (1979) reported strong inhibitory activity of a methanol-acetone extract from *Streptococcus thermophilus* grown in milk against *Bacillus* sp., *Pseudomonas aeruginosa*, *P. fluorescens* and various strains of *L. lactis*.

The hydrophobicity of the compounds has been suggested as an important property in the antimicrobial action, because the hydrophobic regions are required for direct interaction between inhibitor substances with membrane cell components (Perez Espitia *et al.* 2012). In general, AMPs produced by *L. lactis* are cationic and amphipathic; in our work, the antimicrobial substances presented negative net charge similar to an AMP produced by *Bacillus subtilis* (Teixeira, Rosa and Brandelli 2013). The main target of cationic AMPs is the cell envelop; on the other hand, the action mechanism of anionic AMPs has not been elucidated but it believes that their ability to oligomerize and the presence of ions (Ca^{2+}, Mg^{2+} or Zn^{2+}) facilitate membrane insertion (Harris, Dennison and Phoenix 2009). It seems that the binding of anionic antimicrobial peptide to bacterial membranes depends on their phospholipid profile (Becucci *et al.* 2014).

The third step of reversed-phase chromatography (RPC III) yielded enriched fractions of each antimicrobial compound, suggesting the presence of at least two different antimicrobial compounds (Fig. 1a and b). The most active fractions revealed 43-fold and 110-fold increasing in specific activity to compounds present in AI and AII, respectively (Table 2). Agar well diffusion assay of fractions obtained by RPC III of AI and AII showed clear inhibition halos on plates seeded with the indicator strain *L. lactis* LMGT 2122 (Fig. 1c). A different antimicrobial activity was observed for assays using AI and AII, the AI producing larger inhibition halos (Fig. 1c, letters B, C and D).

For improving characterization of the new antimicrobial compounds, mass spectrometry procedures were developed using the fractions AI and AII as samples (Figs S3 and S4, Supporting Information). The MS mass spectrum of AI (Fig. S3a) showed isotopic patterns for some ions, including 1070.05 Da (Intensity 2.9e6), which was subjected to MS/MS analysis (Fig. S3b). This profile showed the mass difference between the peaks correlated with amino acid masses, including hydrophobic and anionic residues. In addition, it was found several identical mass values such as 677.6, 331.4, 256.2 and 206.1 Da in MS and MS/MS profiles. Similarly, the MS mass spectrum of AII sample (Fig. S4a) showed isotopic patterns for ions including 955.9 Da (Intensity 2.1e6), which was subjected to a MS/MS analysis (Fig. S4b), with identical mass values in MS and MS/MS profiles as 699.6, 794.8, 437.3, 405.2 and 313.3 Da.

Comparison of the peak mass lists with databases (Hammami *et al.* 2010) did not show any similarities with known bacteriocins previously described. Antimicrobials with molecular masses similar to these presented by compounds in our study have been produced by *Bacillus* species, which belong to lipopeptides families (Chen *et al.* 2008). Lipopeptides consist of a lipophilic fatty acid chain covalently attached to the N-terminus of a short linear or cyclic peptide synthetized by the multimodule enzymes known as non-ribosomal peptide synthases (NRPS) (Tapi *et al.* 2010). NRPS gene clusters have been identified in *Lactobacillus plantarum* WCFS1 (Kleerebezem *et al.* 2003) and *L. lactis* KF147 (Siezen *et al.* 2010, 2011), suggesting that this type of antimicrobial compound can be synthetized by lactic acid bacteria strains. Although our data did not allow conclusive statements, they provide evidence that the antimicrobial substances presented profile of lipopeptides, as the mass difference between the first and second ion peaks showed in the MS/MS analysis of the 1070 Da peak (392.42 Da in Fig. S3b) that may correspond to the fatty acid moiety of the molecule. In addition, several features become difficult to identify different families of lipopeptides, their structural variability and the co-production of more than one compound by bacteria that cause additional purification problems (Wang *et al.* 2004; Chen *et al.* 2008). However, owing their wide diversity and very attractive functional properties of lipopeptides, they are applied in many domains, highlighting in food industry as emulsifiers, foaming and anti-adhesive agent, where they are very popular (Mnif and Ghribi 2015).

Effect of heat, proteolytic enzymes and Tween 80 on stability of antimicrobial compounds

Antimicrobial compounds present in the fractions AI and AII proved to be resistant to heat and stable to action of proteolytic enzymes (Table 3). Sensitivity to proteolytic enzymes is an important characteristic which distinguishes bacteriocins from other antimicrobial compounds, except a few circular bacteriocins, as gassericin A and reutericin 6 that proved to be resistant to proteolytic degradation (Kawai *et al.* 2001; Maqueda *et al.* 2008). Since, proteinase K is a non-specific serine endopeptidase of broad specificity and target multiple substrate, our

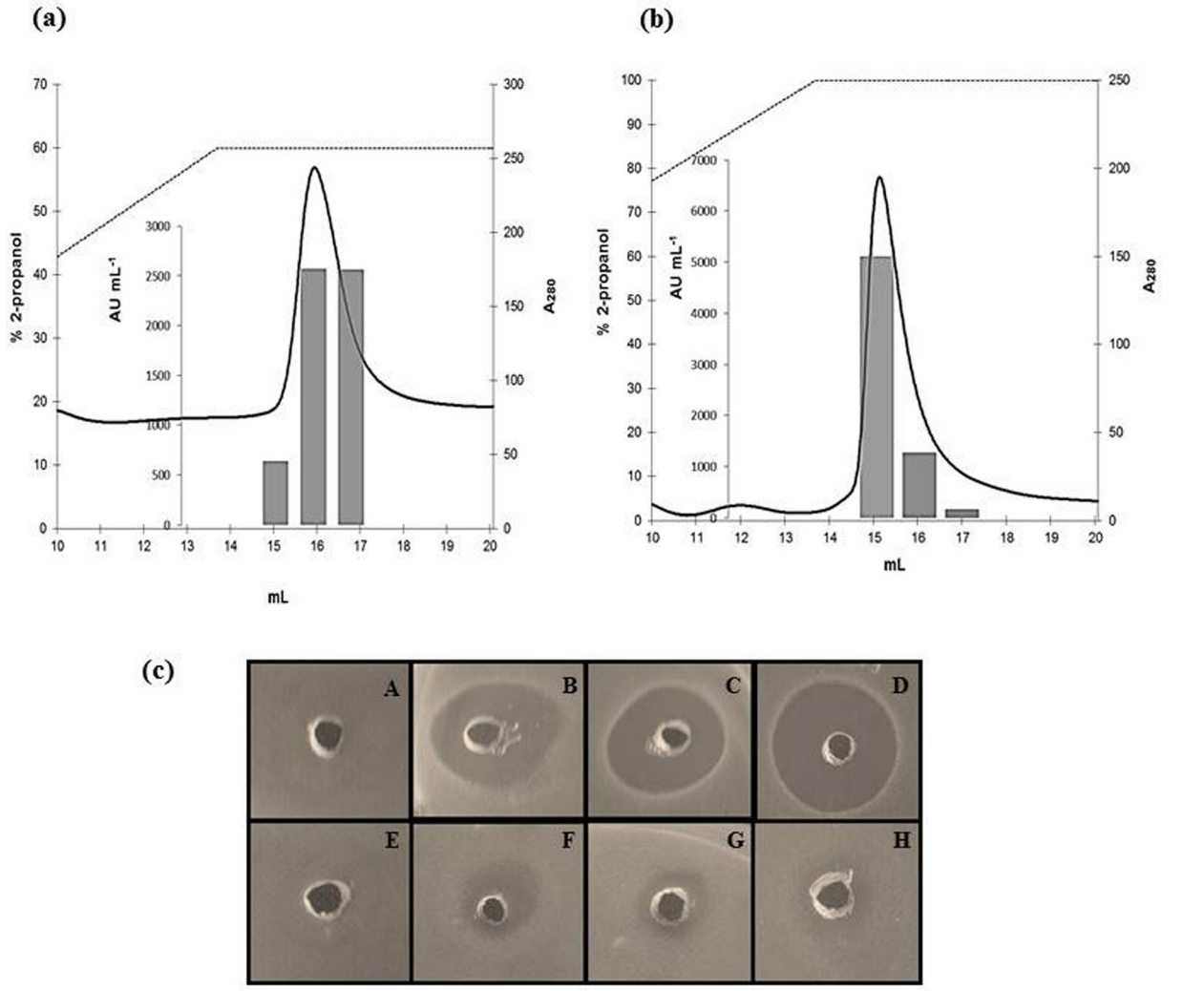

Figure 1. The chromatographic elution profile from RPC III shows (**a**) the elution peak AI and (**b**) the elution peak AII. Solid lines show the absorbance at 280 nm and dotted line the elution gradient of 2-propanol (%, v/v). The antimicrobial activity quantified by microtiter assay system is shown as grey columns. (**c**) Formation of clearing halos around the wells containing the fractions 15 (B), 16 (C) and 17 (D) obtained from RPC III of the elution peak AI, and with the fractions 15 (F), 16 (G) and 17 (H) obtained from RPC III of the elution peak AII in the plates inoculated with *L. lactis* LMGT 2122 as indicator strain. (A) 60% 2-propanol/0.1% TFA (E) 100% 2-propanol/0.1% TFA.

results suggest that the antimicrobial compounds present in the two analyzed fractions not present proteinaceous nature or they could be small peptides not susceptible to the action of this enzyme as observed for a low weight antimicrobial peptide (Singh *et al.* 2014). On the other hand, when the fractions AI and AII were treated with different concentrations of Tween 80, the inhibitory action was reduced, suggesting the involvement of hydrophobic moieties in the activity of these compounds. For treatment with 0.01% of Tween 80, the activities of the fractions AI and AII were reduced in 87% and 75%, respectively (Table 3). After treatment with 0.05% of Tween 80, the antimicrobial activity of compounds present in AI fraction was completely lost, while the activity of compounds in AII fraction was lost after treatment with 1.0% of Tween 80 (Table 3).

This result suggests that Tween 80 may reduce the affinity of these compounds for site of action on the cell, as well as contribute to the formation of a protection barrier of this site in the indicator strain, cause a loss of the three-dimensional structure of molecules that is important to its function or abduct the compounds. Tween 80 is a non-ionic detergent, hydrophilic, mostly composed of oleic acid and has been used for some time in bacterial cultures to assist in growth. It has also been found to promote the entrance of compounds and their exit from the cell (Keren *et al.* 2004). Kimoto, Ohmomo and Okamoto (2002) reported that Tween 80 produced a variation in the fatty acid composition in *L. lactis* strain. They concluded that these fatty acids contributed to enhancing the lipid membrane stability. In contrast, it has been shown previously that Tween 80 increased the sensitivity of indicator strain to the bacteriocin lacticin RM or increased its activity (Keren *et al.* 2004). This suggests that the mode of action of the compounds in the fractions AI and AII might be different than that of bacteriocins, and further research is needed to clarify in detail.

Determination of inhibitory spectrum

The inhibitory spectrum of compounds in the fractions AI and AII produced by *L. lactis* ID1.5 is presented in Table 4. They showed inhibitory activities against several bacteria species Gram-positive (*L. lactis*, *B. subtilis*, *S. pneumoniae* and *Listeria*

Table 3. Effect of heat, proteolytic enzymes and Tween 80 treatments on the antimicrobial activity of the compounds present in the fractions AI and AII produced by *L. lactis* ID1.5.

	Antimicrobial activity (AU mL^{-1})[a]	
Treatment	AI	AII
Untreated	2560	5120
100°C, 15 min	2560	5120
100°C, 30 min	2560	5120
Trypsin	2560	5120
Proteinase K	2560	5120
Tween 80		
0.01%	640	640
0.02%	40	320
0.05%	NI	40
0.1%	NI	40
0.5%	NI	40
1.0%	NI	NI

[a]*Lactococcus lactis* LMGT 2122 was used as indicator strain.
AU mL^{-1}, arbitrary unit for mL; NI, no inhibition.

Table 4. Inhibition spectrum of purified compounds AI and AII produced by *L. lactis* ID1.5

		Antimicrobial activity (AU mL^{-1})[a]	
Indicator	Strain	AI	AII
Lactococcus lactis	ID1.5	640	NI
Lactococcus lactis	IL1403	1280	NI
Lactococcus lactis	LMGT 2115	1280	NI
Lactococcus lactis	LMGT 2122	2560	5120
Bacillus subtilis	DSMZ 347	320	1280
Enterococcus faecalis	v583	NI	NI
Lactobacillus sake	NCDO 2714	NI	NI
Listeria innocua	BL86/26B	NI	1280
Micrococcus luteus	ATCC 4698	NI	NI
Micrococcus luteus	ATCC 10 240	NI	NI
Staphylococcus aureus	2002–05-ME8245–3	NI	NI
Staphylococcus aureus	2002–60-8452	NI	NI
Streptococcus pneumoniae	TIGR4	640	1280
Escherichia coli	ATCC 14 763	NI	NI
Pseudomonas aeruginosa	-	320	1280
Pseudomonas ssp.	-	160	640
Salmonella enterica Thyphimurium	SL 1344	NI	NI
Shigella soneii	ATCC 11 060	NI	NI

[a]NI, no inhibition; AU mL^{-1}; arbitrary unit for mL.

innocua) and Gram-negative (*P. aeruginosa* and *Pseudomonas* sp.). The sensitivity varied considerably among these target strains, when *Pseudomonas* sp appears to be less sensitive for both compounds. *Lactococcus lactis* LMGT 2122, *B. subtilis* DSMZ 347, *L. innocua* BL86/26B, *S. pneumoniae* TIGR 4 and *P. aeruginosa* seem to be the organisms most sensitive to compounds in AII (Table 4). Recently, low molecular weight compound produced by *Lactobacillus* and *Pediococcus* has been described as antimicrobial against Gram-positive and Gram-negative bacteria (Jeevaratnam *et al.* 2015). Interestingly, in our work the compound AI showed antimicrobial activity against all *L. lactis* strains tested (Table 4). Moreover, the purified compound AI was able to inhibit its producer strain, unlike the compound AII that was not active against the producing strain (Table 4). Based on these results, it appears that the amount of compounds present in the fraction AI may have affected the level of producer strain self-immunity. It suggests the compounds in AI might not be produced in high amount or not be secreted during growth of *L. lactis* ID1.5. It is possible that a neutralizing agent is co-produced in this culture, which binds to the antimicrobial, rendering it unable to interact with its producer strain.

Previous work of our research group indicated that *L. lactis* ID1.5 produces a bacteriocin nisin Z. In this study, we reported the production of additional inhibitory compounds present in fractions AI and AII. Antimicrobial compound production is a bacterial defense mechanism, which gives the producer strain a competitive advantage towards non-producer and sensitive strains in the same niche (Pulusani, Rao and Sunki 1979; Kimoto, Ohmomo and Okamoto 2002; Cotter, Hill and Ross 2005). Thereby, microorganisms that simultaneously produce more than one inhibitory substance show additional advantages in terms of capacity for colonization, being important strains for use in biotechnology, for health protective or industrial application.

The results of our study indicate that *L. lactis* ID1.5 produces at least two cell-bound antimicrobial substances not previously reported in other *L. lactis*. Although these compounds have been produced by lactic acid bacteria, showing heat stability and activity against Gram-positive bacteria, several characteristics differ them from the bacteriocins. They are highly hydrophobic, anionic, resistant to proteases and their molecular weight are <2000 Da. As mentioned earlier, these compounds showed antimicrobial activity against *P. aeruginosa* and *S. pneumoniae* TIGR 4, two clinically important respiratory pathogens (Lomovskaya *et al.* 2001). These pathogens can cause difficulty to treat respiratory infection because of their intrinsic resistance to antibiotic therapy with many available antibiotics (Zhanel *et al.* 2004).

The origin of the strain and the diversity in inhibition profiles against several relevant pathogens indicate that *L. lactis* ID1.5 may be suitable for commercial starter cultures for fermented meat products and may be exploited for other applications. Further studies are crucial to investigate the chemical properties and the exact structure of bioactive compounds, as well as to know their distinct mechanism of action.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

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Conflicts of interest. None declared.

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