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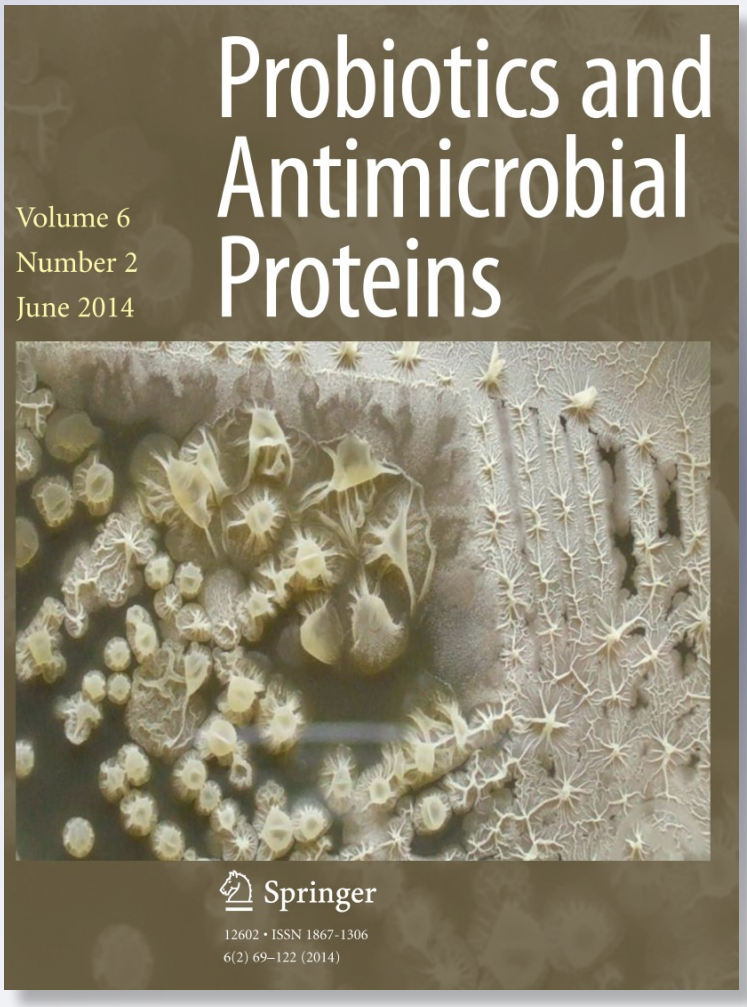

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Lactobacillus pentosus B231 Isolated from a Portuguese PDO Cheese: Production and Partial Characterization of Its Bacteriocin

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Abstract Bacteriocin B231 produced by *Lactobacillus pentosus*, isolated from an artisanal raw cow's milk protected designation of origin Portuguese cheese, is a small protein with an apparent relative mass of about 5 kDa and active against a large number of *Listeria monocytogenes* wild-type strains, *Listeria ivanovii* and *Listeria innocua*. Bacteriocin B231 production is highly dependent on the type of the culture media used for growth of *Lact. pentosus* B231. Replacement of glucose with maltose yielded the highest bacteriocin production from eight different carbon sources. Similar results were recorded in the presence of combination of glucose and maltose or galactose. Production of bacteriocin B231 reached maximal levels of 800 AU/ml during the stationary phase of growth of *Lact. pentosus* B231 in MRS broth at 30 °C. Bacteriocin B231 (in cell-free supernatant) was sensitive to treatment with trypsin and proteinase K, but not affected by the thermal treatment in range of 55–121 °C, or freezing (−20 °C). Bacteriocin production and inhibitory spectrum were evaluated. Gene encoding plantaricin S has been detected in the genomic DNA. Virulence potential and safety of *Lact. pentosus* B231 were assessed by PCR targeted the genes *gel*E, *hyl*, *asa*1, *esp*, *cyl*A, *efa*A, *ace*, *van*A, *van*B, *hdc*1, *hdc*2, *tdc* and *odc*. The *Lact. pentosus* B231 strains harbored plantaricin S gene, while the occurrence of virulence, antibiotic resistance and biogenic amine genes was limited to cytolysin, hyaluronidase, aggregation substance, adhesion of collagen protein, gelatinase, tyrosine decarboxylase and vancomycin B genes.

Keywords *Lactobacillus pentosus* · Bacteriocin · PDO Portuguese cheeses

Introduction

The involvement of lactic acid bacteria (LAB) in food biopreservation processes consists of a very ancient approach and most probably is one of the first ways for food preservation used by humans. Most of the LAB are well accepted as generally recognized as safe (GRAS) and may contribute, as starter cultures or endogenous microbiota, to the formation of important food attributes, not only at an organoleptic level, but also due to their biopreservative effect. Their biopreservative characteristics are mainly explained by the acidification originated from the production of weak organic acids, carbon dioxide or hydrogen peroxide and also the synthesis of bacteriocins, defined as ribosomally produced small peptides displaying antimicrobial activity against closely related bacteria [1, 2]. However, despite over 500 bacteriocins have been characterized, only nisin and pediocin PA-1 are commercialized as food additive and been authorized for application in different countries [3]. As suggested by Cotter et al. [1], bacteriocins can be divided in two classes: class I, lanthionine-containing bacteriocins, and class II, non-lanthionine-containing bacteriocins.

Fermented dairy products such as cheeses, various types of yoghurts and kefir contain large amounts of LAB and

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Table 1 Culture media and incubation conditions used for the study of the LAB populations

Medium	Incubation temp/time	Group targeted	References
Nutrient agar	Aerobic, 10 °C; 5 days	Psychrophilic microflora	[72]
M 17 agar	Anaerobic, 30 °C; 2 days	Mesophilic streptococci	[72, 73]
MRS agar	Aerobic, 30 °C; 2 days	Mesophilic lactic acid bacteria	[72]
MRS agar	Anaerobic, 30 °C; 2 days	Mesophilic anaerobic lactic acid bacteria	[72]
Rogosa agar	Anaerobic, 30 °C; 2 days	Mesophilic lactobacillus	[72]
Slantz and Bartley agar	Aerobic, 37 °C; 2 days	Enterococci	[73]

then may be considered as natural sources of potential bacteriocin-producer microorganisms [4–8]. Bacteriocinogenic *Lact. pentosus* have been isolated from several different products, including fermented meat and fermented cereals [9–13].

In this paper, we report the characterization of a bacteriocin produced by *Lact. pentosus* B231, a microorganism isolated from an artisanal PDO Portuguese cheese prepared from raw cow's milk.

Materials and Methods

Isolation of LAB with Antimicrobial Potential

Thirteen different PDO Portuguese cheeses that have met the regulatory standards, displaying the European union stamp (symbol) and the label of certification, were obtained from a local market. For isolation of LAB, samples were obtained from the interior of the cheeses using sterilized instruments to avoid contamination from the external surface of the products. Ten grams of each cheese sample was mixed with 90 ml of sodium citrate (2 %, w/v) (Oxoid, Basingstoke, UK) and homogenized for 2 min in a Stomacher 400 Lab Blender (Seward, London, UK) at maximum speed. Decimal dilutions were prepared with sodium citrate solution (2 %, w/v), and 0.1 ml of each dilution was plated on the surface of corresponding agar media (Table 1) in duplicate and incubated according to the procedures summarized in Table 1. After the incubation period (conditions summarized in Table 1) and performance of the microbial counting obtained by the use of MRS (Oxoid), M17 (Oxoid) and Rogosa (Oxoid) agars, representative colonies were selected for identification and streaked onto MRS agar and incubated under the same conditions. Further, selected colonies were streaked onto nutrient agar (Oxoid) and incubated in similar manner for further identification. Preidentification of the colonies isolated from SBA agar (Oxoid), presumed to be *Enterococcus* spp., was confirmed in AAB agar (Oxoid), treated for 2 h at 44.5 °C and further streaked onto nutrient agar. All isolates were subjected to Gram staining and tested for the production of catalase and oxidase. Further, gram-positive isolates were tested by the use of galleries from ID System (BBL, Franklin Lakes, USA) and/or API50CHL (BioMerieux SA, Marcy-l'Etoile, France).

Antilisterial activity of the resulting isolates was initially tested according to the spot-on-the-lawn method described by Stecchini et al. [14]. LAB cultures (20 ml) were grown for 18 h in MRS broth at 30 °C and spotted directly on TSA (Biokar Diagnostics, Beauvais F60002, France) agar plates supplemented with 0.5 % yeast extract (Biokar) containing 10^5 cells/ml of *L. monocytogenes* B218 (wild-type strain isolated from a food sample, collection of ESTG, Viana do Castelo, Portugal). Listerial inhibition was considered present when an inhibition halo of at least 5 mm was observed on TSA plates.

Additional confirmation of microbial strain identification was obtained by amplification of the genomic DNA isolated using ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA), according to the manufactures' instructions, with universal primers F8 and R1512 [15]. The amplified fragments using F8 and R1512 primers were purified by the use of the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), and the obtained fragments were sequenced and compared to sequences available at GenBank using BLAST (Basic Local Alignment Search Tool).

Bacterial Strains and Growth Media

Lact. pentosus B231 and various *L. monocytogenes* strains used in this study (Table 1) were stored at −70 °C in MRS or BHI (Oxoid) broth (respectively) supplemented with 20 % (v/v) glycerol (Merck, Darmstadt, Germany). Activation of *Lact. pentosus* B231 was performed by inoculating 100 µl of the stock culture into 5 ml of MRS, followed by incubation at 30 °C for 18 h.

Bacteriocin Activity

Production of bacteriocin was determined by two different methods: the spot-on-the-lawn assay [16] and the well

Table 2 Effect of type of carbohydrates on the bacteriocin production and pH on stability of bacteriocin expresed by *Lact. pentosus* B231

Carbohydrate	Inhibitory activity of CFS
Glucose 20 g/l	++
Maltose 20 g/l	++
Lactose 20 g/l	+
Galactose 20 g/l	+
Sucrose 20 g/l	++
Fructose 20 g/l	++
Mannose 20 g/l	+
Mannitol 20 g/l	−
Glucose + maltose 20 g/l + 20 g/l	+++
pH	
4.50	−
5.00	+
5.50	+++
6.00	+++
6.50	+++
6.70	+++
7.00	++
8.00	−
11.00	−

Activity of CFS expressed as diameter of the inhibition zone: − (no inhibition); + (< 2 cm); ++ (≥ 2 cm and < 3 cm); +++ (≥ 3 cm)

diffusion assay [17] using cell-free supernatant (CFS) from *Lact. pentosus* B231 obtained after its growth for 18 h at 22, 30 and 37 °C in MRS broth, centrifugation (10,000×*g*, 10 min, 4 °C), adjustment of pH to 6.0 by the use of 1 M NaOH solution and filter sterilization (0.22 µm pore membrane) (Orange Scientific, Braine-l'Alleud, Belgium). *L. monocytogenes* B218 was used as indicator strain for the determination of the antimicrobial activity. In addition, serial dilutions of the CFS were prepared in phosphate buffer (100 mM, pH 6.5), and the bacteriocin activity was expressed as arbitrary units per milliliter (AU/ml), which is defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition [18].

Effect of Enzymes, pH and Temperature on Bacteriocin Activity

Lact. pentosus B231 was cultured in MRS broth for 18 h at 30 °C, and CFS was obtained as described above. The CFS was treated with trypsin (Fluka, St. Louis, Missouri, USA), proteinase K (Sigma, St. Louis, Missouri, USA) and catalase (Sigma), at final concentration of 1 mg/ml and incubated at 37 °C for 2 h. After that time period, enzymes were inactivated by boiling the samples for 3 min, followed by immersion on ice.

The effect of the pH on the stability of the antimicrobial substance(s) was performed by the use of CFS obtained from *Lact. pentosus* B231 (as described before) submitted to pH changes with sterile solutions of 0.3 N NaOH and 0.3 N HCl to reach pH of 4.5, 5.0, 5.5, 6.0, 6.5, 6.7, 7.0, 8.0 and 11.0 (Table 2). After incubation for 1 h at the above-mentioned pH values, the samples were readjusted to pH 6.0 and the antimicrobial activity was determined as described before.

The effect of temperature on the stability of the antimicrobial substance(s) produced by *Lact. pentosus* B231 was tested by heating CFS at 55, 80 and 100 °C for 10 min and at 121°C for 15 min. The CFS was further subjected to freezing (−20 °C), and its inhibitory activity was checked after thawing for 12 weeks.

Sterile MRS broth treated with the same enzyme solutions and untreated samples were used as controls. After every treatment, the inhibition activity against *L. monocytogenes* B218 was determined by the well diffusion assay, as described above. All experiments were conducted as three independent experiments.

Effect of Different Carbohydrates and NaCl on Bacteriocin Production

Cells from an 18-h-old culture of *Lact. pentosus* B231, grown aerobically at 30 °C, were harvested by centrifugation (10,000×*g* for 10 min at 4 °C), and the resulting pellet was resuspended in 5 ml of peptone water (Merck). Aliquots of 100 µl of this suspension were used to inoculate 10 ml of the following culture media: (a) MRS broth; (b) MRS broth supplemented with 0.5, 1.0, 2.0 and 4.0 % (w/v) of NaCl; (c) MRS broth redesigned as 10.0 g/l peptone (Biokar), 5.0 g/l yeast extract (Biokar), 10.0 g/l meat extract (Biokar), 1.0 ml/l Tween 80 (Merck), 2.0 ml/l K_2HPO_4 (Merck), 5.0 g/l sodium acetate (Fluka, St. Louis, Missouri, USA), 2.0 g/l ammonium iron (III) citrate (Sigma, St. Louis, Missouri, USA), 0.2 g/l magnesium sulfate (Merck) and 0.05 g/l manganese sulfate (Panreac) adjusted to pH 6.0 with 3 N HCl and 3 N NaOH and supplemented with one of the following carbohydrates: 20 g/l glucose (Merck), 20 g/l maltose (Merck), 20 g/l lactose (Merck), 20 g/l galactose (Merck), 20 g/l sucrose (Merck), 20 g/l fructose (Merck), 20 g/l mannose (Merck) and 20 g/l mannitol (Merck) (Table 2). The incubation was done at 30 °C for 18 h. The CFS was prepared, and the antimicrobial activity was tested as described above. In addition, *Lact. pentosus* B231 was grown in MRS broth for 18 h at 30 °C and CFS prepared as described before. Antibacterial activity was assessed against *L. monocytogenes* B218. The experiments were conducted as three independent experiments.

Partial Purification of Bacteriocin B231

The bacteriocin produced by *Lact. pentosus* B231 was partially purified, using the method described by Todorov [19]. *Lact. pentosus* B231 was grown in 500 ml MRS broth incubated at 30 °C for 18 h, without agitation. The CFS was obtained as described before and treated at 80 °C for 10 min. Following, to the material ammonium sulfate (40 %) was added and incubated overnight at 4 °C on permanent shaking. The precipitate was collected by centrifugation (13,000×*g*, 60 min, 4 °C), dissolved in 14 ml of 25 mM ammonium acetate buffer (pH 6.5) and loaded on a Sep-Pack C18 column (Waters Millipore, MA, USA). The different fractions were eluted with 20 % (v/v) isopropanol, 40 % (v/v) isopropanol and 60 % (v/v) isopropanol, all prepared in 25 mM ammonium acetate (pH 6.5) buffer. The eluted fractions were tested to determine the bacteriocin activity, and the fraction with the highest inhibitory activity was kept at 4 °C until future use.

Growth of *Lact. pentosus* B231 and Bacteriocin B231 Production

An aliquot of 100 µl of an 18-h-old culture of *Lact. pentosus* B231 was inoculated into 100 ml of MRS broth and incubated in 250-ml flasks at 30 °C without agitation for 48 h. Periodically, changes in pH were measured by using a pH meter (Model 3320, Jenway, Essex, UK), cell-free supernatant inhibitory activity (AU/ml) against *L. monocytogenes* B218 was monitored, and changes in OD (600 nm) were assessed (Helios gamma spectrophotometer, UNICAM, UK). The experiments were conducted in triplicate.

Molecular Weight of Bacteriocin B231

Tricine-sodium dodecyl sulfate (SDS) (Sigma, St. Louis, Missouri, USA)-polyacrylamide gel electrophoresis (PAGE) was performed as described by Shagger and Von Jagow [20]. The sample was prepared by mixing CFS from *Lact. pentosus* B231 (obtained as described above) and non-reducing sample buffer [12 % SDS (w/v), 30 % glycerol (w/v), 0.05 % Coomassie blue G-250 (Serva) and 150 mM Tris/HCl (pH 7.0)] in a 3:1 ratio, followed by incubation for 1 h at 37 °C.

The molecular weight standard (low range, Bio-Rad) and 20 µl of the sample were loaded into a gel vertical electrophoresis system (Blue vertical 100, Serva Electrophoresis, Heidelberg, Germany) using a E865 power supply (Consort, Turnhout, Belgium) at a constant voltage (150 V) for 2 h. Half of the gel was stained with a 0.125 % (w/v) Coomassie brilliant blue R-250 (Fluka) solution, and the other half was assayed for anti-*Listeria* activity, as described by Tolinački et al. [21]. The area of the gel containing the tested bacteriocin was placed aseptically on a Petri plate and covered with a layer of BHI agar containing ca. 10^4 cells/ml of *L. monocytogenes* B218 and incubated at 30 °C for 18 h for the observation of inhibition zones.

Listeria monocytogenes Inhibition by CFS

L. monocytogenes B218 was activated overnight at 30 °C in BHI broth. One milliliter of the microorganism culture was inoculated into 100 ml of BHI broth in duplicate. For the first flask, 10 ml of MRS broth was added to it and this worked as the control. For the second flask, 10 ml of filter-sterilized (0.22-µm filter, Orange Scientific), pH-neutralized CFS from *Lact. pentosus* B231 was added to it. The two flasks were incubated at 30 °C for 48 h, and the OD (600 nm) was periodically determined.

The influence of CFS from *Lact. pentosus* B231 (previously grown at 30 °C during 18 h in MRS) on *L. monocytogenes* viability was evaluated during a 2-day period of incubation. For this, *L. monocytogenes* B218 cells (10^7 cfu/ml) were obtained by its cultivation at 30 °C in BHI broth. Measurements of *L. monocytogenes* growth were taken using spectrophotometry (OD at 600 nm), and cell counts were assessed on ALOA agar throughout the 49-h incubation time.

Screening for the Presence of Bacteriocin Genes

Total DNA was isolated from *Lact. pentosus* B231 using ZR Fungal/Bacterial DNA Kit (Zymo Research) according to the manufacturer's instructions. DNA was utilized in PCRs to detect genes responsible for the production of the following bacteriocins: pediocin PA-1, plantaricin S, plantaricin W, plantaricin NC8 [22], nisin [23], enterocin A, enterocin P, enterocin B and enterocin L50B [24–26], curvacin A and sakacin P [27], sakacin G1 and sakacin G2 [28], leuconocin 972, lactacin A, lactacin 481, lactacin B, lactacin M, lactacin GQ, lactacin 3147, sakacin Tα, sakacin Tβ and sakacin X [29]. The PCRs were prepared using primers at a concentration of 10 pM/µL, and the conditions used were the ones previously described elsewhere [22–29], but with adjustment of the annealing temperatures, according to the specification of the used primers. The amplified products were separated by electrophoresis in agarose gels in 0.5× TAE buffer and stained in TAE buffer containing 0.5 µg/ml ethidium bromide (Sigma Diagnostics, St. Louis, Mo., USA).

Characterization of Virulence Potential

Lactobacillus pentosus B231 was tested for the presence of the virulence genes *gel*E (gelatinase), *hyl* (hyaluronidase), *asa*1 (aggregation substance), *esp* (enterococcal surface protein), *cyl*A (cytolysin), *efa*A (endocarditis antigen), *ace*

(adhesion of collagen), *van*A and *van*B (both related to vancomycin resistance), and genes for amino acid decarboxylases: *hdc*1 and *hdc*2 (both related to histidine decarboxylase), *tdc* (tyrosine decarboxylase) and *odc* (ornithine decarboxylase), by using PCR protocols as described by Martin-Platero et al. [30], Rivas et al. [31] and Vankerckhoven et al. [32]. The amplified products were separated by electrophoresis in 0.8–2.0 % (w/v) agarose gels in 0.5× TAE buffer. Gels were stained in TAE buffer added of 0.5 μg/ml ethidium bromide (Sigma-Aldrich Co.). Strains from the collection of University of Sao Paulo, Faculty of Pharmaceutical Sciences, Sao Paulo, SP, Brazil, have been used as positive controls in the determination of the virulence potential of *Lact. pentosus* B231.

Results and Discussion

Isolation and Identification of *Lactobacillus pentosus* B231

The 13 PDO cheeses tested, which were produced in different regions of Portugal from raw milk, presented microbial counts ranging from 10^5 to 10^8 cfu/g. This result is similar to the ones published by other authors who evaluated the same types of cheese studied in this work. Barbosa [33] claimed that mesophilic LAB (10^8 cfu/g) and enterococci (10^7 cfu/g) are the predominant groups in Serpa cheese. In Picante Beira Baixa cheese, LAB was the dominant group of microorganisms [34, 35]. LAB population is also the most predominant in Azeitão cheese, as reported by Mimoso et al. [36].

In this work, it was also found that the dominant microflora was mainly constituted by different LAB based on the morphology and results obtained by microbial growth on different agars (MRS, Rogosa, SBA, Mayeux, and M17). A total of 76 isolated colonies were screened for antilisterial activity using the method described by Stecchini et al. [14].

One of the isolates (B231) obtained from Pico cheese (originated from Azorean islands), isolated on MRS agar presenting antibacterial activity against various *L. monocytogenes* test organisms, was selected for further characterization. Based on morphological, physiological and biochemical test (API50CHL), the isolate B231 was identified as *Lact. pentosus* B231 (95 % level of confidence). By the use of 16 s rRNA sequencing technique, the microorganism identification was confirmed, displaying 98 % homology to *Lact. pentosus* ATCC 8041^T. *Lact. pentosus* seems to be frequently associated with cheeses from different kinds and milk sources as much work has been published identifying this microorganism in cheeses throughout the world, including PDO Salers cheese [37], Feta cheese [38], Halloumi cheese [39] and Slovakian bryndza cheese [40].

Table 3 Cell-free supernatant (CFS) inhibitory spectra activity

Strain	Medium	Inhibition
Listeria monocytogenes NCTC 11994	TSA	+
Listeria monocytogenes (38 wild-type strains, meat and cheese isolated)	TSA	+
Listeria ivanovii B021 (cheese isolated)	TSA	+
Listeria innocua B020 (cheese isolated)	TSA	+
Clostridium perfringens NCTC 13170	TSC	−
Bacillus subtilis (wild type)	TSA	−
Staphylococcus aureus NCTC 6571	TSA	−
Salmonella nottingham NCTC 7382	TSA	−
Enterococcus faecalis NCTC 775	TSA	−
Salmonella enterica subsp. *arizonae*	TSA	−
Pseudomonas aeruginosa NCTC 10662	TSA	−
Escherichia coli NCTC 9001	TSA	−
Bacillus cereus NCTC 7464	TSA	−

Activity of CFS expressed as diameter of the inhibition zone: − (no inhibition); + (inhibition zone)

In addition to *L. monocytogenes*, the antimicrobial substance(s) produced by *Lact. pentosus* B231 was (were) active also against *Listeria ivanovii* subsp. *ivanovii* and *Listeria innocua* (Table 3). The assessment of the inhibitory spectrum is an important characteristic when evaluating possible applications of bacteriocin-producing strains as potential probiotics or starter cultures, as their inhibitory activity plays a relevant role in the competition with other microorganisms in the gastrointestinal tract or in food matrixes, protecting them from the colonization by foodborne pathogens. Our results are important, particularly because *L. monocytogenes* continues to be a major pathogen in dairy products, as several recent listeriosis outbreaks were linked to cheeses [41, 42].

Bacteriocin B231 produced by *Lact. pentosus* B231 was tested against several Gram-positive and Gram-negative microorganisms (listed in Table 3). No microorganism other than *Listeria* spp. was susceptible to bacteriocin B231. However, all the 41 strains of *Listeria* tested, 39 of which *L. monocytogenes*, were sensitive and inhibited by the presence of this bacteriocin (Table 3). Similar results were recorded for the CFS and the semi-purified bacteriocin (data not shown). *L. monocytogenes*, a well-known pathogenic microorganism, is of mandatory control in many food products, as stated in the microbiological criteria regulation (CE) no. 2073/2005. Its inhibition is therefore of great relevance for industry.

Characterization of Bacteriocin B231

Treatment of the CFS obtained from *Lact. pentosus* B231 with proteinase K and trypsin resulted in a complete loss of the antimicrobial activity, confirming the proteinaceous

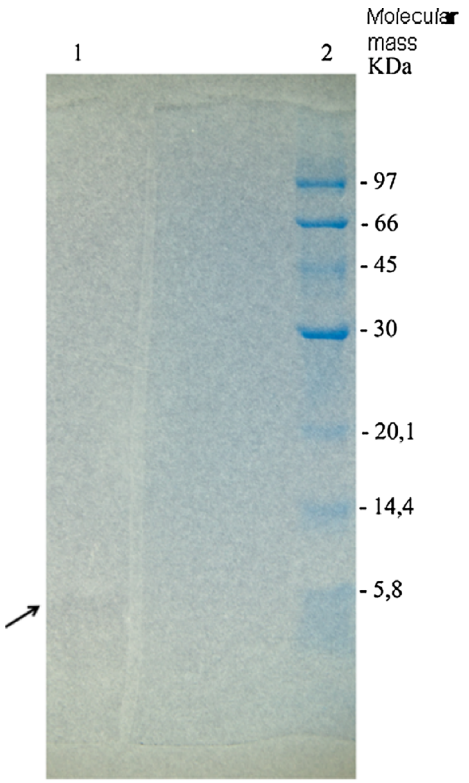

Fig. 1 SDS-PAGE separation of bacteriocin B231 and inhibition of *L. monocytogenes* B218. *Lane 1* semi-purified bacteriocin B231 and *lane 2* molecular weight standards (low range, Bio-Rad)

nature of the antimicrobial substance(s) produced by the studied strain. However, after treatment with catalase, no changes in the antimicrobial activity were observed, clearly excluding the involvement of H_2O_2 in the antagonism process. Moreover, treatment with α-amylase and lipase did not affect the antimicrobial activity, showing that bacteriocin does not contain carbohydrates or lipids in the active molecule. According to de Vuyst and Vandamme [42], most bacteriocins are polypeptides, but others, such as leuconocin S produced by *Leuconostoc paramesenteroides* [43], carnocin 54 produced by *Leuconostoc carnosum* [44] and bacteriocin ST63BZ produced by *Leuconostoc lactis* ST63BZ [45], are typical examples of amylase-sensitive bacteriocins.

Changes in the pH of CFS obtained from *Lact. pentosus* B231 did not significantly affect its antimicrobial activity. Bacteriocin with its pH adjusted in the range of 4.4–8.8 remained stable for 2 h at room temperature and presented similar inhibitory activity as controls. A slight reduction in antimicrobial activity was recorded after pH had been increased from 7.0 to 8.8. Bacteriocin B231 (pH 6.5) remained stable after 10 min at 55, 80 and 100 °C. No decrease in activity was reported after treatment for 15 min at 121 °C. However, the freeze/thaw process slightly lowered the activity of the bacteriocin.

Thermostability of bacteriocins produced by other *Lactobacillus* and *Lactococcus* strains presented results similar to bacteriocin B231 [23, 46–48]. However, lactocin NK24, produced by *L. lactis*, lost 88 % of its activity after 30 min at 100 °C and was completely inactivated after 15 min at 121 °C [49]. In the case of lactocin MMFII produced by *L. lactis*, only 8 % of activity was recorded after 30 min at 110 °C or 25 % after 30 min at 80 and 100 °C [50]. Nisin, produced by *L. lactis* WNC20, was inactivated after 15 min at 121 °C when incubated at pH 7.0, but not under incubation at pH 3.0 at this temperature [51]. Bozacin B14, produced by *L. lactis*, was inactivated after 10 min at 90 °C [52].

The apparent molecular mass of bacteriocin B231 was estimated to be around 5 kDa (Fig. 1). This value is in accordance with most of the well-known and characterized bacteriocins, with molecular mass of <10 KDa [46].

Production of Bacteriocin B231

LAB are fastidious bacteria and as any other microorganisms, growth and metabolism are highly influenced by culture conditions, namely carbon source. In general, bacteriocin B231 was produced when 7 out of 8 carbon sources were tested. Although growth could be observed when mannitol was introduced, the corresponding CFS did not show any inhibitory activity against the tested microorganism (Table 2). Based on these results, production of bacteriocin B231 was carbon source dependent. This was usually strain/bacteriocin specific and not always correlating with the carbon source more efficient for growth promotion. As reviewed by Sanchez et al. [53], the influence of carbon source on bacteriocin expression (namely for nisin or pediocin production) is notorious, as a possible result of signal transduction or the formation of enzymes involved in its modifications. For *Leuc. mesenteroides*, although glucose is the carbon source that results in higher biomass production, fructose is preferred for higher yield of bacteriocin production [54]. Bovicin HC5 is produced best in the presence of glucose, as for many other bacteriocins [55]. When glucose was replaced by other sources of carbohydrate, an increase in bacteriocin production was recorded for both sakacin P [56] and nisin [42]. Bacteriocin ST211Ch production was stimulated in the presence of glucose or sucrose [57], and similar results have been reported for plantaricin UG1 [58], plantaricin KW30 [59] and bacteriocin ST311LD produced by *Ent. faecium* ST311LD [60]. Higher bacteriocin B231 production was recorded when glucose and lactose were used simultaneously.

Bacteriocin B231 production was higher when *Lact. pentosus* B231 was grown between pH 5.5 and 6.7. Similar results have been reported for bacteriocin ST211Ch, produced at the same levels in MRS broth with pH in the range

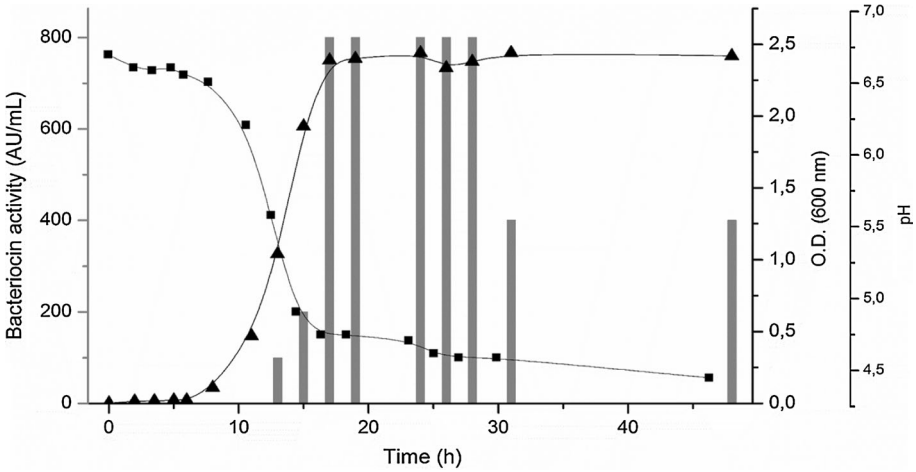

Fig. 2 Production of bacteriocin B231 in MRS broth (30 °C). Antimicrobial activity is presented as AU/ml (*bars*) against *L. monocytogenes* B218. Changes in optical density (*triangle*) and pH (*square*) are indicated

of 5.5 and 6.5 [57]. Much lower growth was recorded for this strain at pH 4.5, and this corresponded to a lower bacteriocin ST211Ch production [57]. This showed that the initial pH of MRS broth has an important role in the bacteriocin production. Similar results were reported for other bacteriocins produced by *Ent. faecium* ST311LD [60] and *Lact. plantarum* [61, 62]. From these results and the literature [60–63], it can be concluded that an initial pH of 6.0 or greater is required for optimal growth of *Lact. pentosus* B231 and bacteriocin production.

Lactobacillus pentosus B231 presented good growth in the presence of up to 4 % of NaCl. Microbial growth and bacteriocin production seemed to be not affected by the presence of up to 1 % of NaCl, whereas higher concentrations had a negative effect on the bacteriocin production. No bacteriocin was produced when *Lact. pentosus* B231 was grown in MRS supplemented with 4 % of NaCl. Previously, concentration of 0.65 mol/l NaCl was reported to stimulate the bacteriocin production by *Lact. pentosus* B96 [11].

The maximum bacteriocin B231 production (800 AU/ml) was recorded after 16–28 h of growth in MRS broth, when the microorganism reached the stationary phase, and a decrease in the inhibitory activity (400 AU/ml) was observed after 32 h of growth (Fig. 2). During the same period of growth, the pH of the medium decreased rapidly from 6.7 to 4.45. On the other hand, the cell density (OD 600) increased from 0.002 to 2.38 in 17 h and remained constant until 48 h of incubation. Low levels of bacteriocin B231 activity (approximately 100 AU/ml) were recorded after 13 h of growth (i.e., log phase) in MRS broth at 30 °C (Fig. 2).

Similar findings were reported for plantaricin ST31 [47], bacteriocin ST311LD produced by *Ent. faecium* ST311LD isolated from spoiled black olives [60], bacteriocin ST4V produced by *Ent. mundtii* ST4V isolated from soy beans [63] and bacteriocins produced by *Ent. faecium* ET05, *Ent. faecium* ET12 and *Ent. faecium* ET88, respectively, isolated from smoked salmon [64].

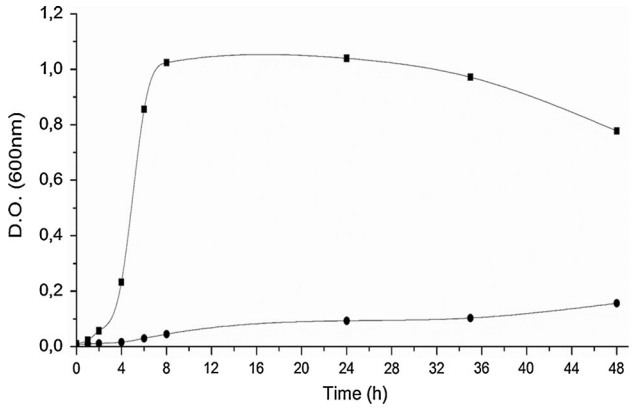

Fig. 3 Effect of cell-free supernatant containing bacteriocin B231 on growth of *L. monocytogenes* B218. *Square* control; *circle* in presence of the antimicrobial peptide

Effect of Bacteriocin B231 on *L. monocytogenes* Growth

The addition of CFS obtained from a 24-h culture of *Lact. pentosus* B231 to a fresh culture of *L. monocytogenes* B218 repressed the pathogen growth over 2 days. However, after 8 h of incubation, some recovery took place and *L. monocytogenes* B218 started to grow, although at a very low level (Fig. 3). These results suggest that the mode of activity of this bacteriocin at the tested concentrations is bacteriostatic. Similar results were reported for bacteriocins ST28MS and ST26MS, which repressed the growth of *Lact. casei* LHS for only 2 h [65]. On the contrary, bactericidal effect tested in similar manners was reported for bacteriocins ST13BR [66] and ST311LD [60].

When ca. 10^7 cfu/ml of *L. monocytogenes* was coincubated with bacteriocin B231, the optical density remained

constant for up to 12 h, with a slight decrease of 0.5 log in viable cells, which reinforces the thesis of the bacteriostatic mode of action of bacteriocin B231.

Screening for the Presence of Bacteriocin Genes

On the basis of the PCRs performed targeting 22 bacteriocin genes, no clear evidence for the presence of these genes in the total DNA of *Lact. pentosus* B231 was obtained. However, when plantaricin S was targeted, positive results were observed. The sequence of the generated amplicons was 100 % identical to the targeted bacteriocin gene (plantaricin S), showing that *Lact. pentosus* B231 is potential plantaricin S producer. However, such a conclusion can be drawn only after appropriate biochemical characterization of the prospective plantaricin S expressed by *Lact. pentosus* B231 bacteriocin. It is important to underline that some of the PCR products are generated corresponding to the size of the target bacteriocin genes (22 tested bacteriocin genes) and to this generated by the positive controls. However, after the sequencing of the generated PCR products, no homology to the targeted bacteriocin genes was observed. In contrast, the sequences of the PCR products obtained by the positive controls exhibited 100 % homology. This highlights the importance of the sequencing of the PCR products in order to confirm the identity of the generated PCR products. It is also important to underline the close relation between *Lact. plantarum* and *Lact. pentosus.* The strain *Lact. pentosus* was isolated from cheese, produced at very geographically isolated island (Azorean islands archipelago). Even if this strain produces bacteriocin described somewhere before, this will be a positive point, showing that bacteriocins encoding gene(s) are highly conservative.

Genes for Virulence, Biogenic Amine Production and Antibiotic Resistance

From tested target genes [adhesion of collagen protein, aggregation substance, cytolysin, endocarditis antigen, enterococcal surface protein, gelatinase, hyaluronidase, histidine decarboxylase, ornithine decarboxylase, tyrosine decarboxylase, vancomycin A and vancomycin B (30–32)] by PCR method, only cytolysin, hyaluronidase, aggregation substance, adhesion of collagen protein, gelatinase, tyrosine decarboxylase and vancomycin B genes generated positive results for DNA of *Lact. pentosus* B231.

In general, the observed frequency of positive results for the virulence factors studied in *Lact. pentosus* B231 was lower than that reported in other studies on *Enterococcus* isolated from foods [67–69] and also in comparison with studies with clinical isolates [69–71]. Despite being less relevant in food isolates, the determination of virulence factors in LAB by molecular and phenotypic procedures is important due to the risk of genetic transfer since these genes are usually coded by conjugative plasmids [70].

Conclusion

The broad spectrum of activity against *Listeria* spp. and the ability of the bacteriocin B231 to persist with anti-*Listeria* activity during long periods of incubation in different conditions make this antimicrobial protein essential for potential industrial application in food industry. Considering the results from PCR, where only plantaricin S gene was detected, further characterization and purification of the expressed protein are needed.

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Conflict of interest The authors' declares that they have no conflict of interest.

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