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ORIGINAL ARTICLE

Lactobacillus plantarum isolated from cheese: production and partial characterization of bacteriocin B391

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Abstract *Lactobacillus plantarum* B391, a strain isolated from an artisanal French cheese, is a producer of a bacteriocin, expressing activity against *Enterococcus faecalis* NCTC 775, *Clostridium perfringens* NCTC 13170 and several *Listeria monocytogenes* strains. High stability was recorded after heat treatment at 121 °C for 20 min and when stored at 4 °C for more than 40 days. A challenge test performed in milk for 11 days showed potential for the control of *L. monocytogenes*. In the presence of the lytic bacteriocin B391, *L. monocytogenes* cells present numerous morphology modifications of cell shape and surface structure as well as in the cell division pattern, resulting ultimately in lysis. The high level of *Listeria* growth inhibition obtained in the presence of *Lb. plantarum* B391, and the stability of B391 bacteriocin for a long period of time, make this strain potentially interesting to use in milk products to increase food safety.

Keywords Bacteriocin · *Lactobacillus plantarum* · *Listeria monocytogenes* · Food-safety · Antimicrobial agents · Milk

Introduction

Bacteriocins, known as small polypeptides able to inhibit the growth of closely related microorganisms, including some food-borne pathogens, are of great interest for the food sector, being the target of numerous research works over the years (Cotter et al. 2005; Todorov 2009). A large number of different small polypeptides identified as bacteriocins have already been isolated and characterized (Cotter et al. 2005; Todorov 2009). However, their availability as pure or semi-purified substances for industrial use is still very limited. Nisin is the oldest, the best known and one of the very few bacteriocins used as a food bio-preservative additive. It has been in use for almost three decades, and is authorized in Europe, the USA, and many other countries (Cotter et al. 2005).

Despite the fact that many bacterial strains are able to produce peptides with antibacterial activity, regarding bacteriocin production, the focus has mainly been on lactic acid bacteria (LAB), not only because of their natural ability to produce bacteriocins, but also because many of these microorganisms are considered as GRAS (generally recognized as safe). One of the intensively studied and characterized bacteriocin producer LAB is *Lactobacillus plantarum*, a facultative heterofermentative LAB, whose potential use as a bacteriocin producer is corroborated from more than 20 different bacteriocins isolated from meat, fish, fruits, vegetables, dairy products or cereals, as recently reviewed by Sabo et al. (2014). *Lb. plantarum* is ubiquitous and frequently present and isolated from the most diverse types of cheeses from all over the world (Belicová et al. 2013; Tzanetakis and Litopoulou-Tzanetaki 1992; Xanthopoulos et al. 2000; Zago et al. 2011). *Lb. plantarum* is also considered by the European Food Safety Authority to be suitable for the qualified presumption of safety (BIOHAZ 2014) and a putative candidate to be used as a probiotic (Todorov and Franco 2010).

In this paper, we report on a bacteriocin with anti-*Listeria,* anti-*Enterococcus* and anti-*Clostridium perfringens* activity produced by the strain of *Lb. plantarum* B391, isolated from an artisanal French type of cheese prepared from raw cow's milk.

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Material and methods

Isolation of the bacteriocin producer

A French-origin Tomme-de-Savoie, a 2- to 4-month ripened raw, skimmed cow's milk cheese, obtained from a local market in Porto, Portugal, was used to isolate bacteriocinogenic LAB. Samples were obtained from the interior of the cheese using sterilized cutting instruments to avoid contamination from outside the product. Ten grams of cheese were 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 maximal 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 MRS agar (Oxoid). After incubation for 48 h at 30 °C, colonies with distinct morphologic characteristics were selected, streaked onto nutrient agar (Oxoid) and incubated in the same conditions for further identification.

Pure cultures were grown on MRS broth for 18 h at 30 °C and the antibacterial activity of the expressed bacteriocins from the selected isolated strains was tested against *L. monocytogenes* B218 using the spot-on-lawn method as described by Guerreiro et al. (2014). Inhibition was considered positive when an inhibition zone of at least 5 mm was obtained.

The isolated strains showing anti-bacterial activity were Gram stained and tested for the production of catalase and oxidase. Further biochemical identification was performed using the API 50 CHL system (Bio Merieux, Marcy-l'Etoile, France). Bacterial identification was confirmed by species-specific primers PCR (Torriani et al. 2001) and 16 s rRNA sequencing (Felske et al. 1997). For this and other biomolecular testing, total genomic DNA was extracted and purified using the ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA), according to the manufacturer's protocol. DNA was quantified by NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA).

Bacterial strains and growth media

Lb. plantarum B391 (bacteriocin producer) and sensitivity test microorganisms used in this study (Table 1) were stored at −70 °C in MRS or BHI (Oxoid) supplemented with 20% glycerol (Merck, Darmstadt, Germany). Recovery of *Lb. plantarum* B391 and sensitivity test microorganisms was performed by inoculating 100 μl of the stock culture into 5 ml of MRS broth or BHI, respectively, followed by incubation at 30 °C for 18 h.

Bacteriocin activity

Production of bacteriocin was determined by the spot-on-lawn assay (Mayr-Harting et al. 1972) using a cell-free supernatant (CFS) of *Lb. plantarum* B391 culture obtained after growing the producer strain for 18 h in MRS broth, centrifugation (10 min, 10,000*g*, 4 °C), correction of pH to 6.0 using 1 M NaOH and filter sterilization using a 0.22-μm membrane (Orange Scientific, Braine-l'Alleud, Belgium). *L. monocytogenes* B218 (wild-type strain isolated from a food sample, collection of ESTG, Viana do Castelo, Portugal) was used as the target strain for identification of antimicrobial activity. In addition, serial dilutions of the CFS were prepared in phosphate buffer (100 mM, pH 6.5), and bacteriocin activity was expressed as Arbitrary Units (AU/ml) and was defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition (Ivanova et al. 1998). Whenever needed, the concentration of CFS was performed using Vivaspin 500 columns, 3000 MWCO PES (Sartorius Stedim Biotech, Germany).

Table 1 Spectrum of activity of bacteriocin produced by *Lactobacillus plantarum* B391

Test strain	Medium	Inhibition
Bacillus cereus NCTC 7464	TSA	–
Bacillus subtilis B101	TSA	–
Clostridium perfringens NCTC 13170	TSC	+
Enterococcus faecalis NCTC 775	TSA	+
Escherichia coli NCTC 9001	TSA	–
Listeria innocua B020	TSA	+
Listeria ivanovii B021	TSA	+
Listeria monocytogenes (36 wild–type strains)	TSA	31 +; 5 –
Pseudomonas aeruginosa NCTC 10662	TSA	–
Salmonella arizonae B210	TSA	–
Salmonella Nottingham NCTC 7832	TSA	–
Staphylococcus aureus NCTC 6571	TSA	–

+ presence of inhibition; − no inhibition

Effect of enzymes, pH and temperature on bacteriocin activity

Lb. plantarum B391 was cultured in MRS broth for 18 h at 30 °C, and CFS was obtained as previously described. Trypsin (Fluka, St. Louis, MI, USA), Proteinase K (Sigma, St. Louis, MI, USA), catalase (Sigma) treatments and pH interference in relation to the stability and activity of the bacteriocin were performed as described by Guerreiro et al. (2014).

The effect of temperature on the stability of the antimicrobial substance produced by *Lb. plantarum* B391 was tested by thermal treatment of CFS at 121 °C for 20 min, at different pH values, without previous neutralization, using 0.1 M NaOH or 0.1 M HCl to correct pH. Stability of the bacteriocin activity over time was determined for a period of 44 days at 4, 22, 37 and 44 °C.

Sterile MRS broth treated with the same enzyme solutions and untreated samples were used as controls. After each treatment, the inhibition activity against *L. monocytogenes* B218 was determined by the spot-on-lawn assay as previously described. All experiments were performed in triplicate.

Bacteriocin mode of action

The lytic mode of action of bacteriocin B391 was confirmed using a chromogenic plate assay according to the method of Mardones and Venegas (2000) with some modifications. Chromogenic *Listeria* Agar (Oxoid) inoculated with 10^7 CFU/ml *L. monocytogenes* B218 cells was used for this assay. Filter-sterilized CFS of *Lb. plantarum* B391 culture (10 μl) obtained after growing the producer strain for 18 h in MRS was spotted on the top of the plate and incubated overnight at 30 °C. Lytic activity was analyzed by observing an increased blue color halo around the bacteriocin spot. Nisin (Sigma) was used as positive control (10 μl of a 20,000-AU/mL solution).

Growth of *Lb. plantarum* B391 and bacteriocin B391 production

A sample of 100 μL of an 18-h-old culture of *Lb. plantarum* B391 was inoculated into 100 mL of MRS broth and incubated in 250-ml flasks at 30 °C for 48 h. Periodically, during the incubation period, changes in pH were measured using a pH meter (Model 3320; Jenway, Dunmow, Essex, UK), CFS inhibitory activity (AU/ml) against *L. monocytogenes* B218 was monitored and changes in OD (600 nm) were measured (Helios gamma spectrophotometer; UNICAM, UK). The experiments were performed in triplicate.

Partial purification of bacteriocin B391

The bacteriocin produced by *Lb. plantarum* B391 was partially purified, using the method described by Todorov and Dicks (2005b). *Lb. plantarum* B391 was incubated in 500 ml MRS broth at 30 °C for 18 h. The CFS was obtained as described before and treated at 80 °C for 10 min, and then precipitated with 40% ammonium sulfate overnight at 4 °C. 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 SepPack C18 column (Waters Millipore, MA, USA). The different fractions were eluted with 20% (v/v) iso-propanol, 40% (v/v) iso-propanol and 60% (v/v) iso-propanol, in 25 mM ammonium acetate (pH 6.5). The eluted fractions were tested for bacteriocin activity and the fraction with the higher activity was kept refrigerated at 4 °C until future use.

Molecular size of bacteriocin B391

Tricine-sodium dodecyl sulfate (Sigma) polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Schagger and Schagger (2006). The sample was prepared by mixing partially purified B391 bacteriocin obtained as previously described and non-reducing sample buffer [12% SDS (w/v), 30% glycerol (w/v), 0.05% Coomassie blue G-250 (Serva), 150 mM Tris/HCl (pH 7.0)] in a 3:1 ratio and incubating for 1 h at 37 °C. The molecular weight standards and 20 μL of the sample were loaded into the 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), and the other half was assayed for anti-*Listeria* activity, as described by Tolinacki et al. (2010). The gel was overlaid with BHI supplemented with 2% agar and containing approximately 10^4 CFU/ml *L. monocytogenes* B218, incubated at 30 °C for 18 h and examined for zones of inhibition.

Adsorption of bacteriocin B391 to producer cells

To test the adsorption of the B391 bacteriocin to the surface of the producer cells, the method described by Todorov et al. (1999) was used. *Lb. plantarum* B391 was cultured in MRS broth for 18 h at 30 °C. The pH of the culture was adjusted to 6.5, the cells were harvested (20,000*g*, 15 min, 4 °C) and washed with sterile 0.1 M phosphate buffer (pH 6.5) two times. The cells were re-suspended in 10 ml of 100 mM NaCl (pH 2.0), agitated for 1 h at 4 °C and harvested (20,000*g*, 15 min, 4 °C). The cell-free supernatant was neutralized to pH 7.0 with sterile 1 M NaOH and tested for activity as described previously.

Challenge test in semi-skimmed cow's milk

A sample of 50 mL of semi-skimmed UHT cow milk (Agros, Portugal) was inoculated with *L. monocytogenes* B218 (approximately 10^3 CFU/ml) and *Lb. plantarum* B391 (approximately 10^6 CFU/ml). Samples have been drawn at regular intervals for a period of 11 days of incubation at 30 °C, and *L. monocytogenes*, and *Lb. plantarum* counts have been performed on Chromogenic Listeria Agar (Oxoid), Slanetz and Bartley agar (Oxoid) and MRS agar (Oxoid), respectively.

Scanning electron microscopy

Filter-sterilized CFS containing bacteriocin B391 (0.5 ml) was added to 4.5 ml of *L. monocytogenes* B218 containing 10^7 cells/ml and incubated at 30 °C. Samples of 1 ml were taken at 30-min intervals during a period of 3 h, filtered through a 13-mm

Nuclepore Track-Etch 0.2-μm membrane (Whatman), and treated for cell fixation in 1.5% (v/v) glutaraldehyde/cacodylate buffer. After fixation, cells were dehydrated in a graded ethanol series and subsequently in hexamethyldisilazane (Fluka). After drying, the membranes were attached to SEM aluminum tubs using LEIT-C Conductive Carbon Cement (Plano) and observed on a scanning electron microscope (Hitachi SU1510) after being coated with a carbon film.

Identification of the genes involved in production of bacteriocin by B391

Total DNA from *Lb. plantarum* B391 was isolated and quantified as described before and submitted to PCR targeting genes related to production of plantaricin S, plantaricin NC8, plantaricin W, Pediocin PA-1, nisin, enterocin A, enterocin P, enterocon B, enterocin L50B, sakacin G2, sakacin G1, sakacin P, sakacin Tα, sakacin Tβ, sakacin X, curvacin A, leuconocin 972, lactacin A, lactacin 481, lactacin B, lactacin M, lactacin GQ, and lactacin 3148 (Barbosa et al. 2014; Biscola et al. 2014; de Souza Barbosa et al. 2015; Holo et al. 2001; Kruger et al. 2013; Macwana and Muriana 2012; Maldonado et al. 2003; Stephens et al. 1998; Todorov et al. 2010). PCR reactions were performed using the GeneAmp® PCR Instrument System 9700 (Applied Biosystems, Foster City, USA) in conditions described by Todorov et al. (2010), Maldonado et al. (2003), Stephens et al. (1998), Holo et al. (2001), Kruger et al. (2013), Macwana and Muriana (2012), de Souza Barbosa et al. (2015), Biscola et al. (2014) and Barbosa et al. (2014). The amplified product was visualized in a 1.0–2.0% (w/v) agarose gel stained with GelRed™ (Biotium, Hayward, CA, USA). A band of interest, corresponding to the correct size of targeted bacteriocins, were purified from the gel using the QIAquick PCR purification kit (QIAGEN) and DNA fragments sequenced in automatic sequencer at the Center for Human Genome Studies, Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, Brazil. Sequences were analyzed using the database of GenBank and the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST).

Characterization of virulence potential

Lb. plantarum B391 was tested for the presence of following virulence genes related to production of gelatinase (*gel*E), hyaluronidase (*hyl*), aggregation substance (*asa*1), enterococcal surface protein (*esp*), cytolisin (*cylA*), endocarditis antigen (*efa*A), adhesion of collagen (*ace*), vancomycin resistance (*van*A and *van*B), and genes for amino acid decarboxylases, such as histidine decarboxylase (*hdc*1 and *hdc*2), tyrosine decarboxylase (*tdc*), and ornithine decarboxylase (*odc*), using PCR protocols of Favaro et al. (2014). The generated PCR products were separated by electrophoresis on 0.8–2.0% (w/v) agarose gels stained with GelRed™.

Results and discussion

Isolation and identification of *Lb. plantarum* B391

Six different colonies growing on MRS were isolated from the Tomme de Savoie cheese sample, but only the one identified as *Lb. plantarum* B391 showed antibacterial activity on the screening performed against *L. monocytogenes* B218. Identification by 16 s rRNA sequencing confirmed the identification as *Lb. plantarum*. In fact, *Lb. plantarum* is frequently associated with cheeses of different types and origins as reviewed by Todorov et al. (2010) and Sharpe (1981).

Bacteriocin B391 was active against more than 86% of the *L. monocytogenes* strains tested, as well as against *L. ivanovii* B021, *L. innocua* B020, *E. faecalis* NCTC 775 and *Clostridium perfringens* NCTC 13170. The spectrum of activity against these microorganisms (Table 1) is relevant, and may indicate a potential industrial use of this bacteriocin, since these bacterial species are frequent contaminants of food products. According to the report of EFSA, 12 EU member states reported 170 food-borne outbreaks in 2013 caused by *C. perfringens*, *C. botulinum* or other *Clostridia.* In relation to *Listeria,* in 2013, 27 EU member states reported 1763 confirmed human cases of listeriosis resulting in 191 deaths. *Listeria* control is mandatory for many food products according to Regulation (CE) no. 2073/2005, and a statistically significant increasing trend of listeriosis in the EU/EEA over the period 2009–2013 can be observed (European Food Safety Authority 2015).

Characterization of bacteriocin B391

Treatment of the CFS obtained from *Lb. plantarum* B391 with proteinase K or trypsin resulted in the complete loss of the antimicrobial activity, confirming the proteinaceous nature of the antimicrobial substance produced by this strain. After treatment with catalase, no changes in the antimicrobial activity were observed, clearly discarding the involvement of H_2O_2 in the antagonism activity.

CFS with pH adjusted in the range of 3.95–8.09 remained active in the whole range of pH tested, the maximum of the activity being at the pH of around 6.0–7.0 (Fig. 1). A similar profile of stability could also be observed in bacteriocins produced by other strains of *Lb. plantarum* (Atrih et al. 2001).

The stability of the bacteriocin when subjected to high temperatures is a typical characteristic for most bacteriocins produced by *Lb. plantarum* (Todorov 2009). Bacteriocin B391 remained stable after 20 min at 121 °C, (Fig. 1), but only at low pH. At pH above 5.0, the bacteriocin is very

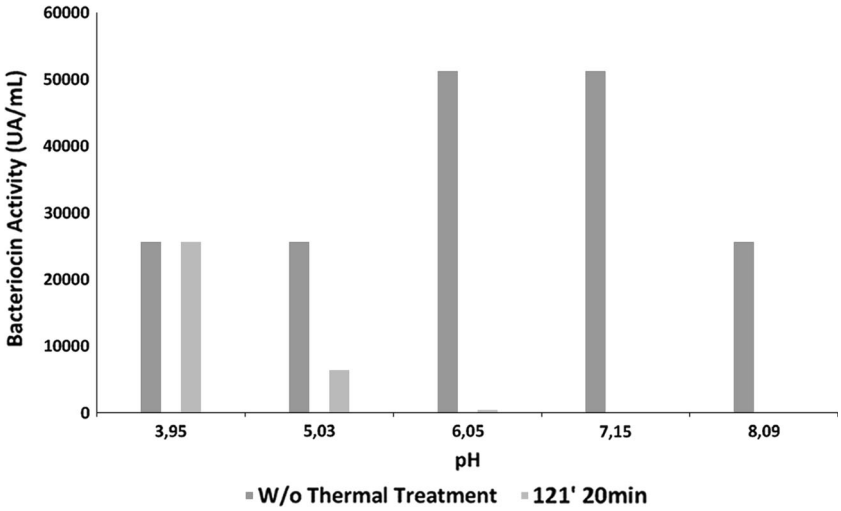

Fig. 1 Antimicrobial activity of B391 CFS against *Listeria monocytogenes* B218, before and after heat treatment (121 °C, 20 min), at different pH values

heat-sensitive, losing almost all the activity when exposed to 121 °C, as can be observed in Fig. 1. Similar results can also be found in the literature: lactocin RN78 (Mojgani 2007), plantaricin LP84 (Suma et al. 1998) or plantaricin C (González et al. 1994) retained activity after heat treatment at high temperatures. This is a common characteristic shared by many class II bacteriocins, once they are small polypeptides.

The apparent molecular weight of bacteriocin B391, when subjected to Tricine SDS-PAGE analysis, on a 16% polyacrylamide gel, is about 6 KDa (Fig. 2). Most of the class I or class II bacteriocins are small polypeptide molecules with a molecular weight not greater than 10 KDa (Nishie et al. 2012). Anti-*Listeria* activity of a protein band with an apparent molecular weight of 6 KDa was confirmed by incubating the acrylamide gel in the presence of an agar layer containing *L. monocytogenes* B218.

The stability of the bacteriocin was studied for a period of 44 days at four different temperatures, 4, 22, 37 and 44 °C (Fig. 3). The stability is clearly dependent on the temperature. Although very stable at 4 °C, it was observed that, as the temperature increases, the activity of the bacteriocin decreases. The level of the decrease in activity obtained after 44 days at 4 °C is the same as the one obtained after a single day at 44 °C.

Production of bacteriocin B391

The growth of *Lb. plantarum* B391, the changes of the medium pH and the production of bacteriocin were assessed for a period of 48 h (Fig. 4). The maximal bacteriocin production (6400 AU/mL) was recorded after 24 h of growth in MRS broth, when the culture reached the stationary phase, and a decrease in activity to 3200 AU/mL was observed after 48 h of growth. A strong acidification profile was observed during the same period of growth, and the pH of the medium decreased rapidly from 6.52 to 4.71. The cell density (OD_{600nm}) increased from 0.179 to 8.416 in 18 h of incubation.

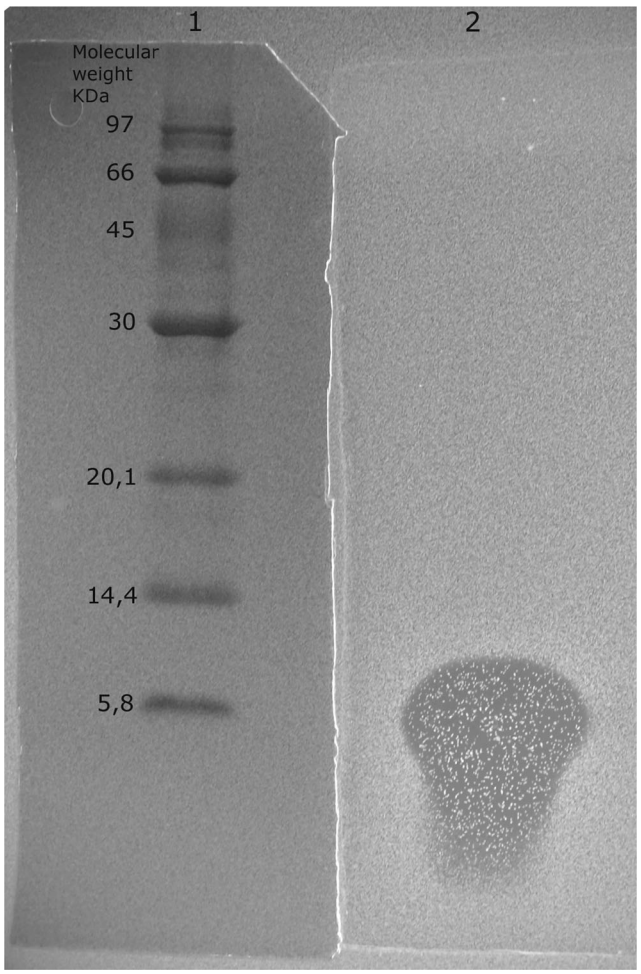

Fig. 2 Tricine SDS-PAGE separation of bacteriocin B391 and inhibition of *Listeria monocytogenes* B218. *Lane 1* molecular weight standards (low range, Bio-Rad and insulin) and *lane 2* semi-purified bacteriocin B391

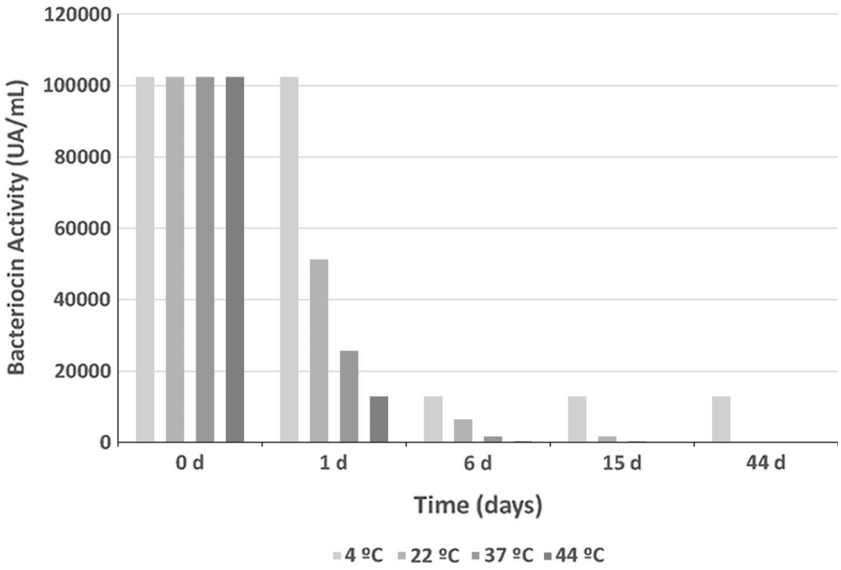

Fig. 3 Activity of the partially purified bacteriocin during a period of 44 days stored at four different temperatures, 4, 22, 37 and 44 °C

Adsorption of bacteriocin B391 to producer cells

The treatment of *Lb. plantarum* B391 cells with NaCl at low pH resulted in no bacteriocin activity, which suggests that the bacteriocin do not adhere to the surface of the producer cells. Some bacteriocins adsorb to some extent to the producer cells in a pH-dependent process (Yang et al. 1992). However, some variability can be observed concerning the behavior of bacteriocins adhering to the cell surface of producer cells. Similar results have also been reported by several authors (Todorov et al. 1999; Todorov and Dicks 2004, 2005a, b).

Bacteriocin B391 mode of action

The lytic mode of action could be confirmed using a chromogenic plate assay using Chromogenic *Listeria* Agar. In the presence of bacteriocin B391, a blue circle staining the edge of the inhibition halo forms, due to the accumulation of X-glucoside cleaved by ß-glucosidase from cell lysis.

L. monocytogenes B218 is rapidly and severely affected by the presence of bacteriocin B391. Several modifications of cell morphology can be seen (Fig.5) as well as roughening of the cell surface with cell debris. Modifications of the cell division pattern can also be observed, revealing some incapacity of division, forming, in some cases, long filaments of irregular-shaped cells. These kind of phenomena are also reported in the literature. A two-peptide bacteriocin, PlnEF, also caused morphological changes of *Lb. plantarum* pl2 cells after 30 min exposure as revealed by scanning and transmission electron microscopy, which showed the formation of blebs protruded in the cell surface, microspheres around the cells, and membrane disruption and channel formation (Zhang et al. 2016).

Scanning and transmission electron microscopy analyses showed that bifidocin A induced alterations in the morphology of *E. coli* cells pore formation, a change in membrane integrity, and complete cell disintegration (Liu et al. 2016).

The morphological cell shape of *E. coli* ATCC25922 and *S. aureus* ATCC25923 was destroyed after incubation with caseicin TN-2 as observed by scanning and transmission electron microscopy (Lü et al. 2014).

Control of *L. monocytogenes* in milk

A challenge test was performed in 50 mL skim cow milk using an initial population of 10^6 CFU/ml of *Lb. plantarum* and 10^3 CFU/ml *L. monocytogenes.* The microbial population was monitored for a period of 11 days and it can be seen in Fig. 6 that the *L. monocytogenes* initial population of 10^3 CFU/ml is strongly affected in terms of growth, as observed from the first day of incubation. No cells of the test microorganism could be detected after 7 days of incubation at 30 °C. No recovery of *L. monocytogenes* could be observed until the end of the incubation.

Detection of bacteriocin genes

In the test for the identification of genes encoding bacteriocin(s) production from total DNA extracted from *Lb. plantarum* ST391, we did not record positive results for any of the tested target genes for plantaricin NC8, plantaricin W, pedioicn PA-1, nisin, enterocin A, enterocin P, enterocon B, enterocin L50B, sakacin G2, sakacin G1, sakacin P, sakacin Tα, sakacin Tβ, sakacin X, curvacin A, leuconocin 972, lactacin A, lactacin 481,

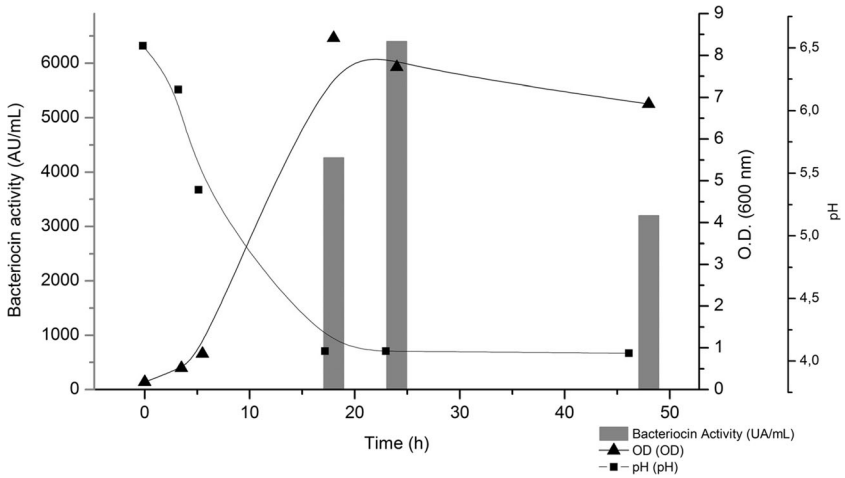

Fig. 4 Production of bacteriocin B391 in MRS broth (30 °C). Antimicrobial activity is presented as AU/mL (*bars*) against *Listeria monocytogenes* B218. Changes in optical density (*triangles*) and pH (*squares*) are indicated

lactacin B, lactacin M, lactacin GQ, or lactacin 3148. However, when PCR was performed targeting the plantaricin S structural gene, an amplicon corresponding in size to that of plantaricin S (not shown) was generated. The sequences of the generated amplicon showing 100% similarity to this was previously reported for plantaricin S (Maldonado et al. 2003). However, the presence of gene/s or even the entire operon does not mean that this bacteriocin is expressed. Several examples of the presence of bacteriocin genes have been used as an argument for claiming that bacteriocins were expressed and present in the supernatant, but only a few works have proved this hypothesis (Todorov et al. 2012).

Detection of virulence, biogenic amines and antibiotic resistance genes

In *Lb. plantarum* B391, PCR testing showed positive results for the presence of *hyl* (hyaluronidase), *gelE* (gelatinase), *cylA* (cytolisin) and *ace* (adhesion of collagen) genes. However, the frequency of positive results for the studied virulence factors was lower compared to that reported in other studies of *Lactobacillus* and especially *Enteroccus* isolated from foods (Barbosa et al. 2010; Gomes et al. 2008; Moraes et al. 2012; Semedo et al. 2003), as well as in studies of *Enterococcus* isolates of clinical origin (Barbosa et al. 2010; Eaton and Gasson 2001; Franz et al. 2001; Semedo et al. 2003). Even

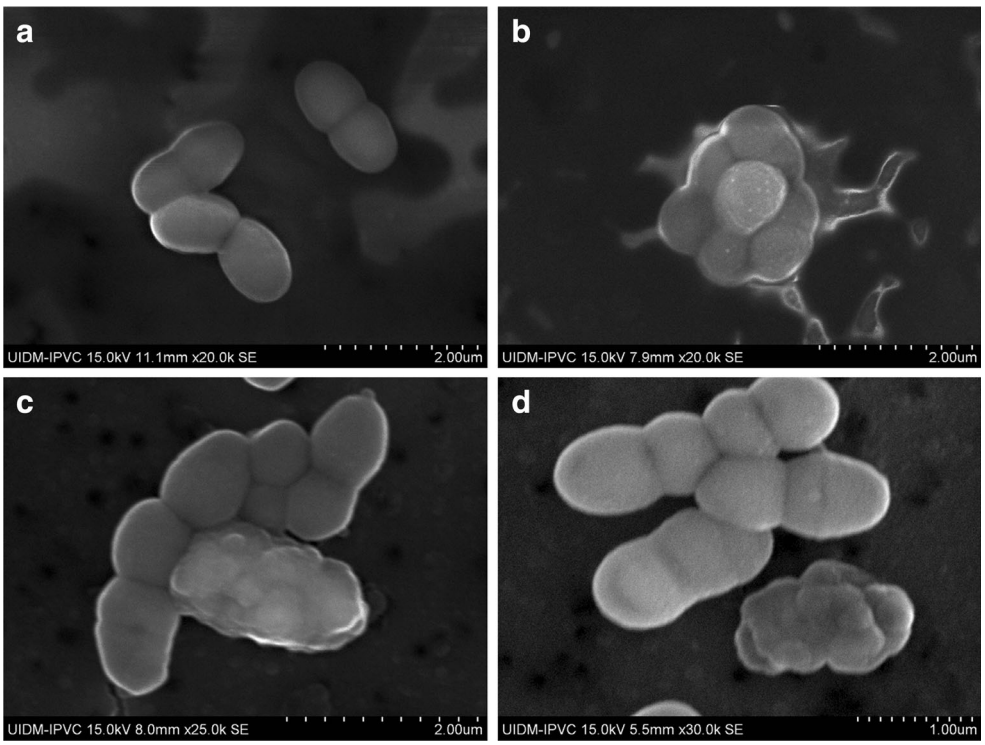

Fig. 5 Scanning electron microscopy of *Listeria monocytogenes*. (**a**) *L. monocytogenes* B218 cells not submitted to the presence of bacteriocin B391. B218 cells submitted to an incubation in the presence of bacteriocin B391 during 60 min (**b**), 90 min (**c**) and 180 min (**d**)

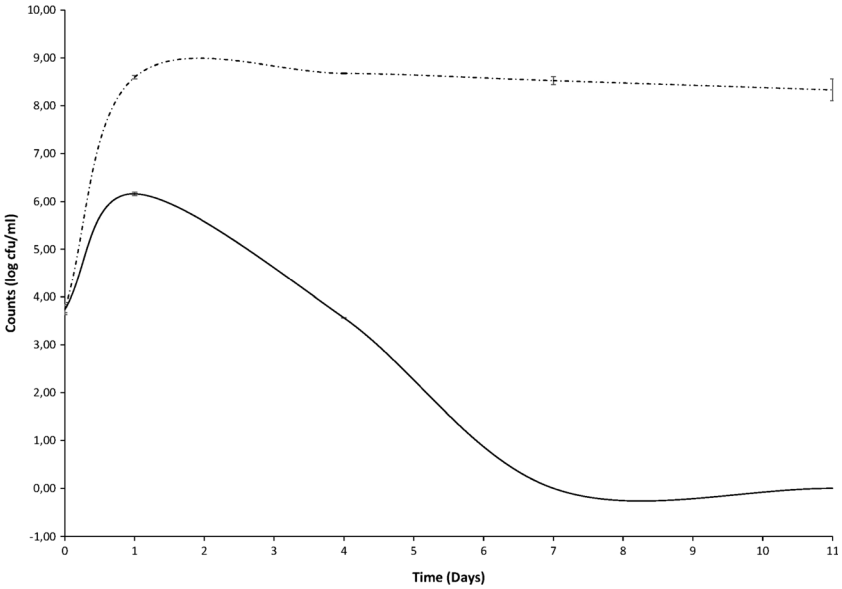

Fig. 6 *L. monocytogenes* B218 growth in the presence of *Lactobacillus plantarum* B391 in semi-skimmed cow's milk. *Dashed line* control (absence of *Lactobacillus plantarum* B391); *Continuous line* in the presence of *Lactobacillus plantarum* B391

if being less relevant in food isolates, the test for the presence of virulence factors in *Lb. plantarum* by molecular and phenotypic procedures is important due to the risk of genetic transfer, because these genes are usually located in conjugative plasmids (Eaton and Gasson 2001).

Special attention needs to be paid to the presence of genes encoding the production of cytolysin. Cytolysin is an exotoxin with bactericidal and hemolytic activity (Haas et al. 2002). The biological role of cytolysin is related to the contribution to evade the host immune system (Franz and Holzapfel 2004), as can lyses of human, rabbit and horse erythrocytes (Chow et al. 1993).

Conclusions

Bacteriocin B391 is an antimicrobial peptide produced by *Lb. plantarum*, highly stable over time and resistant to high temperatures. *Lb. plantarum* B391 showed a low-virulence genes profile. Its broad spectrum of activity against many *Listeria* spp. as well as *E. faecalis* and *Cl. perfringens* makes this antimicrobial protein a potential candidate for bio-safety application in the food industry, and in particular in the dairy industry, since its presence in milk is enough to control the development of *L. monocytogenes* for a long period of time.

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