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Bacteriocinogenic *Lactobacillus plantarum* ST16Pa isolated from papaya (*Carica papaya*) — From isolation to application: Characterization of a bacteriocin

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ABSTRACT

Strain ST16PA, isolated from papaya was identified as *Lactobacillus plantarum* based on biochemical tests, PCR with species-specific primers and 16S rDNA sequencing. *L. plantarum* ST16PA produces a 6.5 kDa bacteriocin, active against different species from genera *Enterobacter*, *Enterococcus*, *Lactobacillus*, *Pseudomonas*, *Streptococcus* and *Staphylococcus* and different serotypes of *Listeria* spp. The peptide is inactivated by proteolytic enzymes, but not when treated with α-amylase, catalase, lipase, Triton X-100, SDS, Tween 20, Tween 80, urea, NaCl and EDTA. However, presence of 1% Triton X-114 deactivates the bacteriocin. No change in activity was recorded after 2 h at pH values between 2.0 and 12.0, and after treatment at 100 °C for 120 min or 121 °C for 20 min. The mode of activity against *Lactobacillus sakei* ATCC 15521, *Enterococcus faecalis* ATCC 19443 and *Listeria innocua* 2030C was bactericidal, resulting in cell lysis and enzyme-leakage. No significant differences in cell growth and bacteriocin production were observed when strain ST16Pa was cultured in MRS broth at 26 °C and 30 °C for 24 h (25 600 AU/ml). However, even though strain ST16PA grows well in MRS broth at 15 °C and 37 °C, a reduction of bacteriocin production was observed (400 AU/ml and 1600 AU/ml, respectively). In addition, effect of MRS medium components, different initial pH and additions of glycerol or vitamins to the media on bacteriocin ST16Pa production was studied.
Peptide ST16PA adsorbs (400 AU/ml) to producer cells. However, bacteriocin ST16Pa was adsorbed at 50% to cells of *L. innocua* 2030C and at 75% to *L. sakei* ATCC 15521 and *E. faecalis* ATCC 19433 when experiments were conducted at 30 °C and pH 6.5. Adsorption of bacteriocin ST16Pa to target cells at different temperatures, pH and in presence of potassium sorbate, sodium nitrate, sodium chloride, ascorbic acid, Tween 80 and Tween 20 were also studied. To the best of our knowledge, this is the first report on detection of *L. plantarum* in papaya.

1. Introduction

Papaya is the fruit of *Carica papaya*, a plant that is native to the tropical regions of Central and South America. This large tree-like plant, with its single stem growing from 5 to 10 m tall, has a spiral arrangement of leaves confined to the top of the trunk under which the fruits grow and mature. The papaya fruit is considered ripe when it feels soft and its skin has developed an amber to orange hue and is commonly used as a food, a cooking component, and in certain traditional medicine practices. It is usually eaten raw, without skin or seeds but sometimes the unripe fruits are cooked.

Lactic acid bacteria (LAB) represent a heterogeneous group of microorganisms that are naturally present in many foods and in the gastrointestinal and urogenital tract of animals. It has been shown that these microorganisms can produce antimicrobial compounds, such as bacteriocins or bacteriocin-like inhibitory substances (De Vuyst & Vandamme, 1994). Bacteriocins of LAB are defined as ribosomally synthesized proteins or protein complexes usually antagonistic to genetically closely related organisms (De Vuyst & Vandamme, 1994; Klaenhammer, 1988; Nes & Johnsborg, 2004). Some bacteriocins are also active against certain Gram negative bacteria, such as *Escherichia coli* and *Salmonella* Thyphimurium (Gong, Meng, & Wang, 2010; Todorov & Dicks, 2005b) and interestingly against *Campylobacter jejuni,* the major cause of gastroenteritis worldwide (Stern et al., 2006). More than 300 different bacteriocins have been described for the genera *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Enterococcus* (Çetinkaya, Osmanağaoğlu,

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& Çökmüş, 2003; De Vuyst & Vandamme, 1994; Olasupo, 1998; Osuntoki, Gbenle, & Olukoya, 2003). They are generally low molecular weight proteins that gain entry into target cells by binding to cell surface receptors. Their bactericidal mechanisms vary and may include pore formation, degradation of cellular DNA, disruption through specific cleavage of 16S rRNA, and inhibition of peptidoglycan synthesis (De Vuyst & Vandamme, 1994; Heu et al., 2001; James, Lazdunski, & Pattis, 1991). A number of bacteriocins have been described for *Lactobacillus plantarum* isolated from fermented meat products (Garriga, Hugas, Aymerich, & Monfort, 1993), milk (Rekhif, Atrih, Michel, & Lefebvre, 1995), cheese (Gonzalez, Arca, Mayo, & Suarez, 1994), fermented cucumber (Atrih, Rekhif, Milliere, & Lefebvre, 1993), olives (Jimenez-Diaz, Rios-Sanchez, Desmazeaud, Ruis-Barba, & Piard, 1993; Todorov & Dicks, 2005a), dough (Todorov et al., 1999), pineapple (Kato et al., 1994), grapefruit juice (Kelly, Asmundson, & Huang, 1996), sorghum beer (Todorov & Dicks, 2004a;2004b; Van Reenen, Dicks, & Chikindas, 1998), molasses (Todorov & Dicks, 2005b), boza (Todorov & Dicks, 2005c; Von Mollendorff, Todorov, & Dicks, 2006), kefir (Powell, Witthuhn, Todorov, & Dicks, 2007), and Amasi (Todorov, Nyati, Meincken, & Dicks, 2007).

Apart from some studies conducted on the effect of nitrogen and carbon sources on the production of plantaricin ST31 (Todorov, Gotcheva, Dousset, Onno, & Ivanova, 2000), plantaricin 423 (Verellen, Bruggeman, Van Reenen, Dicks and Vandamme, 1998), plantaricin UG1 (Enan, Essawy, Uyttendaele, & Debevere, 1996), bacteriocin ST26MS and ST28MS (Todorov & Dicks, 2005b), bacteriocin AMA-K (Todorov, 2008) and plantaricin A (Daeschel, McKeney, & McDonald, 1990), the optimization of bacteriocin production is still considered "terra incognita". Studies conducted on bacteriocins from other LAB, e.g., pediocin PD-1 (Nel, Bauer, Vandamme, & Dicks, 2001), enterocin AS-48 (Abrionel, Valdivia, Galvez, & Maqueda, 2001), enterocin P (Herranz, Martinez, Rodriguez, Hernandez, & Cintas, 2001), sakacin P (Aasen, Moreto, Katla, Axelsson, & Storro, 2000), and bacteriocins produced by *Leuconostoc mesenteroides* L124 (Mataragas, Metaxopoulos, Galiotou, & Drosinos, 2003) have suggested that production is often regulated by microbial growth, pH and temperature. In some cases, higher bacteriocin activity has been recorded at sub-optimal growth conditions (Aasen et al., 2000; Bogovic-Matijasic & Rogelj, 1998; Krier, Revol-Junelles, & Germain, 1998; Matsusaki, Endo, Sonomoto, & Ishizaki, 1996; Mortvedt-Abildgaard et al., 1995; Todorov et al., 2000).

To the best of our knowledge, this is the first report describing the isolation and identification of *L. plantarum* from papaya, as well as being the initial study on the production of a bacteriocin from LAB isolated from this fruit.

2. Material and methods

2.1. Isolation of bacteriocin producing strains

Papaya fruits were collected at the local market (Sao Paulo, SP, Brazil), crushed and homogenized with an equal volume (w/w) of sterile physiological solution (0.85% w/v NaCl). Serial dilutions were plated onto MRS agar (Difco), supplemented with 10 mg/L actidione (Sigma) and the plates were incubated at 30 °C for two days. Colonies for the production of antimicrobial compounds were selected by using the triple-agar layer method (Todorov & Dicks, 2009a). Colonies on MRS agar plates were overlaid with a second layer of MRS agar, supplemented with actidione (10 mg/L) and incubated at 30 °C during 48 h in an anaerobic flask (Oxoid, New Hampshire, England) in the presence of a Gas Generation Kit (Oxoid). Plates with less than 50 colonies were overlaid with 1 mL of active growing cells of *Enterococcus faecalis* ATCC 19443 (ca. 10^6 CFU/mL) imbedded in a thin layer (ca. 10 mL) of semi-solid BHI (BHI, Oxoid) supplemented with 1.0% agar (w/v). The plates were incubated at 37 °C for 24 h. Colonies with the largest inhibition zones were isolated, inoculated into MRS broth (Difco), and re-streaked to obtain pure cultures. Antimicrobial activity was confirmed by using the agar-spot test method, as described by Todorov (2008). The pH of the supernatants was adjusted to 6.0 with sterile 1 M NaOH before testing. Antimicrobial activity was expressed as arbitrary units (AU) per mL and one AU was defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition (Todorov, 2008). The indicator strains used are listed in Table 1.

2.2. Identification of bacteriocin producing strains

The bacteriocin-producing strains were identified according to their physiological and biochemical characteristics as described by Todorov and Franco (2010). Sugar fermentation reactions were recorded by using the API 50 CHL test strips (Biomérieux, Marcy-l'Etiole, France). Confirmation of *L. plantarum* identification was performed using species-specific primers as described by Todorov and Franco (2010).

For future identification and confirmation of the results from species specific primers PCR, robust 16S rDNA sequencing was performed (Felske, Rheims, Wolterink, Stackebrandt, & Akkermans, 1997) using the universal primers 8f (5′-CAC GGA TCC AGA CTT TGA TYM TGG CTC AG-3′) and 1512r (5′-GTG AAG CTT ACG GYT AGC TTG TTA CGA CTT), where Y indicates C + T and M indicates A + C. Amplified fragments were cloned into the pGEM®-T Easy Vector system (Promega, Madison, Wisconsin) and constructs were used to transform *E. coli* DH5α. Plasmid DNAs were isolated using a QIAprep Spin miniprep kit (Qiagen®, Valencia, California, USA) and sequenced using bigdye™ terminator cycle sequencing chemistry (Biosystems,

Table 1
Spectrum of activity of the bacteriocin produced by *Lactobacillus plantarum* ST16Pa.

Test microorganisms	Growth conditions	Sensitive/tested[1]
Acinetobacter baumanii	BHI, 37 °C	1/1
Bacteriodes fragilis	BHI, 37 °C	0/1
Escherichia coli	BHI, 37 °C	2/3
Enterobacter cloacae	BHI, 37 °C	1/2
Enterococcus faecalis	MRS, 30 °C	4/8
Enterococcus faecium	MRS, 30 °C	6/6
Enterococcus mundtii	MRS, 30 °C	1/1
Klebsiella pneumoniae	BHI, 37 °C	1/4
Lactobacillus salivarius	MRS, 30 °C	1/1
Lactobacillus casei	MRS, 30 °C	0/2
Lactobacillus curvatus	MRS, 30 °C	3/4
Lactobacillus delbruekii	MRS, 30 °C	2/2
Lactobacillus fermentum	MRS, 30 °C	0/2
Lactobacillus johnsonii	MRS, 30 °C	1/1
Lactobacillus paracasei subsp. *paracasei*	MRS, 30 °C	0/2
Lactobacillus paraplantarum	MRS, 30 °C	1/1
Lactobacillus pentosus	MRS, 30 °C	1/3
Lactobacillus plantarum	MRS, 30 °C	4/9
Lactobacillus rhmnosus	MRS, 30 °C	1/1
Lactobacillus sakei	MRS, 30 °C	4/4
Lactococcus lactis subsp. *lactis*	MRS, 30 °C	6/6
Listeria innocua	BHI, 37 °C	3/4
Listeria ivanovii subsp. *ivanovii*	BHI, 37 °C	1/1
Listeria monocytogenes	BHI, 37 °C	9/12
Pediococcus acidilactici	MRS, 30 °C	0/3
Pediococcus pentosaceus	MRS, 30 °C	0/2
Proteus mirabilis	BHI, 37 °C	0/2
Pseudomonas aeruginosa	BHI, 37 °C	0/8
Pseudomonas spp	BHI, 37 °C	1/3
Salmonella Typhimurium	BHI, 37 °C	0/1
Salmonella Enterocolitica	BHI, 37 °C	0/1
Staphylococcus aureus	BHI, 37 °C	4/15
Streptococcus spp.	BHI, 37 °C	4/4
Streptococcus agalactiae	BHI, 37 °C	2/2
Streptococcus pneumoniae	BHI, 37 °C	0/6
Streptococcus uberis	BHI, 37 °C	1/1

[1] Number of the test-microorganism that were sensitive to the bacteriocin/total number of the microorganisms tested.

Warrington, England) on an ABI Genetic Analyzer 3130XL Sequencer (Applied Biosystem, SA, Pty, Ltd.).

2.3. Characterization of the bacteriocin — Effect of proteolitic enzymes, chemicals, pH and temperature

Cell-free supernatant of *L. plantarum* ST16Pa, obtained by centrifugation (8000×*g*, 10 min, 4 °C) was adjusted to pH 6.0 with 1 N NaOH. Aliquots of 2 mL were incubated for 2 h in the presence of 1.0 mg/mL (final concentration) of trypsin (Sigma), pronase (Sigma), proteinase K (Sigma), pepsin (Sigma), papain (Sigma), catalase (Sigma), lipase (Sigma) and α-amylase (Sigma) and then tested for antimicrobial activity using the agar-spot test method against *E. faecalis* ATCC 19443 (Todorov & Dicks, 2006c).

In another experiment, the effect of SDS, Tween 20, Tween 80, urea, Triton X-100, Triton X-114, Na-EDTA and NaCl on bacteriocin activity in cell-free supernatants and purified bacteriocin was determined as described by Todorov (2008). The effect of pH on the bacteriocin was determined by adjusting the cell-free supernatant between pH 2.0 and 12.0 with sterile 1 N HCl or 1 N NaOH. After 2 h of incubation at 30 °C, the samples were readjusted to pH 6.5 and the activity determined as described before (Todorov, 2008). The effect of temperature on the bacteriocins was tested by heating the cell-free supernatants to 30, 37, 45, 60 and 100 °C, respectively. Residual bacteriocin activity was tested after 30, 60 and 120 min at each of these temperatures, as described elsewhere (Todorov, 2008).

In all the above mentioned studies, uninoculated MRS broth was exposed to the same conditions (digestive enzymes, chemicals, pH and temperature) as were the cell-free supernatants or the purified bacteriocin and used as a control against *E. faecalis* ATCC 19443.

2.4. Partial purification of bacteriocin ST16Pa

Two liters of a culture of *L. plantarum* ST16Pa grown in MRS broth at 26 °C during 24 h was centrifuged (15 min, 10000×*g*, 4 °C) and proteases in the cell supernatant inactivated by heating for 10 min at 80 °C. Ammonium sulfate was gently added to the supernatant maintained at 4 °C to obtain 40% saturation, and the mixture was stirred during 4 h at 4 °C. After centrifugation for 1 h at 20000×*g* at 4 °C, the resulting pellet was resuspended in 200 mL of 25 mM ammonium acetate buffer (pH 6.5), and loaded on a SepPak C_{18} cartridge (Waters, Millipore, MA, USA). The cartridge was washed with 20% isopropanol in 25 mM ammonium acetate buffer (pH 6.5), followed by a second wash with 40% isopropanol in 25 mM ammonium acetate buffer (pH 6.5) and the bacteriocin was eluted with 60% isopropanol in 25 mM ammonium acetate buffer (pH 6.5). Antagonistic activity against *E. faecalis* ATCC 19443 was determined at each step of the purification process, using the agar spot-test (Todorov, 2008).

2.5. Effect of bacteriocin on growth of test microorganisms

A 20 mL aliquot of bacteriocin-containing filter-sterilized (0.20 μm, Millipore) supernatant (pH 6.0) was added to 100 mL culture of *E. faecalis* ATCC 19433, *Listeria innocua* 2030C and *Lactobacillus sakei* ATCC 15521, respectively in early exponential phase and incubated for 10 h. Optical density readings (at 600 nm) were recorded at 1 h-interval.

2.6. Determination of cell lysis of test microorganisms in presence of bacteriocin ST16Pa

Cell lysis was measured using sterile flat-bottom 96-well TPP plates (Zellkultur testplatte, Switzerland). Cells of *E. faecalis* ATCC 19433, *L. innocua* 2030C and *L. sakei* ATCC 15521 were obtained by centrifugation (8000 x *g*, 4 °C, 10 min) of 10 mL of an 18-h cultures, washed and resuspended in 5 mL of potassium phosphate buffer (20 mM, pH 6.5). Each bacterial suspension (100 μL) was placed individually in microtitre plate wells and 50 μL of potassium phosphate buffer containing different dilutions of semi-purified bacteriocin ST16Pa (as previously described). Plates were incubated at 37 °C for up to 24 h. Absorbance at 655 nm was measured using a Microplate reader (VERSA*max* microplate reader, Molecular Devices, Sunnyvale, CA, USA).

Percentage of cell lysis was calculated as [100 − (At/Ao×100)], where Ao is the initial absorbance (0 h) and At is the absorbance at times 3, 6, 9 or 24 h of incubation.

2.7. Determination of the reduction of viable cells of test microorganisms in presence of bacteriocin ST16Pa

Early stationary phase (18-h-old) cultures of *E. faecalis* ATCC 19433, *L. innocua* 2030C and *L. sakei* ATCC 15521 were harvested (5000×*g*, 5 min, 4 °C), washed twice with sterile saline water and re-suspended in 10 mL of this solution. Equal volumes of the cell suspensions and filter-sterilized (0.20 μm, Minisart®, Sartorius) bacteriocin ST16Pa containing cell-free supernatant were mixed and viable cell numbers were determined before and after incubation for 1 h at 37 °C by plating onto MRS agar. Cell suspensions of *E. faecalis* ATCC 19433, *L. innocua* 2030C and *L. sakei* ATCC 15521 without the addition of the bacteriocin served as controls.

2.8. Determination of cell lysis by measuring the levels of β-galactosidase

Extracellular levels of β-galactosidase activity were monitored. Eleven-h-old cultures of *E. faecalis* ATCC 19433, *L. innocua* 2030C and *L. sakei* ATCC 15521 (100 mL) were harvested and the cells were washed twice with 0.03 M sodium phosphate buffer (pH 6.5) and re-suspended in 16 mL of the same buffer. The partially purified and dried bacteriocin ST16Pa obtained from 2 mL 40% iso-propanol fraction was dissolved into 10 mL sterile MilliQ water (Millipore) and filter sterilized (0.20 μm, Millipore). Cell suspensions of *E. faecalis* ATCC 19433, *L. innocua* 2030C and *L. sakei* ATCC 15521 (2 mL) were treated for 5 min at 25 °C with equal volumes of bacteriocin containing material as described before, followed by the addition of 0.2 mL 0.1 M ONPG (O-nitrophenyl-β-D-galactopyranoside, Sigma) in 0.03 M sodium phosphate buffer (pH 6.8). β-galactosidase activity was stopped by the addition of 2.0 mL 0.1 M sodium carbonate after 10 min incubation at 37 °C. The cells were harvested (8000×*g*, 15 min, 25 °C) and absorbance of the supernatant recorded at 420 nm. Disrupted cells of *E. faecalis* ATCC 19433, *L. innocua* 2030C and *L. sakei* ATCC 15521 (using 0.1 mm diameter glass beads vortexed for 5 min) served as positive lysis controls. All experiments were done in duplicate in two independent occasions.

2.9. Adsorption of bacteriocin ST16Pa to target cells

Adsorption of bacteriocin ST16Pa to target cells was performed according to the method described by Yildirim, Avşar, and Yildirim (2002). The target strains (*E. faecalis* ATCC 19433, *L. innocua* 2030C and *L. sakei* ATCC 15521) were grown overnight in MRS or BHI broth at 37 °C and centrifuged (8000×*g*, 15 min, 4 °C). Cells were washed twice with sterile 5 mM phosphate buffer (pH 6.5) and re-suspended in the same buffer to OD at 600 nm equal to 1.0. The pH was adjusted to 6.5 with sterile 0.1 M NaOH. Each cell suspension was mixed with an equal volume bacteriocin ST16Pa (12800 AU/mL, pH 6.5) and incubated at 37 °C for 1 h. After removal of cells (8000×*g*, 15 min, 25 °C), the activity of unbound bacteriocin ST16Pa in the supernatant was determined as described before. All experiments were done in duplicate.

The percentage adsorption of bacteriocin ST16Pa to target cells was calculated according to the following formula:

$$\%\ \text{adsorption} = 100 - \frac{AU/mL_1}{AU/mL_o} \times 100.$$

AU/mL_1 refers to the bacteriocin activity after treatment; AU/mL_0 refers to the original (before treatment) activity.

2.10. Effect of pH and temperature on the adsorption of bacteriocin ST16Pa

Bacteriocin ST16Pa was added to *E. faecalis* ATCC 19433, *L. innocua* 2030C and *L. sakei* ATCC 15521 as described above, and incubated for 1 h at 4, 15, 30, 37 and 45 °C, respectively (pH 7.0), and at 37 °C at pH 3.5, 5.5 and 7.0. Cells were harvested (8000×*g*, 15 min, 25 °C) and the pH of the cell-free supernatant adjusted to 6.0 with sterile 1 M NaOH. Bacteriocin activity in the supernatant was determined as described before. The experiments were done in duplicate.

2.11. Effect of inorganic salts and organic compounds on adsorption of bacteriocin ST16Pa to target cells

Cells of *E. faecalis* ATCC 19433, *L. innocua* 2030C and *L. sakei* ATCC 15521 were treated with 1% (m/v) Tween 20, Tween 80, NaCl, ascorbic acid, potassium sorbate and sodium nitrate. The pH of all samples were adjusted to 6.5 with 1 M NaOH or 1 M HCl. Bacteriocin ST16Pa was added to the treated cells, as described before, and incubated for 1 h at 37 °C. The cells were harvested (8000×*g*, 15 min, 25 °C) and the activity of bacteriocin ST16Pa in the cell-free supernatant determined as described before. The experiments were done in duplicate.

2.12. Adsorption to producer cells

The ability of the bacteriocin to adsorb to the producer cells was studied as described by Yang, Johnson, and Ray (1992). After incubation for 18 h at 30 °C, the culture of *L. plantarum* ST16Pa in MRS broth was adjusted to pH 6.0 with 1 M NaOH, the cells harvested (10000×*g*, 15 min, 4 °C) and washed with sterile 0.1 M phosphate buffer (pH 6.5). The cells were re-suspended in 10 mL 100 mM NaCl (pH 2.0), stirred for 1 h at 4 °C and then harvested (12000×*g*, 15 min, 4 °C). The cell-free supernatant was neutralized to pH 7.0 with sterile 1 N NaOH and tested for activity using the agar spot-test (Todorov, 2008).

2.13. Growth dynamics of Lactobacillus plantarum ST16Pa, production of bacteriocin and changes of pH

An 18-h-old culture of strain ST16Pa was inoculated (2%, v/v) into MRS broth (Difco), BHI broth (Oxoid), M17 broth (Oxoid), skim milk (0.2 and 10%, w/v) and cheese whey (0.2 and 10%, w/v), respectively and incubated at 15, 26, 30 and 37 °C, respectively, without agitation, for 25 h. In another study, dynamics of bacteriocin ST16Pa production was studied in MRS broth (Difco) at 26 °C during 36 h. Samples were taken every hour and examined for bacterial growth (OD600 nm), changes in culture pH, and antimicrobial activity against *E. faecalis* ATCC 19443. The agar-spot test method was used and the activity expressed as AU/mL as described previously. In a separate experiment, the effect of initial medium pH on bacteriocin ST16Pa production was also evaluated.

2.14. Determination of approximate molecular weight of bacteriocin ST16Pa by SDS-PAGE

Strain ST16Pa was grown in MRS broth (Difco) for 24 h at 26 C. The cells were harvested by centrifugation (8000×*g*, 10 min, 4 °C) and the bacteriocin precipitated from the cell-free supernatants with 40% saturated ammonium sulfate. The precipitate was resuspended in one-tenth volume 25 mM ammonium acetate (pH 6.5), desalted against distilled water by using a 1000 Da cut-off dialysis membrane (Spectrum Inc., CA, USA) and separated by tricine-SDS-PAGE, as described by Schägger and Von Jagow (1987). A low molecular weight marker with sizes ranging from 2.5 to 46 kDa (Amersham International, UK) was used. The gels were fixed and one half overlaid with *E. faecalis* ATCC 19443 (10^6 CFU/mL), embedded in BHI agar (Oxoid), to determine the position of the active bacteriocin, as described by Todorov, Ho, Vaz-Velho, and Dicks (2010).

2.15. Optimization of bacteriocin production

Volumes of 300 mL MRS broth (Difco) were adjusted to pH 4.5, 5.0, 5.5, 6.0 and 6.5, respectively, with 6 M HCl or 6 M NaOH, and then autoclaved. Each flask was inoculated with 2% (v/v) of an 18-h-old culture (OD600nm = 8.0–9.0) of strain ST16Pa, respectively, and incubated at 26 °C for 24 h, without agitation. Changes in culture pH and the activity levels of bacteriocin ST16Pa (AU/mL) was determined as described by Todorov, Ho, et al. (2010).

Strain ST16Pa was cultured in 10 mL MRS broth (Difco) for 18 h at 26 C, the cells harvested by centrifugation (8000×*g*, 10 min, 4 C), and the pellet resuspended in 10 mL sterile saline solution. Four milliliters of the cell suspension was used to inoculate 200 mL of the following growth media: MRS broth, pH 6.4 (without tryptone, meat and yeast extract), supplemented with tryptone (20 g/L), meat extract (20 g/L), yeast extract (20 g/L), tryptone (12.5 g/L) plus meat extract (7.5 g/L), tryptone (12.5 g/L) plus yeast extract (7.5 g/L), meat extract (10.0 g/L) plus yeast extract (10.0 g/L), and a combination of tryptone (10.0 g/L), meat extract (5.0 g/L) and yeast extract (5.0 g/L), respectively; MRS broth, pH 6.4 (without glucose), supplemented with 20.0 g/L glucose, fructose, sucrose, mannose, maltose, lactose and gluconate, respectively; MRS broth (pH 6.4) supplemented with different concentrations K_2HPO_4 and KH_2PO_4 (0.2–10 g/L); and MRS broth (Difco, pH 6.4) supplemented with glycerol (1, 5, 10, 20 and 50 g/L, respectively). In a separate experiment, the vitamins cyanocobalamin (Sigma), L-ascorbic acid (Sigma), thiamine (Sigma) and D,L-6,8-thioctic acid (Sigma) were filter-sterilised and added to MRS broth (Difco) at 1 mg/mL (final concentration). All growth media were incubated at 26 °C for 24 h. Activity levels of bacteriocin ST16Pa were determined as described previously.

3. Results and discussion

3.1. Isolation and identification of LAB from papaya

The average count of LAB in the papaya sample was estimated to be 2.4×10^5 CFU/g. Around 6% of the colonies exhibited an inhibition zone against *E. faecalis* ATCC 19443. Fourteen colonies, selected among those presenting bioactivity against *E. faecalis* ATCC 19443, were successfully identified as being *L. plantarum* by physiological and biochemical tests. The cells presented rods morphology and the carbohydrate fermentation reactions were characteristic of *L. plantarum*. When submitted to API 50 CHL all isolates were positively identified as *L. plantarum* with levels of identification between 99.8 and 100%. Amplification with species-specific primers according to Todorov and Franco (2010) produced a DNA fragment corresponding in size to *L. plantarum* (Fig. 1). Based on the similarity of the biochemical features, morphology, species specific primers PCR and spectrum of activity, all isolates were considered as a replicate of the same strain. For future

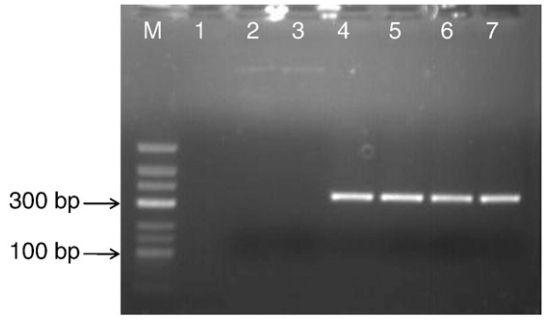

Fig. 1. Agarose gel (1%, w/v) showing DNA fragments obtained after PCR amplification with primers specific for *L. plantarum*. (Line 1) no DNA, (Line 2) *L. pentosus* ATCC 8041 T, (Line 3) *L. paraplantarum* ATCC 700211 T, (Line 4) *L. plantarum* ATCC 14917 T, (Line 5) *L. plantarum* ST202Ch (Todorov, Ho, et al., 2010), (Lines 6 and 7) strain ST16Pa, (M) 50 kb molecular weight marker from Fermentas.

study only one of the isolates, presenting the largest inhibition zone observed against *E. faecalis* ATCC 19443, was used. Identification of this isolate (ST16Pa) by 16S rDNA sequencing indicated 99% homology to *L. plantarum*.

3.2. Characterization of bacteriocin ST16Pa

Bacteriocin ST16Pa presented a large spectrum of activity, inhibiting the growth of many food spoilage bacteria and foodborne pathogens (Table 1). Similar results were recorded for the cell free supernatant and for the semi-purified bacteriocin. It is important to emphasize the bioactivity against *Listeria monocytogenes,* a foodborne pathogen of increasing importance. Antimicrobial activity against Gram-negative bacteria is also a relevant characteristic that was previously detected by several authors in other microorganisms including bacteriocins produced by other *L. plantarum*. Bacteriocins produced by *L. plantarum* ST26MS and ST28MS isolated from molasses showed activity against *Acinetobacter, Escherichia* and *Pseudomonas* (Todorov & Dicks, 2005b). Bacteriocin produced by *Lactococcus lactis* KCA2386 (8.1 kDa); plantaricin 35d (4.5 kDa) produced by *L. plantarum*; bacteriocin ST194BZ (3.0 and 14.0 kDa), ST414BZ (3.7 kDa) and ST664BZ (6.5 kDa) produced by different strains of *L. plantarum*, lacticin NK24 (between 3.0 and 3.5 kDa), produced by NK24 with activities against Gram-negative bacteria have previously been reported (Ko & Ahn, 2000; Lee & Paik, 2001; Messi, Bondi, Sabia, Battini, & Manicardi, 2001; Todorov & Dicks, 2005c; Todorov & Dicks, 2006a, 2006c).

Similar growth rates of *L. plantarum* ST16Pa and production of its bacteriocin (51 200 AU/mL) was recorded when the strain was cultivated either at 26 °C or 30 °C in MRS broth for 24 h. When cultivation temperature was 37 °C after 24 h only 200 AU/mL bacteriocin activity was recorded. A very low growth rates were observed at 15 °C cultivation temperature where a low level of bacteriocin activity (200 AU/mL) was detected after 120 h of incubation.

Treatment of the cell-free supernatant with proteinase K, pronase or α-chymotrypsin resulted in complete inactivation of antimicrobial activity (Table 2). Similar results were observed when cell-free supernatant and semi-purified bacteriocin ST16PA were tested. Treatment with catalase did not affect the activity against the target strains (Table 2), discarding clearly the involvement of H_2O_2 in the antagonism process. Moreover, treatment with α-amylase and lipase did not affect the antimicrobial activity (Table 2), suggesting that bacteriocin ST16Pa does not belong to the controversial group IV of the bacteriocins, which contain carbohydrates or lipids in the active molecule structure. According to De Vuyst and Vandamme (1994), most bacteriocins are polypeptides but others, like Leuconocin S produced by *Leuconostoc paramesenteroides* (Lewus, Sun, & Montville, 1992), and carnocin 54 produced by *Leuconostoc carnosum* (Keppler, Geisen, & Holzapfel, 1994) are typical examples of amylase-sensitive bacteriocins.

Table 2
Stability of bacteriocin produced by *Lactobacillus plantarum* ST16Pa after treatment with enzymes, detergents, urea, heat and pH.

	Concentration	Activity of bacteriocin ST16P*
Enzymes	0.1 and 1.0 mg/ml	
A-amylase		+
Proteinase K, trypsin, pepsin, pronase		−
Lipase		+
pH		
2–6		+
8–12		
Temperature		
1 h		
100, 80, 60, 45, 37, 30, 20, 4 °C for 1 h		+
100, 80, 60, 45, 37, 30, 20, 4 °C for 2 h		+
121 °C for 20 min		+
Detergents/chemicals	1% (w/v)	
Triton X-100, Tween 20, Tween 80, SDS, NaCl, urea, EDTA		+
Triton X-114		−

* Activity of bacteriocin ST16Pa was expressed in + = presence of inhibition zone (>2 mm); − = no inhibition.

The activity of bacteriocin ST16Pa was not affected by 1% SDS, Tween 20, Tween 80, Triton X-100, urea, EDTA, and 1, 2 or 5% NaCl (Table 2). Only the exposition of the free cell supernatant containing bacteriocin ST16Pa to 1% Triton X-114 resulted in the reduction of the bacteriocin activity (Table 2). The sensitivity to detergents, NaCl and urea seems to be bacteriocin-dependent. Bioactivity of plantaricin C19 was not affected by the presence of SDS or Triton X-100 (Atrih, Rekhif, Moir, Lebrihi, & Lefebvre, 2001) contrarily to bacteriocins produced by *Enterococcus faecium* ST311LD (Todorov & Dicks, 2005a), plantaricin 423 (Verellen, Bruggeman, Van Reenen, Dicks, & Vandamme, 1998), pediocin PA-1/AcH (Biswas, Ray, Johnson, & Ray, 1991), lactacin B (Barefoot & Klaenhammer, 1984) and lactocin 705 (Vignolo, Dekairuz, Holgado, & Oliver, 1995).

Bacteriocin ST16Pa was not affected by the pH since it remained active after incubation for 2 h at pH ranging from 2.0 to 12.0 (Table 2). However, when the experiment was conducted at pH 12 with a diluted solution of the bacteriocin (1600 AU/mL), a substantially lower activity was recorded (data not shown). This loss of activity might be ascribed to proteolytic degradation, protein aggregation or instability of proteins at this extreme pH (Aasen et al., 2000; De Vuyst, Callewaert, & Crabbe, 1996; Parente & Riccardi, 1994; Parente, Riccardi, & Addario, 1994). Activity was still present after exposure for 24 h to pH 2.0 to 10.0. Similar observation has been previously reported for pediocin PA-1/AcH (Bhunia, Kim, Johnson, & Ray, 1988; Gonzalez et al., 1994).

Bacteriocin ST16Pa remained stable after 2 h at 25, 30, 37, 45, 60, 80 or 100 °C (Table 2). No decrease in activity of bacteriocin ST16Pa was recorded after heat treatment at 121 °C for 20 min at pH 6.0. Similar results were reported for pediocin PA-1/AcH, which was resistant to heat treatment at 80 °C for 60 min and 100 °C for 10 min, but not to 121 °C (Ray, Motlagh, Johnson, & Bozoglu, 1992). Heat resistance of pediocin PA-1/AcH was pH dependent, as at pH 6.0, 84% of the activity was lost after heating at 121 °C for 15 min and no activity was registered after the same heat treatment at pH 7.0 and 8.0. At pH 4.0, only 11% of the activity was lost. Similar results were recorded for other plantaricins (Todorov & Dicks, 2005a, 2005b, 2005c; Todorov et al., 1999).

3.3. Partial purification of bacteriocin ST16Pa

Since the activity of the bacteriocin in the culture medium was much higher (51 200 AU/mL) than that detected in the surface of the

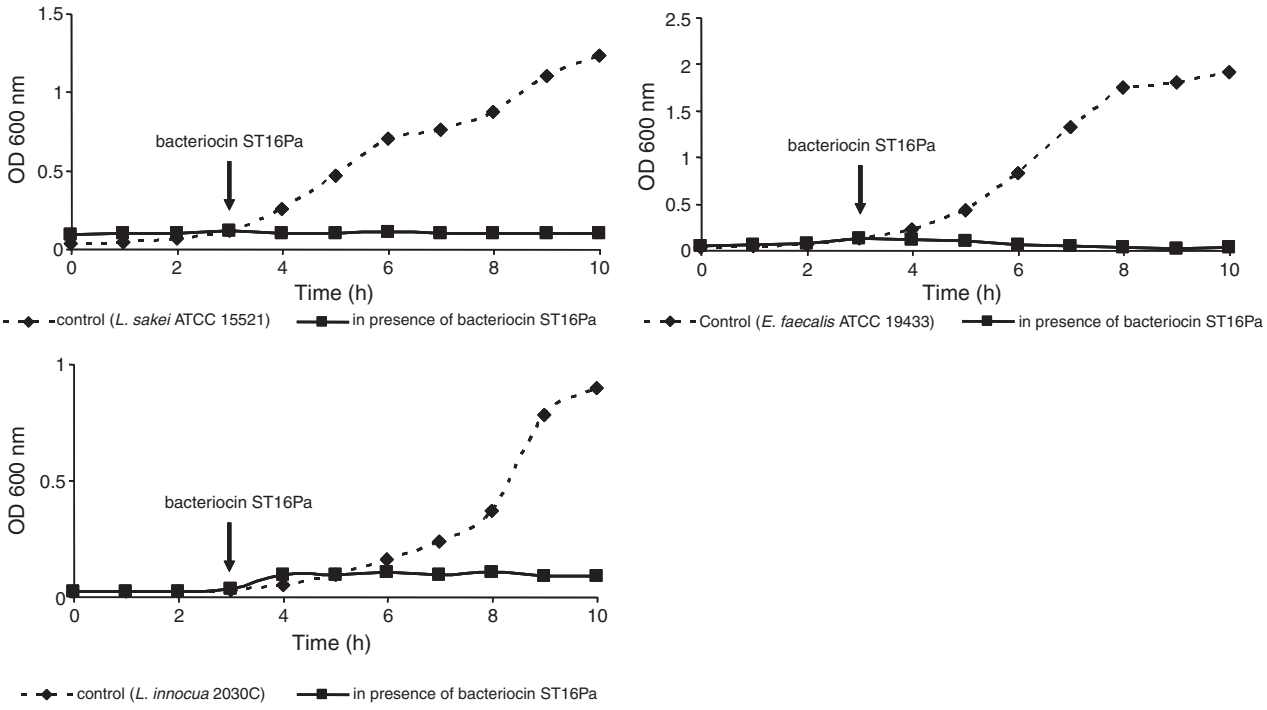

Fig. 2. Effect of bacteriocin ST16Pa on growth of *Lactobacillus sakei* ATCC 15521, *Enterococcus faecalis* ATCC 19443 and *Listeria innocua* 2030C.

bacteriocin-producing *L. plantarum* ST16Pa (400 AU/mL), cell free supernatant from 24-h cultures in MRS broth at 30 °C was used for bacteriocin purification. The activity against *E. faecalis* ATCC 19443 presented by the proteins precipitated with ammonium sulfate and reconstituted in ammonium acetate buffer was similar to that presented by the fractions after chromatography on SepPak C_{18} column and elution with 40, 60 or 80% iso-propanol (1.0×10^8 AU/mL). A similar purification protocol was used for purification of bacteriocins produced by another *L. plantarum* and *Enterococcus mundtii* (Granger, Todorov, Matthew, & Dicks, 2005; Todorov, Wachsman, Knoetze, Meincken, & Dicks, 2005; Todorov et al., 1999).

3.4. Effect of bacteriocin ST16Pa on test microorganisms

Addition of cell free supernatant of a 24 h-old culture of *L. plantarum* ST16Pa to a 3 h-old culture of *E. faecalis* ATCC 19443, *L. innocua* 2030C or *L. sakei* ATCC 15521 (early exponential phase) repressed cell growth of the indicator strains over 10 h (Fig. 2). When the supernatant was added to a 7 h-old culture, a similar inhibition was observed (data not shown). After treatment with bacteriocin ST16Pa, no viable cells of *E. faecalis* ATCC 19443, *L. innocua* 2030C or *L. sakei* ATCC 15521 were detected after 8 h or 10 h, underlining the bactericidal mode of action of this bacteriocin.

The treatment of *E. faecalis* ATCC 19443, *L. innocua* 2030C or *L. sakei* ATCC 15521 with bacteriocin ST16Pa resulted in leakage of β-galactosidase from these cells (Table 3) indicating destabilization of the cell membrane permeability. Similar results were reported for other bacteriocins like buchnericin LB (Yildirim, Johnson, & Winters, 1999; Yildirim et al., 2002), plantaricin 423 (Todorov & Dicks, 2006b), pediocin PA-1/AcH (Bhunia, Johnson, Ray, & Kalchayanand, 1991), mundticin ST4SA (Knoetze, Todorov, & Dicks, 2008) and bacteriocin HV219 (Todorov, Danova, Van Reenen, Meincken, Dinkova, Ivanova & Dicks, 2006).

When *E. faecalis* ATCC 19443, *L. innocua* 2030C or *L. sakei* ATCC 15521 were cultured in MRS broth in the presence of bacteriocin ST16Pa at concentrations ranging from 2.56×10^4 AU/mL to 4.0×10^2 AU/mL, the antagonistic effect could be observed as early as after only 3 h of incubation (Fig. 3). For *L. innocua* 2030C, the percentage of cell lysis (at 3 h incubation time) varied between 19.81 and 30.58%, for *L. sakei* ATCC 15521 from 38.62% to 48.39% and for *E. faecalis* ATCC 19433 from 31.36% to 42.13%, depending on the concentration of bacteriocin ST16Pa used. A more drastic inhibitory effect of bacteriocin ST16Pa was observed at 9 h, 18 h and 24 h of incubation (Fig. 3). After 24 h, the percentage of cell lysis varied from 63.56% to 78.21% for *L. innocua* 2030C, from 83.61% to 99.21% for *L. sakei* ATCC 15521, and from 81.95% to 96.6% for *E. faecalis* ATCC 19433. These results indicate a potential application for bacteriocin

Table 3
Effect of bacteriocin ST16Pa on *L. sakei* ATCC 15521, *L. innocua* 2030C and *E. faecalis* ATCC 19443 as determined by detection of the extracellular levels of β-galactosidase.

	Detection of level of β-galactosidase (OD 420 nm)
L. sakei ATCC 15521 in presence of bacteriocin ST16Pa	0.059 ± 0.02
L. sakei ATCC 15521 non treated cells	0.018 ± 0.01
L. sakei ATCC 15521 mechanically broken cells by glass beads	0.051 ± 0.01
E. faecalis ATCC 19433 in presence of bacteriocin ST16Pa	0.072 ± 0.02
E. faecalis ATCC 19433 non treated cells	0.012 ± 0.01
E. faecalis ATCC 19433 mechanically broken cells by glass beads	0.069 ± 0.02
L. innocua 2030C in presence of bacteriocin ST16Pa	0.076 ± 0.02
L. innocua 2030C non treated cells	0.009 ± 0.01
L. innocua 2030C mechanically broken cells by glass beads	0.072 ± 0.02
Bacteriocin ST16Pa (fraction from SepPakC_{18} purification)	0.004 ± 0.01

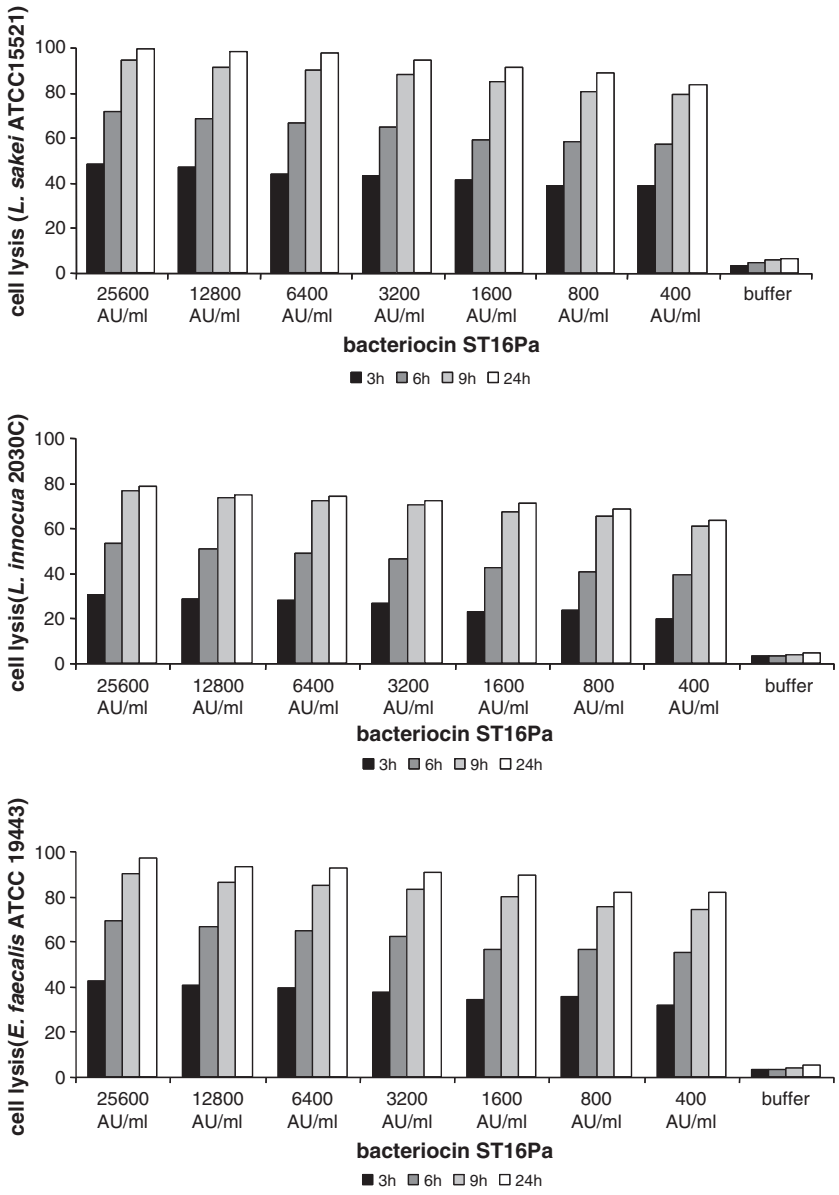

Fig. 3. Determination of the % cell lysis of *Lactobacillus sakei* ATCC 15521, *Enterococcus faecalis* ATCC 19443 and *Listeria innocua* 2030C in the presence of different amount of bacteriocin ST16Pa as detected after 3, 6, 9 and 24 h of incubation. Standard deviation recorded from three repeats was less that 5% and is not indicated.

ST16Pa in the control of listerial or enterococcal food contaminations. It is noteworthy that in these experiments the effect of the bacteriocin was tested against high numbers of the indicator microorganisms (approximately 10^8 CFU/mL). In real food systems, where the level of contamination is much lower, a better effect of bacteriocin ST16Pa can be expected. It is important to underline that high inhibition of *L. innocua* 2030C, *L. sakei* ATCC 15521 and *E. faecalis* ATCC 19443 was observed even with application of very low doses of bacteriocin ST16Pa (Fig. 3). Similar inhibition profiles were observed when bacteriocin ST5Ha produced by *E. faecium* ST5Ha was applied against *L. ivanovii* susbp. *ivanovii* ATCC 19119, *L. sakei* ATCC 15521 and *E. faecalis* ATCC 19443 (Todorov, Wachsman, et al., 2010).

Treatment of cells of *E. faecalis* ATCC 19443, *L. innocua* 2030C or *L. sakei* ATCC 15521 (10^8–10^9 CFU/mL) at stationary phase with bacteriocin ST16Pa resulted in the complete loss of cellular viability (death). After 1 h contact time, no viable cells of *E. faecalis* ATCC 19443, *L. innocua* 2030C or *L. sakei* ATCC 15521 were detected (data not shown), whereas the counts were not modified when these microorganisms were incubated with the control samples. Similar results were obtained when lower concentrations of the contamination microorganisms were used (data not shown). Other bacteriocins, such as the one produced by *Pediococcus acidilactici* HA-6111-2 presented a similar behaviour (Albano et al., 2007).

3.5. Effect of temperature, pH, inorganic salts and organic compounds on adsorption of bacteriocin ST16Pa to target cells

Bacteriocin ST16Pa was adsorbed at 50% to cells of *L. innocua* 2030C and at 75% to *L. sakei* ATCC 15521 and *E. faecalis* ATCC 19433 (Table 4) when experiment was conducted at 30 °C and pH 6.5. Low levels of adsorption (25%) to resistante *P. acidilactici* ET34 were recorded in the same experimental conditions (data not shown). Different levels of adsorption was observed in previous studies using bacteriocin HV219, but in general highest levels of adsorption were observed against sensitive strains compared to strains resistant to the effect of the bacteriocin (Todorov, Botes, Danova, & Dicks, 2007). Similar results have been reported for pediocin N5p (Manca de Nadra, Sandino de Lamelas, & Strasser de Saad, 1998), *viz.* 100% adsorption to sensitive cells of *Oenococcus oeni* X2L, 80% to *Lactobacillus hilgardii* and *Oenococcus oeni* L10, and 70% to *L. hilgardii* 6D . Adsorption of pediocin

Table 4
Effect of temperature, pH and chemicals on adsorption of bacteriocin ST16Pa to *L. innocua* 2030C, *L. sakei* ATCC 15521 and *E. faecalis* ATCC 19433.

	L. innocua 2030C	*L. sakei* ATCC 15521	*E. faecalis* ATCC 19433
Effect of temperatures (°C)			
4	25	25	50
15	50	50	75
30	50	75	75
37	75	75	75
45	75	87.5	100
pH			
3.5	25	50	50
5.5	50	75	50
7.0	50	75	75
Chemicals (1%)			
Tween 80	25	25	25
Tween 20	25	25	50
Ascorbic acid	50	50	50
Potassium sorbate	75	75	75
Sodium nitrate	87.5	75	87.5
NaCl (0.5%, 1.0%)	50	50	50
NaCl (1.5%, 2.0%)	75	75	75

N5p to resistant bacteria was below 20% (Manca de Nadra et al., 1998). Buhnericin LB adsorbed 100% to sensitive cells of *L. plantarum*, *Pediococcus dextranicus*, *O. oeni* and *E. faecalis*, but also 100% to a strain of *Pediococcus cerevisiae* that was resistant to this bacteriocin (Yildirim et al., 2002). In the case of plantaricin 423, adsorption ranged from 17% for *Streptococcus caprinus* ATCC 700066 to 67% for *L. plantarum* LMG 13556, *Lactobacillus curvatus* DF38, *L. innocua* LMG 13568 and *L. sakei* DSM 20017 (Todorov & Dicks, 2006b). Strains sensitive to plantaricin 423 also adsorbed this peptide stronger than the resistant strains (Todorov & Dicks, 2006b).

Optimal adsorption of bacteriocin ST16Pa (50%) to *L. innocua* 2030C and 75% to *L. sakei* ATCC 15521 and *E. faecalis* ATCC 19443 were recorded at pH 6.5 and 7.5. This same adsorption (50% to *L. innocua* 2030C and 75% to *L. sakei* ATCC 15521) was recorded at pH 5.5. At this latter pH, adsorption to *E. faecalis* ATCC 19443 was reduced to 50%. Lower levels of pH (3.5) resulted in the reduction of the adsorption of bacteriocin ST16Pa to 25% to *L. innocua* 2030C and to 50% to *L. sakei* ATCC 15521 and *E. faecalis* ATCC 19433 (Table 4). These results show the potential of the application of this bacteriocin at neutral or moderately acidic pH. Differences in adsorption affected by pH conditions may be due to specific interaction between bacteriocin ST16Pa and the target strain or structural pH dependent modification of the bacteriocin receptors on the target cell surface. In the case of buchnericin LB, optimal adsorption to *L. plantarum* was recorded at pH between 5.0 and 8.0 (Yildirim et al., 2002). Optimal adsorption of plantaricin 423 to *E. faecium* HKLHS was recorded between pH 8.0 and 10.0, and to *L. sakei* DSM20017 between pH 2.0 and 6.0 (Todorov & Dicks, 2006b).

Temperature can also affect bacteriocin ST16Pa adsorption to indicator strains. At 30 °C levels of 50% to *L. innocua* 2030C and 75% to *L. sakei* ATCC 15521 and *E. faecalis* ATCC 19443 adsorption were observed (Table 4). Raising the temperature to 37 °C increased the absorption of bacteriocin ST16Pa to *L. innocua* 2030C up to 75%. The same results were observed with *L. sakei* 15521 and *E. faecalis* ATCC 19443 (Table 4). Higher temperatures (45 °C) resulted in 75% adsorption to *L. innocua* 2030C, 87.5% to *L. sakei* ATCC 15221 and 100% to *E. faecalis* ATCC 19443 (Table 4). However, temperatures of 4 °C and 15 °C resulted in reduction of bacteriocin ST16Pa adsorption to *L. innocua* 2030C, *L. sakei* ATCC 15221 and *E. faecalis* ATCC 19443 (Table 4). Previously, it was shown that temperature had an effect on bacteriocin AMA-K adsorption to *L. innocua* LMG 13568, *L. monocytogenes* ScottA and *L. ivanovii* susbp. *ivanovii* ATCC 19119. At 30 °C and 37 °C, levels of 75% adsorption were observed for all tested *Listeira* species (Todorov, 2008). At 45 °C increased adsorption of bacteriocin AMA-K was recorded to cells of *L. ivanovii* susbp. *ivanovii* ATCC 19119 (87.5%) and to *L. monocytogenes* ScottA (100%) (Todorov, 2008). However, temperatures of 4 °C and 15 °C resulted in reduction to 50% of adsorption of bacteriocin AMA-K to cells of *L. innocua* LMG 13568 and *L. ivanovii* subsp. *ivanovii* ATCC 19119 (Todorov, 2008). It has previously been reported that an increase in temperature from 25 °C to 60 °C had a negative effect on the adsorption of bacteriocin HV219 to *E. faecium* HKLHS. Forty percent adsorption was recorded between 25 °C and 60 °C, with optimum adsorption (80%) at 4 °C. Complete adsorption (100%) to *E. faecalis* E88 was recorded after treatment at 4 °C, 10 °C, 45 °C and 60 °C, whereas a 20% decrease in adsorption was recorded at 37 °C (Todorov, Botes, Danova, & Dicks, 2007). Similar results were observed for other bacteriocins as was the case of buchnericin LB, where identical adsorption levels to cells of *L. plantarum* was recorded after treatment at 0, 10, 25, 50 and 80 °C (Yildirim et al., 2002). Changes in temperature had no effect on the adsorption of plantaricin 423 to *E. faecium* HKLHS (Todorov & Dicks, 2006b).

Decreased adsorption of bacteriocin ST16Pa to *L. innocua* 2030C was observed in the presence of 1% potassium sorbate, 1% sodium nitrate, and 1.5% or 2.0% NaCl. However, these same chemicals did not affect adsorption of bacteriocin ST16Pa to *L. sakei* ATCC 15221. Only presence of 1% sodium nitrate increased adsorption of bacteriocin ST16Pa to *E. faecalis* ATCC 19433. When *L. innocua* 2030C, *L. sakei* ATCC 15521 and *E. faecalis* ATCC 19443 were exposed to bacteriocin ST16Pa in the presence of Tween 20 or Tween 80, a significant reduction of adsorption was recorded (Table 4). Ascorbic acid only affected the adsorption of bacteriocin ST16Pa to *L. sakei* ATCC 15521 and *E. faecalis* ATCC 19433 (Table 4).

Decreased adsorption of bacteriocin AMA-K to *L. innocua* LMG 13568, *L. monocytogenes* ScottA and *L. ivanovii* susbp. *ivanovii* ATCC 19119 was observed in the presence of Tween 20, Tween 80 and different concentrations of NaCl (Todorov, 2008). Ascorbic acid and potassium sorbate did not affect adsorption of bacteriocin AMA-K to cells of *L. innocua* LMG 1568, but this effect was reduced to cells of *L. monocytogenes* ScottA and to *L. ivanovii* subsp. *ivanovii* ATCC 19119 (Todorov, 2008). The presence of 1% sodium nitrate increased the adsorption of bacteriocin AMA-K to cells of *L. innocua* LMG 13568 and *L. monocytogenes* ScottA (Todorov, 2008). Increased adsorption of bacteriocin HV219 to *E. faecium* HKLHS was detected in the presence of Triton X-100, β-mercapto-ethanol, chloroform, NaCl, KH_2PO_4 and $MgCl_2$ (Todorov, Botes, Danova, & Dicks, 2007). Adsorption of bacteriocin HV219 to *E. faecalis* E88 increased in the presence of Na-

acetate, Na_2CO_3, Triton X-100, 80% ethanol, methanol, K_2HPO_4, KH_2PO_4, $MgCl_2$, KCl, Tris and NH_4-citrate (Todorov, Botes, Danova, & Dicks, 2007).

An increase in the adsorption of plantaricin 423 to *E. faecium* HKLHS was observed in the presence of Triton X-100, Triton X-114 and chloroform (Todorov & Dicks, 2006b). Adsorption of bacteriocin HV219 to *E. faecalis* E88 increased in the presence of Na-acetate, Na_2CO_3, Triton X-100, 80% ethanol, methanol, K_2HPO_4, KH_2PO_4, $MgCl_2$, KCl, Tris and NH_4-citrate (Todorov, Botes, Danova, & Dicks, 2007). *L. sakei* DSM 20017 treated with NaCl, K_2HPO_4, KH_2PO_4, $MgCl_2$, KCl, KI, Tris, NH4-citrate, Na_2CO_3, SDS, β-mercapto-ethanol, 80% ethanol and methanol led to a reduction in the adsorption of plantaricin 423 (Todorov & Dicks, 2006b). No change in adsorption of plantaricin 423 was observed in the presence of Na-acetate or EDTA, whereas an increase in adsorption was observed in the presence of Triton X-100, Triton X-114 and chloroform (Todorov & Dicks, 2006b). Adsorption of buchnericin LB to *L. plantarum* was reduced in the presence of NaCl, NH_4Cl, $MgCl_2$, KCl, KI and Tris. Treatment of cells with NH_4-citrate, Na-acetate, $NaCO_3$, EDTA SDS, triton-X, 2-mercapto-ethanol, 80% ethanol and 80% methanol had no effect on adsorption of buchnericin LB to *L. plantarum* (Yildirim et al., 2002). Adsorption of pediocin N5p to *Pediococcus pentosaceus* E5p increased in the presence of $MgCl_2$, $MgSO_4$, $MnCl_2$, $MnSO_4$, whereas NaCl, KCl, KI, NH_4Cl, $CaCl_2$, Na_3PO_4, Na_2SO_4, EDTA and ethanol had no affect on adsorption (Nieto-Lozano, Reguera-Useros, Pelaez-Martinez, & De La Torre, 2002). Organic salts and Na-acetate reduced pediocin N5p adsorption to target cells. Adsorption of pediocin N5p increased by 25% in the presence of SDS (Manca de Nadra et al., 1998).

3.6. Adsorption of bacteriocin to producer cells

Treatment of the *L. plantarum* ST16Pa with 100 mM NaCl (pH 2.0) for 1 h caused adsorption of the bacteriocin to these cells at 400 AU/mL activity level. In contrast, no bacteriocin adsorption was reported for plantaricin ST31 (Todorov et al., 1999), bacteriocins ST194BZ, ST414BZ and ST644BZ (Todorov & Dicks, 2006a, 2006c), bacteriocins ST26MS and ST28MS (Todorov & Dicks, 2005b) or bozacin B14 to the producer strains (Ivanova, Kabadjova, Pantev, Danova, & Dousset, 2000).

3.7. Growth dynamics of Lactobacillus plantarum ST16Pa, production of bacteriocin and changes of pH

Similar growth and bacteriocin production were observed when the strain *L. plantarum* ST16Pa was cultured for 24 h in MRS broth at 25 °C or 30 °C. In all tested temperatures, the activity against *E. faecalis* ATCC 19443 was 51 200 AU/mL. This is in agreement with the results obtained for plantaricin ST31 (Todorov et al., 1999), bacteriocins ST26MS and ST28MS (Todorov & Dicks, 2005b) and mundticin ST4SA (Todorov & Dicks, 2009b). Based on these results, all further experiments were conducted at 25 °C.

The maximal bacteriocin ST16Pa production (25 600 AU/mL) was recorded after 19–30 h of growth in MRS broth (Fig. 4), with a decrease in activity to 12 800 AU/mL observed after 33 h of growth. During the same period of growth, the pH of the medium decreased from 6.31 to 3.71. The cell density (OD_{600nm}) increased from 0.22 to 9.13 in 22 h and stayed more-or-less constant during the following 14 h (Fig. 4). Low levels of bacteriocin ST16Pa activity (approximately 800 AU/mL) were recorded after 3 h of growth in MRS broth at 26 °C.

Several studies have shown that bacteriocin production is dependent on the biomass. Todorov and Dicks (2005c) reported that optimal levels of plantaricin ST194BZ, produced by *L. plantarum* ST194BZ, were obtained in growth media that supported high biomass production, such as MRS. A similar bacteriocin production profile was reported for bacteriocin ST311LD, produced by *E. faecium* ST311LD isolated from fermented olives, in which maximal bacteriocin production was reported after 20 h growth in MRS broth, followed by a decrease in activity in the following 5 h (Todorov & Dicks, 2005a). The decrease in activity of bacteriocin ST16Pa at the end of the monitored period could be explained by the degradation of the bacteriocin by extracellular proteolytic enzymes. Similar decreases have also been observed for bacteriocins produced by *E. faecium* ST311LD (Todorov & Dicks, 2005a), *E. mundtii* ST4SA (Todorov & Dicks, 2009b) and *P. acidilactici* NRRL B5627 (Anastasiadou, Papagianni, Filiousis, Ambrosiadis, & Koidis, 2008). From a metabolic point of view, this trend would be a characteristic of primary metabolite production.

3.8. Determination of aproximative molecular weight of bacteriocin ST16Pa by SDS-PAGE

The molecular weight of the bacteriocin ST16Pa was determined to be near 6.5 kDa, as determined by tricine-SDS-PAGE (Fig. 5), and it is within the range of most bacteriocins reported for other *L. plantarum*.

3.9. Optimization of bacteriocin production

Bacteriocin ST16Pa was produced at 25 600 AU/mL when strain ST16Pa was grown in MRS broth supplemented with 20 g/L of glucose (Table 5). The same level of activity was recorded when glucose was replaced by 20 g/L of maltose (Table 5). An increase in bacteriocin ST16Pa production was recorded in the presence of 30 g/L glucose (102 400 AU/mL), 50 g/L of glucose (51 200 AU/mL) or 20 g/L of mannose (51 200 AU/mL). On the other hand, glucose at 5 g/L or 10 g/L and fructose, lactose and sucrose at 20 g/L as the sole carbon

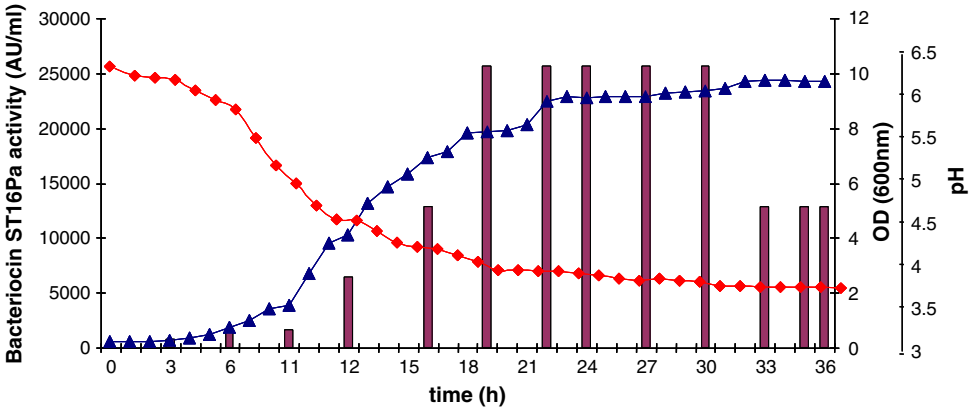

Fig. 4. Production of bacteriocin ST16Pa in MRS broth (pH 6.5 at 26 °C). Antimicrobial activity is presented as AU/mL (bars) against *E. faecalis* ATCC 19443. Changes in optical density (▲) and pH (♦) are indicated. Standard deviation recorded from three repeats was less that 5% and is not indicated.

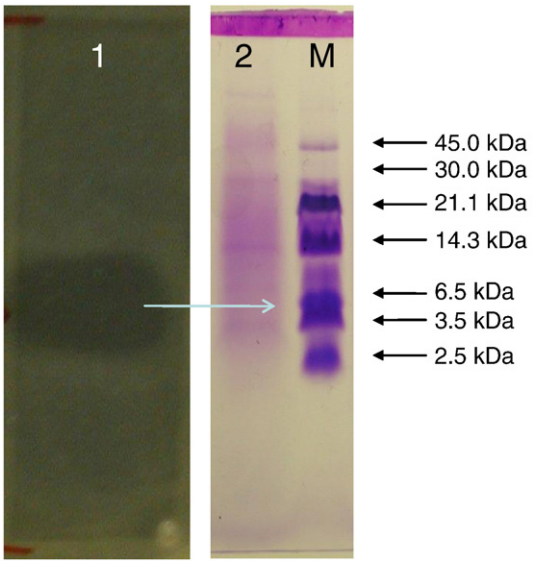

Fig. 5. Tricine-SDS-PAGE of bacteriocin ST16Pa. Lane 1: zone of growth inhibition, corresponding to the position of the peptide band representing bacteriocin ST16Pa. Lane 2: peptide band stained with Coomassie Blue R250 (40% ammonium sulfate saturated), lane M: molecular weight marker, The gel was covered with viable cells of *Enterococcus faecium* ATCC 19443 (approx. 10^6 CFU/mL), imbedded in BHI supplemented with 1% agar. Incubation was performed at 26 °C for 24 h.

source, lowered bacteriocin ST16Pa yields, suggesting that the glucose moiety of sucrose is favored for production (Table 5). Low levels of activity were recorded when the cells were grown in the presence of the same concentration of gluconate (800 AU/mL). These results show that the production of bacteriocin ST16Pa is stimulated by the presence of glucose and mannose. Taking into consideration that glucose forms part of sucrose present in different fruits and the potential costs of industrial application and the production of bacteriocin ST16Pa, we have investigated the effect of different concentration of glucose instead of mannose, the latter being more expensive to obtain. Glucose was shown to be a favorable carbohydrate for production of plantaricin UG1 (Enan et al., 1996), plantaricin KW30 (Kelly et al., 1996), plantaricin ST31 (Todorov et al., 2000) and bacteriocins ST202Ch and ST216Ch (Todorov, Ho, et al., 2010).

Of all of the nitrogen sources tested (Table 5), meat extract or the combination of yeast extract and tryptone yielded an activity of 25 600 AU/mL of bacteriocin ST16Pa, compared with the control (25 600 AU/mL using tryptone, meat extract and yeast extract). A combination of meat extract and tryptone (7.5 and 12.5 g/L), meat extract and yeast extract (10.0 and 10.0 g/L) or yeast extract as a single organic nitrogen source (20.0 g/L) yielded 12 800 AU/mL, a 50% reduction in the bacteriocin activity (Table 5). Supplementation with tryptone (20.0 g/L) increased the bacteriocin production up to 51 200 AU/mL, suggesting that tryptone is required for optimal bacteriocin ST16Pa production (Table 5). These results are in agreement with those reported for plantaricin 423 produced by *L. plantarum* 423. The highest production of plantaricin 423 was obtained in MRS broth supplemented with bacteriological peptone, followed by casamino acid, tryptone and meat extract (Verellen et al., 1998). Stimulation of bacteriocin production by meat extract has also been reported for pediocin AcH Bhunia et al. (1988)) and helveticin J (Joerger & Klaenhammer, 1986).

MRS medium, supplemented with different glycerol concentrations (ranging from 1.0 g/L to 5.0 g/L) did not affect bacteriocin ST16Pa production (25 600 AU/mL). In contrast, when increased concentrations of glycerol (10.0 g/L and 20.0 g/L) were added to the MRS broth, a negative effect on bacteriocin ST16Pa production was observed (Table 5). It was previously shown that glycerol had a negative effect on the production of plantaricin ST31, where glycerol at concentrations of 2.0 g/L or higher resulted in lower antimicrobial activities (Todorov et al., 2000). A similar effect of glycerol was observed in the production of bacteriocin ST202Ch and bacteriocin ST216Ch (Todorov, Ho, et al., 2010). Glycerol is not used as a carbon source and the decrease in bacteriocin production may be due to changes in osmotic stress (Todorov & Dicks, 2005a, 2005b, 2005c, 2005d). These results suggest the need for further research to

Table 5
Effect of the medium components on the production of bacteriocin by *Lactobacillus plantarum* ST16Pa at 24 h, 26 °C against test microorganism *Enterococcus faecalis* ATCC 19433.

Medium component variation from MRS	pH	OD_{600nm}	AU/ml
Glucose 20 g/l	3.87	8.09	25 600
Fructose 20 g/l	4.19	4.20	1600
Lactose 20 g/l	4.39	5.74	12 800
Mannose 20 g/l	3.92	6.99	51 200
Maltose 20 g/l	4.00	10.30	25 600
Sucrose 20 g/l	3.92	10.74	12 800
Gluconate 20 g/l	5.53	2.06	800
Glucose 5.0 g/l	4.44	2.96	200
Glucose 10.0 g/l	4.16	3.52	400
Glucose 20.0 g/l	3.87	8.04	25 600
Glucose 30.0 g/l	3.63	7.11	102 400
Glucose 50.0 g/l	3.61	8.12	51 200
Tryptone 20 g/l	3.97	6.27	51 200
Meat extract 20 g/l	3.89	8.28	25 600
Yeast extract 20 g/l	3.90	9.58	12 800
Tryptone and meat extract 12.5 + 7.5 g/l	3.87	6.82	12 800
Tryptone + yeast extract 12.5 + 7.5 g/l	3.82	8.55	25 600
Meat extract + yeast extract 10.0 + 10.0 g/l	3.84	8.07	12 800
Tryptone + yeast extract + meat extract 10.0 + 5.0 + 5.0 g/l	3.87	8.09	25 600
Glycerol 0 g/l	3.87	8.09	25 600
Glycerol 1.0 g/l	3.84	8.21	25 600
Glycerol 2.0 g/l	3.85	7.03	25 600
Glycerol 5.0 g/l	3.90	8.32	25 600
Glycerol 10.0 g/l	3.85	8.51	12 800
Glycerol 20.0 g/l	3.85	7.91	12 800
KH_2PO_4 2.0 g/l	3.80	8.41	12 800
KH_2PO_4 5.0 g/l	3.79	8.08	25 600
KH_2PO_4 10.0 g/l	3.83	8.54	51 200
KH_2PO_4 20.0 g/l	3.87	8.22	51 200
K_2HPO_4 2.0 g/l	3.87	8.09	25 600
K_2HPO_4 5.0 g/l	3.89	8.92	25 600
K_2HPO_4 10.0 g/l	4.17	7.44	12 800
K_2HPO_4 20.0 g/l	5.03	2.65	800
Vitamin C 1.0 mg/l	3.84	8.75	12 800
Vitamin B_1 1.0 mg/l	3.90	8.86	51 200
Vitamin B_{12} 1.0 mg/l	3.82	8.77	51 200
DL-6,8-thioctic acid 1.0 mg/l	3.81	9.11	25 600
pH 4.5	3.50	4.71	6400
pH 5.0	3.65	7.86	12 800
pH 5.5	3.72	8.78	12 800
pH 6.0	3.76	8.90	25 600
pH 6.5	3.87	8.09	25 600
$MgSO_4$ free	3.80	9.53	12 800
$MgSO_4$	3.87	8.09	25 600
$MnSO_4$ free	4.20	3.53	800
$MnSO_4$	3.87	8.09	25 600
Tri-ammonium citrate 5.0 g/l	3.87	8.09	25 600
Tri-ammonium citrate 10.0 g/l	4.17	8.61	12 800
Tri-ammonium citrate 0.0 g/l	3.73	7.75	25 600
Twen 80 free MRS	3.93	9.05	25 600
Tween 80 1.0 g/l	3.87	8.09	25 600
Tween 80 2.0 g/l	3.95	9.05	51 200
Tween 80 5.0 g/l	3.86	10.17	51 200
BHI 37 g/l	5.09	0.77	–
M17	6.27	0.89	–
Skim Milk 100 g/l	4.62	X	–
Skim Milk 20 g/l	4.71	X	–
Chesse whay 100 g/l	4.52	X	–
Chesse whay 20 g/l	5.07	X	–

elucidate the mechanisms by which glycerol could affect bacteriocin production.

Little is known about the influence of potassium ions on the production of bacteriocins (Todorov & Dicks, 2004a, Todorov, Ho, et al., 2010). Levels of 10.0 g/L and 20.0 g/L KH_2PO_4 increased the production of bacteriocin ST16Pa (51 200 AU/mL), but 2.0 g/L of the same salt reduced bacteriocin production by 50% (Table 5). On the other hand, low levels of K_2HPO_4 (2.0 g/L and 5.0 g/L) resulted in a 25 600 AU/mL bacteriocin ST16Pa production whereas 10.0 g/L K_2HPO_4 resulted in a decrease to 12 800 AU/mL and 20.0 g/L K_2HPO_4 to only 800 AU/mL bacteriocin ST16Pa (Table 5). This variation in production cannot be due to pH changes caused by higher or lower potassium levels, since all media were adjusted to pH 6.5 before inoculation. In the case of plantaricin UG1, 7.0 g/L of K_2HPO_4 resulted in an increased activity (Enan et al., 1996). The optimal level of K_2HPO_4 recorded for plantaricin ST31 was between 2.0 g/L and 5 g/L (Todorov et al., 2000). On the other hand, no difference in antibacterial activity was recorded when *L. plantarum* ST194BZ was grown in the presence of 2.0 g/L of KH_2PO_4 and 2.0 g/L of K_2HPO_4 (Todorov & Dicks, 2005c).

The presence of cyanocobalamin and thiamine (0.001 g/L) stimulated bacteriocin ST16Pa production up to 51 200 AU/mL (Table 5). However, L-ascorbic acid had a negative effect and DL-6.8-thiocitic acid did not seem to affect bacteriocin ST16Pa production (Table 5). In these experiments, cell growth was comparable with the growth recorded in non-supplemented MRS broth. Bacteriocin ST8KF production was stimulated by cyanobalamin and thiamine, but not by L-ascorbic acid and DL-6,8-thioctic acid (Powell et al., 2007). In contrast, no effect was recorded using the same vitamins for bacteriocin ST202Ch and bacteriocin ST216Ch production, except in the presence of DL-6,8-thiocitic acid, which reduced bacteriocin ST216Ch production (Todorov, Ho, et al., 2010).

Bacteriocin ST16Pa production was also lower in the presence of 10.0 g/L tri-ammonium citrate (12 800 AU/mL), compared to growth when 5.0 g/L was used or in its absence (Table 5). A similar effect using tri-ammonium citrate was found for bacteriocin ST8KF production (Powell et al., 2007). Bacteriocin ST202Ch and bacteriocin ST216Ch production was also lower in the absence or presence 10.0 g/L tri-ammonium citrate, compared to growth in 2.0 g/L or 5.0 g/L (Todorov, Ho, et al., 2010).

Reduction in the production of bacteriocin ST16Pa (up to 50%) was recorded with the exclusion of $MgSO_4$ from the MRS medium; omission of $MnSO_4$ in this broth had an even more drastic effect on bacteriocin ST16Pa production (Table 5). Bacteriocin ST8KF production was found to be reduced in the absence of $MgSO_4$, while no production of bacteriocin was observed in the absence of $MnSO_4$ (Powell et al., 2007). No change in the production of bacteriocin ST202Ch was recorded with the exclusion of $MgSO_4$ from the MRS medium. Absence of $MgSO_4$ from the media formula had a negative effect on bacteriocin ST216Ch production. Exclusion of $MnSO_4$ from MRS medium had a negative effect on both the production of bacteriocin ST202Ch and bacteriocin ST216Ch (Todorov, Ho, et al., 2010).

Bacteriocin ST16Pa was produced at the same levels in MRS broth with an initial pH ranging from 6.0 to 6.5 (25 600 AU/mL). Much lower growth was recorded for this strain at pH 4.5, corresponding to a lower production of bacteriocin ST16Pa (Table 5). This shows that the initial pH of MRS broth plays an important role in the production of bacteriocin ST16Pa. Similar results have been reported for other bacteriocins produced by other strains of *L. plantarum* (Daeschel et al., 1990; Kelly et al., 1996; Todorov & Dicks, 2005b; Todorov, Ho, et al., 2010, Todorov et al., 2000). From these results and other reported data (Daeschel et al., 1990; Kelly et al., 1996; Todorov & Dicks, 2005b; Todorov, Ho, et al., 2010; Todorov et al., 2000) it can be concluded that an initial pH of at least 6.0 is required for optimal growth of *L. plantarum* ST16Pa as well as for bacteriocin production.

The absence of Tween 80 from the MRS broth formula had a negative effect on the production of bacteriocin ST16Pa (Table 5). These results are discouraging, since Tween 80 free MRS medium is required for most purification protocols of bacteriocins using an HPLC purification steps. The presence of 2.0 g/L or 5.0 g/L Tween 80 in the MRS broth stimulated bacteriocin ST16Pa production up to 51 200 AU/mL (Table 5). The absence of Tween 80 from the MRS media formula also has a negative effect on the production of both bacteriocin ST202Ch and bacteriocin ST216Ch. Increasing the amount of Tween 80 in MRS broth up to 5.0 g/l had no beneficial effect on bacteriocin ST202Ch production. However, concentrations of Tween 80 up to 2.0 g/L resulted in an increase in the production of bacteriocin ST216Ch, whereas the presence of 5.0 g/L had a negative effect (Todorov, Ho, et al., 2010). Unfortunately, when *L. plantarum* was cultured in BHI (Oxoid), M17 (Oxoid), skim milk (20 g/L or 100 g/L) cheese whey (20 g/L or 100 g/L), no bacteriocin ST16Pa activity was detected even if the strain showed good adaptation to these media and had a reasonable growth rates.

To the best of our knowledge, this is the first report on detection of *L. plantarum* in papaya. Based on the results, including bacteriocin ST16Pa activity and mode of action, we can conclude that bacteriocin producing *L. plantarum* ST16Pa might be useful in the design of novel functional foods.

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