

LACTOBACILLUS PLANTARUM ST16Pa – ARE WE READY TO USE IT AS BIO-PROTECTIVE CULTURE?

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Abstract

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Lactobacillus plantarum ST16PA was isolated from papaya and previously have been shown that 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. In addition *Lb. plantarum* ST16Pa presented a good potential to be consider as a probiotic candidate based on the genetic and physiological tests.

PCR analysis of DNA from *Lb. plantarum* ST16Pa was generated positive results for presence of nisin and enterocin P genes and no evidences for presence of pediocin PA-1, plantaricin S, plantaricin W, plantaricin NC8, enterocin A, enterocin B or enterocin L50B have been obtained. Based on the molecular size (6.5 kDa as determined by Tricin-SDS-PAGE) of the expressed bacteriocin, most probably *Lb. plantarum* ST16Pa express bacteriocin different from nisin or enterocin P.

Semi-purified bacteriocin was presenting a very high activity against *Listeria monocytogenes* (102 400 AU ml⁻¹), *Pseudomonas aeruginosa* (25 600 AU ml⁻¹) and *Enterococcus faecalis* (102 400 AU ml⁻¹). However, when semi-purified bacteriocin have been tested for cytotoxicity, CC₅₀ > 1600 µg ml⁻¹ have been recorded. In addition, *Lb. plantarum* ST16Pa generated positive PCR results on the DNA level for *gelE* (gelatinase), *hyl* (hyaluronidase), *asa1* (aggregation substance), *ace* (adhesion of collagen) and *tdc* (tyrosine decarboxylase), a high virulence profile when been examined for presence of virulence factors.

Key words: *Lactobacillus plantarum*, bacteriocin, virulence factors, cytotoxicity

Introduction

LAB consist of a promising group of bacteriocin-producing microorganisms due to their GRAS (generally recognized as safe) status, which indicates their safe and easy application as food preservatives (Nishie et al., 2012). At the present time, only nisin and pediocin PA-1 are com-

mercially authorized worldwide depending on local law regulation.

Bacteriocins are generally low molecular weight proteins that gain entry into target cells by binding to cell surface receptors. Their bactericidal mechanism varies and may include pore formation, degradation of cellular DNA, disruption through specific cleavage of 16S rDNA, and inhibition

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of peptidoglycan synthesis (James et al., 1991; De Vuyst and Vandamme, 1994).

Lactobacillus plantarum ST16Pa was isolated from papaya and been identified based on biochemical tests, PCR with Species-specific primers and 16S rRNA sequencing. Previously have been shown that *Lb. 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. In addition *Lb. plantarum* ST16Pa presented a good potential to be consider as a probiotic candidate based on the genetic and physiological tests (Todorov et al., 2011; Todorov et al., 2012).

Although many studies have evaluated a wide range of bacteriocins produced by *Lb. plantarum* strains that are active against specific pathogens, few have been studied in a complete and organized manner (Todorov, 2009). A deeper characterization of the safety of producer and its bacteriocins is essential for their successful application in foods

The aim of this work was to characterize the safety of bacteriocinogenic *Lb. plantarum* ST16Pa in terms of presence of genes encoding virulence factors and assessment of cytotoxicity potential.

Material and Methods

Strains and media: *Lb. plantarum* ST16Pa, a bacteriocinogenic strain was isolated from fresh papaya fruit (Todorov et al., 2011) and *L. monocytogenes*, *Pseudomonas aeruginosa* and *Enterococcus faecalis* a test microorganism were cultured in MRS broth and BHI broth (Difco, Detroit, MI, USA), respectively at 30°C and stored at -80°C, in presence of 20% glycerol.

Bacteriocin test and partial purification: Cell-free supernatant obtained from a 24 h culture of *L. plantarum* ST16Pa, prepared as described by Todorov et al. (2011). Bacteriocin was precipitated by addition of ammonium sulfate to the cell-free supernatant to obtain 60% saturation and stirred for 4 h at 4°C. After centrifugation for 1 h at 12000 g at 4°C, the resulting pellet was re-suspended in 100 ml of 25 mM ammonium acetate buffer (pH 6.5), and loaded on SepPak C₁₈ cartridge (Waters, Millipore, MA, USA), and bacteriocin eluted with 60% isopropanol in 25 mM ammonium acetate buffer (pH 6.5). The active fraction was dried under vacuum (Speed-Vac, Savant, France) and the bacteriocin fraction was re-suspended in sterile distilled water and filtered using 0.22 µm pore size filter units (Waters).

Detection of genes: The presence of genes encoding for known bacteriocins produced by *Lb. plantarum* (plantaricin S, plantaricin NC8, plantaricin W, pediocin PA-1, nisin, en-

terocin A, enterocin B or enterocin L50B, enterocin P) was evaluated by PCR with specific primers for these genes. Genomic DNA was extracted from *L. plantarum* ST16Pa using a DNA extraction kit (Zymo Research, USA). PCR reactions were conducted according to Martinez et al. (2013) and Moraes et al. (2012).

Lb. plantarum ST16Pa was tested for virulence genes *gelE* (gelatinase), *hyl* (hyaluronidase), *asa1* (aggregation substance), *esp* (enterococcal surface protein), *cytA* (cytolysin), *efaA* (endocarditis antigen), *ace* (adhesion of collagen), *vanA* and *vanB* (both related to vancomycin resistance), and genes for amino acid decarboxylases: *hdc1* and *hdc2* (both related to histidine decarboxylase), *tdc* (tyrosine decarboxylase), and *odc* (ornithine decarboxylase), using PCR protocols of Moraes et al. (2012). Primers used for assessment of presence of virulence genes are presented in Table 1.

Cytotoxicity: Cytotoxicity was assessed using monkey kidney Vero cells as previously described by Wachsman et al. (2003). The results were calculated by regression analysis and expressed as CC₅₀, which corresponds to the concentration of bacteriocin (µg ml⁻¹) needed to lower the cell viability to 50%.

Results and Discussion

PCR analysis of DNA from *Lb. plantarum* ST16Pa was generated positive results for presence of nisin and enterocin P genes and no evidences for presence of pediocin PA-1, plantaricin S, plantaricin W, plantaricin NC8, enterocin A, enterocin B or enterocin L50B have been obtained. Based on the molecular size (6.5 kDa as determined by Tricin-SDS-PAGE, Todorov et al., 2011) of the expressed bacteriocin, most probably *Lb. plantarum* ST16Pa express bacteriocin different from nisin or enterocin P. The obtained amplicons were purified, sequenced and displayed high homology (> 98%) when compared to sequences previously deposited in the GenBank for nisin and enterocin P. However, the detection of the two genes does not guarantee that *Lb. plantarum* ST16Pa expresses one of these two bacteriocins or even a combination of both. This needs to be confirmed after purification and determination of their molecular masses by mass spectrometry and the amino-acid sequences of the active bacteriocin(s). In our best of knowledge this is a first report on presence of enterocin P in strain of *Lb. plantarum*. Previously have been reported of detection of nisin genes in *E. faecium* (Todorov et al., 2010).

Bacteriocin produced by *Lb. plantarum* ST16Pa has been partially purified by ammonium sulphate precipitation and hydrophobic chromatography on SepPakC₁₈ column. Semi-purified bacteriocin was presenting a very high

activity against *Listeria monocytogenes* (102 400 AU ml⁻¹), *Pseudomonas aeruginosa* (25 600 AU ml⁻¹) and *Enterococcus faecalis* (102 400 AU ml⁻¹). However, when semi-purified bacteriocin have been tested for cytotoxicity, CC₅₀ > 1600 µg ml⁻¹ have been recorded (CC₅₀: Compound concentration required to reduce cell viability, using stationary-phase monolayers of Vero cells, by 50% after 24 h of incubation at 37°C). Possibly, application of this bacteriocin in semi-purified or purified preparations in foods will not have a negative effect for the consumers. Determination of the cytotoxicity is an important parameter in the characterization of bacteriocins in order to recommend their application for food biopreservation or as an alternative to antibiotics in medical practice.

Only a few bacteriocins have been previously characterized regarding their cytotoxicity (Wachsmann et al., 2003; Todorov et al., 2005; Todorov et al., 2008; Todorov et al., 2010).

In addition, *Lb. plantarum* ST16Pa generated positive PCR results on the DNA level for *gelE* (gelatinase), *hyl* (hyaluronidase), *asa1* (aggregation substance), *ace* (adhesion of collagen) and *tdc* (tyrosine decarboxylase), a high virulence profile when been examined for presence of virulence factors (Table 1).

In general, the observed frequency of positive results for the virulence factors studied in *Lb. plantarum* ST16Pa was lower than that reported in other studies on *Enterococcus* isolated from foods (Gomes et al., 2008; Barbosa et al.,

Table 1

Primers sequences utilized in the investigation of presence/absence for virulence factors, vancomycin resistance and biogenic amine production

	<i>Lb. plantarum</i> ST16Pa	Primers (5' – 3')
Virulence genes*		
<i>gelE</i>	+	TATGACAATGCTTTTTGGGAT AGATGCACCCGAAATAATATA
<i>hyl</i>	+	ACAGAAGAGCTGCAGGAAATG GACTGACGTCCAAGTTTCCAA
<i>asa1</i>	+	GCACGCTATTACGAACTATGA TAAGAAAGAACATCACCACGA
<i>esp</i>	–	AGATTTTCATCTTTGATTCTTG AATTGATTCTTTAGCATCTGG
<i>cylA</i>	–	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT
<i>efaA</i>	–	GCCAATTGGGACAGACCCTC CGCCTTCTGTTCTTCTTTGGC
<i>ace</i>	+	GAATTGAGCAAAAAGTTCAATCG GTCTGTCTTTTCACTTGTTTC
Antibiotic resistance		
<i>VanA</i>	–	TCTGCAATAGAGATAGCCGC GGAGTAGCTATCCCAGCATT
<i>VanB</i>	–	GCTCCGCAGCCTGCATGGACA ACGATGCCGCCATCCTCCTGC
Biogenic amines		
<i>hdc1</i>	–	AGATGGTATTGTTTCTTATG AGACCATAACCATAACCTT
<i>hdc2</i>	–	AAATCNTTYGAYTTYGARAARGARG ATNGGNGANCCDATCATYTRTRGNCC
<i>tdc</i>	–	GAYATNATNGGNATNGGNYTNGAYCARG CCRTARTCNGGNATAGCRAARTCNTRTG
<i>odc</i>	+	GTNTTYAAAYGCNGAYAARCANTAYTTYGT ATNGARTTNAAGTTTCRCAYTTYTCNGG

Positive results (+) for genes for virulence and biogenic amines in *Lb. plantarum* ST16Pa. * *gelE* (gelatinase), *hyl* (hyaluronidase), *asa1* (aggregation substance), *esp* (enterococcal surface protein), *cylA* (cytolysin), *efaA* (endocarditis antigen), *ace* (adhesion of collagen), *vanA* and *vanB* (vancomycin resistance), *hdc1* and *hdc2* (histidine decarboxylase), *tdc* (tyrosine decarboxylase), and *odc* (ornithine decarboxylase)

2010; Eaton et al., 2001) and also in comparison to studies with clinical isolates (Eaton et al., 2001; Franz et al., 2001; de Souza 2003). Despite being less relevant in food isolates, the determination of virulence factors in LAB by molecular and phenotypic procedures is important due to the risk of genetic transfer since these genes are usually coded by conjugative plasmids (Franz et al., 2001).

Conclusions

Besides all beneficial properties studied for various LAB, a special attention need to be pay on the possible presence of virulence factors, production of biogenic amines and antibiotic resistance. This virulence determinants have been well detected and studied in Enterococci and Streptococci, however, in last few years report on presence of virulence factors in otherwise GRAS Lactobacilli have been showing the potential upcoming problems. Horizontal gene transfer of virulence factors between pathogenic and LAB, including probiotics is a highly possible scenario in case of uncontrolled application of probiotics.

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