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Safety of *Lactobacillus plantarum* ST8Sh and Its Bacteriocin

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Abstract Total DNA extracted from *Lb. plantarum* ST8Sh was screened for the presence of more than 50 genes related to production of biogenic amines (histidine decarboxylase, tyrosine decarboxylase, and ornithine decarboxylase), virulence factors (sex pheromones, gelatinase, cytolysin, hyaluronidase, aggregation substance, enterococcal surface protein, endocarditis antigen, adhesion of collagen, integration factors), and antibiotic resistance (vancomycin, tetracycline, erythromycin, gentamicin, chloramphenicol, bacitracin). *Lb. plantarum* ST8Sh showed a low presence of virulence genes. Only 13 genes were detected (related to sex pheromones, aggregation substance, adhesion of collagen, tetracycline, gentamicin, chloramphenicol, erythromycin, but not to vancomycin, and bacitracin) and may be considered as indication of safety for application in fermented food products. In addition, interaction between *Lb. plantarum* ST8Sh and drugs from different groups were determined in order to establish possible application of the strain in combination with commercial drugs. Cytotoxicity of the semi-purified bacteriocins produced by *Lb. plantarum* ST8Sh was depended on applied concentration—highly cytotoxic when applied at 25 μg/mL and no cytotoxicity at 5 μg/mL.

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Introduction

Research on lactic acid bacteria (LAB) with probiotic potential is focused on different areas, including re-establishing of the gastrointestinal (GIT) microbiota, prevention and treatment of GIT disorders, stimulation of the immune system, treatment of skin diseases, prevention of some types of cancer, treatment of *Helicobacter pylori,* and involvement in oral health [1]. Parallel to a high number of reports dedicated to the study of beneficial properties of probiotic LAB, a limited number of studies focus on the safety aspects of these strains. Several *Lactobacillus* spp. have been granted GRAS status and are considered as safe for human and other animal applications. Different *Lactobacillus* spp. are associated with the traditional production of different fermented food products from plant, meat, and dairy origin. However, some clinical cases were described and linked to some strains of *Lactobacillus* spp. typical of various fermented foods [2–4].

With the better understanding of the microbial interactions, including horizontal gene transfer and the genetic basis of potential virulence, it is necessary to re-evaluate the perspective related to the safety issues of *Lactobacillus* spp. From traditional point of application, some strains can be considered as safe to be used as starter and beneficial cultures; however, they need to be carefully examined for the potential presence of virulence factors, and to take into account that most probably these strains will be present in high viable cell numbers when applied as probiotics/beneficial cultures. Considering all this, safety aspects of the probiotic LAB need to be carefully examined on a strain by strain basis, and the risk of delivering virulence factors to the host should be excluded.

It is important to check for antibiotic resistance, as probiotic LAB can act as potential reservoirs of (transferable) resistance genes that can result in multidrug resistant strains [5]. Probiotics are frequently prescribed to the consumers under treatment for a variety of illnesses as an accompanying therapy. However, the beneficial effects of the probiotic strain may be reduced by possible interactions with the medication used by these patients/consumers. An important issue is that the interaction between medications or antibiotics and probiotic bacteria in the GIT depends on their concentration in this environment [6, 7]. In this regard, determination of Minimal Inhibitory Concentration (MIC) values in the interaction between probiotic LAB and drugs is an essential aspect in the evaluation of their efficacy. Special attention needs to be given to drugs for treatment of chronic diseases, since, due to their long-term application, they may accumulate in the GIT and affect the viability of probiotic LAB [8].

Production of antimicrobial peptides (bacteriocins) may be a complimentary characteristic for probiotic LAB. Bacteriocins can be involved in the reduction of pathogenic bacteria from the GIT provided these pathogens are sensitive to the produced bacteriocins. Some authors are even suggesting application of bacteriocins in the treatment of some pathogens, including multidrug resistant *Staphylococcus aureus*, *Mycobacterium* spp., and some viral and fungal infections in parallel to applied antibiotics [9]. However, potential cytotoxicity of the bacteriocins may constitute a safety risk and needs more attention. Toxicological studies have showed that nisin intake does not cause toxic effects to the human body with an estimated lethal dose (LD50) of 6950 mg/kg, which is similar to that of salt, when administered orally [10]. In general, some authors have associated LD50 of bacteriocins with digestive enzymes capable of rapidly inactivating these substances such as trypsin and chymotrypsin produced in the pancreas [11–13]. However, different bacteriocins can have a high variation in the molecular mass and amino acid sequence, including even the presence of some non-protein subunits involved in their activity [14]; these features may interfere with their cytotoxicity. Thus, the safety evaluation of each new bacteriocin, both as candidate for a medical or biopreservation application needs to be performed.

In this work, we explore safety aspects of *Lb. plantarum* ST8Sh, a strain isolated from fermented Bulgarian salami "Shpek" [15, 16] related to the presence of genes related to virulence, antibiotic resistance and production of biogenic amines, physiological tests related to expression of some virulence factors, partial purification of expressed bacteriocin/s and determination of its/their cytotoxicity, and inhibitory interactions between *Lb. plantarum* ST8Sh and some selected commercial drugs.

Material and Methods

Strains and Media

Lb. plantarum ST8Sh, a bacteriocinogenic strain isolated from Bulgarian salami "Shpek" [15] and *Listeria monocytogenes* ATCC 7644, *L. monocytogens* ScottA, *Enterococcus faecalis* ATCC 19443, and *Lb. sakei* ATCC 15521 as test microorganisms, were cultured in MRS broth and BHI broth (Difco, Detroit, MI, USA), respectively, incubated at 30 °C and stored at −80 °C, in the presence of 20% glycerol.

Bacteriocin Production and Partial Purification

Lb. plantarum ST8Sh was cultured in MRS broth at 37 °C for 24 h. Cell-free supernatant was obtained after centrifugation at 5000 g for 10 min at 4 °C; pH was corrected to 6.0–6.5 with 1 M NaOH and treated for 10 min at 80 °C. 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 phosphate buffer (pH 6.5), and loaded on SepPak C_{18} cartridge (Waters, Millipore, MA, USA), and bacteriocin eluted with 60 and 80% iso-propanol in 25 mM phosphate 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).

Bacteriocin Test

Titer of the expressed bacteriocin was determined as described by Todorov et al. [15]. The cell free supernatant or semi-purified bacteriocin was serially 2× diluted in 100 mM phosphate buffer pH 6.5, and 10 μL from each dilution has been spotted on the surface of BHI supplemented with 0.7% agar plated with 10^5 CFU/mL of *L. monocytogenes* ATCC 7644, *L. monocytogens* ScottA, *E. faecalis* ATCC 19443, or *Lb. sakei* ATCC 15521 (final concentration). The highest dilution presenting an inhibition zone larger than 2 mm was considered as basis for calculation of Arbitrary Units per mL (AU/ml) taking in consideration the volume of the deposited material and dilution factor.

Cytotoxicity of the Expressed Bacteriocins/s

Human hepatocellular carcinoma cell line (Huh7.5) (5×10^3 cells/well) were seeded into a 96-well plate and incubated for 24 h prior to treatment with semi-purified bacteriocin produced by *Lb. plantarum* ST8Sh. Then the supernatants were removed and substituted by 100 μL of DMEM (Dulbecco's Modified Eagle Medium,

TermoFisher Scientific) supplemented with two different concentrations of semi-purified bacteriocin ST8Sh. After 48 h, culture media was removed and a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 100.0 μL) was added to each well containing the samples. Plate was incubated for 30 min at 37 °C, and MTT crystals were solubilized with 100 μL of DMSO (Dimethyl sulfoxide, Sigma), and light absorbance was measured at 570 nm. Cytotoxicity values are a percentage of the absorbance of the treated sample compared to the control (media without bacteriocin).

Detection of Virulence Genes

Total DNA from *Lb. plantarum* ST8Sh was isolated using the ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA) following the instructions of the manufacturer. All PCR reactions were performed using the GeneAmp® PCR Instrument System 9700 (Applied Biosystems, Foster City, USA). *Lb. plantarum* ST8Sh was tested for virulence genes *gel*E (gelatinase), *hyl* (hyaluronidase), *asa*1 (aggregation substance), *esp.* (enterococcal surface protein), *cyl*A (cytolisin), *efa*A (endocarditis antigen), *ace* (adhesion of collagen), *van*A, *van*B, *van*C1, *van*C2, *van*C2/C3 (related to vancomycin resistance), *erm*A, *erm*B, *erm*C (related to erythromycin resistance), *tet*K, *tet*L, *tet*M, *tet*O, *tet*S (related to tetracycline resistance), *aac*(6′)-Ie-*aph*(2″)-Ia (related gentamycin resistance), *aph*(3′)-IIIa, *ant*(4′)-Ia, *aph*(2″)-Id, *aph*(2″)-Ic, *aph*(2″)-Ib, *ant*(6)-Ia (related to aminoglycosides type antibiotics resistance), *cat*A (chloraphenicol resistance), *bcr*B, *bcr*D, *bcr*R (related to bacitracin resistance), *ccf*, *cob*, *cpd* (related to sex pheromones), *spr*E (serine protease), *int*, *int*Tn (transposom related) and genes for amino acid decarboxylases: *hdc*1 and *hdc*2 (both related to histidine decarboxylase), *tdc* (tyrosine decarboxylase), and *odc* (ornithine decarboxylase), using PCR protocols of Moraes et al. [17] and Fortuna et al. [18]. Primers used for assessment of the presence of virulence genes are presented in Table 1.

Virulence Factors—Phenotypical Tests

A culture of *Lb. plantarum* ST8Sh was subjected to phenotypic tests to identify its virulence activity, according Barbosa et al. [19]. All tests were performed with different time and incubation temperature combinations in order to verify the production of the virulence factors in diverse conditions, detailed in the following, in three independent trials.

Gelatinase production was verified by spotting 1 μL aliquots of the 24 h culture onto the surface of Luria Bertani agar (LB; Becton, Dickinson and Company - BD, Franklin Lakes, NJ, USA) supplemented with 3% (*w*/*v*) gelatin (BD) and incubated at 37 and 42 °C for 48 h, at 25 °C for 72 h, and at 10 and 15 °C for 10 days. After incubation, the plates were maintained at 4 °C for 4 h, and the hydrolysis of gelatin was recorded by the formation of opaque halos around the colonies [20].

Hemolytic activity was assessed by streaking the cultures onto trypticase soy agar (Oxoid) supplemented with defibrinated horse blood at 5% (*v*/v) and incubated at 37 and 42 °C for 24 h, 25 °C for 48 h, and 10 and 15 °C for 10 days. The hemolysis formed by each isolate was classified as total or β-hemolysis (clear halos around the colonies), partial or α-hemolysis (greenish halos around the colonies), and absent or γ-hemolysis [21].

Lipase production was assessed by spotting 1 μL of cultures onto LB plates (BD) supplemented with $CaCl_2$ (Sigma-Aldrich, at 0.2%, *w*/*v*) and Tween 80 (Sigma-Aldrich, at 1%, *v*/v) and incubated at 37 and 42 °C for 48 h, 25 °C for 72 h, and 10 and 15 °C for 10 days. The formation of clear halos around the colonies was recorded as lipase production [22].

DNAse was identified by spotting 1 μL aliquots of the cultures onto the surface of DNAse methyl green agar (BD), and incubated at 37 and 42 °C for 48 h, 25 °C for 72 h, and 10 and 15 °C for 10 days. Positive results were identified by the formation of clear halos around the colonies [23].

Effect of Commercial Drugs and Antibiotics

Lb. plantarum ST8Sh was tested for resistance to drugs, according to de Carvalho et al. [8]. Thirty-three different commercial drugs were purchased in local drugstores (Sao Paulo, Brazil and Belogradtchik, Bulgaria) and solubilized in 5 mL of sterile water to achieve the concentrations indicated in Table 2. Overnight culture of *Lb. plantarum* ST8Sh (MRS broth, 37 °C, 18 h) was mixed into MRS soft agar (1.0%, w/v; Difco) in order to achieve the population of 10^6 CFU/mL. After solidification of the agar, each drug (10 μL) was spotted onto the surface of the plates and incubated at 37 °C for 24 h. The plates were examined for the presence of inhibition zones around the spotted medication, and the inhibition zones larger than 2 mm diameter were subjected to the determination of the minimal inhibition concentration (MIC). Serial twofold dilutions of the drugs were prepared in sterile water, and 10 μL were spotted onto the surface of MRS soft agar plates, previously inoculated with *Lb. plantarum* ST8Sh as described before. The plates were incubated at 37 °C for 24 h and examined for the presence of inhibition zones around the spots. The MIC was calculated, based on the highest dilution that resulted in inhibition halos of at least 2 mm diameter.

In a similar experimental approach, susceptibility of *Lb. plantarum* ST8Sh to antibiotics (Table 3) was tested by the disk diffusion test, using discs from Oxoid (Hampshire, England). The inhibitory effect of the antibiotics was expressed in millimeters of the inhibition zones [8].

Table 1 Primer sequences utilized in the investigation of the presence/absence for virulence factors, antibiotic resistance, and biogenic amine production

	Presence of virulence factor gene on genome of *Lb. plantarum* ST8Sh	Primers (5′–3′)
Virulence genes		
*gel*E	−	TATGACAATGCTTTTTGGGAT AGATGCACCCGAAATAATATA
hyl	−	ACAGAAGAGCTGCAGGAAATG GACTGACGTCCAAGTTTCCAA
*asa*1	+	GCACGCTATTACGAACTATGA TAAGAAAGAACATCACCACGA
esp	−	AGATTTCATCTTTGATTCTTG AATTGATTCTTTAGCATCTGG
*cyl*A	−	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT
*efa*A	−	GCCAATTGGGACAGACCCTC CGCCTTCTGTTCCTTCTTTGGC
ace	+	GAATTGAGCAAAAGTTCAATCG GTCTGTCTTTTCACTTGTTTC
*erm*A	−	TCTAAAAAGCATGTAAAAGAA CTTCGATAGTTTATTAATATTAG
*erm*B	+	GAAAAGTACTCAACCAAATA AGTAACGG TACTTAAATTGTTTA
*erm*C	+	TCAAAACATAATATAGATAAA GCAAATATTGTTTAAATCGTCAAT
*tet*K	+	TTAGGTGAAGGGTTAGGTCC GCAAACTCATTCCAGAAGCA
*tet*L	−	CATTTGGTCTTATTGGATCG ATTACACTTCCGATTTCGG
*tet*M	−	GTTAAATAGTGTTCTTGGAG CTAAGATATGGCTCTAACAA
*tet*O	+	GATGGCATACAGGCACAGAC CAATATCACCAGAGCAGGCT
*tet*S	−	TCGGTATCTTAGCACATGTTG TATYCKAYTATTTGGACGACG
aac(6′)-Ie-aph(2″)-Ia	+	CCAAGAGCAATAAGGGCATA CACTATCATAACCACTACCG
aph(3′)-IIIa	−	GCCGATGTGGATTGCGAAAA GCTTGATCCCCAGTAAGTCA
ant(4′)-Ia	−	CAAACTGCTAAATCGGTAGAAGCC GGAAAGTTGACCAGACATTACGAACT
aph(2″)-Id	−	GTGGTTTTTACAGGAATGCCATC CCCTCTTCATACCAATCCATATAACC
aph(2″)-Ic	−	CCACAATGATAATGACTCAGTTCCC CCACAGCTTCCGATAGCAAGAG
aph(2″)-Ib	+	CTTGGACGCTGAGATATATGAGCAC GTTTGTAGCAATTCAGAAACACCCTT
ant(6)-Ia	−	ACTGGCTTAATCAATTTGGG GCCTTTCCGCCACCTCACCG
*cat*A	+	GGATATGAAATTTATCCCTC CAATCATCTACCCTATGAAT
*vat*E	−	ACGTTACCCATCACTATG GCTCCGATAATGGCACCGAC
*bcr*B	−	AAAGAAACCGACTGCTGATA GCTTACTTGTATAGCAGAGA
*bcr*D	−	AGGATTCGGCCGAATGGCACTTGATTTTAT GTTTCTTCGCGAAATTGCCGTTATAAGTAA
*bcr*R	−	AACAAACAGGGAGCGGCCGCATGGAATTTA TGATGTTCGCGATTTCATTCCCATCTGCTT
*ddl*E	−	ATCAAGTACAGTTAGTCT

Table 1 (continued)

	Presence of virulence factor gene on genome of *Lb. plantarum* ST8Sh	Primers (5′–3′)
aac(6′)-Ii	−	ACGATTCAAAGCTAACTG GCGGTAGCAGCGGTAGACCAAG
*mur*2ed	−	GCATTTGGTAAGACACCTACG AACAGCTTACTTGACTGGACGC
*mur*2	−	GTATTGGCGCTACTACCCGTATC CGTCAGTACCCTTCTTTTGCAGAGTC
ccf	−	GCATTATTACCAGTGTTAGTGGTTG GGGAATTGAGTAGTGAAGAAG
cob	+	AGCCGCTAAAATCGGTAAAAT AACATTCAGCAAACAAAGC
cpd	+	TTGTCATAAAGAGTGGTCAT TGGTGGGTTATTTTTCAATTC
int	+	TACGGCTCTGGCTTACTA GCGTGATTGTATCTCACT
*spr*E	−	GACGCTCCTGTTGCTTCT TTGAGCTCCGTTCCTGCCGAAAGTCATTC
*fsr*A	−	TTGGTACCGATTGGGGAACCAGATTGACC ATGAGTGAACAAATGGCTATTTA
*fsr*B	−	CTAAGTAAGAAATAGTGCCTTGA GGGAGCTCTGGACAAAGTATTATCTAACCG
*fsr*C	−	TTGGTACCCACACCATCACTGACTTTTGC ATGATTTTGTCGTTATTAGCTACT
int-Tn	−	CATCGTTAACAACTTTTTTACTG TGACACTCTGCCAGCTTTAC
*Van*A	−	CCATAGGAACTTGACGTTCG TCTGCAATAGAGATAGCCGC
*Van*B	−	GGAGTAGCTATCCCAGCATT GCTCCGCAGCCTGCATGGACA
*Van*C1	+	ACGATGCCGCCATCCTCCTGC GGTATCAAGGAAACCTC
*Van*C2	−	CTTCCGCCATCATAGCT CTCCTACGATTCTCTTG
*van*C1(2)	−	CGAGCAAGACCTTTAAG GCTGAAATATGAAGTAATGACC
*van*C2/C3	−	CGGCATGGTGTTGATTTCGTT CTCCTACGATTCTCTTG
*hdc*1	−	CGAGCAAGACCTTTAAG AGATGGTATTGTTTCTTATG
*hdc*2	−	AGACCATACACCATAACCTT AAYTCNTTYGAYTTYGARAARGARG
tdc	−	ATNGGNGANCCDATCATYTTRTGNCC GAYATNATNGGNATNGGNYTNGAYCARG
odc	−	CCRTARTCNGGNATAGCRAARTCNGTRTG GTNTTYAAYGCNGAYAARCANTAYTTYGT ATNGARTTNAGTTCRCAYTTYTCNGG

Positive results (+) for genes for virulence, antibiotic resistance, and biogenic amines in *Lb. plantarum* ST8Sh

Results and Discussion

Bacteriocin Partial Purification and Cytotoxicity Test

Bacteriocin produced by *Lb. plantarum* ST8Sh has been partially purified by ammonium sulfate precipitation and hydrophobic chromatography on SepPakC_{18} column. The semi-purified bacteriocin showed an exceptionally high activity against *L. monocytogenes* (102,400 AU/mL) and *E. faecalis* (102,400 AU/mL). Semi-purified fractions (60% iso-propanol and 80% iso-propanol) of bacteriocin ST8Sh were tested on Huh7.5 cells for their cytotoxicity profile. At

Table 2 Effect of commercial drugs on the growth of *Lb. plantarum* ST8Sh, presented as diameter of inhibition zones in millimeters and Minimal Inhibition Concentration (MIC)

Commercial name	Concentration (mg/mL)	Active substance	Medicament class	*Lb. plantarum* ST8Sh Inhibition zone (mm) [MIC (mg/mL)]
Amoxil	100	Amoxicillin	β-Lactam antibiotic (Penicillin)	32 [<0.4]
Arotin	4	Paroxetine	Selective serotonin reuptake inhibitor (SSRI) antidepressant	10 [2.0]
Atlansil	40	Amiodarone	Antiarrhythmic	14 [1.25]
Cataflam	10	Diclofenac potassium	Non-steroidal anti-inflammatory drug (NSAID)	10 [10.0]
Diclofenaco potassico*	10	Diclofenac potassium	NSAID	12 [20.0]
Diclofenaco potassico*	10	Diclofenac potassium	NSAID	10 [20·0]
Dorflex	10	Orphenadrine citrate, Metamizole sodium, Cafein	Analgesic	10 [5.0]
Fenergan	5	Promethazine hydrochloride	Antihistaminic	10 [5.0]
Spidufen	120	Ibuprofen arginine	NSAID	16 [30.0]

Following commercial drugs has no effect on the growth of *Lb. plantarum* ST8Sh — AAS (Acetylsalicylic acid, Analgesic/Antipyretic at 20 mg/mL); Antak (Ranitidine hydrochloride, Histamine H2-receptor antagonist that inhibits stomach acid production (Proton pump inhibitor) at 30 mg/mL); Aspirina (Acetylsalicylic acid, Analgesic/Antipyretic at 100 mg/mL); Celebra (Celecoxib, NSAID at 40 mg/mL); Clorana (Hydrochlorothiazide, Diuretic at 5 mg/mL); Coristina R (Acetylsalicylic acid, Pheniramine maleate, Phenylephrine hydrochloride, Cafein, Analgesic/Antipyretic/Antihistaminic/Decongestant at 10 mg/mL); Doxuran (Doxazosin, Antihypertensive/Treatment of prostatic hyperplasia at 0.8 mg/mL); Dramin (Dimenhydrinate, Antiemetic at 20 mg/mL); Fluimucil (Acetylcysteine, Mucolitic agent at 8 mg/mL); Flutec (Fluconazole, Antifungal at 30 mg/mL); Higroton (Chlorthalidone, Thiazide diuretic at 10 mg/mL); Neosaldina (Metamizole sodium, isometheptenemucate, cafein, Analgesic at 60 mg/mL); Nimesulida (Nimesulide, NSAID at 20 mg/mL); Nisulid (Nimesulide, NSAID at 20 mg/mL); Omeprazol (Omeprazole, Proton pump inhibitor at 4 mg/mL); Redulip (Sibutramine hydrochloride monohydrate, Anorexiant/Sympathomimetic at 3 mg/mL); Seki (Cloperastine, Antitussives (central and periferic mode of action) at 3.54 mg/mL); Superhist (Acetylsalicylic acid, Pheniramine maleate, Phenylephrine hydrochloride, Analgesic/Antipyretic/Antihistaminic/Decongestant at 80 mg/mL); Tylenol (Paracetamol, Analgesic/Antipyretic at 150 mg/mL); Tylex (Paracetamol, Codein, Analgesic at 6 mg/mL); Yasmin (Ethinylestradiol, drospirenone, Contraceptive at 0.6 mg/mL); Zestril (Lisinopril, Antihypertensive (Angiotensin-converting enzyme (ACE) inhibitor) at 4 mg/mL); Zocor (Simvastatin, Hypolipidemic at 2 mg/mL); and Zyrtec (Cetirizine hydrochloride, Antihistaminic at 2 mg/mL)

*Produced by two different companies

concentration of 25 μg/mL, 60% fraction demonstrated to be highly cytotoxic, reducing the cell viability by approximately 80%; however, when this same fraction was tested at a lower concentration (5 μg/mL), no cell cytotoxicity was observed. Regarding the 80% iso-propanol fraction, cell viability was not reduced in both tested concentrations (25 and 5 μg/mL). Moreover, it is essential to pay attention to the cytotoxicity of this and similar bacteriocins in food preparations, due to their potential of concentration depending on the effect on cell viability that may result in severe side effects and tissue damage if ingested by humans or other animal bacteriocins.

Analysis of bacteriocin cytotoxicity is not a routine procedure, and only a few studies on this issue are available in literature. In a previous study, our group demonstrated a high cytotoxicity potential of two other bacteriocins (ST202Ch and ST216Ch) on Huh7.5 cells [24]. Vaucher et al. [25] evaluated the toxicity profile of the commercial bacteriocins on epithelial monkey kidney cells (Vero), and it was demonstrated that at 1.04 μg/mL concentrations, nisin reduced the cell viability by 50%. Therefore, concerning cell cytotoxicity, semi-purified fractions of bacteriocin ST8Sh appear to be safer for practical use than nisin. Although their excellent potential as food preservatives and antibiotic substitutes, bacteriocins can also be toxic to human cells, and so, cytotoxicity assays should be included as routine in research on bacteriocins to be used as biopreservatives.

Screening for the Presence of Virulence Factors

Lb. plantarum ST8Sh showed a low presence of virulence genes. Only 13 genes were detected (related to sex pheromones, aggregation substance, adhesion of collagen, tetracycline, gentamicin, chloramphenicol, erythromycin, but not to vancomycin and bacitracin) and may be considered as safe for application in fermented food products (Table 1). The detected frequency of possible presence of the virulence factors in *Lb.*

plantarum ST8Sh was lower than that reported in other studies on *Lactobacillus* spp. and *Enterococcus* spp. isolated from foods [19, 20, 26–28] and also in comparison to studies with clinical isolates [20, 29, 30]. *Lb. plantarum* ST16Pa [31] isolated from papaya was previously described with positive PCR results for the presence of *gel*E (gelatinase), *hyl* (hyaluronidase), *asa*1 (aggregation substance), *ace* (adhesion of collagen), and *tdc* (tyrosine decarboxylase), thus representing a high virulence profile when compared to the results obtained in the present study for *Lb. plantarum* ST8Sh.

Even when the presence of virulence factors was less relevant to LAB strains isolated from food, compared to LAB from clinical origin, the determination of virulence factors in LAB by molecular and phenotypic procedures is important due to the risk of gene transfer, since these factors are usually encoded by genes located in conjugative plasmids [30]. The LAB comprises a heterologous group of six families and at least 36 different genera with particular metabolism, but with common characteristics including their fermentative ability to produce lactic acid as a major end product of primary metabolism [5, 32]. Several LAB have a long history as beneficial organisms, used in or associated with different fermentation processes and applied as probiotics [1]. However, some LAB are considered as opportunistic pathogens and have been associated with some clinical cases [2–4]. Some enterococci may contain several determinants of pathogenicity, such as colonization factors that promote the adhesion of bacteria to host cells and invasion factors that promote the invasion of epithelial cells disordering the immune system [29, 33]. Different cell wall-anchored surface proteins are related to enterococcal pathogenicity, including aggregation substance, enterococcal surface protein, and collagen binding components [34]. The presence of enterococcal surface proteins, including aggregation substance, *Enterococcus* surface protein, adhesins, and other adhesive molecules, such as *Enterococcus* endocarditis antigen may facilitate close contact between cells for conjugation and subsequent transfer of virulence plasmids [34]. However, on the other side, they can be involved in better adhesion and colonization of the GIT. On the negative side, the aggregation substance protein may have a role in translocation of enterococci into epithelial cells [35] and be involved in the pathogenicity of these bacteria. A cell wall-anchored protein characterized by its ability to form biofilms, e.g., *Enterococcus* surface protein, may therefore be implicated in enterococcal infections associated with biofilms [34].

In the last two decades, the term as quorum sensing (QS) was extensively explored and defined as an intercellular chemical signaling system in bacteria. Related to this, production and detection of compounds known as pheromones to elicit coordinated responses among members of a bacterial community was described [36]. Pheromones produced by Gram-positive bacteria comprise small peptides. These peptides can be related to different key regulatory processes in bacterial cells, including a variety of fundamental behaviors including conjugation, natural competence for transformation, biofilm development, and virulence factor regulation. Even if not much work has been conducted on *Lactobacillus* spp. related to peptide pheromones, we need to be aware that this process is relevant to all bacterial groups. Genes related to production and expression of peptide pheromones can be a part of the natural genome of bacterial species, but can be a result of the horizontal gene transfer within and between species via conjugative plasmids. Generally, conjugation, well studied in *Enterococcus* spp., is controlled via peptide pheromones [36]. The possible presence of bacterial pheromone genes in *Lb. plantarum* ST8Sh needs to be explored in more details in order to give an answer on their exact role in the genus *Lactobacillus* and *Lb. plantarum* particularly. Are these genes a part of the natural genetic heritage of the species or are they appearing as a result of inter-bacterial interaction and horizontal gene transfer?

Based on the performed phenotypic tests, *Lb. plantarum* ST8Sh was not expressing any of the tested virulence factors. These results can be related to the fact that expression of studied virulence factors may be related either to the specific growth condition for the tested *Lb. plantarum* ST8Sh strain, or to the more complex condition/interaction with different factors. Or simply, most probably, the genes encoding this virulence factors are partially inactivated or parts of the operon are damaged or just not present.

Effect of Commercial Drugs on *Lb. plantarum* ST8Sh

Application of probiotics is related to the prevention/prophylaxis of diseases; however, they form part of the active therapy as well. Most frequently individuals or patients taking probiotics are often treated for other illnesses, including chronic clinical cases. However, are we aware of possible interactions between viable probiotics and drugs as chemical substances? As we need to optimize the effect of the probiotics to the host, it is important to determine the effect of drugs on the survival of probiotic strains. As presented in Table 2, the tested *Lb. plantarum* ST8Sh strain was inhibited by non-steroidal anti-inflammatory drugs (NSAID) containing diclofenac potassium, ibuprofen arginine, promethazine hydrochloride, paroxetine, amiodarone, Dorflex, an analgesic that contains orphenadrine citrate, metamizole sodium, and cafein. In addition, *Lb. plantarum* ST8Sh expectedly was sensitive to amoxyl. However, inhibition of *Lb. plantarum* ST8Sh needs to be considered not as qualitative interaction, but in relation of the observed MIC, presented on Table 2. In addition to previous, it is important to mention that the concentration of these drugs/substances in the GIT, together with MIC, is critical for their interaction with the probiotic bacteria [6, 7]. In this regard, considering that the daily dose for Spidufen is 600 mg (Zambon Laboratórios Farmacêuticos Ltda), the MIC

Table 3 Effect of antibiotics on the growth of *Lactobacillus plantarum* ST8Sh, presented as diameter of inhibition zones in millimeters

Antibiotic (μg/disk)	Classification	Inhibition zone (mm)[a]
Ampicillin, 10	Penicillins/β-Lactam (interferes with bacteria cell wall synthesis)	38
Bacitracin, 10	Cyclic polipeptide (inhibits bacteria cell wall synthesis)	22
Cefazolin, 30	1st generation cephalosporin/β-Lactam (interferes with bacteria cell wall synthesis)	23
Cefepime, 30	4th generation cephalosporin/β-Lactam (interferes with bacteria cell wall synthesis)	21
Cefotaxim, 30	2nd generation cephalosporin/β-Lactam (interferes with bacteria cell wall synthesis)	23
Ceftazidim, 30	3th generation cephalosporin/β-Lactam (interferes with bacteria cell wall synthesis)	18
Ceftriaxon, 30	3th generation cephalosporin/β-Lactam (interferes with bacteria cell wall synthesis)	16
Cefuroxim, 30	β-Lactam (interferes with bacteria cell wall synthesis)	19
Ciprofloxacin, 5	Fluoroquinolones (inhibits the bacterial topoisomerase II)	12
Clindamicin, 2	Licosamides (inhibits protein synthesis)	32
Chloramphenicol, 30	Prevents peptide bond formation–inhibits protein synthesis)	27
Erytromicin, 15	Macrolide (inhibits protein synthesis)	21
Furazolidon, 10	Antibiotic/antiparasitic	15
Gentamicin, 10	Aminoglycoside (inhibits protein synthesis)	10
Imipenem, 10	Carbapenem/β-Lactam (interferes with bacteria cell wall synthesis)	36
Neomicin, 30	Aminoglycosides (inhibit protein synthesis)	12
Nitrofurantoin, 300	Nitrofuran derivative (nucleic acid inhibitor)	21
Ofloxacin, 5	Licosamide (inhibits protein synthesis)	12
Penicillin G, 10	β-Lactam (interferes with bacteria cell wall synthesis)	14
Rifampicin, 30	Semisynthetic compound derived from *Amycolatopsisrifamycinica*	25
Rifampicin, 5	Semisynthetic compound derived from *Amycolatopsisrifamycinica*	21
Streptomicin, 10	Aminoglycoside (inhibits protein synthesis)	20
Tetraciclin, 30	(inhibits protein synthesis)	27
Trimetoprim, 5	(Inhibits folatesynthesis)	22

Amicacin 30 μg/disk and Kanamicin 30 μg/disk (Aminoglycoside, inhibits protein synthesis), Metronidazol 50 μg/disk (Nitroimidazole antibiotic, acts on DNA of microorganisms, ameba, and protozoa), Nalidixic acid 30 μg/disk (Syntetic quinolone antibiotic, acts on DNA gyrase), Oxacilin 1 μg/disk (β-Lactam, interferes with bacteria cell wall synthesis), Tobramicin 10 μg/disk (Aminoglycoside, inhibits protein synthesis), and Vancomycin 30 μg/disk (Glycopeptide, inhibits bacteria cell wall synthesis) are not affecting the growth of *Lb. plantarum* ST8Sh

[a] Average diameter of inhibition zones of the test microorganism

value associated to the volume of the human GIT indicates that the recommended daily dose will hardly affect the survival of *Lb. plantarum* ST8Sh. However, more important are the drugs used in the treatment of chronic diseases, since the higher concentrations may be accumulated in the human body, including the GIT. Atlansilis, an anti-arrhytmic drug is normally used in long-term treatments; Fenergan, an antihistaminic drug, and Arotin, a drug from the group of the antidepressants with neuroleptic effect, are also used in long-term treatments and presented an MIC of 1.25, 5.0, and 2.0 mg/mL, respectively. Due to their long-term application, these drugs may accumulate in the GIT and MIC-values which may be reached that may affect viability of *Lb. plantarum* ST8Sh.

The interaction between anti-inflammatory drugs based on diclofenac and LAB detected in this study (Table 2) was also reported previously in other studies. It has been reported that this group of drugs inhibited the growth of *Lb. plantarum* ST8KF and ST341LD, *E. faecium* ST311LD, and *Leuconostoc mesenteroides* subsp. *mesenteroides* ST33LD [31]. In a similar study, potassium diclofenac and ibuprofen inhibited the growth of *Lactococcus lactis* subsp. *lactis* HV219 [6]; *Lb. casei* Shirota and *Lb. casei* LC01 were inhibited by non-steroidal anti-inflammatory drugs (NSAID) containing diclofenac potassium or ibuprofen arginine [8]. In addition, Carvalho et al. (11) reported that *Lb. casei* Shirota was affected by selective serotonin reuptake inhibitors (SSRI), an antidepressant containing paroxetine, and antiarrhythmic medicine containing amiodarone, while *Lb. casei* LC01 was inhibited by hypolipidemic drugs containing simvastatin. Anti-inflammatory drugs, moderate diuretic and neuroleptic containing potassium or sodium diclofenac, ibuprofen, triamterene hydrochlorothiaziden, and thioridazinehydrochlorid acted as inhibitors of *Lb. plantarum*, *Lb. rhamnosus*, *Lb. paracasei,* and *Lb. pentosus* strains isolated from boza and tested for probiotic potential [7]. It is extremely difficult to compare results working with different strains, due to the fact that

drug/bacteria interactions are strain specific, this also being observed when authors explored different strains of the same species in one study [7]. However, it is interesting and also important to compare results obtained with some commercial and/or reference strains as reported by different research groups. Botes et al. [37] found that *Lb. casei* Shirota was inhibited by several commercial antibiotics, in addition to the anti-inflammatory drugs containing meloxican (Coxflam), Ibuprofen (Dolocyl, Adco-Ibuprofen), potassium diclofenac (Cataflam), and prednisolone (Preflam) that also inhibited the strain growth, to a lesser extent. Pinmed, containing paracetamol, codeine phosphate, and promethazine HCl, misclassified as analgesic instead of an antitussive agent, was also inhibitory to *L. casei* Shirota. The same authors also reported the inhibitory effect of Pynmed, more likely due to the presence of alcohol in the formulation than to the drug itself [37]. This is another important point that needs to be taken into account—the composition of the drug preparation and the presence and composition of the accompanying substrate or solvent.

Among the tested drugs in this study, the anti-inflammatory medical preparations were those that affected *Lb. plantarum* ST8Sh more significantly. These results are in agreement with previous studies, investigating other potential probiotics and commercial LAB probiotics [6–8, 37, 38]. Taking into consideration the composition of the mentioned drugs, their inhibitory activity may be related to an increased concentration of potassium ions in the gastric content as a result of the dissociation of potassium diclofenac in the GIT. The excess of potassium ions in the environment is incompatible with microbial cell viability and may have a negative effect on LAB. Other potassium-based drugs may cause a similar negative effect. Individuals under permanent therapy with drugs should be aware that these drugs may reduce the beneficial effects of the probiotic bacteria.

Resistance to Antibiotics

The majority of the investigated antibiotics in this study inhibited the growth of *Lb. plantarum* ST8Sh (Table 3). Growth of *Lb. plantarum* ST8Sh was not affected by the presence of Amicacin (30 μg/disk) and Kanamicin (30 μg/disk) (Aminoglycoside, inhibits protein synthesis), Metronidazol (50 μg/disk) (Nitroimidazole antibiotic, acts on DNA of microorganisms, amoeba, and protozoa), Nalidixic acid (30 μg/disk) (Synthetic quinolone antibiotic acts on DNA gyrase), Oxacilin (1 μg/disk) (β-Lactam, interferes with bacteria cell wall synthesis), Tobramicin (10 μg/disk) (Aminoglycoside, inhibits protein synthesis), and Vancomicin (30 μg/disk) (Glycopeptide, inhibits bacteria cell wall synthesis). Resistance of potential probiotic LAB candidates to antibiotics is a controversial subject, as these strains may be reservoirs of antibiotic resistance genes, and can be transferred horizontally to other bacteria in the human and other animals GIT [5]. However, from another point of consideration, resistance to antibiotics can be considered as a positive aspect, since such LAB strains (carrying resistance to a specific antibiotic) could be applied in combination with such an antibiotic potentially resulting in synergism between the antibiotic and the LAB strain in the treatment of the diseases. Resistance may be inherent to a bacterial genus or species, but may also be acquired through exchange of genetic material, mutations, and the incorporation of new genes [23, 39–41].

Presence of genes related to tetracycline, gentamicin, chloramphenicol, erythromycin antibiotic resistance was detected in *Lb. plantarum* ST8Sh. Lactobacilli have a high natural (constitutive) resistance to different antibiotics, including gentamicin [42]. In addition, many strains of *Lb. plantarum* are intrinsically resistant to the antibiotic, due to the presence of D-alanine-D-alanine ligase related enzymes [43]. Salminen et al. [44] showed that strains of *Lactobacillus* isolated from blood samples had low MIC values to erythromycin in addition to other antibiotics. However, lactobacilli are generally susceptible to antibiotics interfering with protein synthesis, e.g., chloramphenicol, erythromycin, clindamycin and tetracycline, but are more resistant to aminoglycosides (neomycin, kanamycin, streptomycin, and gentamicin) [45–47]. In general, lactobacilli show resistance to most inhibitors of nucleic acid synthesis, including enoxacin, pefloxacin, norfloxacin, nalidixic acid, sulphamethoxazole, trimethoprim, cotrimoxazole, and metronidazole [45, 46]. Herreros et al. [48] reported on resistance to tetracycline in *Lb. plantrum* isolated from Armada cheese. Tetracycline resistance was recorded for several strains of *Lb. plantarum* isolated from raw milk soft cheeses [23]. *Lb. plantarum* isolated from "home-made" Spanish cheese (Serena, Gamonedo and Cabrales) revealed resistance to penicillin G, cloxacillin, streptomycin, gentamycin, tetracycline, erythromycin, and chloramphenicol [49]. Regarding gentamicin, MIC values detected in wine isolates of *Lb. plantarum* were in general very high. A similar observation was reported by Elkins and Mullis [50] in who found intrinsic resistance of lactobacilli to aminoglycosides to be due to membrane impermeability, probably complemented by potential efflux mechanisms. In general, *Lb. plantarum* showed higher MICs for aminoglycosides than other LAB genera and *Lactobacillus* species [51]. Conversely, Zhou et al. [47] found almost all out of ten tested *Lb. plantarum* strains to be resistant to gentamicin.

The antibiotic resistance genes provide elevated competition potential to a strain to survive and constitute a positive attribute to survival and adaptation. From the human point of view, these genes are generally undesirable. Lactobacilli are generally susceptible to antibiotics inhibiting the synthesis of proteins, such as chloramphenicol, erythromycin, clindamycin, and tetracycline, and more resistant to aminoglycosides (neomycin, kanamycin, streptomycin, and gentamicin) [5]. However, strains resistant to these antibiotic agents

have also been identified [5], and several genes providing such resistance have been studied; e.g., a chloramphenicol resistance gene (*cat*) has been detected in *Lb. plantarum* [52]. Also, different erythromycin-resistance genes (*erm*) have been found in many species, as well as a number of tetracycline resistance genes [5]. The *tet*S gene in the probiotic *Lb. plantarum* strain CCUG 43738 was found to be located on a plasmid of 14-kbp [53].

The major financial and societal costs caused by the increase in antibiotic resistance in pathogenic microorganisms are a general issue of concern. The attenuation of this problem is complicated by commercial bacteria that may act as reservoirs for antibiotic resistance determinants found in pathogens [40, 54]. This statement is supported by the fact that the same type of genes encoding resistance to, for example, tetracycline, erythromycin, chloramphenicol, streptomycin, and streptogramin, have been found in commercial lactococci and lactobacilli as well as in potentially pathogenic enterococci and pathogenic streptococci [23]. A most important similarity in resistance genes has also been observed for tetracycline-resistance in *Lb. plantarum* and other LAB [5, 55].

Conclusions

Besides, all beneficial properties studied for various LAB, most considered as GRAS, special attention needs to be given to the possible presence of virulence factors, production of biogenic , and antibiotic resistance. These virulence determinants have been detected and well studied in enterococci and streptococci; however, in the last few years, reports on the presence of virulence factors in otherwise GRAS lactobacilli have indicated potential upcoming problems. Horizontal gene transfer of virulence factors between pathogenic and LAB, including probiotics, appears to be a highly possible scenario in case of uncontrolled application of probiotics. Complex research of all aspects of potential new probiotics strains and antimicrobial peptides is essential in order to ensure safety application of these strains and/or their metabolites.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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