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In Vitro Evaluation of Beneficial Properties of Bacteriocinogenic *Lactobacillus plantarum* ST8Sh

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Abstract *Lactobacillus plantarum* ST8Sh, isolated from Bulgarian salami "shpek" and previously characterized as bacteriocin producer, was evaluated for its beneficial properties. Based on the PCR analysis, *Lb. plantarum* ST8Sh was shown to host a gene related to the production of adhesion proteins such as Mab, Mub, EF, and PrgB. Genetic and physiological tests suggest *Lb. plantarum* ST8Sh to represent a potential probiotic candidate, including survival in the presence of low levels of pH and high levels of ox bile, production of β-galactosidase, bile salt deconjugation, high level of hydrophobicity, functional auto- and co-aggregation properties, and adhesion to cell lines. Application of semi-purified bacteriocin produced by *Lb. plantarum* ST8Sh in combination with ciprofloxacin presented synergistic effect on inhibition of *Listeria monocytogenes* Scott A. Based on observed properties, *Lb. plantarum* ST8Sh can be considered as a potential probiotic candidate with additional bacteriocinogenic properties.

Keywords *Lactobacillus plantarum* · Probiotics · Bacteriocins

Introduction

Lactic acid bacteria (LAB) have been actively studied in the last three decades as potential candidates for biopreservation processes and for their potential as probiotics. The concept of application of beneficial LAB in human health is not new. The idea of exploring the beneficial potential of LAB has been proposed and scientifically justified almost one century ago by Ilia Metchnikov and Stamen Grigorov. Since then, this group of bacteria has been the subject of numerous studies and patents. However, utilization of the LAB and their metabolites has been part of the human tradition and domestic practices since earliest history and in particular in the preparation of various fermented food products, some of which even having been traditionally appreciated as medicine [1–3]. In the second half of the twentieth century, a scientific basis of probiotic concepts was established. According to the World Health Organization/Food and Agricultural Organization (WHO/FAO) probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit to the host" [4]. However, in 2009, according to the International Scientific Association for Probiotics and Prebiotics (ISAPP), the term probiotic is commonly misused in commercial products with frequently no scientific proof of benefits to human and animal health, while, moreover, the term has also been used to describe bacterial components, dead bacteria, or bacteria with uncharacterized health effects [5]. According to the FAO/WHO definition, a probiotic must (i) be alive when administered at a specific level (10^6–10^7 CFU per gram of food product), (ii) have undergone controlled evaluation to document health benefits in the target host, (iii) be a taxonomically defined microbe or combination of microbes (specifying genus, species, and strain level); and (iv) be safe for its intended use [4, 6–9].

The role of bacteriocin production in relation to probiotic properties of LAB is very often misunderstood. It is important to underline that bacteriocin production is regarded as just a complimentary (not mandatory) beneficial feature of probiotic LAB. If a probiotic LAB can produce bacteriocins, this may

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influence its chances for surviving harsh conditions in the gastrointestinal tract (GIT) by outcompeting other (closely related) microbial species present in the same environmental niche. In addition to competition with pathogenic bacteria and reduction of gastrointestinal infections, bacteriocinogenic LAB, like probiotics, can also confer additional health benefits to the host also by alleviation of processes such as inflammatory bowel disease, modulation of the immune system, and defense against recolonization by pathogenic microorganisms [4, 8, 10].

Other beneficial features typical of a wide array of LAB correlate with desirable characteristics of potential probiotics, including aggregation properties, survival in the harsh upper GIT conditions such as low pH and presence of bile salts, adhesion ability to (human intestinal) cell lines, favorable interaction with commonly used non-antibiotic drugs, and survival (non-transferable resistance) against antibiotics. In addition, safety aspects of novel strains of putatively probiotic LAB need to be carefully examined and the potential of delivering virulence factors to host microbiota needs to be excluded.

Application of different *Lactobacillus plantarum* strains was intensively studied in prevention and control of various diseases [11–13]. *Lb. plantarum* strains have been applied for control of gastrointestinal disorders and specific control of *Salmonella* spp., *Escherichia coli*, and *Clostridium difficile*, restoration of the GIT microbial balance after antibiotic treatments and intestinal discomfort, treatment of constipation and diarrhea, enhancement of the GIT barrier function, inducing immune modulatory effects, treatment of chronic venous ulcers, control of inflammation of the GIT, reduction of allergic response, protection against influenza virus infection, maintenance of oral health, treatment of burns, prevention and treatment of cardiovascular disease, cholesterol reduction, and anti-obesity effects [11].

The aim of this work was to investigate some beneficial properties of *Lb. plantarum* ST8Sh, a bacteriocinogenic strain previously isolated from Bulgarian salami "shpek" [14], in order to characterize its potential as a probiotic culture.

Material and Methods

Strains and Media

Lb. plantarum ST8Sh, a bacteriocinogenic strain isolated from Bulgarian salami shpek [14], and the test strains *Listeria monocytogenes* ATCC 7644, *L. monocytogenes* Scott A, *Enterococcus faecalis* ATCC 19443, and *Lactobacillus sakei* ATCC 15521 were cultured in de Man, Rogosa and Sharpe (MRS) broth in the case of LAB and brain-heart infusion (BHI) in the case of *Listeria* spp. (Difco, Detroit, MI, USA), at 30 °C, and stored at −80 °C, in presence of 20% glycerol.

Screening for Probiotic Properties in Presence of Related 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. Presence of genes related to adhesion properties of probiotic bacteria was investigated, using primers targeting *Mub* (adhesion properties) (5′-GTA GTT ACT CAG TGA CGA TCAATG-3′ and 5′-TAA TTG TAA AGG TAT AAT CGG AGG-3′), *Map* (adhesion properties) (5′-TGG ATT CTG CTT GAG GTA AG-3′ and 5′-GAC TAG TAA TAA CGC GAC CG-3′), *EFTu* (adhesion properties) (5′-TTC TGG TCG TAT CGA TCG TG-3′ and 5′-CCA CGT AAT AAC GCA CCA AC-3′), *prgB* (surface protein) (5′-GCC GTC GAC TCG AGG AGA ATG ATA CAT GAA T-3′ and 5′-CCT GCG GCC GCG TCC TTC TTT TCG TCT TCA A-3′), *EF2662*-cbp (choline-binding protein) (5′-GGC GTC GAC CAC TTA AAC TGA TAG AGA GGA AT-3′ and 5′-CGC GCC GCA ATT AAT TAT TAA CTA GTT TCC-3′), *EF1249*-fbp (fibrinogen-binding protein) (5′-GCG GTC GAC AAA CGA GGG ATT TAT TAT G-3′ and 5′-CTG GCG GCC GCG TTT AAT ACA ATT AGG AAG CAG A-3′), and *EF2380*-maz (membrane-associated zinc metalloprotease) (5′-GCG GTC GAC GAC ATC TAT GAA AAC AAT-3′ and 5′-TCC GCG CCG CCT TAA ACT TTC TCC TT-3′) as described by Todorov et al. [9], Kao et al. [15], and Fortina et al. [16].

Hydrophobicity

Cell surface hydrophobicity of *Lb. plantarum* ST8Sh was evaluated on overnight stationary-phase culture. Bacterial culture was centrifuged at 7000×*g* for 5 min at 4 °C, and the cells were washed twice with phosphate buffer (50 mM K_2HPO_4/KH_2PO_4, pH 6.5) and resuspended in the same buffer until A_{560} values (A_0) near 1.0 were obtained. *N*-hexadecane was then added to the cell suspension (1:5), and the mixture was vortexed for 3 min. The suspension was incubated for a period of 1 h at 37 °C, and the A_{560} value (A) of the aqueous layer was measured. Cell surface hydrophobicity was calculated according to the equation: $\%H = [(A_0 - A) / A_0] \times 100$, where A_0 and A are the absorbance values before and after extraction with the organic solvent, respectively. The assay was performed at three independent occasions in duplicates.

Growth at Low pH and in Presence of Ox Bile

Lb. plantarum ST8Sh was grown in MRS broth (Difco) adjusted to pH 3.0, 4.0, 5.0, 6.0, 7.0, 9.0, 11.0, and 13.0 by adding 1 M HCl or 1 M NaOH. In an additional experiment, *Lb. plantarum* ST8Sh was grown in MRS broth containing 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, and 3.0% (*w*/*v*) of ox bile (Sigma). All tests were conducted in sterile flat-bottom 96-well microtiter plates (NUNC, Thermo Scientific). Each well was filled with 180 μL of the bile-containing medium or MRS with modified pH and inoculated with 20 μL of the *Lb. plantarum* ST8Sh culture obtained in MRS broth (Difco) ($OD_{600\ nm} = 0.2$) at 37 °C. Changes in optical density readings were recorded at 600 nm every hour for 12 h, using a microtiter plate reader (BioTek Instruments, Inc. USA). Cultures grown in MRS broth with pH 6.5 and without bile served as control. Experiments were performed in triplicates.

Aggregation and Co-aggregation

For evaluation of auto-aggregation, *Lb. plantarum* ST8Sh was grown in MRS broth for 24 h at 37 °C. After harvesting by centrifugation (7000×*g* for 10 min at 20 °C), the cells were washed twice, resuspended, and diluted in 0.85% sterile saline to reach approximate $OD_{660\ nm} = 0.3$ (BEL Photonics, UV-vis Spectrophotometer, Italy) (OD_0). The cell suspension was incubated for 60 min at 37 °C. The $OD_{660\ nm}$ of the supernatant obtained after the cell suspension was centrifuged at 300×*g* for 2 min at 20 °C was recorded, and auto-aggregation was determined using the following equation [17]: % Auto-aggregation = $[(OD_0 - OD_{60}) / OD_0] \times 100$, where OD_0 refers to the initial OD and OD_{60} refers to the OD determined after 60 min.

For evaluation of co-aggregation, *Lb. plantarum* ST8Sh was grown in 10 mL MRS and *L. monocytogenes* Scott A and *L. monocytogenes* ATCC 7644, *Lb. sakei* ATCC 15521, and *Enterococcus faecium* ATCC 19433 in BHI or MRS, at 37 °C. Cell suspensions were prepared as described before in the auto-aggregation test. The cell suspension of *Lb. plantarum* ST8Sh was mixed with different co-aggregation partner's cell suspensions in equal volumes (1:1). The $OD_{660\ nm}$ was determined, and suspensions were incubated for 60 min at 37 °C. $OD_{660\ nm}$ of the supernatants obtained after the cell mixed culture suspensions were centrifuged at 300*g* for 2 min at 20 °C was recorded, and co-aggregation was determined using the following equation [17]: % Co-aggregation = $[(OD_{tot} - OD_s) / OD_{tot}] \times 100$, where OD_{tot} refers to the initial OD taken immediately after the tested strains were mixed while OD_S refers to the OD of the supernatant after 60 min. Experiments were conducted in triplicates at two separate occasions.

β-Galactosidase Activity

The β-galactosidase activity of *Lb. plantarum* ST8Sh was assessed employing sterile filter paper disks impregnated with *o*-nitrophenyl-β-D-galactopyranose (ONPG disks, Fluka, Buchs, Switzerland), according to the manufacturer's instructions and as previously described by dos Santos et al. [18]. Overnight cultures of *Lb. plantarum* ST8Sh were streaked on MRS agar plates and incubated anaerobically (GasPack System, Oxoid, Basingstoke, Hampshire, UK) at 37 °C for 48 h. A colony of *Lb. plantarum* ST8Sh was picked and emulsified in a tube containing an ONPG disk soaked with 0.1 mL of sterile 0.85% (*w*/*v*) sodium chloride solution. The tubes were incubated at 35 °C and checked at 1-h intervals for up to 6 h. The release of a yellow chromogenic compound, *o*-nitrophenol, served as indication of a positive colony. The test was performed at two independent occasions in duplicates.

Bile Salt Deconjugation

In order to evaluate the ability of *Lb. plantarum* ST8Sh to perform bile salt deconjugation, overnight cultures were streaked on MRS agar plates containing 0.5% (*w*/*v*) of the sodium salts of taurocholic acid (TC), taurodeoxycholic acid (TDC), glycocholic acid (GC), and glycodeoxycholic acid (GDC) (Sigma-Aldrich Co., St. Louis, USA). *Lb. plantarum* ST8Sh was incubated under anaerobic conditions (GasPack System, Oxoid) at 37 °C for 72 h, and the presence of an opaque halo around colonies was considered positive for bile salt deconjugation. The test was performed according to dos Santos et al. [18] at two independent occasions in duplicates.

Adhesion to Caco-2 Cell Line

Caco-2 cells were grown in MEM, supplemented with 10% (*v*/*v*) fetal bovine serum (Sigma), 100 U/mL penicillin, and 100 U/mL streptomycin (Sigma) at 37 °C. Caco-2 monolayers were prepared in 24-well tissue plates (Sigma) and inoculated with 1×10^5 cells per well to obtain confluence. The medium was changed every second day. The cells were washed twice with 1 mL sterile PBS. The medium was replaced by fresh non-supplemented MEM, at least 1 h before the adhesion assay.

Lb. plantarum ST8Sh was inoculated in MEM, supplemented with 2% (*v*/*v*) fetal calf serum (Invitrogen, Eggenstein, Germany) for 24 h at 37 °C. Wells containing the Caco-2 cell monolayers were inoculated with 100 μL of each bacterial suspension (ca. 1×10^6 CFU/mL) and the plates centrifuged at 300*g* and 4 °C for 2 min. The Caco-2 cells were washed twice with 1 mL sterile PBS and then lysed by adding 1 mL 0.5% (*v*/*v*) Triton X-100. Appropriate dilutions for bacterial counts were plated onto MRS agar. Adhesion of bacterial cells to Caco-2 cells was calculated from the initial number of viable cells, number of cells in the supernatant, and number of cells in the lysate after treatment with Triton X-100. *Lactobacillus rhamnosus* GG was used as positive control of adherence to Caco-2 cells. Experiments were done in triplicate.

Bacteriocin Test

Lb. plantarum ST8Sh was grown on MRS at 37 °C for 24 h, and cell-free supernatant was obtained by centrifugation at

10,000*g* for 10 min. The pH of the supernatant was adjusted to 6.0–6.5 with 1 M NaOH and treated for 10 min at 80 °C. Ten microliters of the cell-free supernatant was spotted onto the surface of BHI with 0.7% agar containing 10^5 CFU of *L. monocytogenes* Scott A per milliliter. The plates were incubated at 37 °C for 24 h and checked for the presence of inhibitory zones. Those generated inhibition zones larger than 2 mm in diameter were considered as positive.

Titer of the expressed bacteriocin was determined as described by dos Santos et al. [18]. The obtained cell-free supernatant was serially diluted 2× in 100 mM phosphate buffer pH 6.5 and 10 μL from each dilution spotted on the surface of BHI with 0.7% agar agar with 10^5 CFU/mL final concentration of *L. monocytogenes* Scott A. The highest dilution resulting in an inhibition zone larger than 2 mm was considered as basis for the calculation of arbitrary units per milliliter, taking into consideration the volume of the deposited material and level of dilution.

Bacteriocin Production and Partial Purification

Lb. plantarum ST8Sh was cultured in MRS broth at 37 °C for 24 h. Cell-free supernatant was obtained as described before. 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 12,000*g* at 4 °C, the resulting pellet was resuspended in 100 mL of 25 mM ammonium acetate buffer (pH 6.5) and loaded on a Sep-Pak C_{18} cartridge (Waters, Millipore, MA, USA), and bacteriocin was eluted with 60 and 80% iso-propanol 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 resuspended in sterile distilled water and filtered using 0.22-μm-pore-size filter units (Waters).

Synergistic Interaction Between Bacteriocin and Antibiotic (Ciprofloxacin)

In the first experiment, the minimal inhibition concentration of ciprofloxacin (Sigma) was determined by growing *L. monocytogenes* Scott A in BHI, supplemented with antibiotic concentrations varying from 1.25 to 640 μg/mL. In a subsequent experiment, the combined effect of ciprofloxacin and semi-purified bacteriocin (60% iso-propanol fraction) produced by ST8Sh was determined. All tests were conducted in sterile flat-bottom microtiter plates (NUNC, Thermo Scientific). Each well was filled with 190 μL of sterile BHI medium adjusted to pH 6.0 with 1 M lactic acid, supplemented with final concentrations of 320, 160, 80, 40, 20, 10 and 5 μg/mL ciprofloxacin from left to right of the microtiter plates and final bacteriocin concentrations of 51,200; 12,800; 3200; 800; 200; 50; 12.5; 3.12; 0.78; and 0.20 AU/mL from top to bottom of the plates. Each well was inoculated with 10 μL *L. monocytogenes* Scott A (OD 600 nm = 0.2). Optical density readings (at 655 nm) were recorded every hour for 30 h on a microplate reader (BioTek Instruments, Inc. USA). Cultures grown in BHI broth (Difco) without added bacteriocins and ciprofloxacin served as control.

Results

Screening for Probiotic Properties in Presence of Related Genes

In the present study, our focus was on further beneficial properties of this strain for which we have designed an experimental approach for evaluating its probiotic potential. Based on the performed PCR analysis, the presence of *Map*, *Mub*, *Ef-Tu*, *EF2662*-cbp, and *EF2380*-maz was detected in the genome of *Lb. plantarum* ST8Sh, while the presence of *prg*B and *EF1249*-fbp could not be confirmed.

Hydrophobicity

In addition to the presence of specific proteins, related to binding capacity of probiotic bacteria, the hydrophobic nature of the external surface of microorganisms can have an essential role in the attachment of bacteria to the host tissue. The average hydrophobicity value registered for *Lb. plantarum* ST8Sh was 52%.

Growth at Low pH and in Presence of Ox Bile

Growth and survival in the presence of low levels of pH and presence of ox bile are required characteristics for the future probiotic strains and normally are one of the initial tests in prescreening test for the selection of beneficial LAB. The obtained results indicate that *Lb. plantarum* ST8Sh can grew well in MRS broth with initial pH values of 5.0–9.0 (Fig. 1a), but at pH 4.0, the growth rate was much lower. Even if intensive bacterial growth of *Lb. plantarum* ST8Sh was not recorded, the cells were viable, and when plated on MRS agar, they show good survival (data not shown). In addition, *Lb. plantarum* ST8Sh presented good growth in the presence of ox bile at concentrations ranging from 0.2 to 3.0% (Fig. 1b), suggesting that this strain has good chances to survive in the human GIT.

Aggregation properties

Lb. plantarum ST8Sh was characterized as having an auto-aggregation level of 51.32%; however, auto-aggregations for *L. monocytogenes* Scott A, *L. monocytogenes* ATCC 7644, *Lb. sakei* ATCC 15521, and *E. faecium* ATCC 19433 were

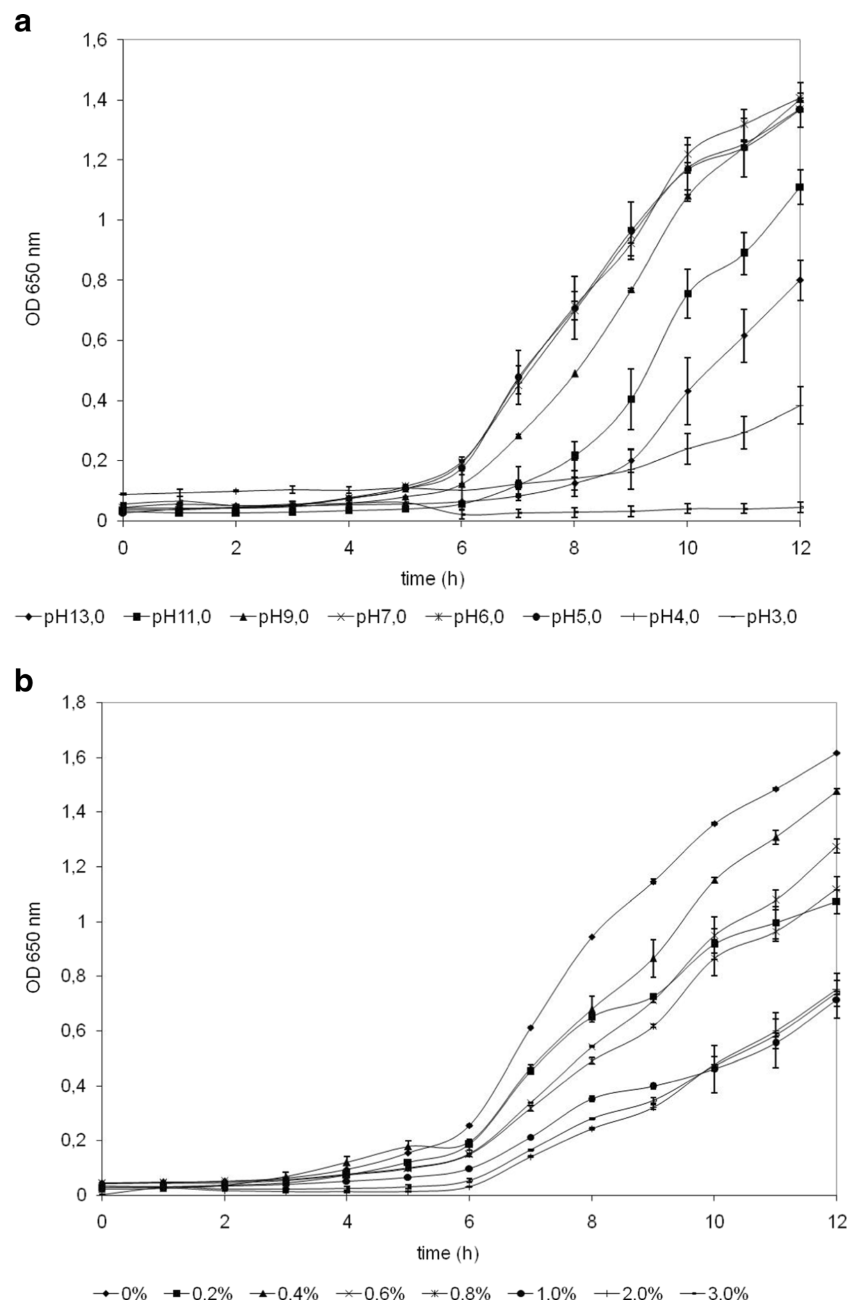

Fig. 1 **a** Comparison of growth of *Lactobacillus plantarum* ST8Sh in MRS broth (Difco) at different pH levels. Each result represents an average of three readings. **b** Comparison of growth of *Lactobacillus plantarum* ST8Sh in MRS broth (Difco) supplemented with different concentrations of ox bile. Each result represents an average of three readings

29.65, 32.74, 44.86, and 33.42%, respectively (Fig. 2). The co-aggregations of *Lb. plantarum* ST8Sh with *L. monocytogenes* Scott A, *L. monocytogenes* ATCC 7644, *Lb. sakei* ATCC 15521, and *E. faecium* ATCC 19433 were 28.54, 31.96, 39.43, and 31.54%, respectively, and also varied according to the strain (Fig. 2).

β-Galactosidase Activity, Bile Salt Deconjugation, and Adhesion to Caco-2 Cell Line

The production of β-galactosidase was detected for *Lb. plantarum* ST8Sh throughout the qualitative in vitro test applying sterile filter paper disks impregnated with ONPG. *Lb. plantarum* ST8Sh was able to grow in the presence of 0.5% (*w*/*v*) of GDC, GC, TDC, and TC sodium salts. Adherence of *Lb. plantarum* to Caco-2 cells was estimated to be 12.8%, similar to that recorded for the reference strain, *Lb. rhamnosus* GG (10.9%) (data not shown).

Bacteriocin Test

Bacteriocin produced by *Lb. plantarum* ST8Sh was semi-purified by ammonium sulfate precipitation and hydrophobic chromatography on a Sep-Pak C_{18} column. Semi-purified

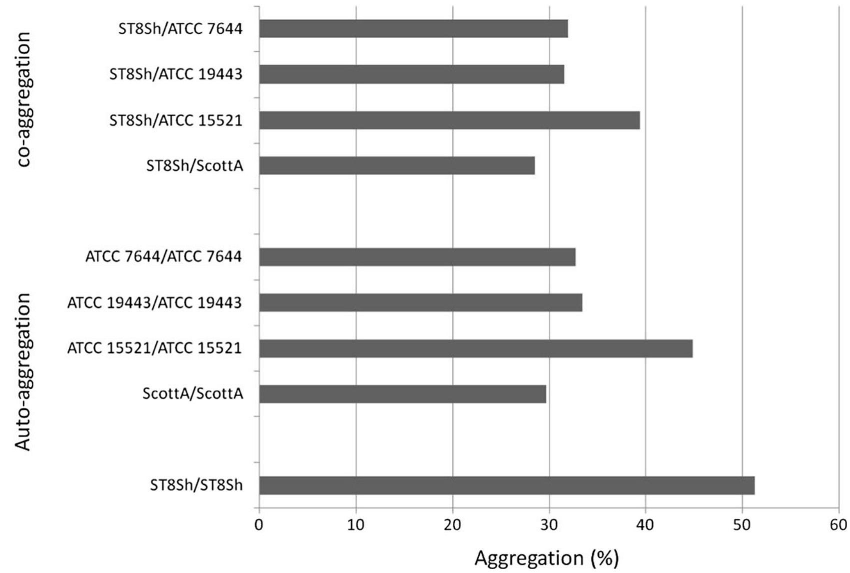

Fig. 2 Aggregation of *Lactobacillus plantarum* ST8Sh, *Enterococcus faecalis* ATCC 19443, *Lactobacillus sakei* ATCC 15521, *Listeria monocytogenes* ATCC 7644, and *Listeria monocytogenes* Scott A. Each result represents an average of three experiments

bacteriocin was presenting a very high activity against *Listeria monocytogenes* (102,400 AU/mL) and *E. faecalis* (102,400 AU/mL). Semi-purified fractions (60% isopropanol) of bacteriocin ST8Sh were used in the following experiments.

Synergistic Interaction Between Bacteriocin and Antibiotic (Ciprofloxacin)

The minimal inhibition concentration (MIC) of ciprofloxacin for *L. monocytogenes* Scott A was determined as 80 μg/mL. Based on the combined application of ciprofloxacin and bacteriocin produced by *Lb. plantarum* ST8Sh, antibacterial activity strongly increased when sublethal levels of ciprofloxacin were used in combination with the studied bacteriocins (Fig. 3). Based on the observed results, ciprofloxacin levels below MIC (e.g., 40 μg/mL) may thus be used when administered in combination with bacteriocins produced by *Lb. plantarum* ST8Sh. Growth inhibition of *L. monocytogenes* Scott A was recorded during the first 10 h and continued for the duration of the experiment. However, cells of *L. monocytogenes* Scott A treated with either bacteriocins produced by *Lb. plantarum* ST8Sh or ciprofloxacin developed resistance after 24 h and can be observed after 30 h as well.

Discussion

In a previous study [14], we reported on the isolation of *Lb. plantarum* ST8Sh from Bulgarian salami shpek and characterization of its bacteriocin with special focus on its mode of action against *L. monocytogenes* Scott A and *E. faecalis* ATCC 19433. Considering the antimicrobial activity of bacteriocin ST8Sh against the test microorganisms and the specific physiological characteristics of *Lb. plantarum* ST8Sh, we have suggested in our previous study that either the bacteriocin or the strain may be used in biopreservation [14]. However, in the present study, our focus was on further beneficial properties of this strain for which we have designed an experimental approach for evaluating its probiotic potential.

Genes related to encoding different adhesion proteins *Mub*, *Map*, *EFTu*, *prgB*, *EF2662*-cbp, *EF1249*-fbp, and *EF2380*-maz [9, 15, 16] were targeted by PCR analysis in total DNA from *Lb. plantarum* ST8Sh. The presence of *Map*, *Mub*, *Ef-Tu*, *EF2662*-cbp, and *EF2380*-maz was detected in the genome of *Lb. plantarum* ST8Sh, while the presence of *prg*B and *EF1249*-fbp could not be confirmed. The *mub* gene encodes extracellular mucus-binding proteins (MUBs), frequently found in intestinal lactobacilli and especially in *Lb. plantarum* [17, 19, 20]. A similar profile was reported for the presence of the *map* gene, associated with the production of a mucus adhesion-promoting protein (MapA). Its mechanism of action, however, has not been fully determined yet [21]. Elongation factor Tu (EF-Tu) is a multifunctional protein produced by certain lactobacilli, including *Lb. plantarum* [19, 20], that can also mediate adhesion and facilitate the colonization of human intestinal mucus. The importance of the *prg*B gene for adhesion of surface proteins was studied by Kao et al. [15]. Although the presence of the studied genes in the genome of probiotic candidates is recommended, it is not considered an essential characteristic in the screening of new potentially beneficial strains. These genes will provide an advantage to potential probiotics in the adhesion process to intestinal cells; however, the expression of these genes is a complex process and can be regulated and limited by several factors. The capacity of probiotic bacteria to adhere to the intestinal mucosa is a relevant

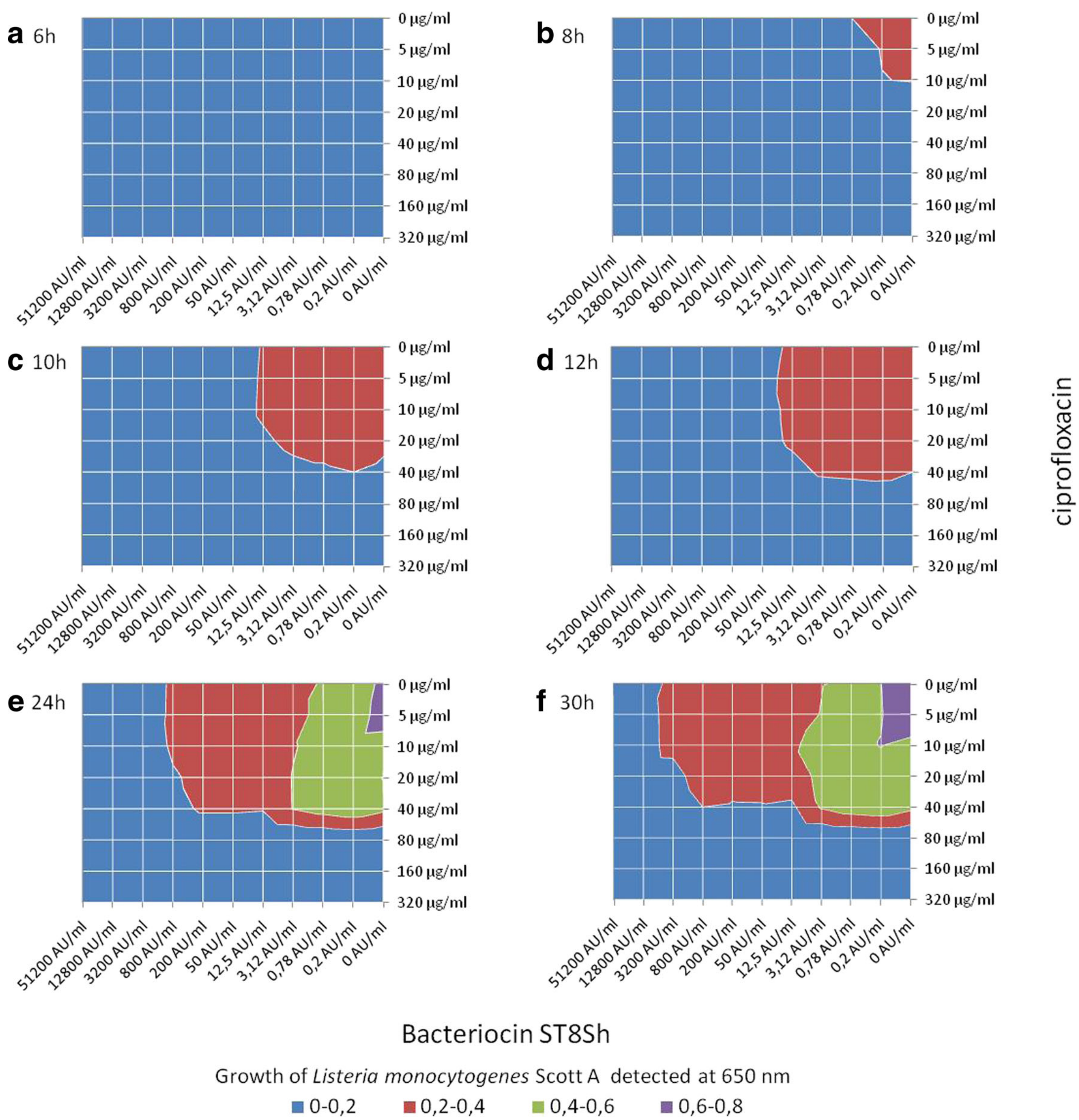

Fig. 3 Inhibitory effect on *Listeria monocytogenes* Scott A by combined effect of bacteriocin produced by *Lactobacillus plantarum* ST8Sh (from 51,200 to 0.2 AU/mL) and ciprofloxacin (from 320 to 5 µg/mL) at 37 °C after 6, 8, 10, 12, 24, and 30 h

characteristic related to consistency of beneficial properties and effects related to the modulation of the immune system and to competitive exclusion of pathogens [22, 23]. Several studies have shown surface proteins with mucus-binding capacity to mediate the adherence of lactobacilli to the intestine [24, 25]. However, various mechanisms may be involved in this process [26, 27].

In addition to the presence of specific proteins, related to binding capacity of probiotic bacteria, the hydrophobic nature of the external surface of microorganisms can have an essential role in the attachment of bacteria to the host tissue. Related to this, the cellular surface hydrophobicity has been recommended as a test, used to estimate the ability of the strain to adhere to epithelial cells [27]. The average hydrophobicity value registered for *Lb. plantarum* ST8Sh was 52%. Vinderola et al. [27] consider values higher than 40% as elevated hydrophobicity, while Zago et al. [28] consider 30% as the cutoff value for high hydrophobicity. Based on these evidences, *Lb. plantarum* ST8Sh can be considered as a strain with high cell surface hydrophobicity. In addition, similar results related to hydrophobicity profile of different *Lb. plantarum* strains was previously reported [9, 17].

Growth and survival in the presence of low levels of pH and presence of ox bile are required characteristics for the future probiotic strains and normally is one of the initial tests in prescreening test for the selection of beneficial LAB. To be able to perform any beneficial properties in the GIT, future probiotics need to survive via low pH in stomach and to be resistant to the presence of ox bile in the intestine. In order to protect LAB from the low pH during passage in stomach, some authors recommend encapsulation of probiotic LAB [29–31]. However, any additional protection and/or treatment of LAB can be related to the increase of the cost and to the reduction of viability of the probiotics.

The obtained results indicate that *Lb. plantarum* ST8Sh can grew well in MRS broth with initial pH values of 5.0–9.0 (Fig. 1a), but at pH 4.0, the growth rate was much lower. Even if intensive bacterial growth of *Lb. plantarum* ST8Sh was not recorded, the cells were viable, and when plated on MRS agar, they show good survival (data not shown). Different strains from *Lactobacillus* genus were shown to be able to grow and survive in the presence of low levels of pH. Strains of *Lb. plantarum*, *Lb. rhamnosus*, *Lb. pentosus*, and *Lb. paracasei* were previously reported as suppressed at pH 3.0 and 4.0, and variable results were recorded for pH of 11.0 and 13.0 [32].

Lb. plantarum ST8Sh presented good growth in the presence of ox bile at concentrations ranging from 0.2 to 3.0% (Fig. 1b), suggesting that this strain has good chances to survive in the human GIT. However, bile salts have different effects in different *Lactobacillus* strains. The growth of the selected strains of *Lb. plantarum*, *Lb. rhamnosus*, and *Lb. pentosus* was less affected by the presence of ox bile, compared to the strains of *Lb. paracasei* and *Lb. rhamnosus* [32]. Similar effects of ox bile and pH on *Lb. plantarum* 423, *Lb. salivarius* 241, and *Lb. curvatus* DF38 were reported by Brink et al. [33]. However, Haller et al. [34], reported that as many as 10% of *Lb. plantarum* cells, but less than 0.001% of *Lb. sakei* and *Lb. paracasei* cells, survived when exposed to HCl (pH 2.0) and bile salts.

Adhesion is considered as one of the primary requirements for the probiotic potential of bacteria. Several mechanisms are involved in this process, including production and expression of adhesion proteins and polysaccharides. However, aggregation plays an important role in this process and needs to be assessed as an important feature for adhesion and biofilm formation. *Lb. plantarum* has been studied intensively, and a number of encoding genes were described related to surface proteins responsible for recognition of or binding to components present in the environment. Some of these genes are homologous to proteins with predicted functions, such as mucus binding, aggregation promotion, and intracellular adhesion [35]. Aggregation properties can be considered as positive or negative related to the bacteriocin spectrum of activity of the investigated strain. If the bacteriocin produced by *Lb. plantarum* ST8Sh does not inhibit the co-aggregation partner, the two strains can co-exist and facilitate biofilm formation and may facilitate the colonization of the host with pathogenic bacteria. However, if the bacteriocin is active against the co-aggregation partner, high co-aggregation will facilitate interbacterial contact and the bactericidal mode of action of bacteriocins against co-aggregating partner, allowing better elimination of the co-aggregation partner from the system. Based on a previous study, auto-aggregation is a strain-specific trait [17]. *Lb. plantarum* ST8Sh was characterized as having an auto-aggregation level of 51.32% (Fig. 2), which is lower than the levels reported by Todorov et al. [32] for other lactobacilli: *Lb. pentosus* ST712BZ (67%) and *Lb. paracasei* ST284BZ (99%). The co-aggregations of *Lb. plantarum* ST8Sh with *L. monocytogenes* Scott A, *L. monocytogenes* ATCC 7644, *Lb. sakei* ATCC 1552, and *E. faecium* ATCC 19433 were 29.65, 32.74, 44.86, and 33.42%, respectively, and also varied according to the strain (Fig. 2), but the lowest levels were observed for *Listeria* and *Enterococcus* strains. Low levels of co-aggregation may play an important role in preventing the formation of biofilms and preventing the persistence of pathogenic species in the GIT.

The production of β-galactosidase was detected for *Lb. plantarum* ST8Sh throughout the qualitative in vitro test applying sterile filter paper disks impregnated with ONPG. The production of β-galactosidase by probiotic LAB, including *Lb. plantarum* ST8Sh, can contribute to the alleviation of the symptoms of lactose intolerance due to lactase deficiency [36], which includes intestinal distress, excessive flatulence, or diarrhea, and is highly prevalent among some human groups [37].

Lb. plantarum ST8Sh was able to grow in the presence of 0.5% (*w*/*v*) of GDC, GC, TDC, and TC sodium salts. The ability to survive in the presence of bile is a desirable feature for probiotic bacteria, as it favors their viability at the intestinal part and is being recommended in the selection of new probiotic candidates [27, 38]. The ability of LAB to deconjugate bile salts is a related feature to LAB, important in reducing the toxicity of these compounds [39]. The role of LAB, involved in the deconjugation of bile salts, is related to the formation of bile acids that are less soluble and less absorbed from the intestinal lumen, and the deconjugation of bile salts favors their excretion via feces, which may imply the lowering of serum cholesterol levels to the host. This feature has been reported by Jones et al. [40], showing the cholesterol-lowering effect in vivo of a bile salt hydrolase-active *Lactobacillus reuteri* strain, in a double-blind, placebo-controlled clinical trial.

Adherence of *Lb. plantarum* to Caco-2 cells was estimated to be 12.8%, similar to that recorded for the reference strain, *Lb. rhamnosus* GG (10.9%). Other studies had also evaluated the binding of LAB to colon carcinoma cells. Todorov et al. [41] reported on adherence of different LAB isolated from smoked salmon to Caco-2 cells to be from 7.8 to 13.9%. In other studies, Todorov et al. [32, 42] evaluated LAB isolated from boza and observed that adherence of studied LAB to Caco-2 cells was in similar manner (0.26–9.0%), in comparison to the 3.2 to 14.4% adhesion values reported by Tuomola and Salminen [43] (1998) and the 0.08 to 0.74% values reported by Bertazoni-Minelli et al. [7].

Based on the combined application of ciprofloxacin and bacteriocin produced by *Lb. plantarum* ST8Sh, antibacterial activity strongly increased when sublethal levels of ciprofloxacin were used in combination with the studied bacteriocins (Fig. 3). Based on the observed results, ciprofloxacin levels below MIC (e.g., 40 μg/mL) may thus be used when administered in combination with bacteriocins produced by *Lb. plantarum* ST8Sh. Growth inhibition of *L. monocytogenes* Scott A was recorded during the first 10 h and continued for the duration of the experiment. However, cells of *L. monocytogenes* Scott A treated with either bacteriocins produced by *Lb. plantarum* ST8Sh or ciprofloxacin developed resistance after 24 h and can be observed after 30 h as well. Most probably, the bacteriocin produced by *Lb. plantarum* ST8Sh was degraded by proteolytic enzymes produced by *L. monocytogenes* Scott A or was inactivated due to aggregation or adhesion processes.

Previously, a synergistic effect after combined application of ciprofloxacin and bacteriocin ST44AM, produced by *Pediococcus pentosaceus* on inhibition of *Listeria ivanovii* subsp. *ivanovii* ATCC 19119, was reported by Todorov and Dicks [44]. In a similar study, the effect of sublethal concentrations of enterocin CRL35, a cationic peptide, on the activity of erythromycin, chloramphenicol, and tetracycline was reported at sublethal concentrations and little growth inhibition was recorded [45]. Most probably, this synergistic effect can be explained as a result of the fact that *Listeria* spp. membrane depolarization is necessary, but not sufficient to produce cell death, and another concentration-dependent step, not described at present, may be implicated. Pleurocidin and derivatives, which are antimicrobial peptides from eukaryotic organisms, lost their ability to damage cell membranes at sublethal concentrations, while maintaining their capacities to inhibit macromolecular synthesis [46].

Conclusions

In our previous study, we have reported on bacteriocinogenic potential of *Lb. plantarum* ST8Sh isolated from Bulgarian salami shpek. In the present study, we evaluated the basic characteristics required for a probiotic potential of this strain based on its functional behavior. In addition, combined application of expressed bacteriocin by *Lb. plantarum* ST8Sh and ciprofloxacin on inhibition of *L. monocytogenes* Scott A was evaluated and a synergistic effect between the two applied antimicrobials was observed. What will be the future of *Lb. plantarum* ST8Sh? To be validated as a functional probiotic strain, additional research needs to be performed on its effect on immune system and observation on interaction with healthier and pathological human cells. Safety aspects of *Lb. plantarum* ST8Sh need to be investigated, including possible presence of virulence factors, interaction of the strain itself with non-antibiotic drugs, resistance to antibiotics, and cytotoxicity of the expressed bacteriocins. However, this will be a part of the future studies.

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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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