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RESEARCH

Bacteriocin and Antioxidant Production, a Beneficial Properties of Lactic Acid Bacteria Isolated from Fermented Vegetables of Northwest Bulgaria

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Abstract

Lactiplantibacillus plantarum ST01BG, ST07BG, ST10BG, and ST15BG; *Latilactobacillus curvatus* ST02BG; *Lacticaseibacillus paracasei* ST04BG; *Pediococcus pentosaceus* ST05BG; *Leuconostoc mesenteroides* ST06BG; and *Enterococcus faecium* ST11BG were isolated from home-made fermented vegetables from Northwest Bulgaria and identified by biochemical, physiological, and biomolecular analyses, including partial 16S rRNA sequencing. The strains were designated as bacteriocin producers and the expressed antimicrobials partially characterized with a focus on their proteinaceous nature, stability to different pH and temperatures. The bacteriocins were effective in inhibiting different strains of *Listeria* spp., *Enterococcus* spp. (including vancomycin resistant enterococci) and *Staphylococcus* spp. These strains can be considered safe, based on the evaluation of hemolytic activity, production of biogenic amines, mucin degradation, antibiotic susceptibility/resistance, and gelatinase enzyme production. Moreover, the strains can be considered potentially beneficial based on their stability and survival under simulated gastrointestinal tract conditions (stomach and duodenum), the production of diacetyl, and specific levels of hydrophobicity. Special attention was given to antioxidant properties (DPPH radical, hydroxyl radical, superoxide anion radical scavenging activity, Fe^{+2} ion chelating activity, and anti-lipid peroxidation) of the strains. Antioxidant properties were found to be strain specific. The beneficial attributes (antimicrobial and antioxidant) of these cultures to fermented food products may enable the reduction of chemical additives in line with consumers' demand for more natural and chemical-free food commodities.

Keywords Lactic acid bacteria · Bacteriocins · Antioxidants · Safety · Fermented vegetables

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Introduction

Isolation of beneficial lactic acid bacteria (LAB) has become a trend in the last two decades, aiming at selecting microbial cultures with application as probiotics, starters, or adjunct cultures for food fermentation processes. In the selection process, most research projects have focused on the production of antimicrobial peptides [1, 2], technological properties in accordance with the specificity of the fermentation process, or probiotic potential and health promoting properties [3]. It has been clearly established that bacteriocins produced by different LAB can be considered beneficial property for probiotic strains [4, 5], that they can be applied as powerful agents involved in increasing food safety of different fermented food products [6], and be involved in the control of some human and animal pathogens [7]. Moreover, recent research suggested that bacteriocins can be applied by the pharmaceutical industry as alternatives to antibiotics for adjunctive therapy [8, 9]. It was suggested that some bacteriocins may play a key role in *quorum sensing* interactions, being more than just simple killing metabolites [9]. Kaur and Kaur [10] suggested that some bacteriocins can be regarded as tools to control cancer cells, an application that should be explored more deeply in the coming years.

Antioxidant properties are another characteristic expressed by some LAB that have attracted attention also with the aim to reduce chemical additives in fermented and processed food products or explore their role as postbiotics in cosmetic products [11]. In fact, different LAB strains with antioxidant properties were suggested and applied in food industry [11] showing that appropriate bacterial cultures can be exploited for conducting fermentation processes and, in addition, play a role in reduction of different radicals, contributing to food safety and adding health benefits for the consumers [12].

Different antioxidants (chemical compounds and natural metabolites) play a key role in protection against free radicals and can be actively involved in the control of several diseases. Moreover, capacity of the endogenous antioxidant system of humans and animals is not sufficient for detoxification of all known threats, thereby requiring exogenous supplementation to reduce oxidative stress generated by different free radicals. For a decade, the pharmaceutical industry was supplying the health market with different additives with antioxidant properties, the majority of them being products of synthetic chemistry. However, regarding the trends for using natural additives in pharmaceutical products, screening for effective and safe natural antioxidants needs to be developed in line with current legal requirements [13, 14]. Different dietary supplements with antioxidant properties were suggested and applied with the aim of controlling and/or reducing oxidative stress. It was also suggested to use microorganisms with antioxidant systems as effective tools for maintaining low levels of free radicals [15]. Moreover, these can be explored and applied in human and animal health promoting practices, including in the control of some diseases [16]. Previously, specific antioxidant properties were reported for some LAB in oxidative stress process control [17–20]. Moreover, some authors even suggested that specific LAB, including the representative strains from the species such as *Lacticaseibacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus helveticus*, and *Lacticaseibacillus rhamnosus*, can be applied as probiotics with specific antioxidant properties [21]. However, need of new natural antioxidant metabolites that can be used for replacing synthetic chemically designed products, some of which are already prohibited for application in food and pharmaceutical products in certain countries, is obvious fact [22].

The objective of this study was to screen for safe LAB strains, naturally present in fermented food products, for their antimicrobial (bacteriocins) and antioxidant properties.

Material and Methods

Isolation: Screening for Potential Producers of Bacteriocins

In the isolation process of LAB with bacteriocinogenic and additional beneficial properties, the primary selection focused on building a collection of strains with antimicrobial properties. For this purpose, samples of artisanal fermented vegetables, prepared at the end of the Bulgarian fall season, were collected from the villages of Belogradchik district (Northwest Bulgaria). In the selection process, priority was given to the producers using their own grown vegetables, conforming to the requirements for organic farming. Samples were collected by sanitized tools and kept in sterile plastic bags at refrigeration temperatures and proceeded for isolation of bacterial cultures during the following 48 h. Samples (25 g) were macerated in 225 mL of sterile saline solution (0.85% NaCl, *w*/*v*) during 5 min in stomacher (Seward, West Sussex, UK) and serially 10 × diluted with the sterile saline solution. From each dilution, 100 µL was plated on MRS agar (Difco, Franklin Lakes, NJ, USA) and covered with an equal volume of 2% agar (*w*/*v*) (Difco). Plates were incubated for 24–48 h at 37 °C and the CFU/g determined. Plates showing well-distinguished individual colonies were selected and covered with an additional layer of BHI (Difco) supplemented with *Listeria monocytogenes* ATCC 15313 or *Enterococcus faecalis* ATCC 19443 at final concentration of approx. $10^{5–6}$ CFU/mL. Plates were incubated for an additional 24 h at 37 °C

and observed for formation of inhibitory zones around the bacterial colonies. Selected representatives of interest (colonies with clear inhibitory zones) were selected and purified according to well-accepted microbiological practices and analyzed for morphology, Gram staining, and catalase reaction. Obtained isolates were tested for the production of bacteriocins according to the recommendations of Valledor et al. [23]. The process included cultivation of the isolates in MRS broth for 24 h at 37 °C, the cell free supernatant (CFS) obtained after centrifugation (5000 × g, 15 min, 20 °C) being separated in two aliquots, and the pH of one corrected to 6.0. Both aliquots were treated for 10 min at 80 °C in order to reduce the potential effect of eventually produced proteases or H_2O_2. Next, 10 µL of each sample was spotted on the surface of BHI supplemented with 1% agar (*w*/*v*) and *L. monocytogenes* ATCC 15313 or *E. faecalis* ATCC 19443 at final concentration of approx. $10^{5–6}$ CFU/mL. Plates were incubated for 24 h at 37 °C and checked for the formation of inhibition zones. Zones larger than 3 mm were considered positive indication for the potential production of bacteriocins, taking into account the presence of inhibition zones between CFS with corrected and not corrected pH. For the evaluation of levels of activity of expressed bacteriocins, recommendations from Valledor et al. [23] were followed. The CFS aliquots, obtained as described before and with pH corrected to 6.0 and treated for 10 min at 80 °C, were 2 × serially diluted in 100 mM potassium phosphate buffer with pH 6.5. From each dilution 10 µL was spotted on the surface of previously prepared BHI supplemented with 1% agar (*w*/*v*) and test microorganism at final concentration of approx. $10^{5–6}$ CFU/mL. Plates were incubated at 37 °C for 24 h and observed for inhibition zones. Bacteriocin activity was expressed in AU/mL = $(D^n \times 1000)$/p, taking into account dilution level of the observable inhibition zone (at least 3 mm in diameter) (*n*), volume of deposited CFS (*p*) in microliters, and that *D* is the serial dilutions were prepared. Pure cultures obtained in isolation processes and bacterial test strains were grown in appropriate culture medium (MRS or BHI) for 24 h at 37 °C and stored in cryogenic tubes in the presence of 30% glycerol (*v*/*v*) at – 20 °C and – 80 °C.

Taxonomical Identification

Selected isolates with bacteriocinogenic properties were differentiated and taxonomically identified based on physiological, biochemical, and bio-molecular test recommended by de Vos et al. [24]. All isolates were evaluated after microscopy and Gram staining, for catalase reaction, growth at recommended pH and temperatures [24], and the isolates meeting criteria for LAB were subject for further differentiation and bio-molecular identification. Isolates of interest were cultured in MRS for 24 h at 37 °C and DNA isolated according to manufacturer's instructions using ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA). The concentration and purity of the obtained DNA were evaluated spectrophotometrically on NanoDrop (Thermo Fisher, Waltham, MA, USA).

The DNA concentrations were adjusted to a final concentration of 50 ng/µL and differentiation was based on repPCR with primer (5′-$(GTG)_5$-3′), performed on Veriti 96-well thermal cycler (Thermo Fisher, Waltham, MA, USA) and amplicons visualized in 1.5% agarose (*w*/*v*) gel with SYBR®Safe DNA gel stain (Sigma-Aldrich, St. Louis, MO, USA) and 1 × TAE buffer on GH-200 Genera Biosystems (Victoria, Australia)/Elite 300 Plus Power Supply (Wealtec Bioscience Co., Ltd., Taiwan) and visualized on OmegaLumG gel documenter (Aplegen, Inc., San Francisco, CA, USA).

The identification of the strains of interests was a complex approach based on recorded biochemical and physiological tests according to recommendations from de Vos et al. [24] in addition to partial 16S rRNA sequencing, performed as commercial service by primers according to recommendations from Todorov et al. [4] and Valledor et al. [23]. All obtained sequences were analyzed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Bacteriocinogenic Activity

Effect of Enzymes, pH, and Temperature on Bacteriocin Stability

With the aim to confirm that produced antimicrobials by selected strains can be classified as bacteriocins, CFS from the strains of interests, obtained as described previously, were treated with the proteolytic enzymes (proteinase K and α-chymotrypsin), and, in addition, also with α-amylase, catalase, and lipase (Sigma-Aldrich) [25]. Enzymes were added (0.1 mg/mL) to CFS and the reactions performed at 37 °C for 1 h, and enzymatic reactions were stopped by heat treatment at 98–100 °C for 5 min and then cooled down to room temperature. Residual bacteriocin activity for each of the samples was tested against *L. monocytogenes* ATCC 15313 as described before [25]. All experiments were performed in duplicate. As controls, CFS was applied without enzymatic treatment and enzyme solutions in their recommended buffers.

The effects of temperature and pH on the stability of the expressed bacteriocins were evaluated following recommendations of Fugaban et al. [25]. CFS from the cultures of interest, grown in MRS for 24 h at 37 °C, treated for 10 min at 80 °C, and pH corrected to 6.5, were separated in different aliquots and heat treated for 60 and 120 min at 4, 25, 30, 37, 45, 60, 80, and 100 °C and for 15 min at 121 °C. After cooling down to room temperature CFS

aliquots were tested for residual bacteriocin activity against *L. monocytogenes* ATCC 15313.

For the evaluation of the effect of pH, CFS from the cultures of interest, grown in MRS for 24 h at 37 °C and obtained as described before and treated for 10 min at 80 °C, were separated in different aliquots and the pH adjusted to 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0 by sterile 6 M NaOH or 6 M HCl [25]. Samples were incubated at 37 °C for 1 h and pH readjusted back to 6.0. Residual bacteriocin activity was evaluated as described before against *L. monocytogenes* ATCC 15313.

As controls, CFS samples without exposure to different temperatures and changes of pH (tested with pH corrected initially to 6.0) were analyzed as in previous experiments.

Spectrum of Activity of Produced Bacteriocins

In the evaluation of spectrum of activity for the bacteriocins produced by the studied strains, the bacterial species (comprising several strains each, and listed in Table 1) were applied. All test microorganisms were grown on the recommended growth media. CFS from the bacteriocinogenic strains grown in MRS at 37 °C for 24 h, prepared as described before, were spotted (10 µL) onto the plates (MRS or BHI) supplemented with 1% agar (*w*/*v*) and the applied (indicator) test microorganisms at final concentration of 10^5 CFU/mL. Tests were performed in duplicate, and plates were incubated at 30 °C and 37 °C for 24 h. Inhibition zones larger than 3 mm were considered positive.

Safety Evaluation of the Selected LAB

The principle "safety is priority" is a driving point in selection of putative beneficial strains, as probiotics or starters and adjunct cultures for food industry. In the present study, the bacteriocinogenic strains were evaluated for their specific resistance patterns to common and recommended antibiotics by EFSA (European Food Safety Authority) [26], for hemolytic and gelatinase activity, production of biogenic amines, and mucus degradation properties. All tests associated with safety evaluation of studied strains were performed in at least in two independent occasions in triplicate.

Evaluation for the Biogenic Amines Production

Production of biogenic amines was performed following recommendations from Bover-Cid and Holzapfel [27]. Previously selected bacteriocinogenic strains were initially cultured in MRS broth, additionally supplemented with precursors for production of biogenic amines, in proportions 1:100 (tyrosine, ornithine, lysine, and histidine, all from Sigma-Aldrich) and 0.005% pyridoxal-5-phosphate (*w*/*v*) (Sigma-Aldrich) for 24 h at 37 °C for at least 5 subsequent times according to the recommendations of Bover-Cid and Holzapfel [27]. Plates with MRS supplemented with 2% agar (*w*/*v*) and corresponding previously specified precursors for production of biogenic amines were prepared; pH indicator and 10 µL of each evaluated microbial culture were individually spread on the plates surface. All plates were incubated for up to 7 days at 37 °C and daily observed for alteration of the medium's color from yellow to violet as evidence for

Table 1 Spectrum of activity for bacteriocins produced by *Lactiplantibacillus plantarum* ST01BG, ST07BG, ST10BG, and ST15BG; *Latilactobacillus curvatus* ST02BG; *Lacticaseibacillus paracasei* ST04BG; *Pediococcus pentosaceus* ST05BG; *Leuconostoc mesenteroides* ST06BG; and *Enterococcus faecium* ST11BG against several test organisms

Test organisms	ST01BG	ST07BG	ST10BG	ST15BG	ST02BG	ST04BG	ST05BG	ST06BG	ST11BG
Listeria monocytogenes	7/7*	7/7	7/7	7/7	4/7	7/7	7/7	5/7	7/7
Listeria innocua	2/2	2/2	2/2	2/2	2/2	2/2	2/2	1/2	2/2
Enterococcus faecium	5/5	5/5	5/5	5/5	2/5	4/5	4/5	3/5	5/5
Enterococcus faecalis	3/3	2/3	2/3	2/3	1/3	3/3	3/3	1/3	3/3
Staphylococcus aureus	2/4	2/4	1/4	2/4	1/4	0/4	3/4	1/4	1/4
Lactiplanibacillus plantarum	2/6	1/6	1/6	2/6	4/6	2/6	2/6	1/6	5/6
Latilactobacillus curvatus	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
Pediococcus pentosaceus	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
Leuconostoc mesenteroides	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Vancomycin resistant enterococci (VRE)	12/14	11/14	12/14	1/14	1/14	0/14	0/14	0/14	12/14
Lactococcus lactis	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
Escherichia coli	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Salmonella spp.	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7
Klebsiella spp.	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

*Number of strains sensitive to the tested bacteriocins from the total number of evaluated strains from the mentioned species

decarboxylation of the supplemented biogenic amine precursor to form biogenic amines. *Lb. plantarum* ATCC 14917 (negative control) and *Escherichia coli* ATCC 25922 (positive control) were included in the experimental procedures, respectively.

Hemolytic Activity of the Selected LAB

In the evaluation of the specific hemolytic activity of the strains, recommendations from Fugaban et al. [28] were followed. The strains were cultured in MRS broth for 24 h at 37 °C, aliquots were streaked on trypticase soy agar supplemented with 5% defibrinated sheep blood (*v*/*v*) (Synergy Innovation, Seongnam-si, Republic of Korea), and plates were incubated for 24 h at 37 °C. *Bacillus cereus* ATCC 27348 served as positive control for β-hemolytic activity, associated with destruction of the red blood cells and formation of distinct halos around the formed bacterial colonies. *Streptococcus pneumoniae* ATCC 49619 was selected as control for α-hemolytic activity, associated with partial reduction of the red blood cells and formation of green or brown halos around the growing colonies, while *Lp. plantarum* ATCC 14917 served as control for γ-hemolytic activity, associated with lack of hemolysis and no discoloration around the growing colonies.

Gelatinase Enzyme Production by the Selected LAB

For the exploration of potential production of gelatinase, the bacteriocinogenic cultures were grown in MRS broth for 24 h at 37 °C. Previously prepared tubes, containing 10 mL MRS broth supplemented with 5% gelatin (*w*/*v*), were inoculated with 100 µL (approx. 10^5 CFU/mL) of each strain and incubated for 24 h at 37 °C. For determining liquification of gelatin as consequence of production of gelatinase, tubes were cooled for 1 h at 4 °C. Positive results for production of gelatinase were shown by retaining of the liquid phase after refrigeration. *Bacillus amyloliquefaciens* ST109, a producer of gelatinase, served as positive control [28], and *Lp. plantarum* ATCC 14197 and an untreated medium as negative controls.

Antibiotics Susceptibility/Resistance of the Selected LAB

For the evaluation of the antimicrobial susceptibility of the strains, the disc diffusion assay was applied (Oxoid, Basingstoke, Hampshire, UK). All selected strains were cultured in MRS broth for 24 h at 37 °C and seeded into MRS supplemented with 2% agar (*w*/*v*) to reach a final concentration of around 10^5 CFU/mL. Antibiotics were selected according to EFSA recommendations [26]. Antibiotic discs were deposited on the surface of previously prepared and seeded plates and incubated for 24 h at 37 °C. Plates were evaluated for formation of inhibition zones around the antibiotic containing discs and diameter of the inhibition zones (mm) recorded. Results were interpreted (susceptible or resistance) based on the guidelines provided by the manufacturer along with the recommendations of Charteris et al. [29].

Mucin Degradation by the Selected LAB

The studied bacteriocinogenic strains were grown in MRS broth for 24 h at 37 °C. For the purpose of the experiment, recommendations from Abe et al. [30] were followed. MRS (prepared manually according to the commercial product composition, but without glucose) supplemented with 1.5% agar (*w*/*v*), 0.3% of HGM (*w*/*v*) (hog gastric mucin, type III, Sigma-Aldrich), and with or without 1% of glucose (*w*/*v*) was prepared. Ten microliters of each bacterial culture was dropped on the surface of the previously prepared plates and incubated in duplicate under anaerobic (using the gas pack, Mitsubishi Gas Chemicals Company, Tokio, Japan) and aerobic conditions for 72 h at 37 °C. Subsequently formed colonies were stained for 30 min with 0.1% of amido black (*w*/*v*) in 3.5 M of acetic acid. Plates were then washed with 1.2 M of acetic acid and observed for the mucin lysis zone around the colonies.

Stability and Survival of the Selected LAB in the Simplified GIT Model

For the evaluation of survival to the gastrointestinal tract (GIT) environment, strains were exposed to the *in vitro* conditions of a simplified GIT model according to Haberer et al. [31] with some modification and optimization. Bacterial cultures were grown in 10 mL MRS broth for 24 h at 37 °C and cells harvested by centrifugation (4000 × *g*, 5 min, 20 °C) were washed twice with sterile saline (pH 6.0, 0.85% NaCl, *w*/*v*), re-suspended in the original volume in saline with adjusted pH 2.5, and the obtained suspensions were homogenized for about 1 min. These suspensions were incubated for 60 min at 37 °C, conditions representing the stomach passage. Previously prepared and sterilized by autoclaving 4 mL of a bile salt solution (10% ox-gall, *w/v,* Difco) and 17 mL of synthetic duodenum juice pH 6.0 ($NaHCO_3$ 6.4 g/L, KCl 0.239 g/L, and NaCl 1.28 g/L) were added to the treated bacterial suspensions and incubated for additional 2 h at 37 °C. These conditions are chosen for simulating the small intestine passage. At all experimental steps, samples were withdrawn, and viable cells were estimated (CFU/mL) by preparing serial dilutions in sterile saline solution, plating on MRS supplemented with 2% agar (*w*/*v*) and incubating for 48 h at 37 °C. For calculation of CFU/mL, applied dilutions of all experimental steps were considered and results were compared to the original numbers of the evaluated bacterial population.

Beneficial Properties

Surface Hydrophobicity of the Selected LAB

For the evaluation of cell surface hydrophobicity, cells obtained from the overnight cultures of the strains, grown in MRS broth at 37 °C for 24 h, were collected by centrifugation (7000 × g, 5 min, 20 °C), washed twice with 50 mM potassium phosphate buffer, pH 6.5, and re-suspended in the same buffer at turbidity around 1.0 (at 560 nm) (value A_0). *N*-hexadecane (Sigma-Aldrich) was then added to the obtained cell suspension at proportions (1:5) and homogenized by vortexing for 2 min. After incubation for 1 h at 37 °C, turbidity of the aqueous phase was measured (at 560 nm) (value *A*). The cell surface hydrophobicity was estimated according to the equation:

$$\%H = [(A_o - A)/A_o] \times 100,$$

where *A*o and *A* are the absorbance values before and after extraction with the organic solvent, respectively, according to dos Santos et al. [32]. The assays were performed in triplicate.

Diacetyl Production by the Selected LAB

Production of diacetyl was determined by growing each culture in 10 mL MRS broth for 24 h at 37 °C, followed by additional growth in reconstituted skimmed milk (1%) for 24 h at 37 °C. Next, cultures were supplemented with 5 mL of 0.5% of α-naphthol (solution prepared with ethanol) and 5 mL of creatin-KOH solution (0.3 g of creatin in 40% of KOH solution), according to recommendations of de Moraes et al. [33]. The obtained suspensions were well homogenized by vortexing for 30 s, followed by incubation for 30 min at room temperature. Diacetyl production was indicated by a pink color change around the top surface of the solution [33].

Antioxidant Activity for the Selected LAB

Determination of the DPPH Radical Scavenging Effect For the evaluation of the DPPH radical scavenging activity, experimental procedures recommended by Li et al. [34] and Duz et al. [16] were followed. Cells of the cultures grown in MRS at 37 °C for 24 h were harvested by centrifugation (3000 × g for 10 min at 20 °C) and resuspended in PBS (Lonzo, Walkersville, MD, USA); the turbidity of the suspension was adjusted to McFarland scale level 5 (corresponding to an approx. of 11 log CFU/mL). After centrifugation (10,000 × g for 15 min at 20 °C) of 0.5 mL of the previously prepared suspension, cells were resuspended in 1 mL PBS. Intact bacterial cells, prepared as described before, were mixed with 1 mL of freshly prepared DPPH solution (0.05 mM, in ethanol) and the samples stored for 1 h in darkness. As blank, PBS was used instead of a cell suspension. A solution of 1 mg/mL ascorbic acid (Sigma-Aldrich) in distilled water served as positive control. After incubation, supernatant of the experimental setup was obtained by centrifugation (10,000 × g for 10 min at 20 °C) and OD measured spectrophotometrically at 517 nm. DPPH radical scavenging activity (%) was calculated according to recommendations of Li et al. [34] and Duz et al. [16]:

$$\text{Scavenging activity (\%)} = \{1 - [(A_{\text{sample}} - A_{\text{blind}})/A_{\text{blank}}] \times 100,$$

where blind = PBS solution; blank = PBS and DPPH solutions.

Determination of Fe^{+2} Ion Chelating Activity Suggestions from Decker and Welch [35] were followed for determining the Fe^{+2} ion chelating activity. Previously prepared intact cells resuspended in PBS (1 mL suspension) were supplemented with 0.05 mL of a 2 mM $FeCl_2$ solution. Reaction was initiated by addition of 0.2 mL of 5 mM ferrozine and the samples were incubated for 10 min at room temperature in dark. Supernatant obtained by centrifugation (10,000 × g for 10 min at 20 °C) was examined spectrophotometrically at 562 nm for recording the reduction in optical density. One mg/mL EDTA instead of cell suspension was used as positive control. For the calculation of the chelating activity, the following formula was applied:

$$\text{Ferric ion chelating activity (\%)} = [1 - (OD_1/OD_2)] \times 100,$$

where OD_1 was the sample and OD_2 was the control solution.

Hydroxyl Radical Scavenging Activity For the evaluation of hydroxyl radical scavenging activity, recommendations of Wang et al. [36] were followed. Previously prepared intact cells resuspended in PBS (1 mL suspension) were transferred to glass test tubes and supplemented with 1 mL brilliant blue (0.435 mM), 2 mL $FeSO_4$ (0.5 mM), 1.5 mL H_2O_2 (3%, *w*/*v*), and incubated for 1 h at 37 °C. The supernatant was collected after centrifugation (3000 × g for 5 min at 20 °C) and absorbance measured at 624 nm. One mg/mL of ascorbic acid (Sigma-Aldrich) was used as positive control for comparison. For the evaluation of the hydroxyl radical scavenging effect of the studied bacteria, calculations were made according to recommendations of Wang et al. [36].

Hydroxyl radical scavenging activity (%)

$$= [(A_0 - A_1/(A - A_1))] \times 100,$$

where A_0 was the absorbance of the solution that contains a certain concentration of the sample and A_1 refers to the absorbance of the solution without the sample. A refers to the absorbance of the solution that did not contain the sample and the Fenton reaction system [36].

Superoxide Anion Radical Scavenging Activity In the evaluation process of the superoxide anion ($O^{2'-}$) radical scavenging activity [19], spectrophotometry with improved pyrogallol autoxidation was applied. The process was performed under alkaline conditions, where the superoxide anion radical was produced with pyrogallol (1,2,3-benzentriol) autoxidation systems. Previously prepared intact cells were resuspended in PBS (1 mL suspension) and the suspension adjusted to OD of 0.8 as working solution concentration and supplemented with 4.5 mL Tris–HCl solution (50 mM, pH 8.2). This suspension was incubated for 20 min at 25 °C in a water bath. Subsequently, 0.4 mL pyrogallol (0.25 M, preheated to 25 °C) was added and the suspension incubated for 4 min at 25 °C. By adding 0.1 mL of 8 M HCl, reaction was stopped. An equal volume of 50 M Tris–HCl buffer (pH 8.2) was applied to replace the sample for the control, while 1 mg/mL of ascorbic acid and BHA served as positive controls. For the calculations, absorbance was measured at 320 nm and the superoxide radical scavenging activity was expressed as

Superoxide anion radical scavenging activity (%)

$$= [(A_0 - A_1)/A_0] \times 100,$$

where A_1 was the absorbance of the samples and A_0 was the absorbance of the solution that did not include the sample [19].

Anti-Lipid Peroxidation Recommendations from Hsu et al. [37] were followed in the determination of anti-lipid peroxidation. Equal volumes of egg yolk (Difco) and PBS (0.2 M, pH 7.2) were mixed and homogenized in a magnetic stirrer. Obtained suspension was further diluted with PBS (in proportion egg yolk/PBS, 1:25, *v*/*v*) and 1 mL of the suspension was added to 0.5 mL cell suspension, prepared as previously described, and adjusted to McFarland 5, supplemented with 1 mL PBS and 1 mL of iron sulfate (25 mM). The final suspension was mixed and stirred for 15 min at 37 °C. In the next step, 1 mL of trichloroacetic acid (20%, *w*/*v*) was added and kept static for 10 min. Three microliters of supernatant, obtained by centrifugation (3000 × g for 10 min, 20 °C), was mixed with 2 mL of thiobarbituric acid (0.8%, *w*/*v*) by stirring in a boiling water bath for 10 min, followed by cooling down to room temperature. The supernatant was obtained by centrifugation (3000 × g for 10 min, 20 °C) and the absorbance was measured at 532 nm (As). The blank comprised 0.5 mL PBS to replace the sample (A_0). Lipid peroxide inhibition rate (%) = $[(A_0 - As)/A_0] \times 100$ [37].

Statistical Analyses

All performed experiments were repeated at least in duplicate at two independent occasions and the results represent the average of individual data with calculated standard deviations.

Results and Discussion

Screening for Potential Producers of Bacteriocins

The microbial load of the studied fermented vegetables ranged between 10^5 and 10^6 CFU/mL, suggesting relatively low microbial populations regarding the type of products. Based on the isolation procedures (MRS, anaerobic conditions, 37 °C) and preliminary observations of colony morphology, microscopical observations after Gram staining, and catalase reaction of isolates, LAB appeared to predominate the microbiota recovered from the fermented vegetables in comparison from isolated Gram-negative and catalase positive colonies. Interestingly representatives of *Bacillus* spp. (based on colony morphology) were detected only in isolated cases and can be considered uncommon in these food samples. LAB are natural inhabitants of fermented vegetables and were reported as the predominant microbiota in production of sauerkraut [38], kimchi [39], olives [40], and pickles [41]. Strains of LAB can be applied as starter cultures of the fermentation of different vegetables and are directly involved in the formation of the final food products features, playing an essential role in product safety, associated with production of different antimicrobials, including organic acids, low molecular antimicrobials, diacetyl, and bacteriocins [42]. Fermentation processes for different vegetables are not only for the preparation of traditional recipes but are also appreciated in different regions of the world, being one of the oldest ways of preservation of food commodities [43]. From the perspective of modern microbiology, LAB are one of the principal contributors to these processes [42].

In the screening processes, priority was given to the isolation of bacteriocinogenic strains. Based on the applied 3-level approach, preselection of colonies with potential antagonistic properties was selected and further evaluated for the production of bacteriocins. From the initially 73 isolated colonies showing inhibitory zones in the applied screening procedure versus *L. monocytogenes* ATCC 15313

Table 2 Principal characteristics and 16S rRNA sequencing results identification of studied bacteriocinogenic strains

Strain	Morphology	Gram-staining	Catalase production	Identification by 16S rRNA
ST01BG	Short rods, individual, straight	+	-	*Lactiplantibacillus plantarum*
ST02BG	Rods, individual, slightly curved	+	-	*Latilactobacillus curvatus*
ST04BG	Long rods, individual or in pair	+	-	*Lacticaseibacillus paracasei*
ST05BG	Cocci, grouped in tetrades	+	-	*Pediococcus pentosaceus*
ST06BG	Very short rods, coccobacillus, curved	+	-	*Leuconostoc mesenetroides*
ST07BG	Short rods, individual, straight	+	-	*Lactiplantibacillus plantarum*
ST10BG	Short rods, individual, straight	+	-	*Lactiplantibacillus plantarum*
ST11BG	Cocci, individual or in short chains	+	-	*Enterococcus faecium*
ST15BG	Short rods, individual, straight	+	-	*Lactiplantibacillus plantarum*

and *E. faecalis* ATCC 19443, only 9 were confirmed to be bacteriocin producers based on initial tests following recommendations of Valledor et al. [23]. This included testing of the CFS with adjusted to neutral pH and exposed to proteolytic enzymes. It was interesting that no more than one bacteriocinogenic isolate was obtained from the same fermented sample, neither from producer. Most probably this reveals that specific bacteriocinogenic cultures established themselves as predominant in each evaluated food product which might be associated with their specificity and involvement in the fermentation process, but also supporting the competing ability with other microorganisms in the ecosystem. However, in other studies with fermented products, more than one bacteriocinogenic strain was isolated from the same production batch [44–46]. From an ecological point of view this is an interesting finding that requires additional investigation and clarifying role of the bacteriocinogenic cultures in the development and stability of the natural fermentation food systems.

Identification of the Selected Bacteriocinogenic LAB

For each fermented vegetable lot, one culture presenting antimicrobial activity was selected for further studies and was identified based on a complex approach including recommended biochemical and physiological tests by de Vos et al. [24] and 16S rRNA sequencing. These strains were identified as *Lp. plantarum* ST01BG, ST07BG, ST10BG, and ST15BG; *Latilactobacillus curvatus* ST02BG; *Lacticaseibacillus paracasei* ST04BG; *Pediococcus pentosaceus* ST05BG; *Leuconostoc mesenteroides* ST06BG; and *Enterococcus faecium* ST11BG (Table 2). In case of the 4 isolates identified as *Lp. plantarum*, comparison of the individual cultural characteristics, sugar fermentation profile obtained via API50CHL (BioMerieux, Marcy-lÉtoile, France), and RAPD-PCR profile suggested that these isolates represent different strains (data not shown). The presence of different LAB in the fermented vegetables can be associated with beneficial and/or spoilage properties associated with the specificity of each microbial culture. Involvement of *Lp. plantarum* strains for the production of sauerkraut, pickles, or olives is well studied and clearly shows its role in vegetable fermentation processes [38–41]. Other representatives from the former genus *Lactobacillus*, recently reclassified in several genera [47], including *Lb. curvatus* and *Lc. paracasei* are also associated with different fermented food products and indicate not only to appropriate starter cultures for different food products [38–41], but have also been suggested as probiotics with beneficial properties for human and animal applications [48]. *Pediococcus* spp. are involved in the production of silage and play a role in the acidification and biotransformation of plant material [44] and bioconservation, including in the reduction of some spoilage and pathogen microorganisms [49].

The presence of different *Enterococcus* spp. in food products is frequently generating controversial discussion about safety. It is well documented that several *Enterococcus* spp. can be associated with nosocomial infections and several have been clearly identified as vancomycin resistant strains [50]. Nonetheless, *Enterococci* are part of the natural microbiota of humans and animals and play an essential role in health and may contribute to the microbial balance in the GIT [51]. Moreover, some strains were identified and applied as probiotics for decades [52]. Suvorov [53] underlined the safety of particular enterococci and advocated their applications with arguments regarding their suitability as starter cultures in fermentation processes and as probiotics for humans and animals. For centuries, *Enterococci* have been part of the starter cultures for different Mediterranean fermented food products, including dairy and products from fruits and vegetable origin [51]. The role of *Enterococci* in fermentation processes is associated with biotransformation, formation of specific sensory characteristics, production of different antimicrobial metabolites, and contribution to safety of the final fermented products [54].

Leuconostoc mesenteroides strains were isolated from different fermented food products, including fruits and vegetables [55], and their biological role in the fermentation processes was reviewed by Paula et al. [56]. Bacterial strains that do not exist can be clearly called beneficial or spoilage, since microbial behavior is well dependent on several circumstances, environmental conditions, genetic heritage, and conditions for expression of these genes. Examples can include the production of exopolysaccharides by different *Leuconostoc* strains, considered beneficial for the production of some dairy products, contributing to the consistence of the products and organoleptic properties; for some probiotics, these exopolysaccharides are metabolites with clear beneficial properties, involved in immunological response [57]; however, these polysaccharides are factors reducing the quality of some industrial products such as wine, beer, or juice, also causing spoilage in the sugar industry [58]. Different strains of *Leuconostoc* spp. were reported to be involved in the fermentation of sauerkraut [59], and kimchi and pickled vegetable products [60].

Bacteriocinogenic Activity

Effect of Enzymes, pH, and Temperature on Bacteriocin Stability

For the classification of bacteriocins, the proteinaceous nature of the inhibitory agent serves as principal characteristic. For the confirmation of proteinaceous characteristics of the inhibitory metabolite produced by studied LAB strains, the effect of selected proteinases on antimicrobial activity of the inhibitory compounds was evaluated. After exposure to proteinases, the evaluated antimicrobials lost their activity (data not shown). Additionally, the presence of α-amylase, catalase, or lipase did not affect the antimicrobial activity of these antimicrobials. Controls, CFS obtained from studied strains without the presence of mentioned enzymes, did not show changes in antimicrobial activity. Based on these results, it can be concluded that the antimicrobials produced by the evaluated strains are of proteinaceous nature and that carbohydrates or lipids and H_2O_2 are not involved in the antimicrobial activity. It was previously reported that some bacteriocins can be described as complex molecules, where in addition to the proteinaceous moiety, specific carbohydrates, or lipids can be involved in the formation of an active antimicrobial complex [61, 62]. Moreover, Ouwehand et al. [63] classified such complex sub-molecular structures as class IV bacteriocins. The fact that all tests were performed with CFS with pH adjusted to neutral is a relevant argument that the observed inhibitory action was not associated with organic acids produced by these strains. Moreover, acid tolerance of *L. monocytogenes* has been reported [64]. Hydrogen peroxide can also be excluded as potential antimicrobial produced by these strains, since treatment with catalase did not affect antimicrobial activity, in addition to treatment of the CFS obtained from studied strains for 10 min at 80 °C; also, H_2O_2 is not stable at high temperatures.

Stability of bacteriocins at different temperatures and pH levels is a characteristic relevant not only for the practical application of antimicrobials but providing information for the chemical structure of the antimicrobial protein. Since most of the studied bacteriocins are described as small polypeptides with molecular mass below 10 kDa [65], they were expected to be stable at different temperatures. Indeed, no changes in antimicrobial activity were shown after exposure to a range of temperatures (4, 25, 30, 37, 45, 60, 80, and 100 °C for 2 h), and even after 15 min at 121 °C (data not shown). This was already reported for several bacteriocins produced by different LAB, including representatives from genera *Lactobacillus* (later reclassified [47]), *Leuconostoc*, *Pediococcus*, and *Enterococcus* [25, 66–69]. Stability of the bacteriocins after exposure to 121 °C for 15 min is an interesting characteristic, since it opens the possibility for application of bacteriocins as biopharmaceutics, where safety requirements are associated with sterilization of commercial pharmaceutical preparations.

The evaluated levels of pH (2.0, 4.0, 6.0, 8.0, 10.0, and 12.0) also did not affect antimicrobial activity of these bacteriocins (data not shown). Most of the studied bacteriocins, reported in the literature, are stable over a wide pH range [25, 66–69]; however, some examples suggest that extreme pH levels may change structure of the antimicrobial peptides and interfere with their bioactivity [70]. From a practical point of view, stability of bacteriocins over wide ranges of pH may open better possibilities for their application as effective antimicrobials, since the pH of different fermented foods may determine the type of microorganisms involve in those processes. Moreover, for the pharmaceutical sector, pH is an important factor and a parameter that needs to be considered in formulations of new therapeutical preparations.

Spectrum of Activity

In the evaluation of beneficial properties of bacteriocinogenic strains, it is relevant to evaluate the spectrum of activity of the expressed antimicrobials. Indeed, antimicrobial properties are considered beneficial characteristics and may be advantageous for probiotic strains, but not when it is associated with pathogens or spoilage organisms. It is important that expressed bacteriocins will not affect other beneficial organisms present in the fermentation system (starter or adjunct cultures), neither micro-organism with beneficial properties when probiotics with antimicrobial properties are applied. From this point of view, evaluation of the spectrum of activity is relevant, showing potential areas of application of bacteriocins or bacteriocin-producing strains.

In this study, bacteriocins showed inhibitory effects against most of the tested strains of *Listeria* spp., *Staphylococcus* spp., and *Enterococcus* spp., but only a few strains of the tested *Lactobacillus* spp., *Pediococcus* spp., *Leuconostoc* spp., and *Streptococcus* spp. were sensitive (Table 1). By definition, bacteriocins are effective against strains closely related to producer species [9] and, as expected, none of the tested Gram-negative strains included in the test panel were affected (Table 1). The fact that only few of the LAB were inhibited by these bacteriocins and their producing strains can be considered beneficial, since this opens the possibility for their effective application in food fermentation processes.

Safety Evaluation

Safety evaluation on strain specific basis should be regarded as priority and no compromises should be made in the application of microbial cultures as beneficial organisms, including probiotics. For some microbial species long history of safe applications and absence of serious incidents and/or GRAS (generally recognized as safe) status are often given as argument for their application in food processing. Development in the assessment of microbial safety has shown this to be a strain-specific feature; this is generally considered essential step in the characterization of newly isolated microbial cultures proposed for human and veterinary application. Influence of environmental conditions, use of uncontrolled application of antibiotics and other biocides, and horizontal gene transfer between species traditionally not inhabiting the same ecological environment, but co-existing in ecological niches, are only some of the factors related to mutations and genetical changes in microorganisms.

Biogenic Amines Production

Production of biogenic amines is a natural process of microbial metabolism associated with decarboxylation of amino acids. Production of biogenic amines is typically associated with food fermentations; however, it can be considered safety issue when detrimental high levels of specific metabolites are produced and accumulate in a food product. Biogenic amines are associated with (e.g., scromboid) food poisoning with effects on the nervous system [27]. Their presence in food products is regulated by national and international regulatory bodies by clearly established tolerance levels.

In our study, the selected LAB strains were shown to be negative regarding production of biogenic amines when grown at 30 °C and 37 °C in the presence of the relevant precursors (tyrosine, ornithine, lysine, and histidine). Most *Enterococcus* spp. are typically positive for tyramine production [27]. Biogenic amines are organic bases, produced by the microorganisms from the decarboxylation of specific amino acids, generally with low molecular weight, and exerting strong biological activity associated with some negative consequences for humans and animals and being harmful when ingested [71]. Production of biogenic amines is strongly related to microbiological (inter- and intra-species) diversity; however, it is also clearly related to physicochemical parameters that promote bacterial growth in the food matrix and associated ecosystems [72]. Moreover, production and accumulation of biogenic amines, especially in fermented food products, can be a health hazard for the consumers when their concentrations increase as consequence of microbial metabolism during fermentation and storage. According to the regulatory agencies, permitted limits for biogenic amines in food products are strictly defined [73].

Hemolytic Activity

Hemolysin is an exotoxin that attacks blood cell membranes and causes cell rupture and a high number of endocarditis incidences have been associated with ß-hemolytic strains. Strains considered safe should show no evidence for hemolytic activity and are designated as γ-hemolytic [23]. All investigated strains in this study were shown to γ-hemolytic with no lysis of the blood cells observed. This test is normally applied as an initial discrimination approach being easy to perform and not requiring specific equipment. Several human pathogens are ß-hemolytic, for example, *Streptococcus pyogens* [74], where lysis of red blood cells is one of the principal pathotoxin characteristics. Most of the former *Lactobacillus* spp. are typically γ-hemolytic; however, some examples of ß- and α-hemolytic activity were reported for some strains [74]. Strains belonging to the *Enterococci* are a good example of the diversity regarding the type of hemolysis. Some *Enterococcus* strains were clearly shown to be hemolytic, with β-hemolytic features, while some were α-hemolytic and others γ-hemolytic [75]. Moreover, the *Enterococci* are still considered one of the most controversial groups of the LAB, since several strains are clearly characterized as human and animal pathogens, associated with different clinical cases, and carrying numerous antibiotic-resistance genes, including vancomycin [75]. However, from the other side, scientific evidence supports the safety of several *Enterococcal* strains with long traditional application in preparation of fermented food products [54] and as probiotics [51]. Thus, evaluation on strain-specific basis needs to be performed regarding all potential factors associated with the safety of newly isolated microbial cultures, for building a beneficial and safety portfolio.

Gelatinase Enzyme Production

Gelatinases are a group of proteolytic enzymes, targeting gelatin as substrate for their mode of action. The role of

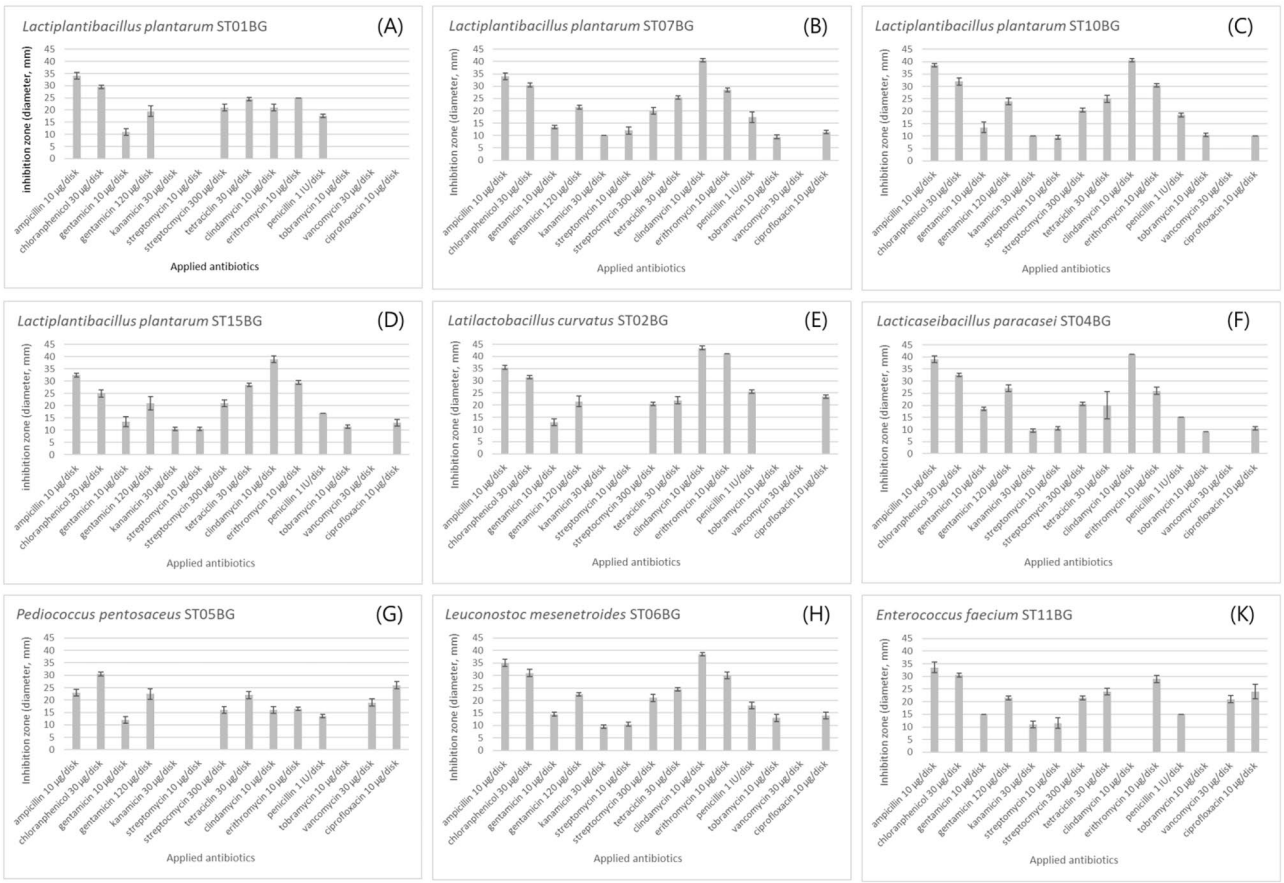

Fig. 1 Antibiotics resistance/susceptibility of *Lactiplantibacillus plantarum* ST01BG, ST07BG, ST10BG, and ST15BG; *Latilactobacillus curvatus* ST02BG; *Lacticaseibacillus paracasei* ST04BG; *Pediococcus pentosaceus* ST05BG; *Leuconostoc mesenteroides* ST06BG; and *Enterococcus faecium* ST11BG. Results represent an average value with SD indicated

gelatin is associated with locomotion functions of the body. Microbial cultures can produce gelatinases, permitting them to hydrolyze such substrates and gain energy. Moreover, in clinical microbiology, gelatinase activity is frequently associated with potential pathogenicity. Certain bacterial species/strains, particularly pathogens, can produce extracellular gelatinases (proteinases) involved in the hydrolysis of gelatin polypeptides [76]. Contrary to the pathogenicity of expression of gelatinases for the human and animal health, gelatinases have applications such as components of detergents for laundry, additives in food industry, or applied as ingredients for clarification of juices or broths, giving gelatinase-producing microorganisms appropriate biotechnological importance. In this study, the evaluated LAB strains were negative regarding the production of gelatinase enzymes and can be considered safe regarding this factor.

Antibiotic Susceptibility/Resistance of the Studied LAB

The studied LAB strains showed moderate antibiotic resistance (Fig. 1). Interaction between LAB and antibiotics is a topic raised by several researchers. Antibiotic resistance can be a serious health threat, and this may have strong relevance for the safe application of any bacterial culture. Uncontrolled use of antibiotics in different areas, in the second half of the twentieth century, resulted in selection and spread of antibiotic-resistance genes. In the last decades several regulations were implemented to reduce the emergence of antibiotic-resistance strains; in fact, the medical sector is already facing serious problems with vancomycin-resistance enterococci, methicillin-resistant *Staphylococcus aureus*, and other pathogens of relevance to humans and animals [52]. Not all mechanisms involved in the generation, selection, and spread of antibiotic-resistance determinants are clearly mapped and studied, but antibiotic-resistance genes can be horizontally and vertically transmitted between microbial cultures, and this fact might be one of the principal players in antibiotic-resistance issues [77]. On the other side, Suvorov [53] provided some arguments regarding the low probability of occurrence of antibiotic horizontal gene transfer between *Enterococci* under non-laboratory conditions. However, despite of the opposing arguments, recommendations

from the WHO (World Health Organization) regarding safety issues and potential health risks of antibiotic-resistance spread need to be considered guidelines. Applications of LAB strains showing resistance to antimicrobials must be avoided. On the other hand, applications of probiotics as preventive or therapeutic agents presume that, in some cases, they could be applied in combination with some antimicrobials (antibiotics, sulfonamides, or bacteriocins). Regarding the functionality and efficacy of probiotics, a moderate antibiotic resistance can be regarded as a positive characteristic since it will allow the combined application of the probiotic (live bacterial culture) with antibiotics (or other therapeutic antimicrobials) and to explore their complementary or synergetic mode of actions. However, such suggestions need to be scientifically evaluated assuring that genetic determinants associated with transferable antibiotic resistance in probiotic bacterial strains are not associated with easy transmittable genetic determinants, such as plasmids.

Mucin Degradation by Studied LAB

The mucin layer plays an essential role in the functionality and protections of the GIT. It is involved in the active transport of different metabolites between the GIT lumen and the epithelial cells; it plays an essential role in the adherence of beneficial microbes and provides protection against pathogens [78]. Degradation of the mucus layer is considered a clinical disorder with consequences associated with predisposition of the host to the microbial disbalance (dysbiosis) and is associated with pathological health syndromes [79]. In this study, all evaluated strains were found unable to digest mucus (in presence or absence of glucose), and therefore can be considered safe regarding this factor.

Stability and Survival in the Simplified GIT Model

According to the widely accepted definition for probiotics [80], they are live organisms providing beneficial properties for the host when present in sufficiently large numbers. A point of this definition is the fact that probiotics need to be viable when reaching a specific part of the human (or other animals) body to perform their health-promoting properties. Thus, as suggested by several research studies, the viability and persistence of putative probiotics in the human (or animal) GIT need to be considered an essential pre-requisite in the selection process [80]. Bacterial cultures can be protected from the effect of low pH in the stomach or of bile salts in the intestinal parts; however, strains with natural resistance and persistence in the GIT are the first option for the selection of putative probiotics.

In this study, selected LAB strains were isolated from fermented vegetables, an environment characterized by low pH values, and the presence of NaCl added in the preparation process as principal preservative. The stability of several fermented food products is based on the preservative effect of low pH and of NaCl; these products include fish, meat, and dairy, in addition to the different fermented vegetables prepared all around the world. Parameters typical of natural environment from where the studied bacterial strains were isolated represent selective factors related to the survival potential of those strains studied under the simulated GIT conditions *in vitro*, and serve as indication of their potential for application under in vivo conditions. Tolerance of the strains to the effect to pH and bile salts is reflected in the data shown in Fig. 2. According to the results, the assay revealed that after 1 h exposure to simulated stomach conditions (pH 2.5), *P. pentosaceus* ST05BG presented the highest tolerance to acid stress with changes in viable cell numbers from 10.35 $\log_{10}$ CFU/mL to 8.67 $\log_{10}$ CFU/mL (reduction of 1.67 $\log_{10}$CFU/mL), and quite similar for *Lc. paracasei* ST04BG with reduction from 10.42 $\log_{10}$CFU/mL to 8.69 $\log_{10}$CFU/mL (reduction of 1.74 $\log_{10}$CFU/mL). Moreover, the lowest tolerance was recorded for *E. faecium* ST11BG where reduction in the viable cells varied from 10.12 $\log_{10}$CFU/mL to 7.12 $\log_{10}$CFU/mL (reduction of 3.00 $\log_{10}$ CFU/mL) and for *Lp. plantarum* ST15BG with reductions from 9.99 $\log_{10}$ CFU/mL to 6.72 $\log_{10}$ CFU/mL (reduction of 3.27 $\log_{10}$CFU/mL) (Fig. 2). In the next stage of the experiment, bacterial cultures were exposed to simulated small intestine conditions and the presence of duodenum juice and bile salts; the changes in viable cell counts were for *Lc. paracasei* ST04BG from 10.42 $\log_{10}$ CFU/mL to 9.69 $\log_{10}$ CFU/mL (reduction of 0.73 $\log_{10}$ CFU/mL) and for *P. pentosaceus* ST05BG from 10.34 $\log_{10}$ CFU/mL to 9.60 $\log_{10}$ CFU/mL (reduction of 0.74 $\log_{10}$ CFU/mL), suggesting their higher survival potential as probiotic candidates (Fig. 2). However, highest reductions in the viable cells after exposure to duodenum juice were recorded for *Lp. plantarum* ST15BG, with reduction from 9.99 $\log_{10}$ CFU/mL to 6.66 $\log_{10}$ CFU/mL (reduction of 3.34 $\log_{10}$ CFU/mL), and for *E. faecium* ST11BG, with reduction from 10.12 $\log_{10}$ CFU/mL to 6.79 $\log_{10}$ CFU/mL (reduction of 3.33 $\log_{10}$ CFU/mL) (Fig. 2). According to the different regulatory agencies, 10^6 CFU/g has been suggested as the minimum therapeutic dose of viable probiotic microorganisms for exhibiting a health benefit [80]. According to this criterion, these results suggest that all evaluated strains can be considered appropriate bacterial cultures (Fig. 2).

Beneficial Properties

Hydrophobicity

The studied LAB strains presented variable although relatively low levels of hydrophobicity, ranging from 1.72% for *Lb. curvatus* ST02BG to 12.35% for *Lp. plantarum* ST15BG

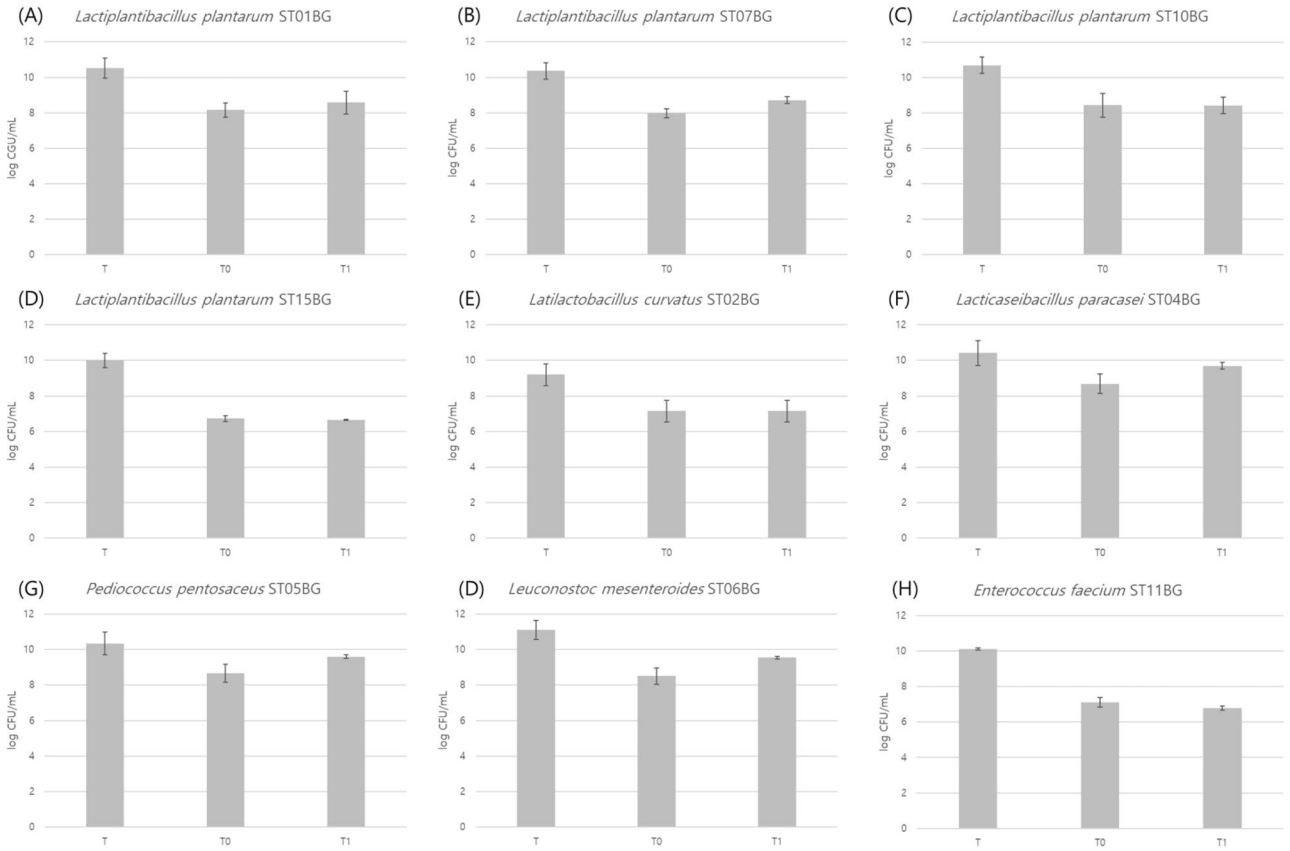

Fig. 2 Evaluation of the survival of *Lactiplantibacillus plantarum* ST01BG, ST07BG, ST10BG, and ST15BG; *Latilactobacillus curvatus* ST02BG; *Lacticaseibacillus paracasei* ST04BG; *Pediococcus pentosaceus* ST05BG; *Leuconostoc mesenteroides* ST06BG; and *Enterococcus faecium* ST11BG. The strains were exposed to simulated GIT conditions. T, initial applied microbial cells concentration (CFU/mL); T0, CFU/mL after exposure to the stomach conditions; T1, CFU/mL after exposure to the duodenum environment model conditions. Results represent an average value with SD indicated

(Fig. 3), based on the adhesion to *n*-hexadecane. It is well known that cell surface hydrophobicity is associated with a non-specific interaction between the host and the microbial cells and different surface proteins, while other components of the cell wall are also involved in these processes, normally with a weak initial interaction, in several cases reversible, and precedes subsequent specific better adhesion with involvement of more specific mechanisms and the role of lipoteichoic acids [81]. It was suggested that bacterial cultures showing a high hydrophobicity generally exert strong interactions and better adhesion to the mucosal cells. In previous studies, Todorov et al. [45] reported hydrophobicity values between 75 and 80% for *Lc. rhamnosus* ST461BZ and ST462BZ and *Lp. plantarum* ST664BZ strains and also demonstrated that their strains had a hydrophobicity value higher than that recorded for *Lc. rhamnosus* GG (55%), a commercial well-known probiotic strain. Hydrophobicity is an approximate indication of the potential of the bacterial cultures to adhere to the mucous in the GIT; however, this correlation is still not fully confirmed. Hydrophobicity may contribute to the adhesion processes but is not a prerequisite for strong adherence. Moreover, this property is strain specific, and hydrophobicity may vary among genetically closely related species and even among strains of the same species [82].

Diacetyl Production

Of the nine evaluated strains, only *Lp. plantarum* ST07BG, ST10BG, and ST15BG; *Lc. paracasei* ST04BG; and *Le. mesenteroides* ST06BG generated positive results for diacetyl production. This property is considered to be a beneficial feature of the LAB, suggesting an association with its participation on the formation of flavor in dairy products and contributing to antimicrobial properties. Jay [83] suggested that diacetyl plays an important role as an effective inhibitor of different microbial groups, such as yeasts, molds, and some Gram-negative and Gram-positive bacteria [83]. Myrvik and Volk [84] reported on antibacterial activity of diacetyl against *Mycobacterium tuberculosis* (at 3 µg/mL,

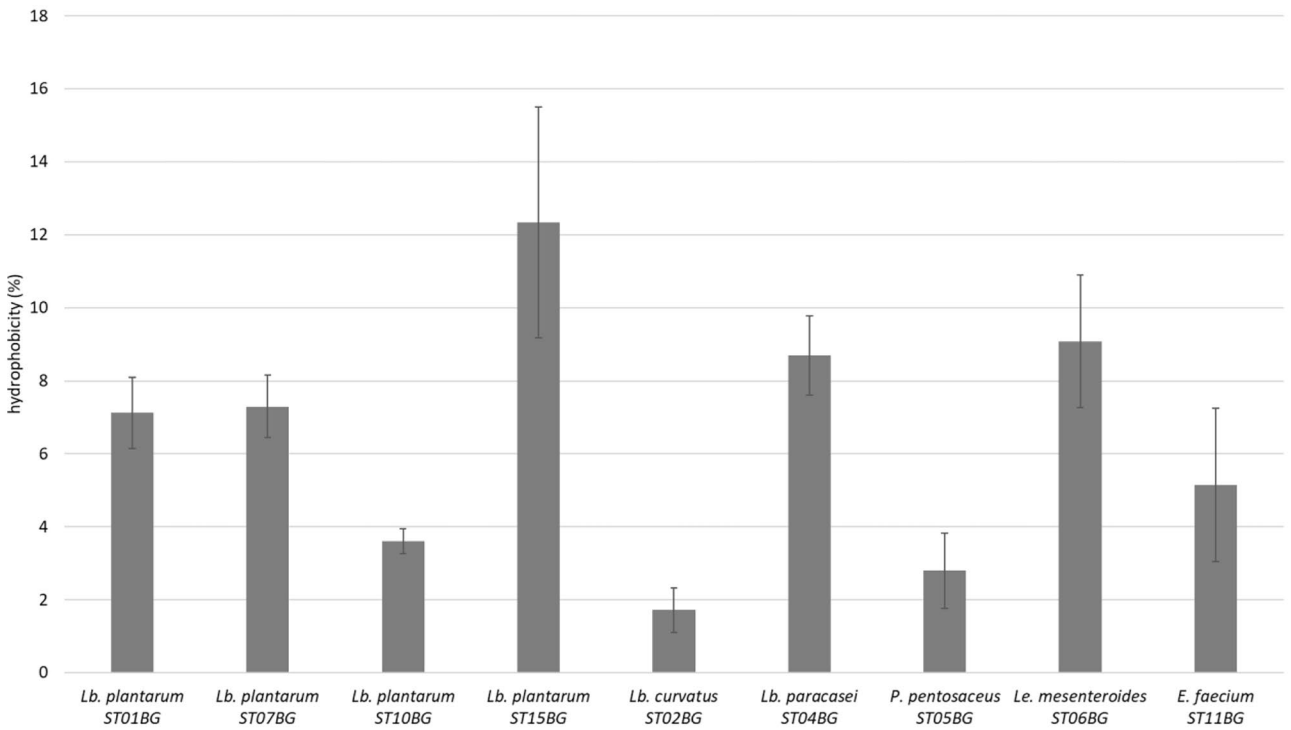

Fig. 3 Values determined for hydrophobicity of *Lactiplantibacillus plantarum* ST01BG, ST07BG, ST10BG, and ST15BG; *Latilactobacillus curvatus* ST02BG; *Lacticaseibacillus paracasei* ST04BG; *Pediococcus pentosaceus* ST05BG; *Leuconostoc mesenteroides* ST06BG; and *Enterococcus faecium* ST11BG. Results represent an average value with SD indicated

the lowest inhibitory concentration) and against *E. coli* (at 31 µg/mL). Moreover, Kulshrestha and Marth [85] reported that 1000 µg/mL of diacetyl was effective against *E. coli* and *Stap. aureus*.

Antioxidant Activity

Antioxidants are defined either as metabolites secreted by different organisms or as chemical compounds produced by synthetic chemistry, and are naturally involved in the processes of prevention, halting, or reduction of oxidative damage by different radicals. Antioxidants are known for their role as effective participants in protection processes in the human (and animal) host against negative effects of free radicals, not only those defined as subproducts of synthetic chemistry but also the products of cell metabolism. Moreover, the role of microbial antioxidants was also associated with a reduction in the progression of several diseases [86]. Some probiotics with antioxidant properties can be considered beneficial by the effective reduction of the negative effect of different radicals and thus playing an essential role in the restoration of homeostasis in the organism during oxidative stress [13, 14]. Specific strains, including some LAB with antioxidant capabilities, can be applied for the prevention of some diseases associated with the negative effect of free radicals [15, 17–20]. All tested LAB strains in this study were associated with different antioxidant activity levels, an additional argument for their characterization as beneficial strains and potentially effective probiotics.

Determination of the DPPH Radical Scavenging Effect The DPPH radical scavenging research approach is one of the frequently applied antioxidant methods associated with sensitivity, reproducibility, easy to perform, and less time consuming compared to other assays. In the register of the DPPH radical scavenging capacity, the antioxidant activity increases proportionally with the reduction of the purple color formed when the DPPH radical is added to the experimental medium. In our experiments the highest radical scavenging property was demonstrated for *P. pentosaceus* ST05BG (70.63%), while the lowest activity was recorded for *Lp. plantarum* ST10BG (36.96%), compared to that of ascorbic acid (88.22%), applied as positive control (Fig. 4A).

It is clear that even strains belonging to the same species can show different antioxidant properties; in other words, these properties are strain-specific characteristics. It was previously suggested that antioxidant activity recorded for some LAB strains was associated with the presence of specific cell surface metabolites, including extracellular polysaccharides [87]. Moreover, for representatives of *Bifidobacterium*

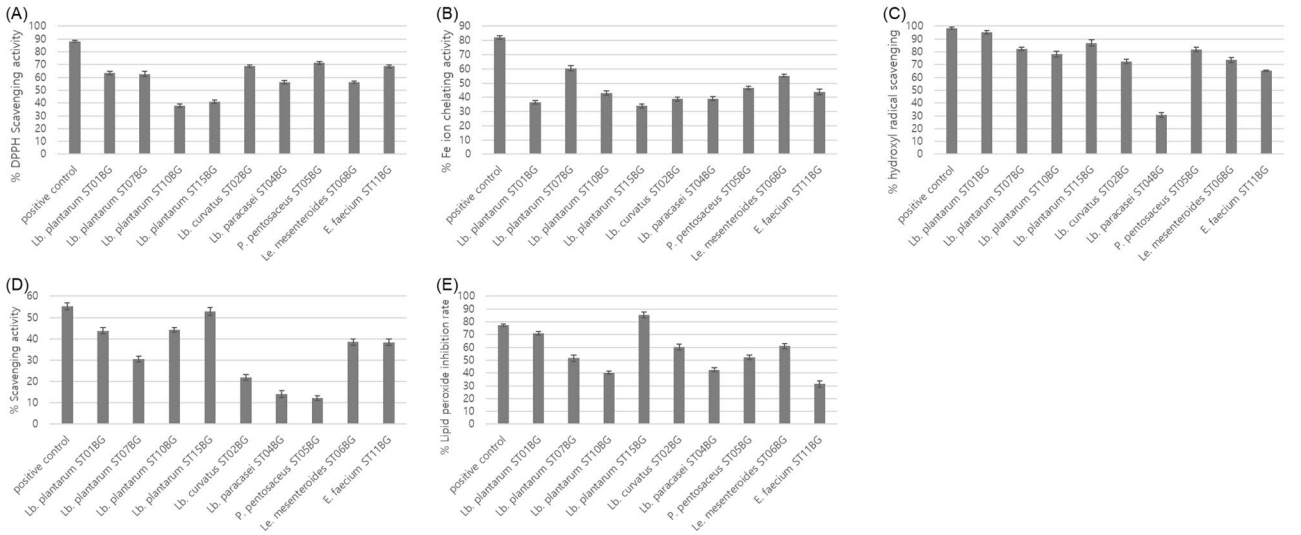

Fig. 4 Antioxidant activities for *Lactiplantibacillus plantarum* ST01BG, ST07BG, ST10BG, and ST15BG; *Latilactobacillus curvatus* ST02BG; *Lacticaseibacillus paracasei* ST04BG; *Pediococcus pentosaceus* ST05BG; *Leuconostoc mesenteroides* ST06BG; and *Enterococcus faecium* ST11BG. **A** DPPH activity; **B** ferric ion chelating activity; **C** hydroxyl radical scavenging activity; **D** superoxide anion radical scavenging activity; **E** lipid peroxide inhibition rate. Results represent the average of at least 3 repetitions and showing the SD

spp., antioxidant properties were linked to the presence of lipoteichoic acid [88]. Recorded levels of DPPH in the studied strains ranged between 70.63% for *P. pentosaceus* ST05BG and 36.96% for *Lp. plantarum* ST10BG (Fig. 4A). Even between the 3 evaluated *Lp. plantarum* strains, DPPH values varied (62.53% for strain ST01BG, 61.14% for ST07BG and ST10BG) (Fig. 4A), indicating that antioxidant properties are strain specific. Similar values were reported by Rwubuzizi et al. [89] and Duz et al. [16]. Moreover, applying a similar research approach, Zhang et al. [90] reported lower DPPH radical scavenging activities of 23.99% and 27.50% for *Lc. casei* subsp. *casei* SY13 and *Lb. delbrueckii* subsp. *bulgaricus* LJJ, respectively. High levels of DPPH were reported for *Lc. casei* EMP2 (78.5%) and for different *Lb. delbrueckii* subsp. *bulgaricus* strains (between 56.3 and 77.7%) [91]. Furthermore, when comparing the results reported in different research studies, it is important that they should be based on similar cell concentrations. Wang et al. [92] showed that DPPH values can increase in a concentration-dependent manner in relation to the CFU/mL applied in the experimental procedures and suggested 10^6–10^9 CFU/mL as optimal cell density.

Determination of Fe^{+2} Ion Chelating Activity Catalysis of reactions involves transition of different metals, resulting in the release of specific reactive oxygen species, including hydroxyl radical and superoxide anion. In the current study, the strains ferric ion chelating activities with the highest values was recorded for *Lp. plantarum* ST07BG (59.10%), and the lowest chelation activity for *Lp. plantarum* ST15BG (33.00%), compared to the positive control, ethylene diamine tetra-acetic acid (EDTA) (1 mg/mL) (82.27%).

Ferrous ions can exhibit specific antioxidant properties, however, that can be related to the breakdown of lipid peroxides, generally associated with the formation of specific off-flavors during the storage of dairy and meat food products [16]. The chelating activities of LAB were suggested to be associated with the physiological chelators located on the bacterial cell wall [93]. In the study of Duz et al. [16], metal (Fe^{+2}) ion chelating effects between 20 and 75% were reported, where the positive standard (EDTA) (1 mg/mL) generated values of 92%. Moreover, Rwubuzizi et al. [89] reported levels between 98.20 and 59.10% for different strains of *Streptococcus salivarius*. In comparison, levels between 33.00 and 59.10% were recorded in our study (Fig. 4B).

Different mechanisms can be involved in the antioxidant and free radical scavenging properties of LAB cultures, including probiotics; however, they may exhibit some degree of metal chelating activity as well [94]. Such antioxidant activities of LABs were associated with the expression of specific iron binding proteins [95]. These properties can be beneficial not only reflecting the antioxidation potential but also as a positive characteristic in the processes of exclusion of heavy metals from the human and animal host by reducing toxicity. Moreover, it was suggested that for some *Str. thermophilus* and *Lc. casei* strains, antioxidant properties most probably are associated with the elimination of the

transition metal ions and by this way, reducing the negative effect of high levels of free O_2 [93, 96].

Hydroxyl Radical Scavenging Activity An important role of microorganisms based on *in vitro* radical scavenging activity was reported for different LAB strains [97]. In our study, 9 LAB strains isolated from fermented vegetable food samples demonstrated variable hydroxyl radical scavenging activities with highest levels ranging from 94.5% for *Lp. plantarum* ST01BG to 64.90% for *E. faecium* ST11BG and the lowest of only 29.60% for *Lc. paracasei* ST04BG, as compared to the control, ascorbic acid, with results of 98.34% (Fig. 4C). The obtained values were similar to those reported by Duz et al. [16], where levels of hydroxyl radical scavenging activities range from 82% for *Lp. plantarum* IH16L and *Lb. sakei* IH15L and 78% for *Lb. curvatus* IH1L. Rwubuzizi et al. [89] reported levels between 94.60% for *Str. salivarus* and 36.60% for *Lp. plantarum*, respectively, the last value being much lower than that obtained for *Lp. plantarum* strains in the present study. An antioxidant role of LAB was suggested by Virtanen et al. [98], pointing that consumption of functional foods enriched by LAB can reduce the accumulation risk of reactive oxygen species (ROS) possibly as a consequence of the degradation of hydrogen peroxide and superoxide via microbial antioxidant systems. Another study reported on high levels of hydroxyl radical scavenging properties for different strains of *Lp. plantarum*, isolated from traditional fermented Chinese food products, and proving the relation to the levels of viable cells [34]. As potential mechanism in these antioxidant processes, Duz et al. [16] reported that LAB strains (*Lp. plantarum* IH16L, *Lb. sakei* IH15L, and *Lb. curvatus* IH1L) can exhibit strong hydroxyl radical scavenging activity, suggesting the association with their affinity in binding metal ions, including Fe^{2+}. Therefore, thanks to the potential of LAB of reducing heavy metal contamination in processed food and feed, including fermented products, suitable strains can be applied for increasing the safety of these products.

Superoxide Anion Radical Scavenging Activity Defense mechanisms against oxidative stress associated with different intracellular enzymes are generally only released after degradation of bacterial cells. Some of these enzymes are also associated with superoxide anion scavenging ability [99]. In the current study, the recorded superoxide anion radical scavenging activity was higher for *Lp. plantarum* ST15BG (51.61%) and lower for *P. pentosaceus* ST05BG (11.39%) (Fig. 4D). Relatively lower hydroxyl radical scavenging activities were reported by Duz et al. (2020) with *Lp. plantarum* IH28L showing the higher value of 43%. Rwubuzizi et al. [89] found that superoxide anion radical scavenging activity was higher for *Str. salivarius* ST61HK (54.62%) and lower for *E. faecium* ST651ea (18.7%).

The superoxide dismutase (SOD) enzymatic activity is clearly related to the in vivo antioxidant defense mechanism [100], and Mn-SOD was identified in LAB strains [95]. Compared to the former evaluated antioxidant properties, the SOD enzymatic activities recorded in the present study were relatively lower (between 51.61% for *Lp. plantarum* ST15BG and only 11.39% for *P. pentosaceus* ST05BG), in accordance with the results of Duz et al. [16] and Rwubuzizi et al. [89]. A possible hypothesis to justify these lower SOD activities can be that the investigated LAB strains most probably were more able to remove metal ions and by these mechanisms to reduce oxidative stress; most probably they were not specialized for higher SOD production to wedge the oxidative chain reactions. Ji et al. [100] reported on SOD enzymatic activities for 11 LAB (5 *Lactobacillus* spp. and 6 *Leuconostoc* spp.) strains with levels of about 35% for most of the evaluated *Leuconostoc* strains.

Anti-Lipid Peroxidation The process of lipid peroxidation is described as the oxidation of cellular membrane phospholipids to peroxide derivatives [101]. In the current study, *Lp. plantarum* ST15BG (83.65%) and *E. faecium* ST11BG (29.80%) showed the highest and lowest anti-lipid peroxidation values, respectively (Fig. 4E). The recorded levels of anti-lipid peroxidation were higher compared to the results reported by Rwubuzizi et al. [89] and were associated with strains isolated from the oral cavity of Caucasian volunteers and Korean fermented products, with a maximum value of 69.43% reported for *Lb. gasseri* ST16HK. Lower lipid peroxidation inhibition values were reported also by Duz et al. [16], ranging between 63.29 (for *Lb. curvatus* GH1L) and 40.18% (for *Lb. plantarum* GH2.7L). These authors also mentioned that lipids are the principal target for the oxygenation processes in biological systems. Ou et al. [102] reported on the antioxidative properties of *Str. salivarius* subsp. *thermophilus* ATCC 19258 (57%) and *Lb. delbrueckii* subsp. *bulgaricus* ATCC 11842 (42%) strains isolated from yogurt. Ural [91] suggested that the types of lipids are important in the evaluation of lipid peroxidation inhibition, reporting that *Lc. rhamnosus* SMC6 and *Lb. delbrueckii* subsp. *bulgaricus* 12L applied to blood plasma (containing biological lipid) presented anti-lipid peroxidation activity values of 39.2% and 30.2%, respectively [91]. Moreover, in a study of Duz et al. [16], egg yolk was suggested as lipid source to demonstrate the anti-lipid peroxidation activity of the selected LAB strains. Zhang et al. [103] suggested a similar experimental approach and the use of egg yolk for determining anti-lipid peroxidation capacities of *Lb. curvatus* SR6 and *Lb. paracasei* SR10-1, isolated from traditional fermented Chinese meat products, and showing values of, respectively, 55% and 63.93%.

Conclusions

In current study we have evaluated beneficial properties of some isolated obtained from fermented vegetable artisanal produced in Northwest Bulgaria. This research does not have as aim to state that studied fermented food products can be considered "super foods," but want to serve as example how fermented food products can be served as source of isolation of beneficial microorganisms. Beneficial properties of several LAB strains have been widely reported, and numerous strains were suggested and/or applied as probiotics or starter cultures. In the last decade, prospective research and screening for new strains with more than one beneficial property were accelerated for the implementation of multifunctional starters in food fermentations and, where possible, with probiotic or bioprotective properties additional to their function as starter cultures. Safety has always been and will be an essential priority in the selection of new functional microbial cultures. However, in addition, combination between production of antimicrobial proteins (bacteriocins) and antioxidants can be considered an interesting application scenario for the selection of new starter cultures. Beneficial properties of LAB cultures can add value to fermented food products and may lead to the reduction of chemical additives in line with consumers' demand for more natural food commodities free of artificial preservatives. Appropriate selection of safe microbial cultures with antimicrobial and antioxidant properties is the first step in an extensive research project, in which technological and desired beneficial properties should be validated in situ and in vivo.

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Data Availability All data generated or analyzed during this study are included in this published article and comply with research standards.

Declarations

Ethical Approval This article does not contain any studies with human or animal subjects.

Conflict of Interest The authors declare no competing interests.

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