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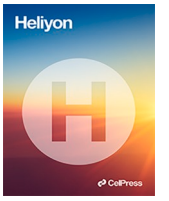

Research article

Beneficial, safety, and antioxidant properties of lactic acid bacteria: A next step in their evaluation as potential probiotics

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ABSTRACT

The role of lactic acid bacteria (LAB) as probiotics as health promoting factors for human or veterinary practice has gained increasing interest during the last three decades. This is reflected in screening approaches of LAB strains in line with minimal requirements for a "probiotic" with regard to safety and functionality. The latter might also include natural antioxidant properties, thereby constituting an additional benefit in substituting synthetic antioxidants. The *in vitro* antioxidant assays conducted in this study included the scavenging of the 2,2-diphenyl-1-picryl-hydrazil (DPPH) free radical, metal (Fe^{+2}) ion chelation, determining the scavenging properties of the hydroxyl and superoxide radicals, and anti-lipid peroxidation.

Analysis of DPPH free radical scavenging property for the microorganisms included in current study, showed *Streptococcus salivarius* ST59HK to exhibit the highest activity at a level of 85.24%. The greatest Fe^{+2} chelation activity with 98.2% was recorded for *Str. salivarius* ST62HK while the lowest was recorded for *Str. salivarius* ST48HK at 71.5%. The greatest and minimal hydroxyl radical scavenging levels were detected for *Str. salivarius* ST59HK (98.6%) and *Lactiplantibacillus plantarum* ST63HK (35.60%), respectively. Superoxide anion radical scavenging activity was highly exhibited by *Str. salivarius* ST61HK (54.62%) and the least exhibited by *Enterococcus faecium* ST651ea (18.7%). Lastly, the strains *Lactobacillus gasseri* ST16HK and *E. faecium* ST7319ea showed the highest and lowest anti-lipid peroxidation levels with 69.43% and 26.15%, respectively. Anti-oxidative properties appear to be strain specific and thus some of these strains could be potentially applied as natural antioxidants in fermented food products.

1. Introduction

Selection of the appropriate probiotic strains includes numerous *in vitro* research steps. The rationale for applying a probiotic strain is to provide health benefits to the consumers, to prevent development of clinical complications of an infection, or even to complement modern medical approaches in the treatment of some diseases. The beneficial application of probiotic strains has been supported by evidence for the prevention and/or treatment of e.g., diabetes [1] asthma [2], Inflammatory Bowel Disease (IBD) [3], improvement of

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GIT functions [4] reducing risks and consequences of traveler's diarrhea [5]. The beneficial role of selected strains belonging to different lactic acid bacteria (LAB), bifidobacteria and new generation probiotics, including representatives of the genera such as *Clostridium* and *Akkermansia*, has been confirmed by scientific evidence [6].

However, preliminary screening and evaluation of principal functional properties of new probiotic candidates is decisively important. Initially these must be performed *in vitro*, for selection of promising candidates with specific beneficial properties. Subsequently these should be evaluated and validated in appropriate animal models and in controlled human clinical studies. Some of the key points in the preliminary *in vitro* tests exploring potential probiotic properties are generally associated with confirmation of the safety of evaluated strains, their adequacy for and adaptation to the human and/or animal GIT, oral cavity, skin, lung or other parts of the body, and should also consider appropriate survival, and production of metabolites or expression of properties that can be associated with their positive effect on the host's wellbeing.

Suggestions to apply some probiotics as natural antioxidants were explored in recent years and some LAB strains were validated for their antioxidant potential [7–9]. As chemical compounds antioxidants may avoid, halt, or decrease oxidative damage and are widely applied in the food and pharmaceutical industry [10]. From a physiological point of view antioxidants are involved in protection against free radicals and can potentially impede the progression of numerous diseases. However, related with the restricted antioxidant capacity of the endogenic antioxidant capacity, the body (humans and animals) demands exogenous regulation and supplementation in order to reduce oxidative stress generated by different free radicals. The need for appropriate antioxidants is generally realised. In addition to chemical additives with antioxidant properties, new, effective, and safe natural antioxidants should however be developed in line with current legal requirements [11,12]. In the last decade, dietary antioxidant supplements were suggested and applied as important instruments in managing of the oxidative stress. Microorganisms have their own antioxidant systems that were suggested as potential tool for maintaining low free radical levels [13]. It has been suggested that these microbial properties can be explored and applied for health promotion, and, even more, in the control of some diseases [14]. In a previous study, antioxidant properties of LAB and their health beneficial properties associated with the control of oxidative stress were reported [15–18]. Different LAB representatives, including the species *Lacticaseibacillus casei, Lactobacillus acidophilus, Lactobacillus helveticus* and *Lacticaseibacillus rhamnosus* were suggested as useful probiotics with specific antioxidant properties [19]. The pharmaceutical industry is already exploring the antioxidant properties of different synthetic antioxidants such as butylated hydroxy-anisole (BHA), butylated hydroxytoluene (BHT), and n-propyl gallate (PG), all of which with proven sturdy antioxidant performance against various oxidation systems. However, strong negative effects, including liver damage and carcinogenic consequences, were reported for some chemical antioxidants and served as arguments for their restriction or prohibition in certain countries for application in food products [20].

Thus, there appears a definitive need to find specific strains and/or natural products with antioxidant properties as safer and natural antioxidants and thereby to either substitute or decrease the use of synthetic antioxidants. This study aimed to determine the antioxidant activities for several LAB strains as natural antioxidants by using different methods complemented with additional safety tests.

2. Materials and methods

2.1. Bacterial cultures

Lactobacillus gasseri ST16HK, *Streptococcus salivarius* ST48HK, ST59HK, ST61HK, and ST62HK, *Lactiplantibacillus plantarum* ST63HK and ST66HK, *Latilactobacillus sakei* ST69HK, *Enterococcus faecium* ST651ea, ST7119ea, and ST7319ea were previously isolated and evaluated as potential probiotics [21,22]. All strains were kept at −20 °C in presence of 20% glycerol and sub-cultured in MRS broth (Difco, Franklin Lakes, NJ, USA) at 37 °C for 24 h right before every experimental procedure.

2.2. Proteolytic activity and formation of free amino acids

The studied LAB strains were cultured in MRS broth at 37 °C for 24 h and centrifuged at 10,000×*g* for 30 min at 4 °C. The cells were washed 3 times with 0.85% saline, resuspended in 10 mL of sterile skim milk and incubated at 37 °C for 24 h. The cultures in the skim milk were centrifuged under the same conditions (10,000×*g*, 30 min, 4 °C). The protein concentration in the obtained supernatant was determined by the Bradford assay, using bovine serum albumin as standard (Bio-Rad Laboratories, BioRad, Hercules, CA, USA). Ten percent of sterile skim milk (Difco) incubated under the same conditions were used as control. For the spectrophotometric analysis, 20–50 μL of each selected strain were added to the 1 mL of OPA (*o*-phthaldialdehyde) reagent. The OPA solution was prepared by measuring the final volume as 100 mL with water by mixing 50 mL of 0.1 mol/L of sodium borate (2% *w/v* SDS), 80 mg of OPA which was dissolved in 2 mL of methanol, and 0.2 mL of 2-mercaptoethanol/ethanethiol. The OPA and strain solution were then mixed and incubated at 20 °C (room temperature) for 2 min and the absorbance was measured at 340 nm in the spectrophotometer [23]. Experiment was performed in triplicates and SD calculated.

2.3. Mucin degradation

The LAB strains were inoculated in MRS broth, followed by incubated at 37 °C for 24 h. Ten microliters of the bacterial cultures were dropped on the MRS agar surface previously prepared by adding 1.5% of agar and 0.3% of HGM (hog gastric mucin, type III, Sigma-Aldrich, San Luis, MO, USA) with or without 1% of glucose into MRS. The plates were incubated anaerobically at 37 °C using the gas pack (Mitsubishi Gas Chemicals Company, Tokio, Japan) for 72 h and then stained with 0.1% of amido black in 3.5 mol/L of acetic

acid for 30 min. It was then washed with 1.2 mol/L of acetic acid and the results were observed by the mucin lysis zone around the colonies [24].

2.4. Antioxidant activity

2.4.1. Determination of the DPPH radical scavenging effect

Bacterial cultures were grown in MRS at 37 °C for 24 h, cells were obtained by centrifugation (3000×*g* for 10 min at 20 °C), resuspended in PBS (Lonzo, Walkersville, MD, USA) and adjusted to corresponding of around 11 log CFU/mL based on comparison to McFarland standards. 0.5 mL of the obtained suspension was centrifuged (10,000×*g* for 15 min at 20 °C) and the pellet suspended in 1 mL PBS. For the evaluation of the DPPH radical scavenging activity recommendations from Li et al. [25] and Duz et al. [14] were applied. Previously prepared intact cells were mixed with 1 mL freshly prepared DPPH solution (0.05 mmol/L, in ethanol) and samples stored in darkness for 1 h. For preparation of the blank, the cell suspension was replaced with PBS. A mixture of 1 mg/mL ascorbic acid (Sigma-Aldrich) in distilled water served as positive control. Supernatants from the all samples were obtained by centrifugation (10,000×*g* for 10 min at 20 °C) and examined spectrophotometrically at OD 517 nm. Estimation of the DPPH radical scavenging activity (%) of tested strains was calculated according to Li et al. [25] and Duz et al. [14] as follows: Scavenging Activity (%) = {1 − [(A_{sample} − A_{blind})/A_{blank}]} × 100, where Blind = PBS solution; Blank = PBS and DPPH solutions.

2.4.2. Determination of Fe^{+2} ion chelating activity

In the evaluation of Fe^{+2} ion chelating activity, recommendations from Decker and Welch [26] were applied. Previously prepared 1 mL intact cells, resuspended in PBS, were added to 0.05 mL of a 2 mmol/L $FeCl_2$ solution. For the initiation of the reaction, 0.2 mL of 5 mmol/L ferrozine were added and the samples kept in dark for 10 min. As positive control, 1 mg/mL EDTA was used for comparison. After 10 min of incubation, the supernatant from the studied samples were obtained by centrifugation (10,000×*g* for 10 min at 20 °C) and evaluated spectrophotometrically at 562 nm for determining of the reduction in optical density. Estimation of the chelating activity were performed as: Ferric ion chelating activity (%) = [1 = (OD_1/OD_2)] × 100, where OD1 was the sample, and OD_2 was the control solution.

2.4.3. Hydroxyl radical scavenging activity

For this study recommendations of Wang et al. [27] were applied as previously described. The intact cells, prepared as described before, and 1 mL of PBS suspension were transferred to glass test tubes previously filled with 1 mL Brilliant blue (0.435 mmol/L), 2 mL $FeSO_4$ (0.5 mmol/L), 1.5 mL H_2O_2 (3%, *w/v*) and incubated at 37 °C for 1 h. As positive control, 1 mg/mL of ascorbic acid (Sigma-Aldrich) was used for comparison. The samples were centrifuged (3000×*g* for 5 min at 20 °C) and absorbance of the obtained supernatants were measured at 624 nm. For calculations of the hydroxyl radical scavenging effect of the bacteria recommendations of Wang et al. [27] were applied: Hydroxyl Radical Scavenging Activity (%) = [(A_0 − A_1)/(A − A_1)] × 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 added research sample. A refers to the absorbance of the solution without research sample and the Fenton reaction system.

2.4.4. Superoxide anion radical scavenging activity

For the evaluation of the superoxide anion ($O^{2\cdot-}$) radical scavenging activity assay, recommendations of Wu et al. [17] were implemented, applying spectrophotometry with improved pyrogallol autoxidation. Superoxide anion radical was produced under alkaline conditions with pyrogallol (1,2,3-benzentriol) autoxidation systems. Previously prepared intake cells (0.1 mL microorganism sample), adjusted to 0.8 OD working solution concentration, were mixed with 4.5 mL Tris-HCl solution (0.05 mol/L, pH 8.2) and the suspension was incubated for 20 min at 25 °C in a water bath. Next, 0.4 mL pyrogallol (0.25 mol/L, preheated to 25 °C) were added and the mixture was incubated for 4 min at 25 °C. The reaction was stopped by adding 0.1 mL of 8 mol/L HCl.

For the calculations, absorbance was measured at 320 nm. As control, an equal amount of 50 mmol/L Tris-HCl buffer (pH 8.2) was applied to replace the sample, while 1 mg/mL of ascorbic acid and BHA served as positive controls. The superoxide radical scavenging activity was calculated as follows: Superoxide anion radical scavenging activity (%) = [(A_0 − A_1)/A_0] × 100, where A_1 was the absorbance of the samples, A_0 was the absorbance of the solution that did not include the sample [17].

2.4.5. Anti-lipid peroxidation

For the determination of anti-lipid peroxidation, the method suggested by Hsu et al. [28] was applied. Fresh egg yolk (Difco) was supplemented to an equal volume of PBS (200 mmol/L, pH 7.2) and the suspension homogenised in a magnetic stirrer. The obtained suspension was further diluted with PBS (egg yolk/PBS, 1:25, *v/v*) and 1 mL egg yolk suspension, 0.5 mL microorganism sample solution adjusted to 11 log CFU/mL (prepared as described before), 1 mL PBS, and 1 mL of iron sulphate (25 mmol/L), and mixed and stirred at 37 °C for 15 min. Next, 1 mL trichloroacetic acid (20%, *w/v*) was added to the previous suspension and kept static for 10 min, followed by centrifugation (3000×*g* for 10 min, 20 °C). Thiobarbituric acid (2 mL; 0.8%, *w/v*) was added to 3 mL of the supernatant and the mixture heated by stirring in a boiling water bath for 10 min and then cooled to room temperature. The supernatant was obtained by centrifugation (3000×*g* for 10 min, 20 °C) and the absorbance 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] × 100. While 1 mg/mL of ascorbic acid served as positive controls.

2.5. Statistical analyses

All experiments were performed at least in duplicates at two independent occasions and results presented as average of individual data with calculated standard deviations.

3. Results and discussion

Evaluation of the safety properties of putative probiotic strains should be on a scientific basis researchers responsibility and in line with standards set by regulatory authorities. Properties such as hemolytic and lecithinase activity, production of biogenic amines, virulence factors and antibiotic resistance based on microbial physiological investigations represent some of the principal safety criteria for selection of new strains before further analysis of their potential functionality and applicability [29]. In addition, biomolecular approaches are now considered essential for confirming the absence of genes associated with potential hazard properties for strains intended as probiotics [30,31]. Moreover, in the last decade, full genome sequencing is considered compulsory for the portfolio of the new probiotic candidates [32,33].

The evaluated strains in this study were previously isolated from soybean paste [22] and the oral cavity of healthy Caucasian volunteer [21], and identified as recommended by Bergey's Manual [34] and by 16S rRNA sequencing, they were subjected to evaluation as potential probiotic candidates, with features including stability, and beneficial and safety properties [21,22]. However, in this study, additional safety and antioxidant properties were evaluated for the selected LAB strains.

Biological functions of the studied strains resulted in the digestion of proteins in the growth medium, resulting in the formation of free amino acids. Different levels of free amino acids were recorded, and results were summarized in Table 1.

The presence of free amino acids can have a direct influence on bacterial growth, however, can be involve in the different inflammatory processes. Thus, the fact that free amino acids are present in the environment can be associated with significant consequences for the microorganisms and the host. Appropriate systematic studies will need to be performed to evaluate the consequences of the proteolytic properties of studied strains and role of the changes in the bioavailability of the particular amino acids and their biological performance. Different amino acids, including glycine, were reported to be involved in downregulating the expression of inflammatory factors, comprising tumor necrosis factor (TNF), in processes of increasing nitric oxide bioavailability [35,36], and in supporting glutathione synthesis (GSH) to exert anti-oxidative stress [37,38]. Yu et al. [39] suggested the correlation between glycine and endothelial metabolism and immunostimulant processes [39]. Biosynthesis of collagen, one of the essential proteins in the body, is essentially related with the bioavailability of proline [40]. Different research projects showed that glutamine is associated with the restoration of vascular endothelial role by downregulating inflammatory responses, reducing oxidative stress, improving mitochondrial function, and regulating the expression of heat shock proteins [41–44].

Proteolytic properties of LAB can be associated with the availability of essential amino acids (tryptophan, methionine, and phenylalanine) to macro-organisms and representatives of the associated microbiome. Essential amino acids cannot be synthesized via anabolic processes but can be released by proteolytic processes and to be available for their biological roles. It was suggested that tryptophan metabolism plays a critical role in coronary heart disease; the free tryptophan concentration in the serum was found to be reduced while the L-kynurenine/tryptophan ratio was elevated in patients with coronary heart disease [45]. Excess and deficiency of methionine affects normal vascular growth [46]. Probiotics with high ability in the formation of free amino acids may have beneficial properties and play an extended role in different aspects of human and animal health. However, appropriate animal model studies are needed to confirm the suggested beneficial properties and health promoting benefits.

Colonization of the GIT of humans or animals is presumed to be essential for a probiotic strain in order to exert beneficial properties to the host [47]. In the last decade the probiotic concept was extended, and the beneficial role of specific micro-organisms was suggested to be associated with a positive effect on the skin for which some authors even suggested cosmetic products to be applied as

Table 1
Proteolytic activity and formation of free amino acids by strains isolated from the oral cavity of a healthy volunteer and Korean traditional fermented soybean paste.

Strain	Specific activity (U/mg PTN)	SD
Control	816.278581	207.320594
Enterococcus faecium ST651ea	736.792174	236.495859
Enterococcus faecium ST7119ea	830.561711	29.5405017
Enterococcus faecium ST7319ea	636.777482	275.308515
Lactobacillus gasseri ST16HK	632.722154	280.407219
Streptococcus salivarius ST48HK	580.413734	140.723111
Streptococcus salivarius ST59HK	583.317614	195.914379
Streptococcus salivarius ST61HK	558.737171	96.8210615
Streptococcus salivarius ST62HK	1047.67631	61.0568157
Lactiplantibacillus plantarum ST63HK	1280.81569	87.404496
Lactiplantibacillus plantarum ST66HK	1080.03003	59.3827913
Latilactobacillus sakei ST69HK	800.570798	10.2802046

One unit (U) of protease activity was defined as the amount of enzyme required to produce an increase in of 0.001 between absorbance at 340 nm in fermented milk and the control. The specific activity was calculated by dividing the proteolytic activity (U) by the respective protein (PTN) content (mg) [23].

vectors for application of beneficial strains [48].

Moreover, some probiotics associated with the removal of toxins and/or heavy metals from the host are suggested to have low adherence to the GIT and having a short transition time [49]. Mucin contributes in the protection and functionality of the GIT, including active transport of different metabolites, adherence of beneficial microbes and protection against pathogens [50]. In general, degradation of the mucus layer is associated with predisposition of the host to clinical complications and microbial dysbiosis [51]. Even if some new generation probiotics such as *Akkermansia municiphila*, a known as mucin metabolizing species were proposed [52], safety evaluations still need to be accessed on strain specific basis. All 9 strains were found unable to digest mucus (in presence or absence of glucose), and therefore these strains can be considered safe regarding this factor.

By definition, antioxidants are characterized as chemical compounds/metabolites involved in processes of prevention, halting or reduction of oxidative damage. Moreover, antioxidants are effectively involved in the protection of the human body from free radicals (natural compounds or subproducts of synthetic chemistry) and can be associated with a reduction in the progression of several diseases. Normally, an endogenous antioxidant system has limited antioxidant capacity, and the body requires additional exogenous regulation and specific supplements to reduce generated oxidative stress for a healthy balance. Thus, probiotics with effective antioxidant properties may provide valuable support for the restoration of homeostasis [11,12]. Antioxidant supplements (metabolites or microorganisms with such properties) are considered as important instruments for potential control of oxidative stress. Some microorganisms, including some LAB strains, possess antioxidant systems for stabilizing free radical levels [13] and their beneficial application can contribute to general health and the prevention of diseases [15–18]. The antioxidant capacities of LABs were proposed and explored in research projects evaluating intact cells [18]. Moreover, Virtanen et al. [53] suggested a direct correlation between a microorganism's antioxidant activities and its proteolytic activity. All the LAB strains in this study showed antioxidant properties in the performed experiments, thereby providing additional knowledge in support of the effectiveness of these strains as potentially effective probiotics.

The ease, speed, sensitivity, and reproducibility of the DPPH radical scavenging method makes it one of the most commonly used antioxidant methods related to other assays. The antioxidant activity raises in ratio with the removal of purple color produced when the DPPH radical was added to the medium. The elimination of the purple color of the DPPH radical after addition is directly linked to the increase in antioxidant activity of the strain. Recorded radical scavenging property for the studied microorganisms, demonstrated that *Str. salivarius* strain ST59HK exhibited the highest activity (85.24%) while *E. faecium* ST651ea showed the lowest activity (66.44%) in comparison to that of ascorbic acid (94.7%) which was used as positive control (Fig. 1).

Previous studies suggested an association of antioxidant activity of some LAB with the production of specific cell-associated surface metabolites, including extracellular polysaccharides [54] or, for *Bifidobacterium* spp., lipoteichoic acid [55]. The levels of DPPH in our study were higher than 66% for all evaluated strains (*Streptococcus* spp., *Enterococcus* spp., and *Lactobacillus* spp., Fig. 1), a value similar to that reported by Duz et al. [14]. Ji et al. [56] reported for DPPH regarding 11 *Lactobacillus* strains, values close to 50%. In a similar evaluation Zhang et al. [57] reported the DPPH radical scavenging activities for *Lactobacillus* strains (*Lacticaseibacillus casei* subsp. *casei*

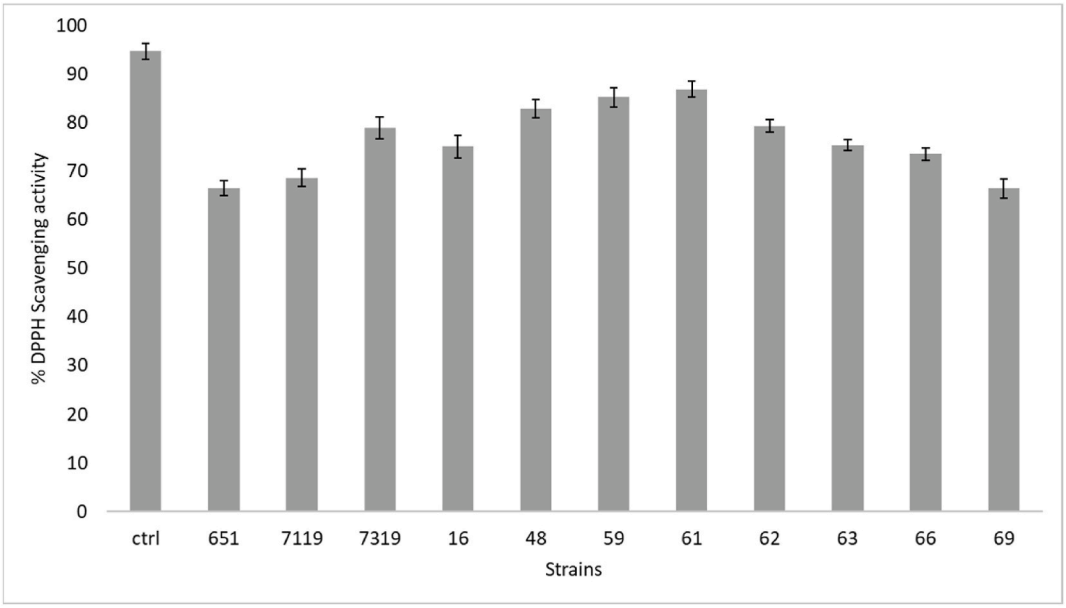

Fig. 1. Evaluation of the DPPH activity for strains isolated from oral cavity of a healthy volunteer and Korean traditional fermented soybean paste. Ctrl: control, ascorbic acid; 651: *Enterococcus faecium* ST651ea; 7119: *Enterococcus faecium* ST7119ea; 7319: *Enterococcus faecium* ST7319ea; 16: *Lactobacillus gasseri* ST16HK; 48: *Streptococcus salivarius* ST48HK; 59: *Streptococcus salivarius* ST59HK; 61: *Streptococcus salivarius* ST61HK; 62: *Streptococcus salivarius* ST62HK; 63: *Lactiplantibacillus plantarum* ST63HK; 66: *Lactiplantibacillus plantarum* ST66HK; 69: *Latilactobacillus sakei* ST69HK. Results represent average of at least 3 repetitions and SD were presented.

SY13 and *Lactobacillus delbrueckii* subsp. *bulgaricus* LJJ) as 23.99% and 27.50%, respectively, lower that detected in our experiments. Ural [58] reported the DPPH activity of *Lb. casei* EMP2 as 78.5%, and for different *Lb. delbrueckii* subsp. *bulgaricus* strains between 56.3% and 77.7%. Moreover, Wang et al. [59] suggested that DPPH can also be associated with cell concentrations applied in the experimental protocols and demonstrated that at cell densities between 10^6 and 10^9 CFU/mL DPPH increased in a concentration-dependent manner. AlKalbani et al. [7] reported that the DPPH levels can be associated with peptides released as a consequence of proteolysis. Moreover, Talib et al. [60] described DPPH activities in kefir associated *Lactobacillus* spp. and pointed on biological activity when evaluated in combination of phenolic and flavonoid composts.

Catalysis of reactions by transition metals results in the release of reactive oxygen species, such as hydroxyl radical and superoxide anion. In the present our study, all strains exhibited ferric ion chelating activities with *Str. salivarius* ST62HK (98.2%), while the lowest chelation activity was detected for *Str. salivarius* ST48HK (71.5%) related to the positive control, ethylene diamine tetra-acetic acid (EDTA) (1 mg/mL) (93.8%).

The role of the ferrous ions is not only associated with specific antioxidant properties but can also be related to the catalysis and metabolization of lipid peroxides and thereby explain the production of off-flavors during the storage of some food products [14]. Moreover, iron could be involved in the formation of free radicals because of Fenton reactions, reflecting the reduction in Fe^{+2} concentration and the resulting protective effect against oxidative damage [60,61]. The chelating activities of LAB may also be associated with the physiological chelators mapped on the bacterial cell wall [62]. Duz et al. [14] reported metal (Fe^{+2}) ion chelating effects between 20% and 75% for all tested strains, compared to the positive standard (EDTA) (1 mg/mL), 92%. In comparison, levels between 66% and 87% were recorded in our study (Fig. 2).

Probiotics can be associated with production of antioxidants and free radical scavenging properties; however, they may also exhibit some degree of metal chelating activity as well [63]. Yamamoto et al. [64] associated such an antioxidant activity in LAB with the expression of iron binding protein. It was suggested that for some LAB and particularly *Streptococcus thermophilus* strains [62] and *Lb. casei* strains [64,65] antioxidant properties could be associated with the elimination of the transition metal ions and a reducing negative effect of high O_2 levels.

Zhang et al. [66] reported that due to the formation of the hydroxyl radicals in Fenton reactions, it is possible to assess the radical scavenging activity of antioxidants like microorganisms in *in vitro* Fe^{2+}/H_2O_2 systems. In similarity with the positive standard, (ascorbic acid, 1 mg/mL), the strains showed a slight difference at 89.2% in their scavenging activity. In this study, 11 LAB strains isolated from the oral cavity and soybean paste demonstrated strong hydroxyl radical scavenging activities with 98.6% for *Str.*

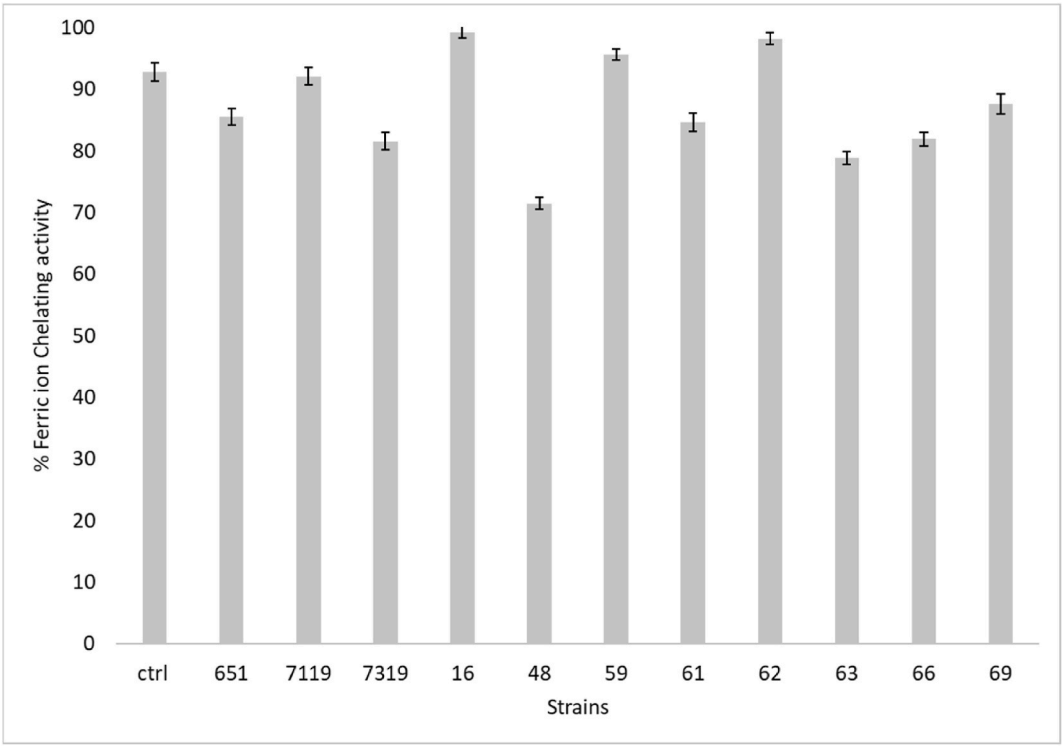

Fig. 2. Evaluation of the ferric ion chelating activity for strains isolated from the oral cavity of a healthy volunteer and Korean traditional fermented soybean paste. Ctrl: control, EDTA; 651: *Enterococcus faecium* ST651ea; 7119: *Enterococcus faecium* ST7119ea; 7319: *Enterococcus faecium* ST7319ea; 16: *Lactobacillus gasseri* ST16HK; 48: *Streptococcus salivarius* ST48HK; 59: *Streptococcus salivarius* ST59HK; 61: *Streptococcus salivarius* ST61HK; 62: *Streptococcus salivarius* ST62HK; 63: *Lactiplantibacillus plantarum* ST63HK; 66: *Lactiplantibacillus plantarum* ST66HK; 69: *Latilactobacillus sakei* ST69HK. Results represent the average of at least 3 repetitions with the SD also shown in the graph presented.

salivarius ST59HK and 35.60% for *Lb. plantarum* ST63HK, exhibiting the highest and lowest antioxidant activities, respectively.

In their study, Duz et al. [14] reported similar levels of hydroxyl radical scavenging activities ranging from 82% for *Lb. plantarum* IH16L, 82% for *Lb. sakei* IH15L to 78% for *Latilactobacillus curvatus* IH1L. In comparison with the control (89%), most of the strains in our essays presenetd similar levels of hydroxyl radical scavenging activity (Fig. 3). As supported by our results by Virtanen et al. [53] have suggested that consumption of LAB as part of functional foods can reduce the risk of ROS accumulation and that this can be associated with the degradation of hydrogen peroxide and superoxide. The high levels of hydroxyl radical scavenging properties of numerous *Lb. plantarum* strains (isolated from traditional Chinese fermented food products) were reported, also by showing a relationship with levels of viable cells [25]. Duz et al. [14] suggested that intact *Lb. plantarum* IH16L, *Lb. sakei* IH15L and *Lb. curvatus* IH1L cells presenetd strong hydroxyl radical scavenging activity, most possibly associated with their specific ability to attach metal ions such as Fe^{2+}.

Intracellular enzymatic enzymes that are involved in defending the body against oxidative stress such as catalase are important to LAB and can only be obtained by degradation of bacterial cells. Some of these enzymes are also responsible for superoxide anion scavenging [67]. In this study, superoxide anion radical scavenging activity was highly exhibited by *Str. salivarius* ST61HK (54.62%) and least exhibited by *E. faecium* ST651ea (18.7%) (Fig. 4).

Similar although slightly lower levels were reported by Duz et al. [14], where most strains showed values between 21% and 7% with the highest activity (for *Lb. plantarum* IH28L) with a higher hydroxyl radical scavenging activity at a level of 43%.

The superoxide dismutase enzymatic activity is a relevant *in vivo* enzymatic antioxidant cell protection mechanism [56]. MnSOD was clearly identified in LAB [63]. SOD enzymatic activities were recorded in lower proportions in our study, compared to other antioxidant properties, as well for LAB strains evaluated by Duz et al. [14]. This can be associated with the fact that the LAB strains in our study most probably were able to remove metal ions but not to increase the SOD enzymatic level in order to oppose the oxidative chain reaction. Ji et al. [56] evaluated the SOD enzymatic activities in 5 *Lactobacillus* spp. and 6 *Leuconostoc* spp. and reported that most *Leuconostoc* strains exhibited a scavenging activity of over 35%, however, the enzymatic activity of *Lactobacillus* strains was exhibited in a strain specific manner.

The oxidation of cellular membrane phospholipids into peroxide derivatives is known as lipid peroxidation [68]. In our study, the strains *Lb. gasseri* ST16HK (69.43%) and *E. faecium* ST7319ea (26.15%) showed the highest and lowest anti-lipid peroxidation,

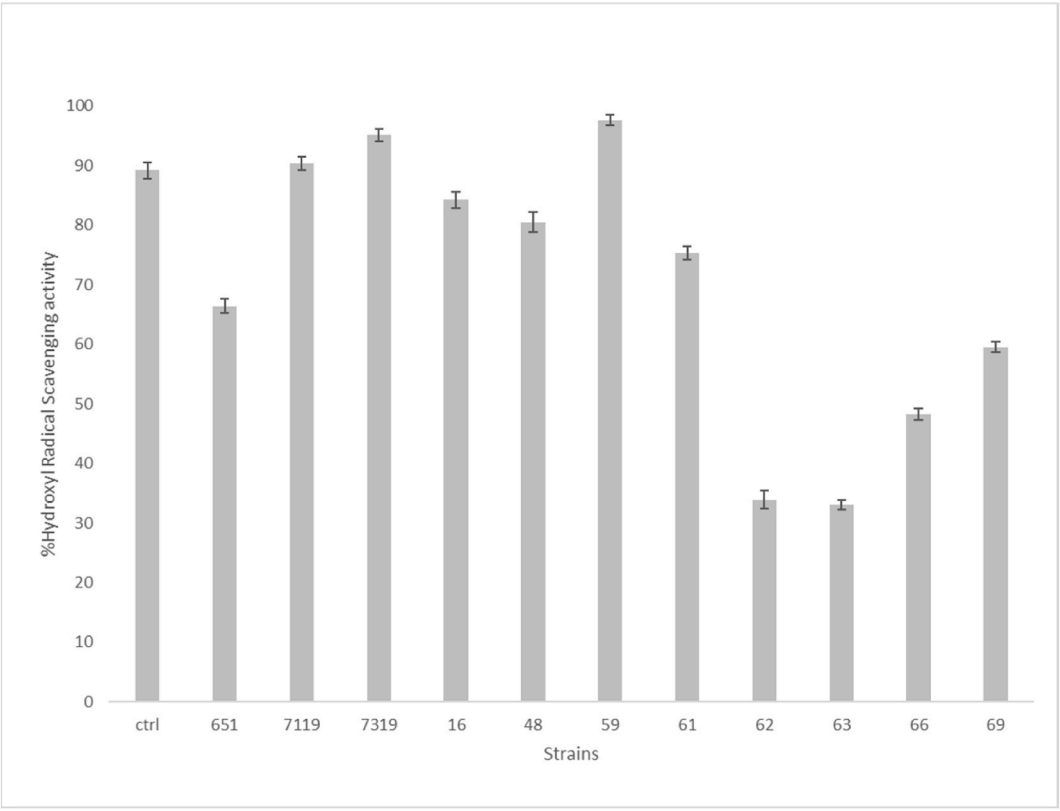

Fig. 3. Evaluation of the hydroxyl radical scavenging activity for strains isolated from the oral cavity of a healthy volunteer and Korean traditional fermented soybean paste. Ctrl: control, ascorbic acid; 651: *Enterococcus faecium* ST651ea; 7119: *Enterococcus faecium* ST7119ea; 7319: *Enterococcus faecium* ST7319ea; 16: *Lactobacillus gasseri* ST16HK; 48: *Streptococcus salivarius* ST48HK; 59: *Streptococcus salivarius* ST59HK; 61: *Streptococcus salivarius* ST61HK; 62: *Streptococcus salivarius* ST62HK; 63: *Lactiplantibacillus plantarum* ST63HK; 66: *Lactiplantibacillus plantarum* ST66HK; 69: *Latilactobacillus sakei* ST69HK. Results represent the average of at least 3 repetitions with the SD also shown.

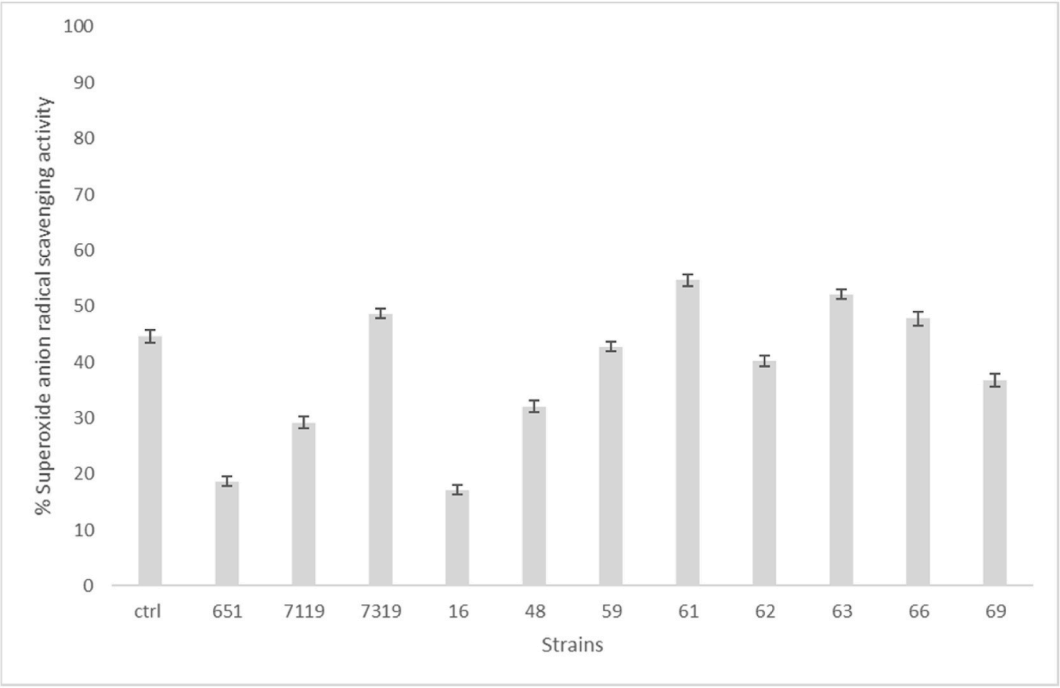

Fig. 4. Evaluation of the superoxide anion radical scavenging activity for strains isolated from the oral cavity of a healthy volunteer and Korean traditional fermented soybean paste. Ctrl: control, ascorbic acid; 651: *Enterococcus faecium* ST651ea; 7119: *Enterococcus faecium* ST7119ea; 7319: *Enterococcus faecium* ST7319ea; 16: *Lactobacillus gasseri* ST16HK; 48: *Streptococcus salivarius* ST48HK; 59: *Streptococcus salivarius* ST59HK; 61: *Streptococcus salivarius* ST61HK; 62: *Streptococcus salivarius* ST62HK; 63: *Lactiplantibacillus plantarum* ST63HK; 66: *Lactiplantibacillus plantarum* ST66HK; 69: *Latilactobacillus sakei* ST69HK. Results represent the average of at least 3 repetitions and the SD is also shown.

respectively. In comparison, the control generated 62.17%, like that recorded for *Str. salivarius* ST59HK, but lower than value generated by *Lb. gasseri* ST16HK (Fig. 5).

Similar results were reported by Duz et al. [14], where lipid peroxidation inhibition rate ranged between 63.29% and 40.18% for *Lb. curvatus* GH1L and *Lb. plantarum* GH2.7L, respectively. It was suggested that the most considerable effect of free oxygen species on biological systems is that observed for lipids. Such a fact is known as lipid peroxidation. Ou et al. [69] evaluated antioxidative properties of bacterial strains isolated from yogurt (*Str. salivarius* subsp. *thermophilus* ATCC 19258 and *Lb. delbrueckii* subsp. *bulgaricus* ATCC 11842) and reported values of 57% and 42%, respectively. Ural [58] reported on lipid peroxidation inhibition of *Lb. rhamnosus* SMC6 and *Lb. delbrueckii* subsp. *bulgaricus* 12L with blood plasma (containing biological lipid) and reported 39.2% and 30.2% respectively [58]. In a study conducted by Duz et al. [14], egg yolk was applied as the lipid source in the demonstration of the anti-lipid peroxidation activity of LAB isolated from dry sausages. Moreover, Zhang et al. [70] reported in a similarly conducted experiment on anti-lipid peroxidation capacities of *Lb. curvatus* SR6 and *Lacticaseibacillus paracasei* SR10-1, isolated from Chinese traditional fermented meat products, for which values of 55% and 63.93% were reported, respectively.

Taking into consideration results associated with antioxidant properties, can be clearly stated that strain specificity is the key word regarding obtained data. However, *Str. salivarius* ST59HK was showing highest levels for all 5 different essays, DPPH activity (86.54%), ferric ion chelating activity (96.27%), hydroxyl radical scavenging activity (98.57%), superoxide anion radical scavenging activity (42.92%), and lipid peroxide inhibition rate (64.01%), a value, higher or similar to that recoded to the controls (Figs. 1–5). Will be appropriate if this strain, already shown that can be considered as safe [21] to be further evaluated in fermented food product and/or in animal model study to validate observed *in vitro* antioxidant properties in *in vivo* experimental scenario.

4. Conclusions

Since establish of the principals of probiotic concept and scientifically proof that different microorganisms can be involved in the health promoting processes for humans and other animals, several research projects were focus on selection of appropriate bacterial strains. To be a probiotic, selected strain needs to provide some health promoting benefits for the consumers, and this needs to be proven via appropriate clinical studies. In addition, any strain, intended to be applied as probiotic or beneficial culture in food production properties need to be covering rigidus safety assessment evaluation and to ensure that cannot be presenting any hazard for the consumers or environmental. The concept of "safety first" needs to be considered as researchers' responsibility and priority. Moreover, probiotics can present additional beneficial properties, and when applied in fermentation processes, improve safety and organoleptic properties of the food products. Production of antioxidant metabolites by LAB need to be regarded as advantageous property for strains

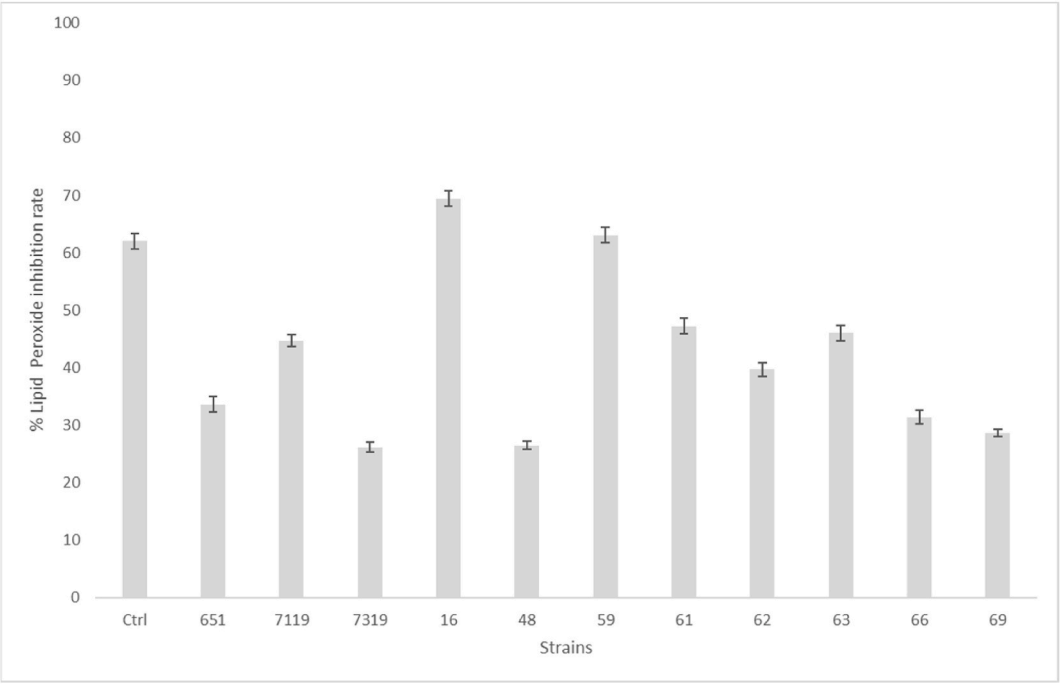

Fig. 5. Evaluation of the lipid peroxide inhibition rate for strains isolated from the oral cavity of a healthy volunteer and Korean traditional fermented soybean paste. Ctrl: control, ascorbic acid; 651: *Enterococcus faecium* ST651ea; 7119: *Enterococcus faecium* ST7119ea; 7319: *Enterococcus faecium* ST7319ea; 16: *Lactobacillus gasseri* ST16HK; 48: *Streptococcus salivarius* ST48HK; 59: *Streptococcus salivarius* ST59HK; 61: *Streptococcus salivarius* ST61HK; 62: *Streptococcus salivarius* ST62HK; 63: *Lactiplantibacillus plantarum* ST63HK; 66: *Lactiplantibacillus plantarum* ST66HK; 69: *Latilactobacillus sakei* ST69HK. Results represent average of at least 3 repetitions and with the SD also presented.

evaluated as probiotics for humans and other animals and/or beneficial strains in food processing industry. Thus, can be extending spectrum of application of such as LAB and open realistic opportunities for the reduction of application of chemically designed additives and improving health status of food products. In current study we have shown that evaluated strains have antioxidant properties and they have clear potential to be applied as functional strains. However, these antioxidant properties will be expressed when strains will be part of the food fermentation system or will be applied as probiotics (in humans or other animals)? This hypothesis merit further investigation and confirmation of the beneficial antioxidant properties of the evaluated *in vitro* strains in current study. Moreover, scientific dream of selecting a microbial strain that can be effective as starter and biopreservative culture, providing antioxidant properties and act as probiotics can be realization of Hippocrates dream that one day food will be a medicine and medicine will be a food.

Author contribution statement

Svetoslav Todorov: Conceived and designed the experiments; Analyzed and interpreted the data; Fund requisition, contributed reagents, materials, analysis tools or data; Drafting the article; Critically revising its important intellectual content.

Ronaldo Rwubuzizi: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Drafting the article.

Hamin Kim: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Wilhelm Heinrich Holzapfel: Fund requisition, contributed reagents, materials, analysis tools or data; Critically revising its important intellectual content.

Data availability statement

Data will be made available on request.

Additional information

No additional information is available for this paper.

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Declaration of interest's statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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