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Comparative evaluation of the probiotic and antioxidant potential of indigenous Lactic Acid Bacteria[#]

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Abstract

This study was undertaken to assess the probiotic and antioxidant potential of five indigenous cultures of lactic acid bacteria viz, Lactobacillus acidophilus, Lactiplantibacillus plantarum, Lacticaseibacillus rhamnosus, Lactobacillus bulgaricus and Streptococcus thermophilus. The probiotic properties in terms of acid and bile tolerance, cell surface hydrophobicity, autoaggregation, coaggregation and bile salt hydrolase activity were assessed in vitro. The DPPH assay indicated highest antioxidant activity of 45.33 per cent for Streptococcus thermophilus. All five cultures exhibited significant antioxidant potential. Lacticaseibacillus rhamnosus, Lactobacillus acidophilus and Lactiplantibacillus plantarum showed higher probiotic potential in terms of acid and bile tolerance, cell surface hydrophobicity, autoaggregation and coaggregation. None of the cultures exhibited bile salt hydrolase activity.

Keywords: *Lactic acid bacteria, probiotic, antioxidant*

Being one of the earliest groups of bacteria studied, Lactic Acid Bacteria (LAB) have a very long history of application. Lactic acid bacteria are gram positive, catalase negative non spore forming useful bacteria that can convert lactose to lactic acid. These are used to manufacture various products, especially fermented milk products. The main features contributing to the popularity of LAB are its Generally Regarded as Safe (GRAS) status, simple and versatile metabolism and ability to metabolize various carbon sources. They not only can synthesise lactic acid as the major

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end product but also produce a wide range of metabolites that beneficially affect the nutritional, sensorial, and technological properties of fermented food products. The functionality of LAB is primarily attributed to their metabolites, the major one being lactic acid. Others include acetic acid, ethanol, aroma compounds, exopolysaccharides, enzymes, bacteriocins, *etc.* (Leroy and De Vuyst, 2004). Fermentation by LAB also results in the release of biologically active peptides which are known to have functions like immunomodulatory, angiotensin converting enzyme inhibitory and antioxidant activities (Abubakr *et al*., 2012). Some LAB are also able to produce antioxidases which results in their antioxidant activity (Zhang *et al*., 2017). Fermented milk with health-promoting probiotic properties is one of the oldest functional foods.

Some species in LAB are known to possess probiotic potential which is considered to be more beneficial from a health point of view. *Lactobacillus*, *Bifidobacterium*, *Leuconostoc*, *Streptococcus*, *Enterococcus*, *Pediococcus*, and yeasts like *Saccharomyces* possess probiotic attributes (Fijan, 2014). According to FAO/ WHO, probiotics are live microorganisms that when administered in adequate amount confer a health benefit on the host. The health benefit is generally acquired by improving or restoring the gut flora. The LAB have been used as probiotics to manage intestinal disorders such as lactose intolerance, acute gastroenteritis, constipation, and inflammatory bowel diseases. Immunomodulating, serum cholesterol lowering, anticarcinogenic, antihypertensive, antidiabetic effects of LAB has also been reported. In addition to these, probiotics also find use in the stabilization of gut flora, recolonisation of bowel following antibiotic treatment, treatment of food allergies, as vaccine adjuvants and improved weight gain (Goldin, 1998).

The probiotic attributes used for the selection of microorganisms are safety, viability/ activity in delivery vehicles, acid tolerance, bile tolerance, resistance to pepsin and pancreatin, ability to adhere to gut epithelial tissue, gastrointestinal tract colonization potential, capacity to stimulate a host immune response, antimicrobial resistance and antimicrobial activity (Pundir *et al*., 2013; Balamurugan *et al*., 2014). According to Sharma *et al*. (2021), acid tolerance, bile salt tolerance, bile salt hydrolase activity, cell surface hydrophobicity, antibiotic susceptibility, antimicrobial activity, haemolytic activity, and production of biogenic amines may be assessed for selecting putative probiotic candidates.

Materials and methods

Lactic acid bacteria cultures

Lactic acid bacteria - *Lactobacillus acidophilus, Lactiplantibacillus plantarum, Lacticaseibacillus rhamnosus, Lactobacillus bulgaricus* and *Streptococcus thermophilus. Lactobacillus acidophilus* (MTCC 307) and *Lactobacillus bulgaricus* (304) were procured from National Collection of Dairy Cultures (NCDC) and others from the stock culture of Dairy Microbiology Department of Verghese Kurien Institute of Dairy and Food Technology, Mannuthy.

Lactobacilli were propagated in de Man Rogosa Sharpe (MRS) broth and Lactococi in M17 broth. One set was maintained as glycerol stock at -20°C by mixing equal volumes (50 µl) each of overnight grown culture and sterilized 50 per cent glycerol. Another set of cultures was propagated and preserved in sterilised reconstituted skimmed milk tubes and stored in the refrigerator. The purity of the cultures was always ascertained before use by Gram's staining and catalase test. The culture of pathogenic bacteria (*Escherichia coli*) was maintained in nutrient broth.

Acid tolerance

The acid tolerance of the cultures was determined as per Pundir *et al*. (2013). The cultures were inoculated in sterile MRS broth tubes with pH adjusted to 2.0 and 3.0. After incubation at 37°C optical density was measured at an hour interval for three hours. The pH adjusted broth inoculated with culture was taken as control.

Bile tolerance

The bile tolerance of the cultures

was determined as per Pundir *et al*. (2013). The cultures were inoculated in sterile MRS broth tubes in which the percentage of bile was adjusted to 0.3 and 0.6. the tubes were then incubated at 37°C. After incubation at 37°C optical density was measured at an hour interval for three hours. The bile adjusted broth inoculated with culture was taken as control.

Bacterial cell surface hydrophobicity

The adhesion potential of cultures in terms of cell surface hydrophobicity was determined by Bacterial Adhesion to Hydrocarbons assay using the procedure followed by Collado *et al*. (2008) with some modifications. The cultures were incubated in MRS broth at 37°C for 16 h. After refrigerated centrifugation at 4°C for 10 min at a speed of 12000 rpm, cells in the stationary phase were collected as pellets. The pellets were washed three times using phosphate buffered saline and then resuspended in the same buffer to achieve an optical density (OD) of 0.25±0.05 at 600 nm. An equal volume of xylene was added to 5 mL of this suspension and mixed thoroughly by vortexing for five minutes followed by an immediate measurement of OD at 600nm. The vortexed samples were then held at 37ºC for 1h for phase separation. The aqueous phase of the cell culture was pipetted out and the OD at 600 nm was once more measured. The cell surface hydrophobicity (CSH) in percentage was calculated using the formula.

$$\text{CSH (\%)} = \frac{\text{Initial OD- Final OD}\times 100}{\text{Initial OD}}$$

Autoaggregation

The autoaggregation potential of the cultures was determined as per Kos *et al*. (2003). The freshly activated culture was added to MRS broth at the rate of one per cent inoculation and incubated at 37°C for 18h. The cells were harvested by refrigerated centrifugation at 5000 g for 15 min. The cell pellets obtained were washed twice with phosphate buffered saline (PBS) and resuspended in the same buffer to attain a final optical density of 0.60±0.02 at 600 nm. Four millilitres of this cell suspension was mixed thoroughly by vortexing and then 0.1 millilitre of the undisturbed upper suspension was transferred to another tube with 3.9 mL of PBS. The absorbance (A1) of this suspension was measured at 600 nm. The sample was left undisturbed at 37°C, and the OD of samples (A2) was taken exactly after one hour and six hours. The autoaggregation in percentage was expressed as follows:

$$\text{Auto-aggregation (\%)} = [(A1 - A2)/ (A1)] \times 100$$

Where A1: initial optical density, A2: optical density after incubation.

Co-aggregation

Co-aggregation of cultures was assessed by the method followed by Anandharaj *et al*. (2015), with slight modifications. Cells were harvested by centrifuging 10 mL culture at 5,000 rpm for 10 minutes. Two millilitres each of LAB strain and pathogenic strain (*Escherichia coli*) were mixed and incubated at 37ºC for five hours. The optical density of the resultant mixture was taken at 600 nm with either LAB strain or pathogenic strain as a control. The co-aggregation percentage was estimated using the formula given below.

$$\text{Co-aggregation \%} = [(A_{\text{pathogenic bacteria}} + A_{\text{LAB)}} - 2(A_{\text{mixed strain}})/ (A_{\text{pathogenic bacteria}} + A_{\text{LAB}})] \times 100$$

Where,

$A_{\text{pathogenic bacteria}}$ -OD_{600nm} Pathogenic Bacteria

A_{LAB} -OD_{600nm} LAB

$A_{\text{mixed strain}}$ -$OD_{600\ nm}$ of LAB + Pathogen

Bile salt hydrolase (BSH) activity

A direct plate assay for the detection of BSH activity was carried out according to Lee *et al*. (2011). The active cultures were streaked on pre-solidified MRS agar containing 0.5% (w/v) bile and 0.37 g/L of $CaCl_2$. The plates were then incubated anaerobically in the anaerobic jar at 37ºC for 48 h. BSH activity of the cultures was indicated by the formation of distinctive precipitate around the colonies.

Antioxidant potential

The antioxidant potential of the

cultures in skimmed milk was determined as per the procedure followed by Ogunyemi *et al*. (2021) with slight modifications. The cultures were inoculated in sterilised skim milk and incubated overnight. One gram of fermented skim milk was dissolved in 10 mL ethanol and kept in a shaker incubator for two hours. After incubation, it was centrifuged at 5000 rpm for 20 min and the supernatant was filtered using Whatman no. 1 filter paper. Then 0.5 mL of filtrate was added with 3.5 mL ethanol and 1 mL DPPH reagent (2.4 mg in 100 mL ethanol). Simultaneously, a blank was prepared using 4 mL ethanol and 1 mL DPPH reagent. The absorbances of the solutions were measured at 517 nm after incubation at 37ºC for 30 min.

% Antioxidant activity =
(Absorbance of blank – Absorbance of sample) x 100
Absorbance of blank

Result and discussion

The probiotic potentials of cultures were analysed in terms of acid tolerance, bile tolerance, cell surface hydrophobicity, autoaggregation, coaggregation and bile salt hydrolase activity. For effective transit through the stomach and small intestine, potential probiotic strains need to be able to endure acidic conditions and bile secretions (Anandharaj *et al*., 2015). To be used as a probiotic, bacteria should withstand a low pH of around 3.0 for two hours (Gotcheva *et al*., 2002). The acid tolerance of LAB cultures determined by evaluating their growth in acidic pH is shown in Fig. 1. Among the 5 cultures, *Lacticaseibacillus rhamnosus* showed the highest acid tolerance followed by *Lactobacillus acidophilus*. According to Biswas *et al*. (2019), *Lacticaseibacillus rhamnosus* was found to tolerate prolonged acidic conditions which support current observations. Bile tolerance of the cultures is depicted in Fig. 2. The level of bile salt in the intestine is around 0.3 per cent and can reach extremely up to 2 per cent during the start of digestion. Therefore, bile resistance for probiotic potential is usually assessed in 0.1-0.5 per cent bile (Gotcheva *et al*., 2002). *Lactiplantibacillus plantarum* exhibited considerable bile tolerance in 0.6 per cent bile. As per Hamon *et al*. (2011) this species was having some strains with reasonable growth in broth containing bile which strengthens our findings. *Lacticaseibacillus rhamnosus* also showed good bile tolerance and *Lactobacillus acidophilus* showed slight tolerance at 0.3 per cent level but not at 0.6 per cent. *Lactobacillus bulgaricus* and *Streptococcus thermophilus* were found to be sensitive to bile even at 0.3 per cent.

As probiotics are meant to inhabit the intestine of the host, aggregation ability and cell surface hydrophobicity are advantageous. Bacterial cell surface hydrophobicity was established by adherence to apolar solvent xylene. The bacterial cell surface hydrophobicity of cultures in chloroform and xylene are shown in Fig. 3. *Lacticaseibacillus rhamnosus* (83.24 in xylene and 65.78 in chloroform) was found to have the highest cell surface hydrophobicity succeeded by *Lactobacillus acidophilus* in xylene as well as chloroform. Strains of *Lacticaseibacillus rhamnosus* showed high hydrophobicity in a study by Harty *et al*. (1993) which is similar to the present observation. Also, *Lactobacillus acidophilus* was found to have good hydrophobicity (Reid *et al*., 1992). Higher hydrophobicity of bacterial cell surface can be attributed to the presence of (glycol-) proteinaceous material on the cell surface (Collado *et al*., 2008). Autoaggregation seems to be important for the adhesion of probiotic organisms to the intestinal epithelial cells while coaggregation hinders colonisation by pathogenic microorganisms by creating a barrier (Sabir *et al*., 2010). The autoaggregation potential of cultures are presented in Fig. 4. Maximum percentage of autoaggregation (78.405% in 6h and 35.34% in 1h) was demonstrated by *Lacticaseibacillus rhamnosus* followed by *Lactobacillus acidophilus*. The high autoaggregation of *Lacticaseibacillus rhamnosus* strains can be attributed to their surface proteins (Polak-Berecka *et al*., 2014). The coaggregation of cultures with *E. coli* was assessed and the results were shown in Fig. 5. *Lactobacillus acidophilus* and also *Lacticaseibacillus rhamnosus* and *Lactiplantibacillus plantarum* were found to have good coaggregation potentials of 26.67, 25.89 and 25.65 per cent respectively. Similarly, *Lactobacillus acidophilus* showed

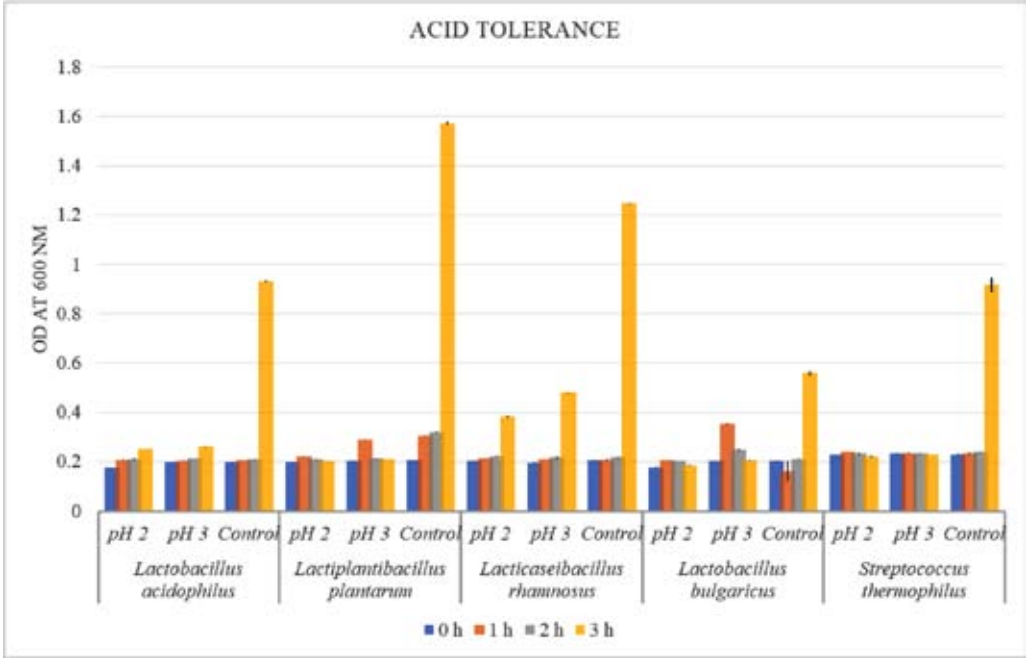

Fig. 1. Optical density at 600 nm of lactic acid bacteria at different pH

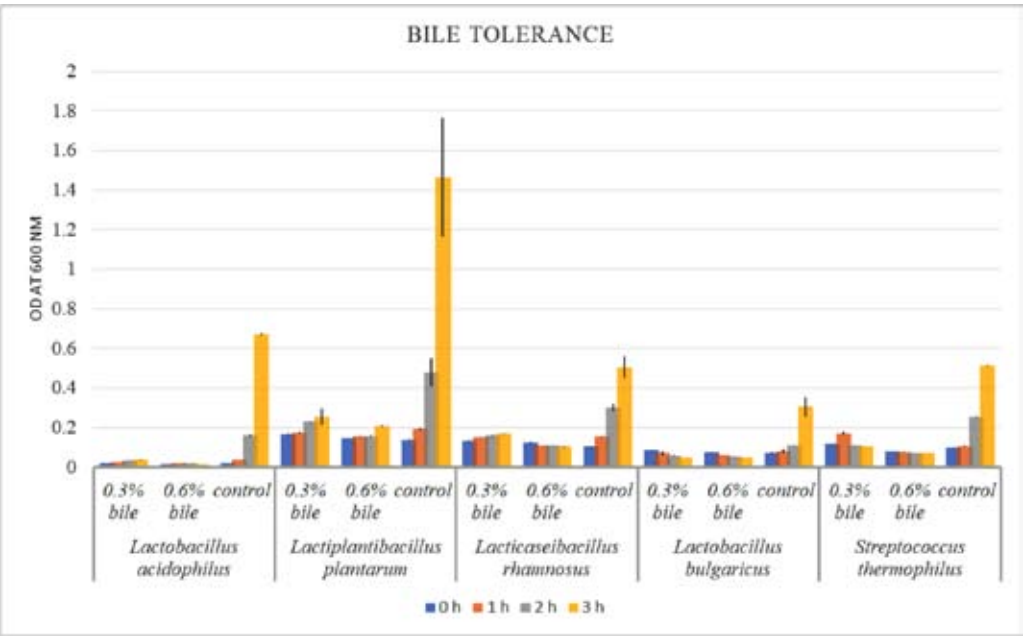

Fig. 2. Optical density at 600 nm of lactic acid bacteria at different concentrations of bile

very high coaggregation with *E. coli* in a study carried out by Ekmekci *et al*. (2009). Bile salt hydrolase (BSH) activity is responsible for deconjugating bile salts in the intestine and helps in colonisation (Anandharaj *et al*., 2015). None of the cultures showed a positive result in bile salt hydrolase activity.

The cultures were assessed for their antioxidant activity in fermented skim milk and

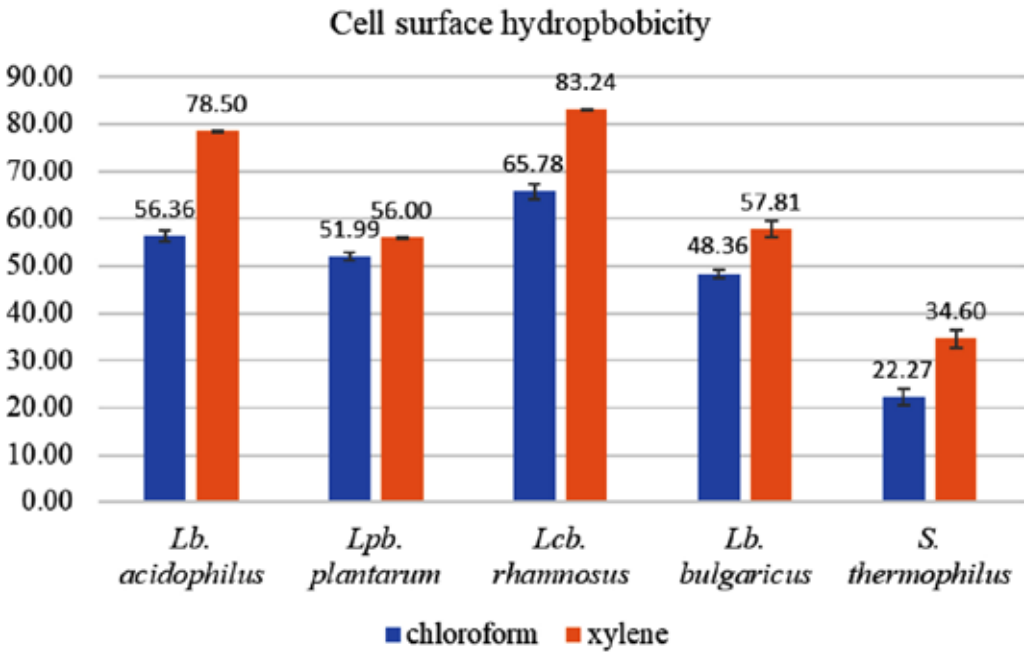

Fig. 3. Bacterial cell surface hydrophobicity of lactic acid bacteria

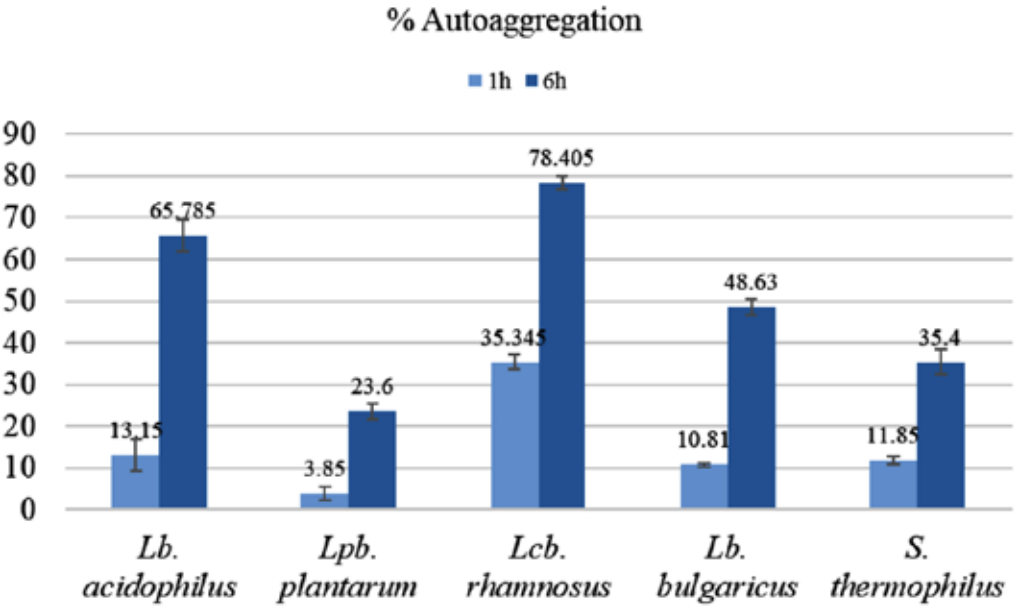

Fig. 4. Autoaggregation of lactic acid bacteria (%)

the results were depicted in Fig. 6. All the cultures were found to have good antioxidant potential among which *Streptococcus thermophilus* has the highest activity of 45.33 per cent followed by *Lactobacillus bulgaricus* and *Lactobacillus acidophilus*. Fermentation with *Streptococcus thermophilus* resulted in highest antioxidant effect in a study conducted by Lee *et al*. (2015) which is in agreement with our observation.

Conclusion

Lacic acid bacteriae *viz. Lactobacillus acidophilus, Lactiplantibacillus plantarum,*

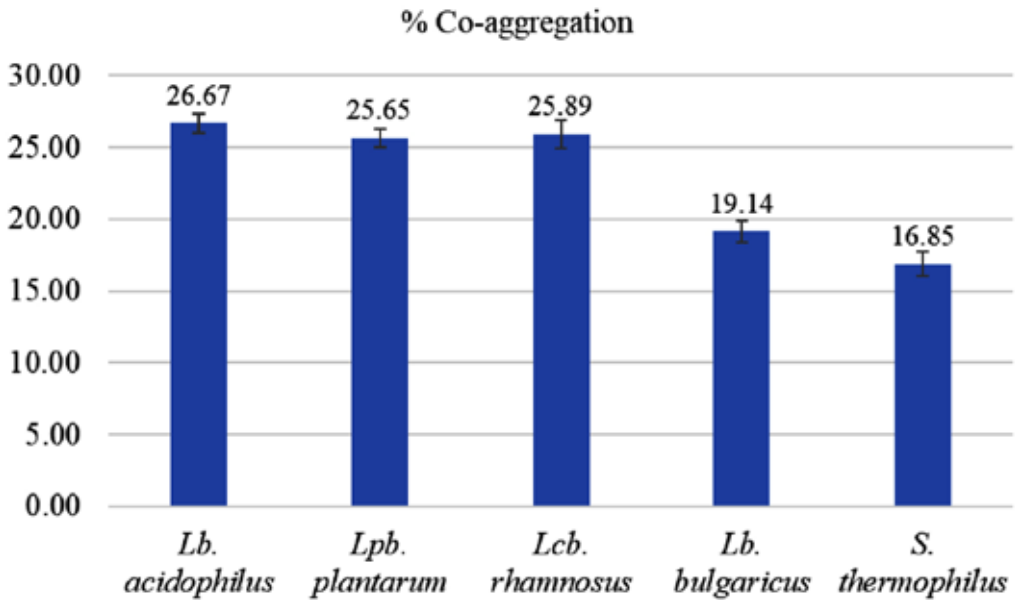

Fig. 5. Coaggregation of lactic acid bacteria (%)

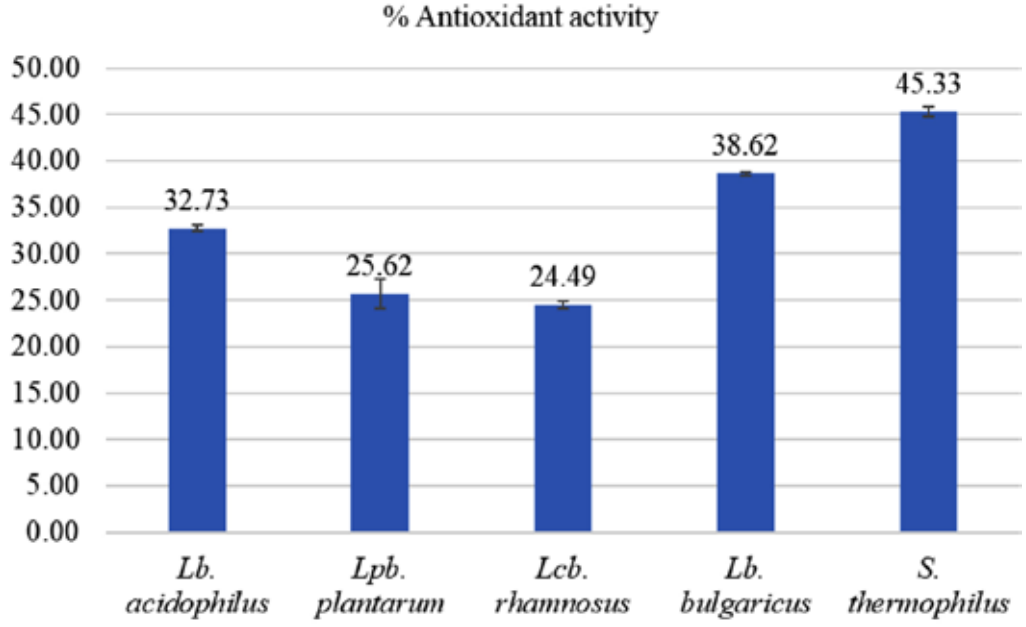

Fig. 6. Antioxidant activity of lactic acid bacteria

Lacticaseibacillus rhamnosus, Lactobacillus bulgaricus and *Streptococcus thermophilus* were assessed for probiotic attributes and antioxidant potential. It was found that among the five cultures, *Lacticaseibacillus rhamnosus* showed good probiotic attributes along with *Lactobacillusacidophilus* and *Lactiplantibacillus plantarum* and hence these three have the potential to be used as probiotics. But further studies are to be done including safety tests in order to use these organisms as probiotics. Even though *Streptococcus thermophilus* and *Lactobacillus bulgaricus* lack some of the probiotic attributes like acid and bile tolerance, they were having good antioxidant potential. With the growing awareness, health-conscious

consumers are getting receptive to 'Probiotic movement' and the study emphasis the need for culture screening for its probiotic attributes even though it belongs to LAB.

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Conflicts of interest

There were no conflicts of interest reported by the authors

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