In vitro evaluation of functional attributes of LABs for the development of turmeric based probiotic beverage

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Abstract

The plant-based fermented beverages have received considerable interest for their rich nutrition, high content of soluble and insoluble fibers and for their high fermentability by probiotic lactic acid bacteria (LAB). In the present study, three LAB strains, namely *Lactobacillus fermentum, Lactobacillus lactis and Lactobacillus plantarum*, were used to develop a functional beverage utilizing juice of fresh turmeric rhizome as substrate for their growth and development. The survivability of probiotics in the beverage was evaluated in terms of their kinetic growth and viability in the samples was maintained during storage of 30 days. Various studies correlating their functional attributes of the developed beverage was performed like Total Titrable acidity, pH, TPC, hydrophobicity, Total Sugar estimation, transit tolerance toward acid and alkali, cholesterol assimilation, lipid peroxidation and various antioxidant analyses. The results revealed significant enhancement in probiotic properties and fermented characteristics after fermentation of LABs with fresh turmeric juice. Among investigated LABs, *Lactobacillus plantarum* was the best potential probiotic strain for the production of fermented turmeric based beverage.

Keywords: Probiotics, Turmeric, Functional Food, Antioxidant analysis, Plant based beverage, Lactobacillus

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Introduction

The increasing cost of health care, the steady increase in life expectancy and the desire of the improvement of the quality of lives are driving factors for research and development in the area of functional foods [1]. Among functional food probiotic manufacturing are of intense research and development area. Recent research has underlined the importance of a vital and healthy microbial population of the gastro intestinal tract (GIT) [2]. Increased research efforts during the last three decades, have confirmed the beneficial association of LAB with the human host. The growing recognition of the importance of this recognition is proved to be effective means to maintain and promote health attributes [3]. Since, the level of consumer awareness of different types of probiotics has improved in recent years an emerging area of innovation is to develop non-dairy probiotic beverages as functional food [4, 5]. These types of products accounted for 11.5% of the global market in 2010. The development of functional beverages is one of the most dynamic and challenging task in food industry [6].

Recent research studies revealed that plant-based beverage could promote good health since they contain beneficial nutrients such as minerals, vitamins, dietary fibers and antioxidants while lacking the dairy allergens that might prevent consumption by certain segments of the population. Moreover, fermentation reduces the amount of non-digestible material in plant foods, leading to improved bioavailability of minerals and trace elements [7]. Turmeric is an important tropical spice mainly and known for its colour, aroma and antioxidant properties [8, 9]. The yellow colour in turmeric are mainly due to the presence of three major pigments; curcumin 1,7-bis(4-hydroxy-3-methoxyfenil)-1,6-heptadiene-3,5-dione), demethoxy-curcumin and bis-demethoxy-curcumin. These curcuminoids are known to have high antioxidant activities [10]. The major disadvantages associated with its administration are its high physical and metabolic

instability and poor aqueous solubility at neutral and basic pH values limiting its systemic bio-availability [11].

Despite potential sensory challenges, the turmeric juice has emerging interest world-wide mainly because of its remarkable chemical composition which includes protein, especially globulins and albumins, carotene, fatty acids, flavonoids and phenolics [12]. So far not much work appears to have been done on fermentation of turmeric and development of probiotc beverage [13]. However, a systematic approach is needed in order to identify the factors, which would facilitate the growth of LAB initially so that these findings can form a platform for fermentation of turmeric juice. Therefore, our research team tried to explore the potential of indigenous probiotic strains to ferment the turmeric juice to enhance their bioactivity. The probiotic potential of the investigated strains is well reported in the literature [14-16]. Thus, the present work laid emphasis on development of herbal formulation based functional beverage, fortified with the LABs as probiotic ingredients. The overview of the fermented beverage will be depicted in Fig.1. The strain viability in fermented turmeric beverage was evaluated. The probiotic potential of investigated LABs were evaluated as functional attributes of the fermented beverage in terms of TPC, pH, Total Titrable acidity, Total hydrophobicity, gastric transit tolerance, cholesterol assimilation, lipid peroxidation and various antioxidant analysis. The storage studies have also been investigated for evaluating the shelf life of developed probiotic drink.

Materials and Methods

Reagents and Equipments

The glassware's were purchased from Borosil. The reagents used in this study were purchased from Himedia, India. The spectrophotometer (Perkin-Elmer), pH meter (Orion), colony counter for viable cell enumeration (Hanna instruments) were used as mentioned.

Turmeric Rhizome

Fresh Turmeric rhizome was purchased from a local superstore in Greater Noida city. Turmeric was partially sterilized using 100 ppm of potassium metabisulfite and incubated overnight before beverage preparation.

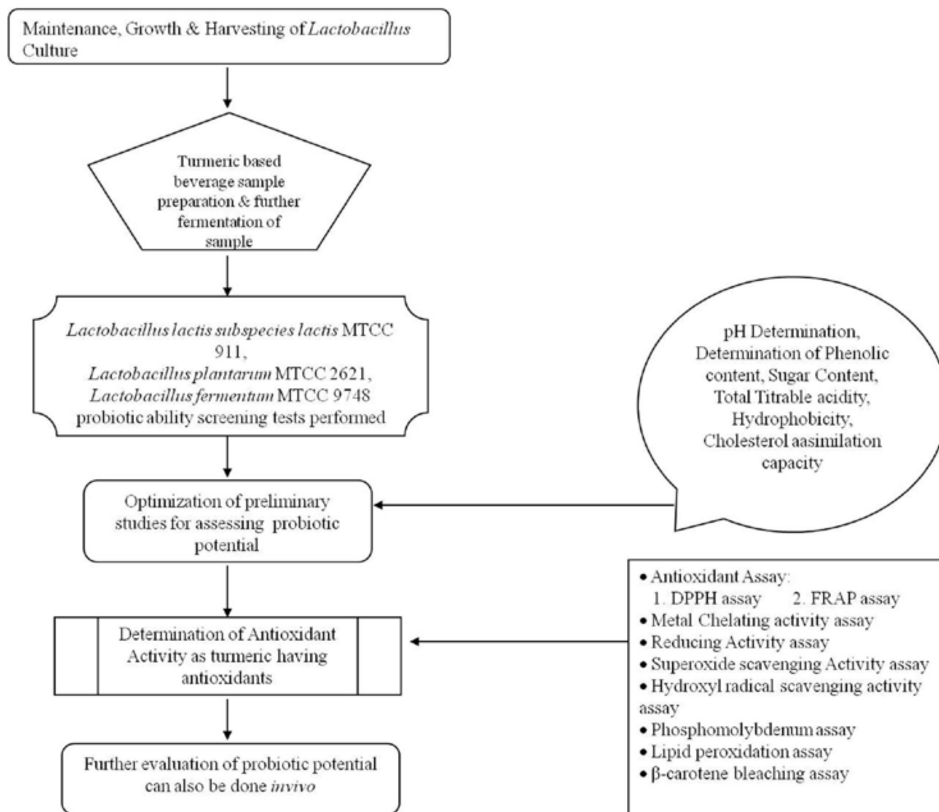

Figure 1. Generalized strategy for the development of fermented turmeric probiotic based beverage.

Culture

Lactobacillus lactis subspecies lactis MTCC 911, *Lactobacillus plantarum* MTCC 2621, *Lactobacillus fermentum* MTCC 9748 was procured from Microbial Type Culture Collection Chandigarh, India. The culture was maintained at -80˚C in 20% glycerol stocks and grown in ManRossa de Sharpe (MRS) broth at 37˚C.

Inoculum Development

Overnight grown cells of all LABs mentioned above were used as inoculums such that a cell concentration of 2.0 x 10^9 (CFU)/ml could be attained in the fermented beverage.

Fermentation of turmeric based beverage

To prepare the turmeric based beverage edible portions (10 g) of turmeric rhizome were cut into small pieces and homogenized using a homogenizer for 2 minutes and filtered through Muslin cloth. Homogenized sample (3 parts) was mixed with 1 part of sucrose and 10 parts of autoclaved distilled water. The three starter cultures were inoculated at 5% (v/v) in turmeric mixture. All fermentative flasks were maintained at 30°C in dark condition. The experimental design comprised of four treatments with three different starters *L. lactis*, *L. plantarum*, *L. fermentum* and mixed culture of these three LABs as co-culture. One treatment having turmeric mixture without starter culture acted as control for these studies.

Analytical Methods

Kinetic growth

To evaluate the survival of LAB in fermented beverage the kinetic growth was observed in two medium (MRS broth and turmeric mixture). An overnight LAB culture was inoculated (5% (v/v) by MRS broth and turmeric mixture and then incubated aerobically at 30°C. The samples were collected at 2-h intervals up to 18 h, and the optical density was measured at 600 nm using a spectrophotometer.

Viable cell enumeration

To evaluate the functionality of fermented beverage and the enumeration of viable cells in turmeric based mixture the estimation of colony forming unit number on MRS-agar plates was evaluated after incubation at 37$^{\circ}C$ for 48 h.

Titrable acidity and pH

To monitor the progress of fermentation as well as to estimate the lactic acid concentration in fermented beverage the pH and total titrable acidity was calculated. pH was measured through pH meter and titrable acidity was determined by titrating 10 ml of each samples with 0.1 mol·l^{-1} NaOH, using phenolphthalein as an indicator.

Detection of Enterobacteria and moulds

Microbiological analyses of the turmeric based fermented beverage were carried out at the end of 14 h to check for the presence of *Enterobacteria* and moulds. Serial dilutions were prepared in peptone water and 100 µl of the broth was spread on the following media: (1) Eosin Methylene Blue (EMB) agar for enumeration of *Enterobacteria* such as *Escherichia coli* (2) Malt Extract Agar (MEA) for the presence of moulds.

Functional attributes of the turmeric based fermented beverage

Total phenolic content (TPC)

The TPC was estimated according to the Folin-ciocalteu procedure [17]. 100µl of fermented and non-fermented turmeric samples and blank (water) was added into a test tube followed by 50µl of Folin-ciocalteu reagent and then 150µl of 20% sodium bicarbonate solution and mixed well. After 30 min of incubation at 37°*C*, absorbance of the solutions was determined at 765 nm against the blank. The TPC was expressed as mg of gallic acid equivalent per ml of sample.

Total Sugar Estimation (TSE)

The total sugar estimation was carried out by using phenol sulphuric acid method. 1 ml of fermented and non-fermented turmeric samples and blank (water) was added into a test tube followed by 1 ml of 5% phenol and 5 ml of concentrated sulphuric acid. After 30 min of incubation at 30°C, absorbance of the solutions was determined at 490 nm against the blank. The total sugar concentration was expressed as mg of glucose released per ml of sample.

Total Hydrophobicity estimation

The Fermented samples and control samples (2ml) were centrifuged at 15,000 rpm for 10 minutes, and the cell pellet were washed and resuspended in sterile saline (0.85%), and absorbance was measured at 600 nm. 3 ml of this suspension was mixed with 1 ml toluene followed by vortexing and incubation at room temperature for 2 min. The aqueous phase was separated and the absorbance was determined at 600 nm. The Total hydrophobicity was measured by following formula:

$$\text{(\% H) Hydrophobicity} = \frac{\text{O.D. of Initial cell suspension} - \text{O.D. of Lower aqueous phase} \times 100}{\text{O.D. of Initial cell suspension}}$$

Transit tolerance of LABs in fermented beverage

Simulated gastrointestinal juice and simulated intestinal juice were prepared according to the method [18]. Fermented samples (1%) were separately inoculated into simulated gastric juice at pH 2.0 following mixing for 10 s, the mixtures were incubated anaerobically at 37°*C*. Gastric transit tolerance was studied by determining total viable counts in gastric juice withdrawn at 0, 1 and 3 h. After 3 h of incubation in gastric juice, 1 ml of culture was inoculated into 9 ml simulated intestinal juice (pH 8.0) and incubated at 37°C anaerobically. The intestinal transit tolerance was studied by determining the total viable counts in intestinal juice withdrawn at 0, 12 and 24 h.

Enzyme Tolerance

To evaluate the survival of LAB of fermented beverage in gastric enzymes, enzyme tolerance was performed. Two digestive enzymes trypsin and lysozyme were taken as reference for this study. 1 ml of the fermented and control samples was taken into a separate test tubes followed by 500µl of gastric enzymes and after serial dilution 100µl of the samples were plated on MRS agar plates immediately. Rest of the samples was incubated at 37°C for 2 hrs. and tolerance was assayed by plating these samples on MRS plates. The (CFU)/ml were observed after 24-48 hours on MRS agar plate.

Analyses of antioxidant activities of turmeric based fermented beverage

DPPH radical scavenging

The free-radical scavenging activity was measured using 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay was performed according to the reported procedure [19]. 5 ml of DPPH methanol solution (0.025 $mol \cdot l^{-1}$ DPPH in methanol) was mixed with 0.5 ml fermented samples and incubated for 30 min (in dark) at room temperature, the decrease in the absorbance at 517 nm was measured. The blank contained methanol instead of DPPH methanol solution, while the control contained DPPH methanol solution without sample. The percentage inhibition was measured according to the following formula and analyzed.

$$\text{\% scavenging} = \frac{1 - (\text{Absorbance of sample} - \text{Absorbance of blank}) \times 100}{\text{Absorbance of control}}$$

Phosphomolybdenum assay

The antioxidant activity of fermented samples was evaluated by phosphomolybdenum method as reported in literature [20]. An aliquot of 0.1 ml of fermented turmeric samples and blank was taken in test tube followed by 1 ml of reagent solution (0.6 mol·l^{-1} sulphuric acid, 28 m mol·l^{-1} sodium phosphate and 4 m mol·l^{-1} ammonium molybdate). The tube was capped and incubated in a water bath at 95°C for 90 min. After the incubation, samples were cooled to room temperature and the absorbance of the mixture was measured at 765 nm against a blank.

Hydroxyl radical scavenging activity

The effect of fermented samples on hydroxyl radicals was assayed by using the deoxyribose method [21]. The assay was performed by using the reaction mixture which contained 450 µl of 0.2 mol·l^{-1} sodium phosphate buffer (pH 7.0), 150 µl of 10 m mol·l^{-1} 2-deoxyribose, 150 µl of 10 m mol·l^{-1} $FeSO_4$-EDTA, 150 µl of 10 m mol·l^{-1} H_2O_2, 525 µl of H_2O, and 75 µl of fermented sample solutions. The reaction was started by the addition of H_2O_2. After incubation at 37°C for 4 h, the reaction was stopped by adding 750 µl of 2.8% trichloroacetic acid and 750 µl of 1% TBA in 50 m mol·l^{-1} NaOH, the solution was boiled for 10 min and then cooled in water. The absorbance of the solution was measured at 520 nm. Ascorbic acid (0.05-0.250 mmol·l^{-1}) was used as positive controls. The ability to scavenge the hydroxyl radical was calculated using the following equation.

$$\%\ \text{Hydroxyl radical scavenging activity} = 1 - (\text{Absorbance of sample} - \text{Absorbance of blank}) \times 100$$

Superoxide radical scavenging activity

Superoxide radical scavenging activity of fermented samples was determined by the nitroblue tetrazolium reduction method [22]. 1ml of nitroblue tetrazolium (NBT) solution (l mol·l^{-1} NBT in 100 m mol·l^{-1} phosphate buffer, pH 7.4), 1 ml NADH solution (l mol·l^{-1} NADH in 100 m mol·l^{-1} phosphate buffer, pH 7.4) and 0.1 ml of the fermented samples and ascorbic acid (0.50-0.50 mmol·l^{-1}) were mixed. The reaction was started by adding 100 µl of PMS solution (60 µ mol·l^{-1} PMS in 100 m mol·l^{-1} phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25°C for 5 min, and the absorbance at 560 nm was measured against blank samples, containing all the reagents except the PMS. The positive and negative controls were subjected to the same procedures as the sample, except that for the negative control, only the solvent was

added, and for the positive control, sample was replaced with ascorbic acid. All measurements were made in triplicate. The abilities to scavenge the superoxide radical were calculated using the following equation

% Superoxide radical scavenging activity = 1-(Absorbance of sample-Absorbance of control) × 100

Metal Chelating activity

The fermented samples were assessed for their ability to compete with ferrozine for iron (II) ions in free solution. The chelating ability of ferrous ions was estimated as reported in literature [23]. 0.5 ml of fermented turmeric samples were added to a solution of 2 $mmol \cdot l^{-1}$ $FeCl_2.4H_2O$ (0.05 ml). The reaction was initiated by the addition of 5 $mmol \cdot l^{-1}$ ferrozine (0.2 ml), the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured at 562 nm against the blank performed in the same way using $FeCl_2$ and water. EDTA served as the positive control, and a sample without EDTA served as the negative control. All tests were run in triplicate and averaged. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the formula

% chelating activity = 1- (Absorbance of sample -Absorbance of control) × 100

Antioxidant activity by ß-carotene bleaching method

The antioxidant activity of fermented samples was evaluated using the β-carotene-linoleate model system [24]. 2 mg of β -carotene were dissolved in 10 ml chloroform and 1 ml β -carotene solution was mixed with 20 mg of purified linoleic acid and 200 mg of Tween 40 emulsifiers. Chloroform was then evaporated under a gentle stream of nitrogen and the resulting mixture was immediately diluted with 50 ml of distilled water. To an aliquot of 5 ml of this emulsion, 0.2 ml of each fermented sample were added and mixed well. The absorbance at 470 nm, which was regarded as t_0, was measured, immediately, against a blank consisting of the emulsion without β -carotene. The capped tubes were placed in a water bath at 50°*C*, and the absorbance was measured after every 15 min up to 120 min. For the positive control, sample was replaced with ascorbic acid. A negative control consisted of 0.2 ml of distilled water or solvent instead of extract or reference antioxidant was used. All samples were assayed in triplicate. The antioxidant activity (AA) was measured in terms of successful bleaching of ß-carotene by using the following equation:

$$AA = ((1-A_0-A_t/A_0-A_{0t})) \times 100$$

Where, A_0 and A_0 are the absorbance values measured at zero times during the incubation for each fermented samples and control, respectively. A_t an A_{0t} was the absorbance values measured for each fermented samples and control respectively, after incubation for 120 min.

Analysis of Lipid peroxidation

Lipid peroxidation assay was performed according to modified protocol as described in literature [25] to measure the lipid peroxide formed, using mice liver homogenates as lipid-rich media [26]. Liver homogenate (0.5 ml of 10%, v/v) and 0.1 ml of fermented sample were added to a test tube and final volume was made up to 1 ml with distilled water. 0.05 ml of $FeSO_4$ (0.07 $mol \cdot l^{-1}$) was added to induce lipid peroxidation and incubated for 30 min followed by the addition of 1.5 ml of 3.5 M acetic acid (pH adjusted to 3.5 with NaOH) and 1.5 ml of 0.06 $mol \cdot l^{-1}$ TBA in 0.04 M sodium dodecyl sulphate and 0.05 ml of 1.2 $mol \cdot l^{-1}$ of TCA was added, and the resulting mixture was vortexed and then heated at *95°C* for 60 min. To eliminate this non-MDA interference, another set of samples was treated in the same way, incubating without TBA, to subtract the absorbance for fraction and reference compounds. After cooling, 5 ml of butan-1-ol was added to each tube and centrifuged at 3000 × g for 10 min. The absorbance of the organic upper layer was measured at 532 nm. Ascorbic acid samples act as positive control. Inhibition of lipid peroxidation (%) by the sample was calculated according to the following formula:

$$\%\ \text{lipid peroxidation} = 1 - \frac{(\text{Abs. of solu}^n\ \text{with extract} - \text{Abs. of solu}^n\ \text{without liver homogenate}) \times 100}{\text{Absorbance of control without extract}}$$

Antioxidant analysis using FRAP assay

FRAP assay was performed according to the methods of Benzie and Strain with slight modification [27]. The working FRAP reagent (3 ml) was added to 100 µl of fermented samples and mixed. The working FRAP reagent was prepared by mixing 2.5 ml of 2,4,6- tripyridyl-s-triazine (TPTZ) solution (10 $mmol \cdot l^{-1}$ TPTZ in 40 $mmol \cdot l^{-1}$ HCl), 25 ml of 0.3 $mmol \cdot l^{-1}$ of acetate buffer and 2.5 ml of 20 m $mol \cdot l^{-1}$ $FeCl_3.6H_2O$ solution. The mixture was incubated at *37°C* for 4 min and the absorbance was measured at 593 nm. using spectrophotometer. The change in absorbance at zero and 4 min was used to calculate the FRAP value. The FRAP value was calculated using the equation as follows:

$$\text{FRAP value (mgFeSO}_4\text{/ml)} = (\wedge \text{A 593nm sample}/ \wedge \text{A 593nm std.}) \times [Fe^{2+}]\ \text{standard}$$

Results and discussion

Kinetic growth curve

To assess the growth potential of LABs in turmeric mixture the kinetic growth was observed in turmeric mixture and MRS broth as standard growth medium which is shown in Fig. 2. The samples were collected at 2 h intervals up to 18 h, and the optical density was measured at 600 nm using a spectrophotometer. The turmeric mixture was prepared as described in Turmeric fermentation. The log phase of *L. lactis* was observed after 4 h of inoculation, while *L. plantarum* and *L. fermentum* were observed at 6 h. The similar growth pattern was observed in coculture of all three investigated bacteria in MRS medium. The growth of cell cultures was continued until 14 h for all LABs after inoculation, when they reached stationary phase. *L. plantarum* had the highest growth rate, followed by *L. lactis* and *L. fermentum*. In turmeric mixture (Fig. 2), the growth rate of LAB was found lower than the MRS broth and could not separated according to the growth phases (lag phase, log phase, stationary phase). These results may be attributed to various components present in turmeric's rhizome as well as might be high poly-phenolic content which can inhibit the growth of bacterial cells [28].

pH analysis

To assess the progress of fermentation the pH of turmeric beverage was monitored as shown in Fig. 3. The pH of all treatments with LAB (initial values ranging from pH 6.8-6.6) decreased in the first 14 hrs of fermentation (value ranging from pH 5.4-4.9) while in treatments without LAB pH drop was observed due to the natural microbial population present in turmeric rhizome. At the end of the fermentation, the pH drop of LAB treatments was greater than treatments without LAB. This might be attributed to the production of lactic acid. pH value of the fermented samples was stable during 30 days of cold storage in refrigerator.

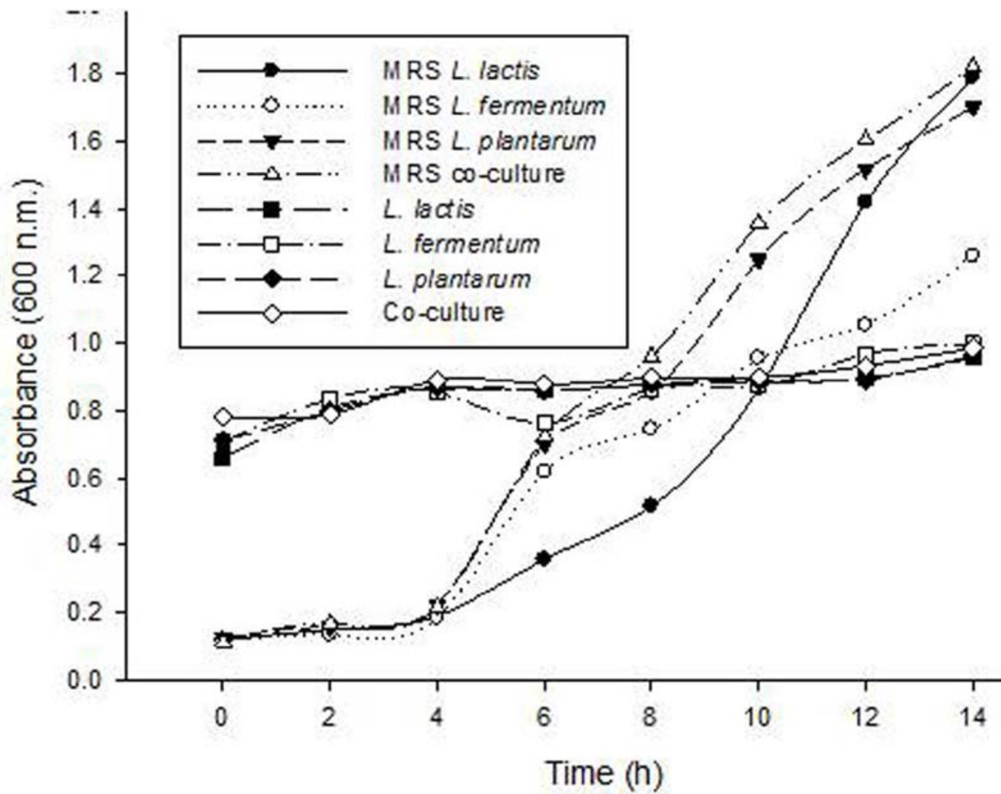

Figure 2 Kinetic Growth curve of LABs in MRS broth and fermented turmeric beverage.

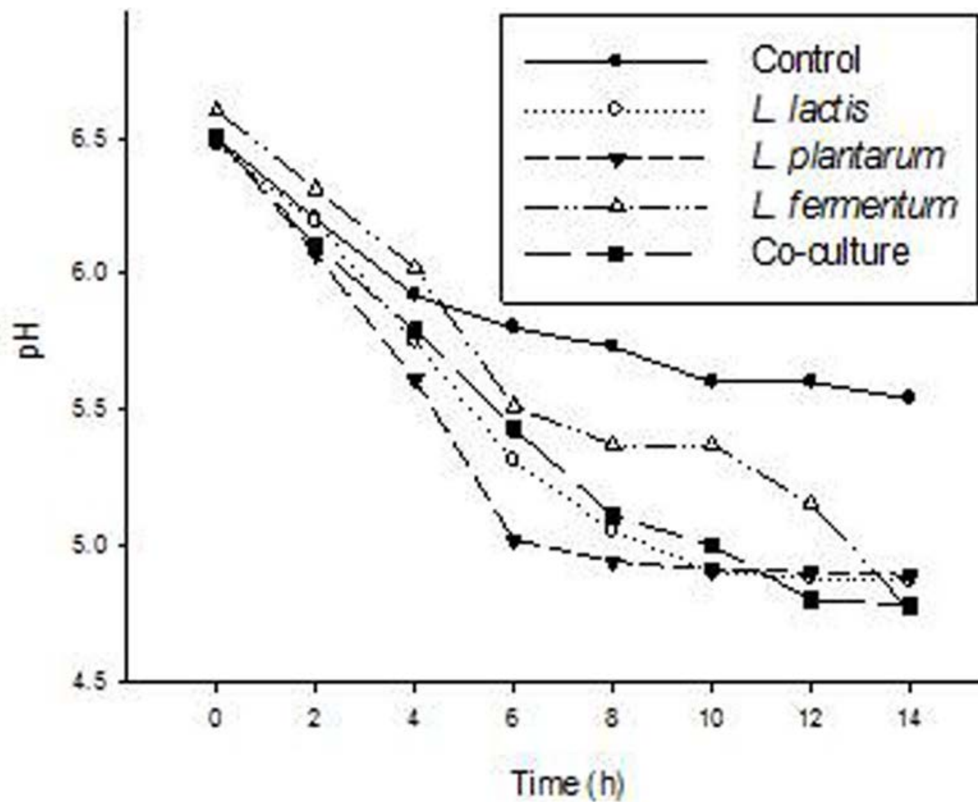

Figure 3 pH analysis of fermented turmeric beverage.

Analysis of total titrable acidity (TTA)

The results obtained from the analysis of total titrable acidity are shown in Fig. 4. The initial lactic acid concentration of LAB treatments was in the range of 2.7-3.6%. The concentration of lactic acid was increased linearly till the end of fermentation for LAB treatments and as well as unfermented samples. The highest lactic acid concentration was observed in *L. plantarum*. This might be attributed to the production of lactic acid and other organic acids produced from native flora (such as yeast and fungi) of turmeric rhizome. Hence, these acids lead to decrease in pH values in treatments with and without LAB. After 10 days, pH values showed slight changes due to the production of CO_2 which dissolved in the water and attained buffering properties. During the refrigerated storage of 30 days the total titrable acidity in all fermented samples was found to be higher as compared to unfermented sample. The lowest titrable acidity was observed in co-culture among all fermented cultures of investigated LABs.

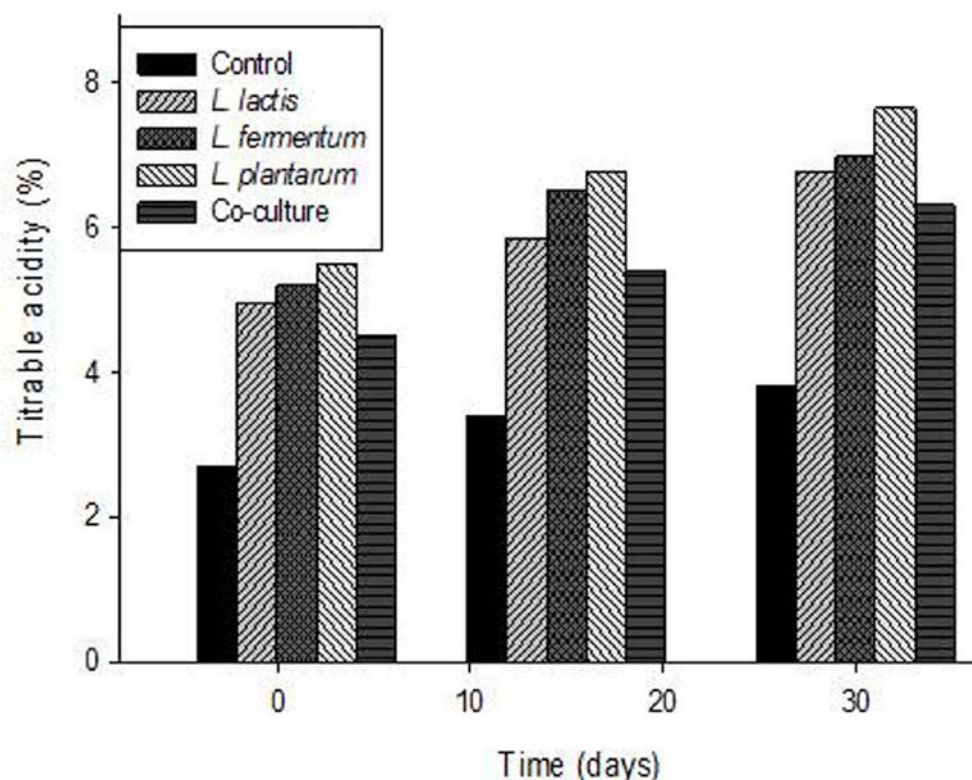

Figure 4 Analysis of Total titrable acidity of fermented turmeric beverage.

Estimation of Total Phenolic Content

The results obtained from the analysis of total phenolic content are shown in Fig. 5. The total phenolic content in all fermented cultures was found to be more in comparison to unfermented. Before fermentation, the TPC value of unfermented sample was 4.0µg gallic acid per ml. TPC was enhanced after fermentation for the *L. plantarum* 6.5µg/ml and *L. fermentum* 5.1µg/ml and *L. lactis* 5.6µg/ml and co-culture 5.0µg/ml gallic acid per ml. Among three investigated LABs, co-culture fermented samples showed minimum enhancement in total phenolic content. During the storage the phenolic content of the fermented samples has been increased which leads to prevention of deterioration of probiotic characteristics. The TPC was enhanced on 15th day *L. plantarum* 6.8 µg/ml and *L. fermentum* 5.2µg/ml and *L. lactis* 5.8 µg/ml and co-culture 5.1 µg gallic acid per ml and on 30th day *L. plantarum* 8.8 µg/ml and *L. fermentum* 5.5 µg/ml and *L. lactis* 7.0 µg/ml and co-culture 8.2 µg gallic acid per ml.

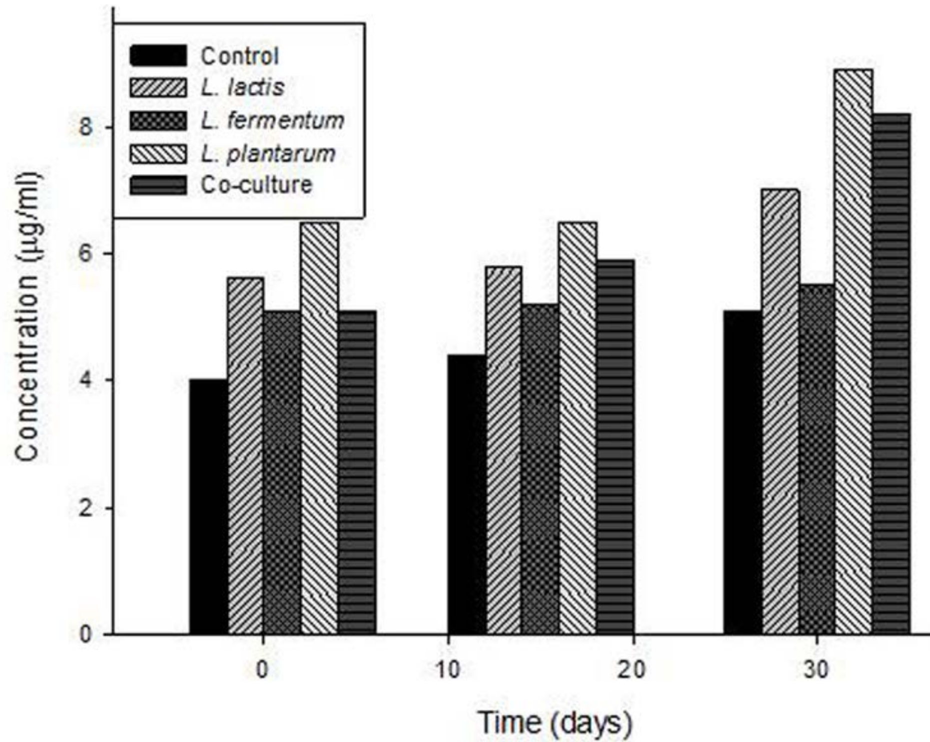

Figure 5 Analysis of Total phenolic content of fermented turmeric beverage.

Estimation of Total Sugar

The results obtained from the analysis of total sugar are shown in Fig. 6. The total sugar content in unfermented sample was found to be 18.5 mg/ml while in the fermented sample the sugar content was found to be increased for *L. plantarum* 30.98 mg/ml, *L. lactis* 28.6 mg/ml and *L. fermentum* 25.89 mg/ml, respectively. The lowest content was observed in co-culture among all of fresh and stored fermented samples. During the refrigerated storage the total sugar content was found to be decreased on 15th days of storage the sugar content was found to be 22.3, 25.8, 26.9 mg/ml in *L. plantarum*, *L. lactis* and *L. fermentum*, respectively. This might be due to the metabolic activity of LABs present in the sample. There was enhancement in sugar content was observed on 30th days of storage and the values were found to be 50.7, 43.4, 51.7 mg/ml in *L. plantarum*, *L. lactis* and *L. fermentum,* respectively.

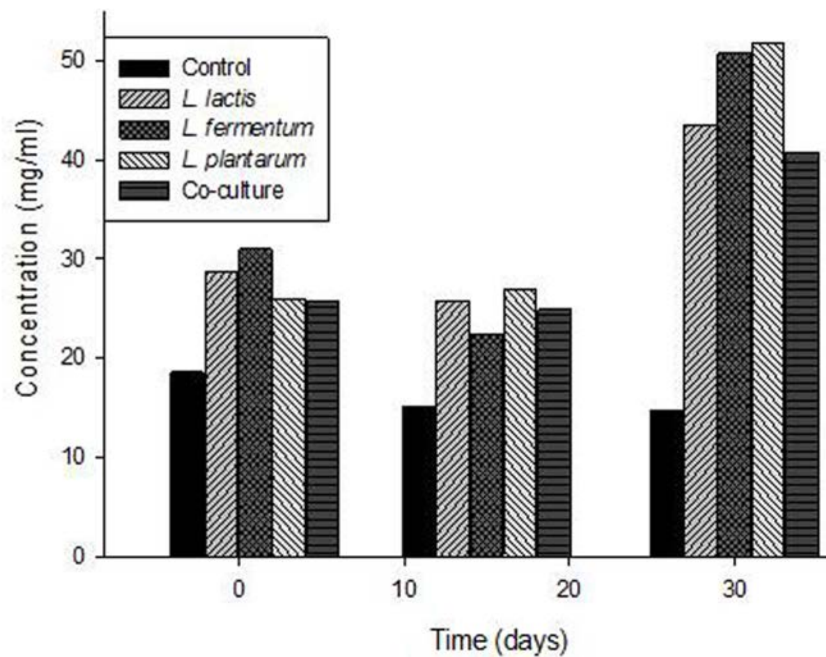

Figure 6 Analysis of Total sugar content of fermented turmeric beverage.

Analysis of Total Hydrophobicity

The cell surface hydrophobicity (%H) was calculated for the assessment of the *invitro* adhesion capacity of plasma membrane. The results obtained from the analysis of hydrophobicity are shown in Fig. 7. The unfermented sample has approximately 48-54 % hydrophobic nature with respect to toluene while there was increase in the hydrophobic property after fermentation with various LABs. *L. plantarum* has high adhesion capacity of 88%. The total hydrophobicity in all fermented cultures was found to be higher in comparison to unfermented sample. There were not significant differences in % hydrophobicity in fermented samples during storage studies.

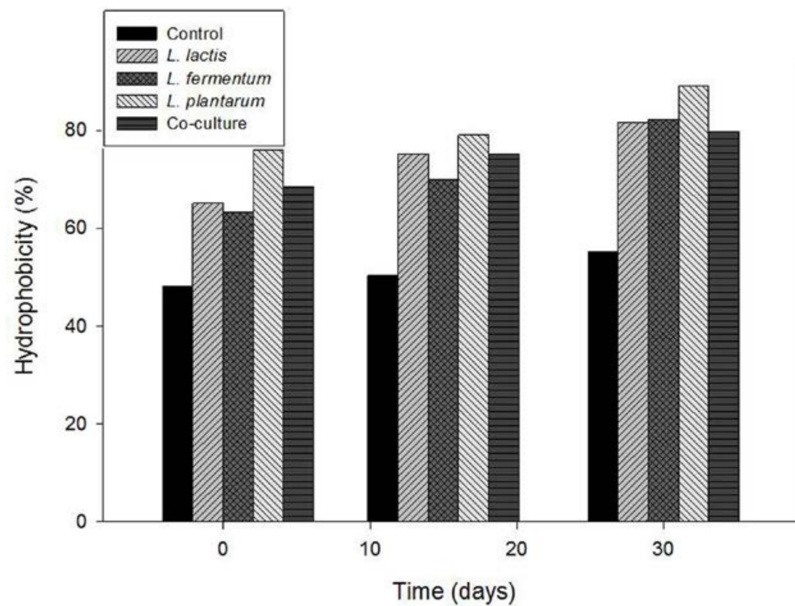

Figure 7 Total Percent (%) hydrophobicity of fermented turmeric beverage

Analysis of cholesterol assimilation of LABs in fermented beverage

It has been reported that probiotic bacteria exert a hypocholesterolemic effect [29, 30] therefore the cholesterol assimilation assay was performed. The results obtained from the analysis of cholesterol are shown in Fig. 8. The results showed that *L. plantarum* could assimilate 16.0 mg/ml, *L. lactis* 18.0 mg/ml and *L. fermentum* 27.0 mg/ml, of cholesterol from the media, respectively. The lowest assimilation capacity was observed in co-culture among all fermented cultures. During the refrigerated storage, the cholesterol assimilation capacity of all fermented samples was found to be decreased. On 30th day of storage *L. plantarum* could assimilate 12.0 mg/ml, *L. lactis* 13.0 mg/ml and *L. fermentum* 17.0 mg/ml, of cholesterol from the media. Our results indicated that fermented turmeric have the ability to assimilate cholesterol, which supports their potential as functional beverage.

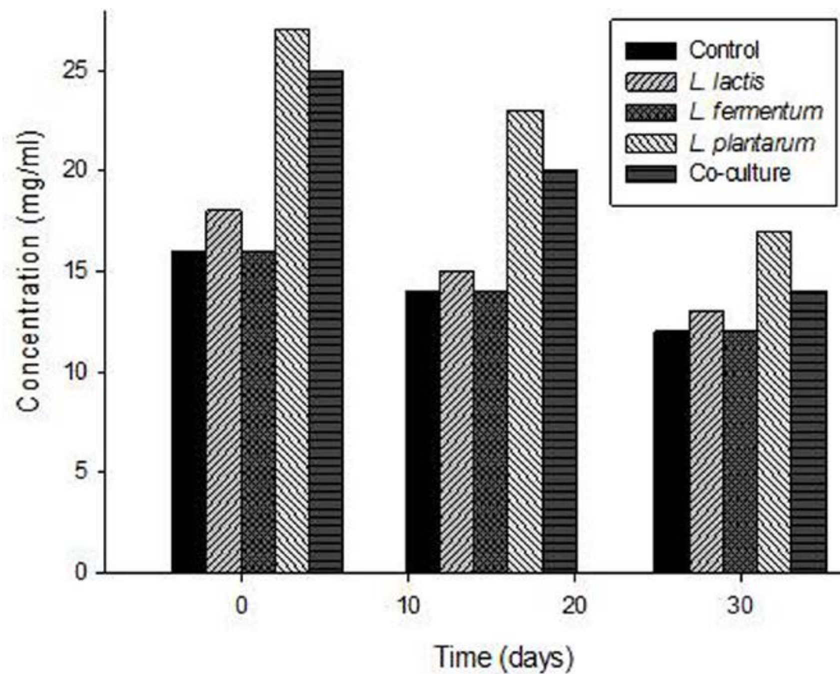

Figure 8 Analysis of cholesterol assimilation on fermented turmeric beverage

Determination of Viable cell count and shelf life

The gradual increases in the viable counts (CFU)/ml during fermentation were observed after adding inoculum of various LABs in turmeric juice. There was a general trend towards a slight decrease in the number of viable bacteria between storage day15 and day 30. There were no dramatic differences in the viable counts of all strains in the fermented turmeric beverage samples during storage (Table 1). After the completion of fermentation samples were withdrawn to check for the presence of *Enterobacteria* and moulds. There was a complete absence of any *Enterobacteria* or moulds in the fermented beverage.

Table 1. Effect of fermentation on viable cell count of investigated LABs in fermented turmeric beverage

Samples	Viable Cell count (log CFU/ml)		
	Fresh Sample (1day)	15 days	30 days
Unfermented	2.11 ± 0.007	1.07 ± 0.010	0.825 ± 0.028
L. lactis	8.26 ± 0.057	8.19 ± 0.006	8.10 ± 0.004
L. plantarum	8.91 ± 0.002	8.76 ± 0.045	8.60 ± 0.031
L. fermentum	8.49 ± 0.028	8.32 ± 0.014	8.22 ± 0.005
Co-culture	8.37 ± 0.055	8.29 ± 0.017	8.14 ± 0.021

Effect of enzyme tolerance on viable cell count in fermented Turmeric beverage: Bacterial cell membrane is susceptible for degradation of proteolytic enzymes. The enzyme tolerance is carried out for the assessment of the susceptibility towards enzymes. Trypsin and Lysozyme were taken as standard proteolytic enzymes and the results are depicted in (CFU)/ml and summarized in (Table 2). The results revealed that all the investigated LABs shows good survival rate after the treatment of proteolytic enzymes. The highest survival rate was observed in *L. plantarum* and the lowest survival rate was observed in co-culture. During storage there was slight increase in the viable cell count which shows good indication for maintaining their probiotic potential.

Table 2. Analysis of enzyme tolerance of investigated LABs in fermented turmeric beverage

Samples	Viable Cell count (log c.f.u.//ml)											
	Fresh Sample (1day)				15 days				30 days			
	Trypsin		Lysozyme		Trypsin		Lysozyme		Trypsin		Lysozyme	
	0h	2h	0h	2h	0h	2h	0h	2h	0h	2h	0h	2h
Unfermented	2.10± 0.016	2.06 ± 0.025	2.09± 0.088	1.76 ± 0.074	1.02 ± 0.095	1.00 ± 0.080	1.05 ± 0.073	1.02 ± 0.053	0.76 ± 0.027	0.23 ± 0.034	0.82 ± 0.029	0.16 ± 0.065
L. lactis	8.23 ± 0.011	7.61 ± 0.036	8.20 ± 0.073	6.91 ± 0.058	8.10 ± 0.062	5.31 ± 0.065	8.11 ± 0.057	4.32 ± 0.059	7.97 ± 0.041	3.58 ± 0.049	7.82 ± 0.028	2.98 ± 0.037
L. plantarum	8.90 ± 0.005	7.81± 0.012	8.88 ± 0.028	7.45± 0.021	8.75 ± 0.015	6.20 ± 0.013	8.75 ± 0.019	5.88 ± 0.014	8.55 ± 0.008	3.91 ± 0.009	8.50 ± 0.020	3.16 ± 0.032
L. fermentum	8.43 ± 0.075	7.32 ± 0.055	8.45 ± 0.080	7.72 ± 0.045	8.31 ± 0.064	5.42 ± 0.061	8.30 ± 0.058	4.78 ± 0.054	8.20 ± 0.071	4.31 ± 0.068	7.98 ± 0.034	2.71 ± 0.026
Co-culture	8.35 ± 0.055	5.38 ± 0.034	8.30 ± 0.067	4.76± 0.045	8.29 ± 0.026	4.62 ± 0.037	8.18 ± 0.064	3.75± 0.013	8.10 ± 0.029	2.16± 0.031	8.00 ± 0.024	1.33 ± 0.089

Evaluation of transit tolerance of LABs in fermented beverage

The transit tolerance studies were carried out for the *in vitro* assessment of the survival of investigated LABs in gastrointestinal environment. The fermented and control samples were incubated in the simulated gastric juice (pH 2.0), and the results revealed that after fermentation with all investigated LABs showed good acid tolerance. After 3 h. incubation in simulated gastric juice *L. plantarum* showed tolerance up to a range 8.49-5.92 (CFU)/ml, *L. lactis* showed tolerance up to 8.24-4.37 (CFU)/ml, *L. fermentum* showed tolerance up to 8.91-5.35 (CFU)/ml, and co-culture showed tolerance up to 8.37-2.33 (CFU)/ml, respectively. Incubation in the simulated intestinal juice up to 12 h also did not significantly affect the viability of these fermented samples except co-culutre. The results are summarized in (**Table 3**). *L. plantarum*, *L. lactis*, *L. fermentum* survived with viable counts of about 1.99, 1.13 and 3.87 (CFU)/ml, respectively in the simulated intestinal juice for 24 h., but co-culture did not tolerate incubation in the simulated intestinal transit. The highest tolerance level was observed in *L. plantarum*.

Table 3. Transit tolerance studies of fermented turmeric beverage

Samples (Turmeric based fermented beverage)	Tolerance to simulated gastric juice at pH 2.0 (log c.f.u.//ml)			Tolerance to simulated intestinal juice at pH 2.0 (log c.f.u.//ml)		
	0h	1h	3h	0h	12h	24h
Unfermented	2.10 ± 0.007	1.39± 0.002	0.876± 0.120	0.876± 0.120	0.265± 0.009	0.000±0.000
L. lactis	8.24± 0.009	7.68± 0.100	4.37± 0.011	4.37± 0.011	3.61± 0.020	1.13± 0.041
L. plantarum	8.91± 0.002	7.55± 0.018	6.21± 0.078	6.21± 0.078	4.73± 0.021	2.87± 0.005
L. fermentum	8.49± 0.028	6.33± 0.080	5.92± 0.013	5.92± 0.013	3.77± 0.025	1.99± 0.062
Co-culture	8.37± 0.055	5.81± 0.020	1.98± 0.042	1.98± 0.042	0.816± 0.002	0.11± 0.030

Evaluation of antioxidant potential of fermented beverage

Estimation of Metal chelating activity

Chelation of iron plays the main role for assessing antioxidant potential of fermented beverage. The reducing power of various fractions to reduce iron ion Fe (III) into Fe (II) is shown in Fig. 9. The metal chelating activity in all fermented samples was found to be higher in comparison to unfermented samples. The results revealed that the chelating activity of *L. plantarum* was found to be 6.5 mg/ml, *L. lactis* 6.0 mg/ml *L. fermentum* 7.5 mg/ml, and co-culture 6.8 mg/ml, respectively. The lowest content was observed in *L. lactis* among all of the fermented samples. There was not significant variation in the metal chelating activity during storage which shows good antioxidant potential of fermented beverage. The ascorbic acid was taken as standard antioxidant system for this study.

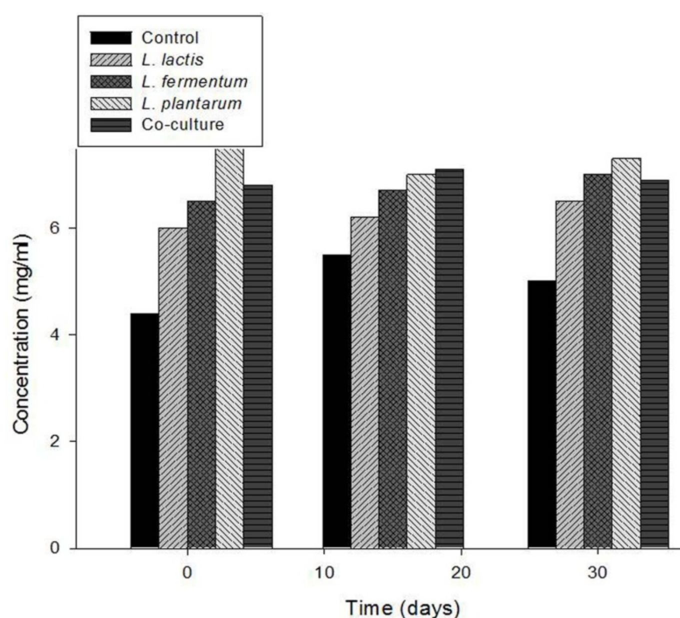

Figure 9 Analysis on metal chelating activity of fermented turmeric beverage.

Evaluation of Phosphomolybdenum activity

The basic principle to assess the antioxidant capacity through phosphomolybdenum assay includes the reduction of Mo (VI) to Mo (V) by the plant extract possessing antioxidant compounds. The results obtained from the analysis of phosphomolybdenum assay are shown in Fig. 10. The phosphomolybdenum activity in all fermented samples was found to be higher in comparison to unfermented sample. In the present study *L. plantarum* was more effective in reduction of Mo (VI) to Mo (V) *i.e.* 5.7 µg/ml whereas as in *L .fermentum* and co-culture the values were found to be 5.2 µg/ml and 5.5 µg/ml, respectively, the lowest content was observed in *L. lactis* 4.4 µg/ml among three investigated bacteria. There was insignificant variation in the reduction capacity of Mo (VI) to Mo (V) during storage which shows good antioxidant potential of fermented beverage. The present studies indicated the close agreement from number of reports that fermentation directly enhanced antioxidant activity [31, 32].

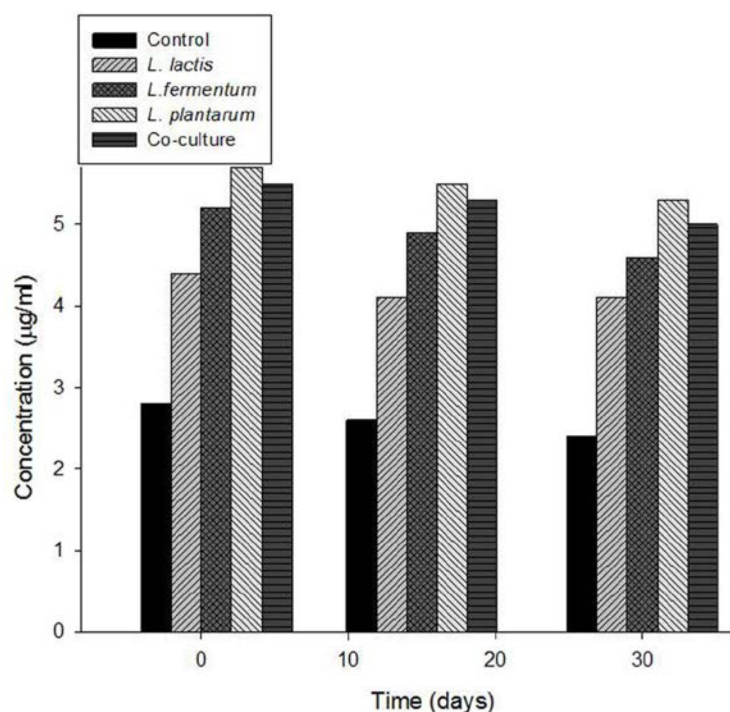

Figure 10 Analysis of Phosphomolybdenum activity of fermented turmeric beverage

Evaluation of Antioxidant activity by DPPH scavenging

DPPH is a stable free radical, which has been widely used in phytomedicine for the assessment of scavenging activities of bioactive fractions. The scavenging activities of fermented samples were determined using free radicals of 1,1-diphenyl 1-2-picryl-hydrazyl (DPPH) and presented in terms of scavenging activity (%). The results are presented in Fig. 11. The scavenging activity of unfermented sample was 31.5-35.7 %. During fermentation the scavenging activity of all the fermented samples was found to be dramatically increased as well as the storage samples also possess enhanced antioxidant activity. During fermentation the scavenging activity enhanced from 31.5% to 72.5%. The highest scavenging activity was found to be for *L. plantarum* 68.2% and the lowest scavenging effect was seen from *L. lactis* 49.2%. During 30 days of storage, the enhancement of scavenging activity of all the fermented samples was observed which might be due to increase in phenolic content of the investigated LABs [33]. The results are compared with ascorbic acid as standard which shows approximately 98% scavenging activity.

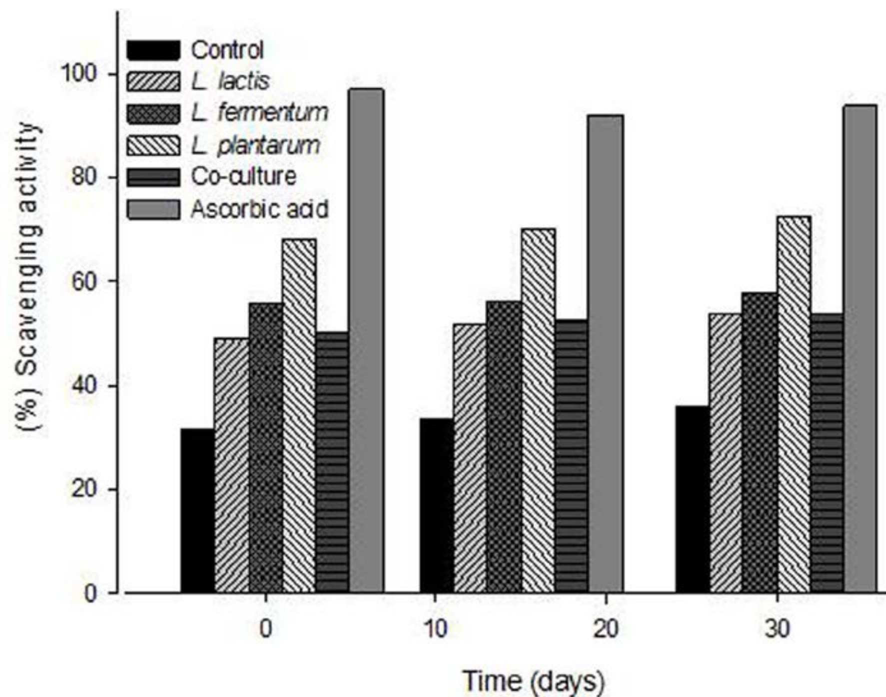

Figure 11 Analysis of DPPH scavenging activity of fermented turmeric beverage

Antioxidant activity determination by FRAP assay

The FRAP, a method for measuring total reducing power of electron donating substances, was assessed. The results obtained from the (ferric reducing ability of plasma) antioxidant activity are shown in Fig. 12. The total reducing power was assessed in terms of scavenging activity (%). The scavenging activity of fermented samples increases with time as compared to unfermented sample. The FRAP value of the fermented turmeric beverage with *L .fermentum* was more effective in reduction of Mo (VI) to Mo (V) *i.e.* 32.4% whereas as in *L. plantarum* and *L. lactis* the values were found to be 25.9 % and 18.6 %, respectively. The lowest activity was observed in co-culture 15.5 %. There was no significant enhancement in the FRAP values of all fermented samples were observed during storage.

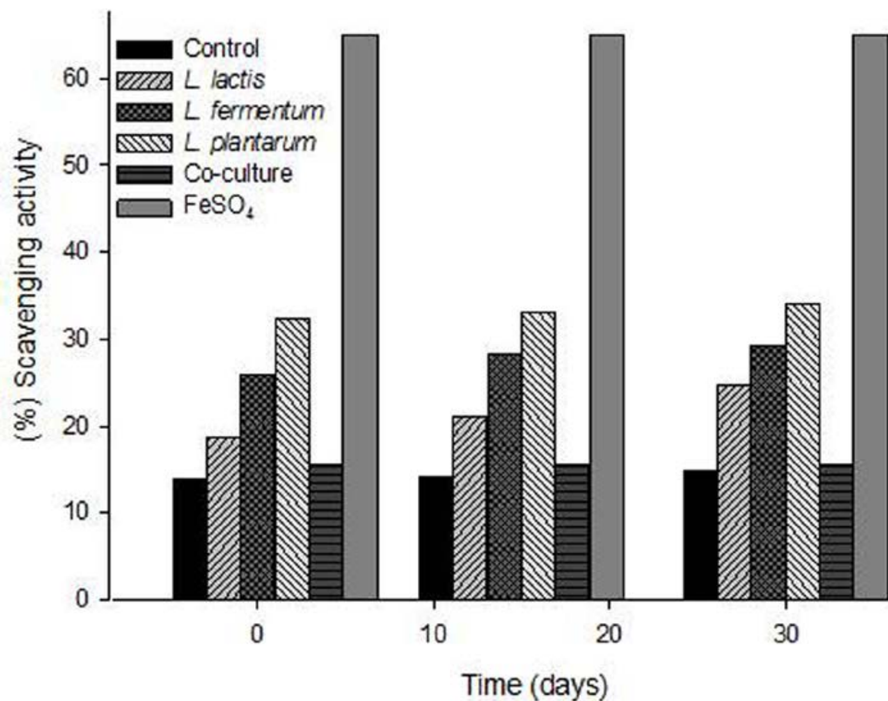

Figure 12 Ferric reducing activities of plasma antioxidant activities of fermented turmeric beverage.

Estimation of β-carotene bleaching activity

The antioxidant potential of the developed beverage was assessed through β-carotene bleaching method. The results obtained from the analysis of β-carotene bleaching activity are shown in Fig. 13. All fermented as well as unfermented samples inhibited the oxidation of linoleic acid and subsequent bleaching of β-carotene. The bleaching activity in fermented samples was found to be higher in comparison to unfermented sample. Among the fermented beverage *L. plantarum* showed greater inhibitory activity *i.e.* 80 mg/ml followed by *L. fermentum* 75 mg/ml and *L. lactis* 74 mg/ml. The lowest bleaching activity was observed in co-culture *i.e.* 71 mg/ml among all fermented samples. The literature revealed that the bleaching activity is time dependent therefore the bleaching activity was decreased for all the samples during different storage conditions [34]. Despite this fact, the results suggested that fermented turmeric beverage possessed effective antioxidant constituents.

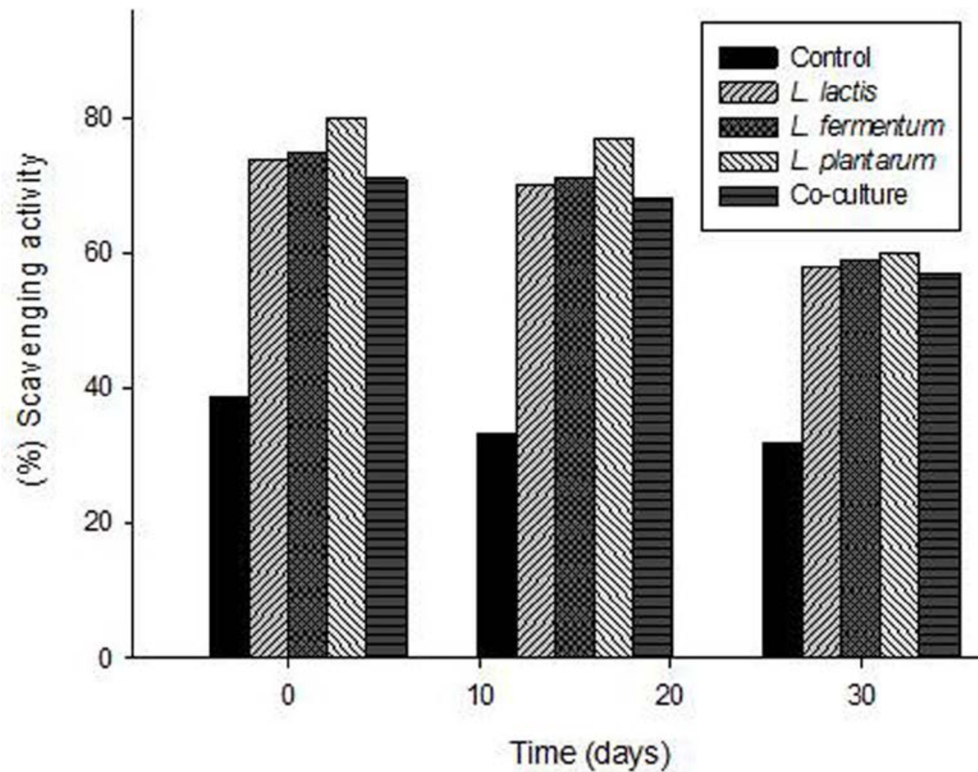

Figure 13 Effect of fermentation on β-carotene bleaching activity of fermented turmeric beverage

Evaluation of Lipid peroxidation activity

Lipids peroxides are likely involved in many pathological events, including inflammation, metabolic disorders, oxidative stress and cellular aging. The effect of fermented beverage samples on non-enzymatic peroxidation is depicted in Fig. 14. There was not significant variation was found in lipid peroxidation activity in all fermented samples. The lipid per oxidation activity in *L. plantarum* was found to be slightly higher 43.8 % as compared to other investigated LABs. The lowest percentage was observed in *L. fermentum* 29.6 % among all fermented samples. There was continuous decrease was observed in the lipid peroxidation activity during storage studies.

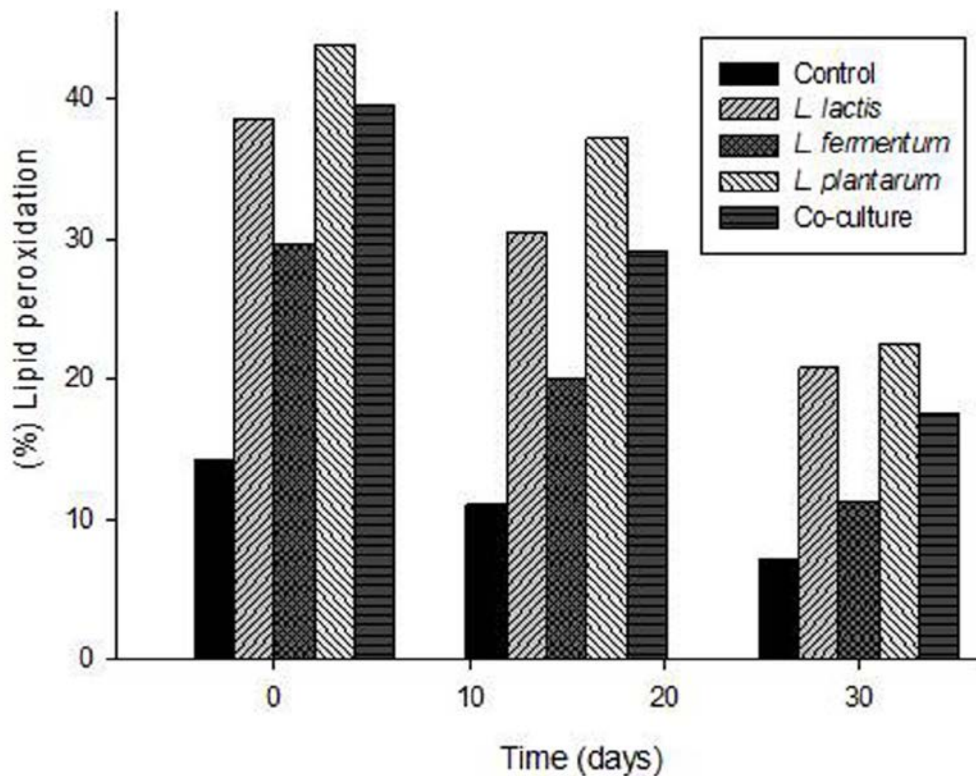

Figure 14 Effect of fermentation on lipid peroxidation activity of fermented turmeric beverage

Determination of Hydroxyl radical scavenging activity

Among the oxygen radicals, hydroxyl radical is the most reactive and induces severe damage to biomolecules such as protein, DNA and lipids cause's lipids peroxidation. The results obtained from the analysis of hydroxyl radical scavenging activity are shown in Fig. 15. The hydroxyl radical scavenging activity in *L. plantarum* was found to be 81 µg/ml followed by co-culture 73 µg/ml and *L. lactis* 71 µg/ml. The lowest scavenging activity was observed in *L. fermentum* among all fermented cultures. The scavenging activity was decreased for all the samples during different storage conditions.

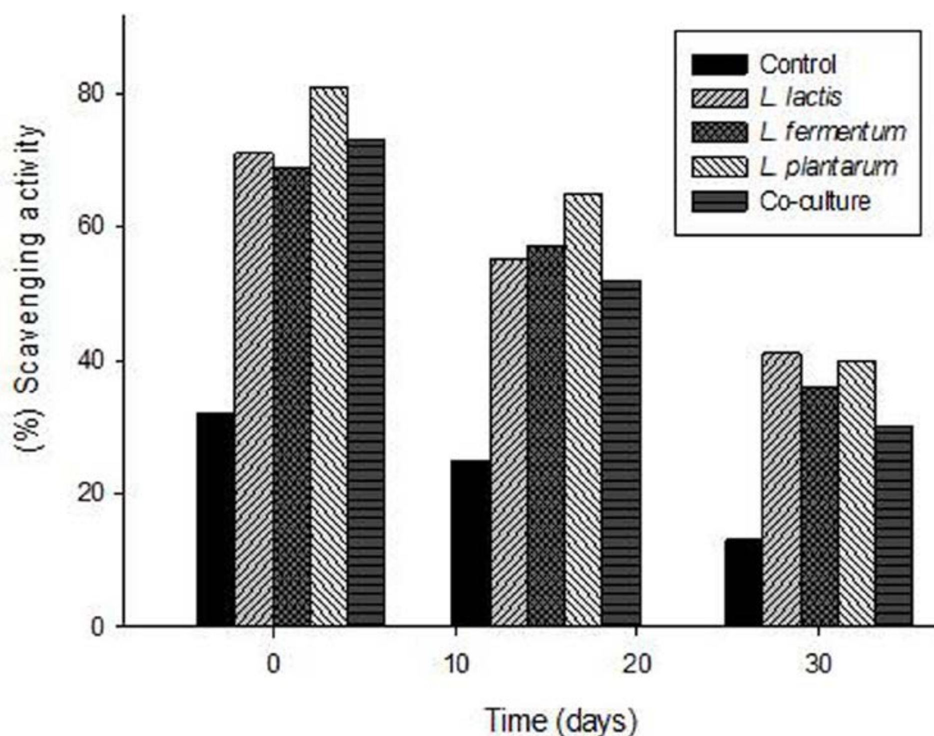

Figure 15 Effect of fermentation on hydroxyl ion activity of fermented turmeric beverage

Determination of superoxide radical scavenging activity

During normal metabolism of oxygen, various free radicals as well as superoxide radicals are produced continuously. The high level of this superoxide radical leads to severe damage to cellular ingredients, which causes various metabolic disorders. The scavenging of the fermented as well as unfermented samples on superoxide radicals are shown in Fig. 16. The superoxide scavenging activity in all fermented samples was found to be higher in comparison to unfermented sample. The highest scavenging of superoxide radicals was exhibited by *L. plantarum* 37.8 % followed by co-culture 37.4%, *L. lactis* 34.1%. The lowest scavenging activity was observed in *L. fermentum* among all fermented cultures. The scavenging activity was enhanced during storage studies in all fermented samples and the highest activity was found to be *L. plantarum* 59.9%.

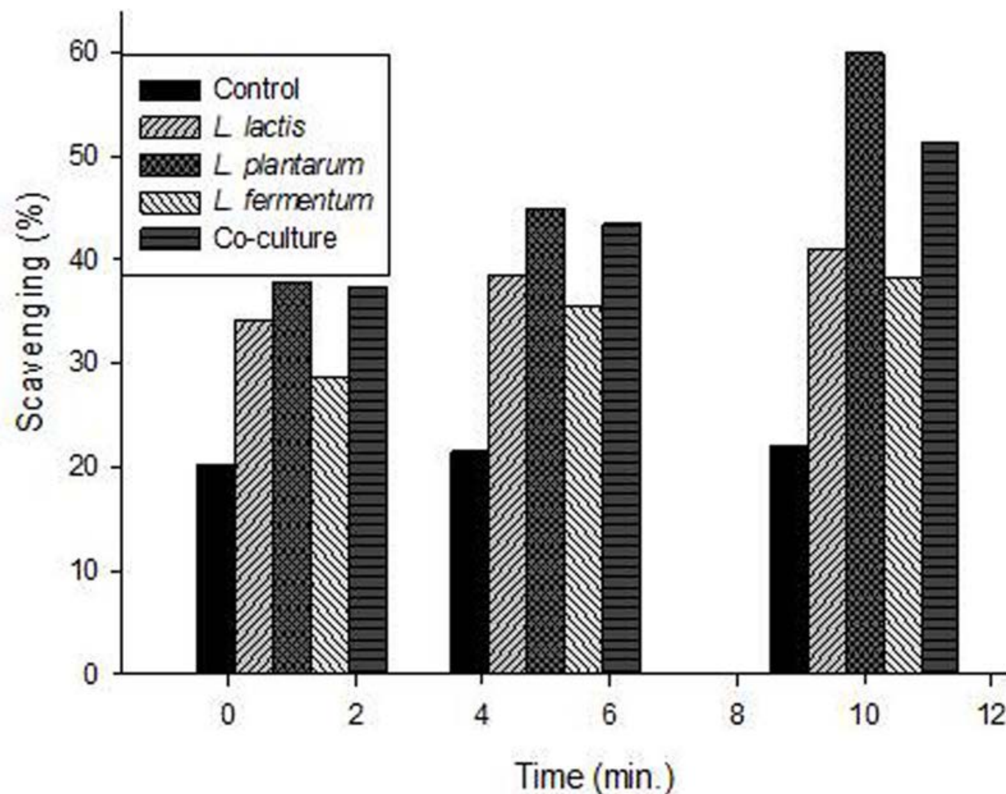

Figure 16 Analysis of Superoxide radical scavenging activity of fermented turmeric beverage.

Conclusion

The development of functional food is a challenging task for the food industry. This work aimed to investigate the capabilities of three strains of lactic acid bacteria, *L. lactis, L. plantarum and L. fermentum* for the development of probiotic beverage utilizing juice of fresh turmeric rhizome. Results showed that all test strains have ability to produce high amounts of lactic acid and they exhibited fairly good transit tolerance to acid as well as alkali with the viable cell numbers of 10^6 c.f.u./ml after 14 hours of fermentation. The strains can be survived at low pH. The results revealed the enhancement of TPC of fermented turmeric beverages therefore, fermentation of

turmeric by LAB could potentially enhance antioxidant potential which is measured by different antioxidant analysis. The lactic acid bacteria present in fermented turmeric beverages were found to have the ability to assimilate cholesterol from their medium. These findings suggest that they may aid in lowering overall cholesterol in the blood. The studies also revealed high hydrophobicity (88%) in *invitro* conditions that leads to the assumption that they may be able to colonize the intestinal tract and compete with the resident flora. The best results were obtained with *L. plantarum* among other investigated bacteria. The studies can correlated with other studies going on the immunomodulatory and other probiotic properties of *L. plantarum* [35, 36]. The turmeric-based beverage is a non-dairy vegetarian product therefore it can be considered as very good alternative to dairy based beverages that can be used to maintain a healthy lifestyle by reducing the risk of diseases. Further studies are required to perform to fully a sensory panel's subjective analysis to correlate with the objective results of the present studies. The time and dose dependent studies can be performed for assessment of the bioavailability of curcumin and absorption of antioxidants in *invitro* as well as *invivo* conditions for their commercialization.

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Conflict of interest

The authors have no conflict of interest for the present studies, manuscript preparation and submission to this journal.

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