



# Techno-Functional Assessment of Riboflavin-Enriched Yogurt-Based Fermented Milk Prepared by Supplementing Riboflavin-Producing Probiotic Strains of *Lactiplantibacillus plantarum*

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## Abstract

Vitamin enrichment in fermented dairy products through the intervention of vitamin-producing probiotic strains during fermentation is a novel approach in the field of probioceuticals. In this study, riboflavin-enriched yogurt-based fermented milk was prepared by mixing 1% (v/v) riboflavin-producing strain [ $1.2 \times 10^8$  CFU/mL of *Lactiplantibacillus plantarum* MTCC 25432 or *L. plantarum* MTCC 25433 or *L. plantarum* MTCC 25434] with 2% (v/v) traditional yogurt cultures [*Streptococcus thermophilus* NCDC 295 and *L. delbrueckii* subsp. *bulgaricus* NCDC 293; each of  $1.3 \times 10^7$  CFU/mL]. The yogurt-based fermented milk prepared with traditional yogurt cultures (2%, v/v) was served as a control. The prepared yogurt-based fermented milk samples were analyzed and compared for riboflavin content, antimicrobial activity, physicochemical, and functional properties. As a result, the yogurt-based fermented milk prepared with *L. plantarum* MTCC 25432 produced a significantly higher amount of riboflavin (2.49 mg/L) as compared with MTCC 25433 (2.33 mg/L), MTCC 25434 (2.14 mg/L), and control (1.70 mg/L). The probiotic supplementation to yogurt cultures maintained the pH and titratable acidity in the range of 4.1–4.4 and 1.0–1.05% (lactic acid/100 mL), as recommended by Indian yogurt standards. The rheological, texture, and antimicrobial properties of yogurt-based fermented milk were enhanced with the addition of riboflavin-producing probiotic strains. Moreover, all yogurt-based fermented milk samples prepared in this study were acceptable as per the sensory evolution scores. In conclusion, the use of riboflavin-producing *L. plantarum* strains along with standard yogurt cultures could be the best approach to enhancing riboflavin content in yogurt-based fermented milk and fulfilling the daily riboflavin requirement in humans.

**Keywords** Probioceuticals · Riboflavin · Yogurt · Antimicrobial · Techno-functional · *L. plantarum*

## Introduction

Nowadays, the production and consumption of fermented milk have increased due to its therapeutic potential. Yogurt is a functional dairy-based product that is consumed widely due to its delicious taste, texture, and health benefits, such as

immunomodulation and gut health. It is produced from the fermentation of milk (cows, goats, and buffalos) [1]. Yogurt contains a significant amount of protein, calcium, minerals, and traces of vitamins (folic acid, riboflavin, and cobalamin). It is one of the promising strategies for the fortification of vitamins to mitigate vitamin deficiency in humans [2].

Riboflavin (vitamin B<sub>2</sub>) is an essential vitamin that undergoes modifications in a living cell to provide flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) [3, 4]. FMN and FAD act as a substituent to flavoproteins which play a major role in several metabolic reactions such as the breakdown of carbohydrates, proteins, fats, photosensitization, oxidative stress response, and vitamin activation (folate and pyridoxine) [5, 6]. Flavins can transfer electrons in both one and two-electron transfer reactions [5, 6]. Besides this, flavoproteins are involved in DNA repair, circadian cycling, body development, light sensing, etc.

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[7]. This makes riboflavin an essential vitamin in nature. Plants, fungi, and bacteria can synthesize their riboflavin. However, higher animals and human lack this capacity [8]. Approximately 50% of the population of developing countries has mild riboflavin deficiency [9], which could be due to inadequate dietary intake and poor malabsorption [10]. Riboflavin deficiency, such as ariboflavinosis, comprises impaired vision, reduce growth rate, high level of homocysteine, liver and skin damage, and change in cerebral glucose metabolism [11].

Recently, vitamin-producing lactic acid bacteria (LAB) attracted much attention from researchers to enhance the level and bioavailability of vitamins in food [12]. Several studies reported the use of riboflavin overproducing strain for the manufacturing of riboflavin-enriched bread, pasta, soymilk, and soy curd [13–16]. However, their use to improve riboflavin levels in yogurt has not been reported so far. In this study, we investigated the ability of riboflavin-producing *L. plantarum* strains (MTCC 25432, 25433, and 25434) to improve riboflavin content in yogurt-based fermented milk and the techno-functional feasibility thereof. The strains reported here were previously been isolated based on riboflavin production ability [15] and reported for probiotic properties, safety [15, 16], and preparation of riboflavin-enriched soymilk and soy curd [15, 16].

## Materials and Methods

### Bacterial Cultures

*Lactiplantibacillus plantarum* MTCC 25432, *L. plantarum* MTCC 25433, and *L. plantarum* MTCC 25434 were obtained from the Microbiology Laboratory, National Institute of Food Technology Entrepreneurship and Management (NIFTEM), India. All the cultures were deposited at Microbial Type Culture Collection (MTCC) (Chandigarh, India) under safe deposit. The cultures were activated in MRS (De Man, Rogosa, and Sharpe) broth (HiMedia, Mumbai, India) at 37 °C for 24 h. Standard yogurt starter cultures, i.e., *Streptococcus thermophilus* NCDC 295 and *L. delbrueckii* subsp. *bulgaricus* NCDC 293, were obtained from the National Collection of Dairy Cultures, India, and activated as per the standard procedures.

### Milk Coagulation Activity

The milk coagulation activity of *L. plantarum* strains was determined as per the method described by Georgieva et al. [17] with minor modifications. The bacterial suspension ( $1.2 \times 10^8$  CFU/mL in PBS, pH 7.3) obtained from overnight grown cultures were each inoculated (1%, v/v) separately in 10% (w/v) reconstituted skim milk, 10% (w/v) skim milk

supplemented with 1% (w/v) yeast extract and or 1% (w/v) glucose and incubated at 37 °C for 16–24 h. The strains able to coagulate milk  $\leq 16$  h were defined as fast coagulating strains, and  $\geq 16$  h were slow coagulating strains.

### Preparation of Yogurt-Based Fermented Milk

A 1% (v/v) suspension ( $1.2 \times 10^8$  CFU/mL in PBS, pH 7.3) of overnight grown riboflavin-producing strain (*Lactiplantibacillus plantarum* MTCC 25432 or *L. plantarum* MTCC 25433 or *L. plantarum* MTCC 25434) was inoculated in lukewarm (~42 °C) full cream cow milk containing 2% (v/v) yogurt cultures (*Streptococcus thermophilus* NCDC 295 and *L. delbrueckii* subsp. *bulgaricus* NCDC 293; each of  $1.3 \times 10^7$  CFU/mL in PBS, pH 7.3) and incubated at 42 °C for up to 20 h (Fig. 1). The pH and titratable acidity were determined at intervals of 0, 4, 8, 12, 16, and 20 h of fermentation. Simultaneously, the samples withdrawn at 8 h of fermentation were stored at 4 °C for further analysis. The yogurt-based fermented milk obtained by the addition of the starter culture (2%, v/v) served as a control.

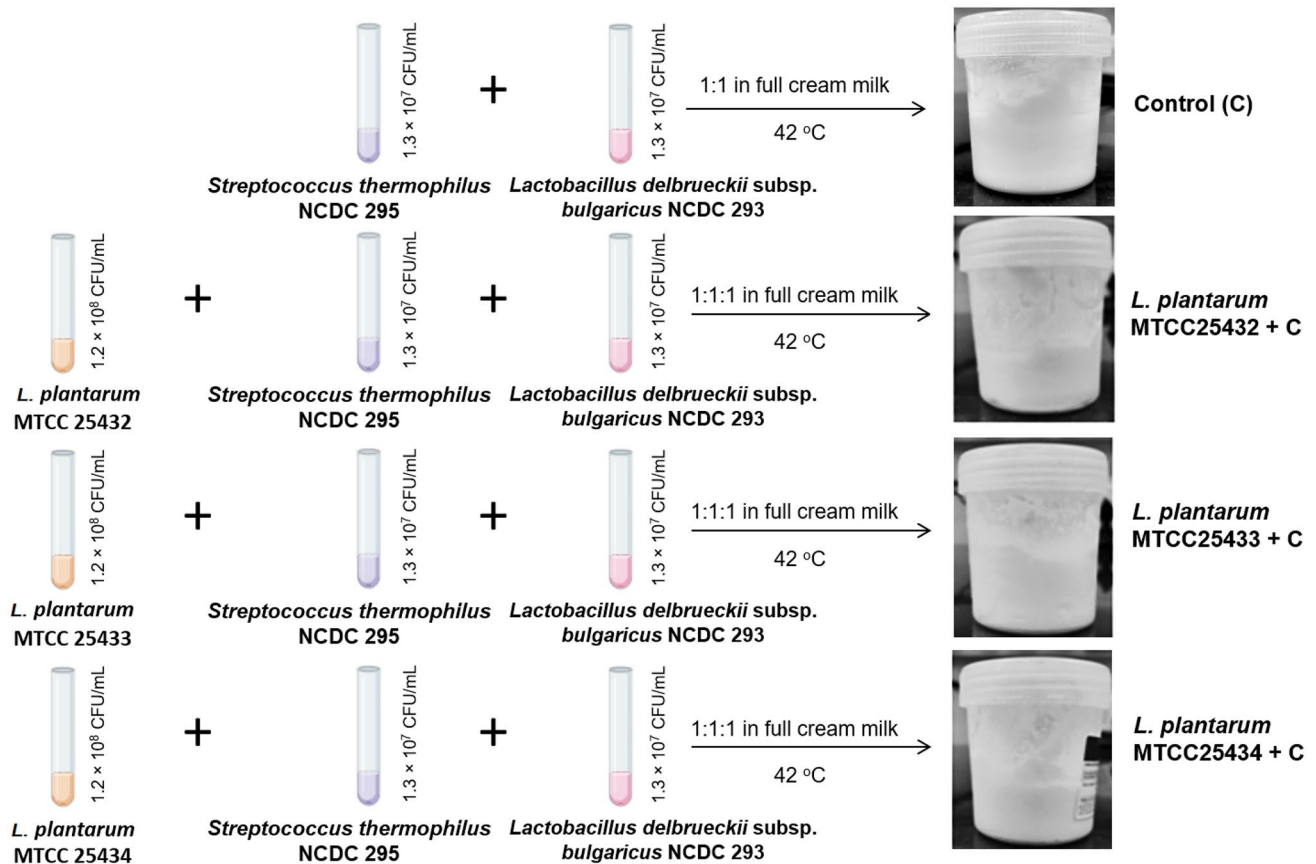
### Physicochemical Analysis of Yogurt-Based Fermented Milk

#### Moisture

The moisture content of the yogurt-based fermented milk samples was analyzed at 0-, 7-, and 14-day intervals by weight difference method [18] with slight modifications. In brief, a yogurt-based fermented milk sample of known weight was subjected to drying at 105 °C for 5 h. After drying, the weight was recorded, and moisture content was calculated as per the given formula. Moisture (%) = weight before drying – weight after drying / weight before drying  $\times$  100.

#### Titratable Acidity

The titratable acidity (TA) of the yogurt-based fermented milk samples was determined using a routine laboratory method. Briefly, a known quantity of yogurt-based fermented milk samples (0, 7, and 14 days) were mixed with 2–3 drops of phenolphthalein indicator and titrated using 0.1 N NaOH until the development of light pink color. The volume of NaOH consumed to change the color of yogurt-based fermented milk was recorded. Titratable acidity (lactic acid) was calculated using the following formula. Percentage of TA (lactic acid) =  $90 \times N \times \text{titer volume} \times 100 / \text{sample volume} \times 1000$  [16].



**Fig. 1** Diagrammatic representation of yogurt-based fermented milk prepared with standard starter cultures and in combinations with the different strains of *Lactiplantibacillus plantarum*

## pH

The pH of the yogurt-based fermented milk samples (withdrawn at 0, 7, and 14 days) was measured with the help of calibrated digital pH meter (Model-MT 103, Manti Lab, India). The total soluble solids (TSS) of the yogurt-based fermented milk samples (withdrawn at 0, 7, and 14 days) were measured at 25 °C by using a digital refractometer (ATAGO RX-5000 $\alpha$ , Tokyo, Japan) and expressed in terms of Brix.

## Color

Color properties of yogurt-based fermented milk samples were measured by using a handheld chroma meter (CR-400, Konica Minolta, Japan) at 25 °C. The results were recorded in the  $L^*$ ,  $a^*$ , and  $b^*$  scales [18].  $L^*$  represents the lightness index (0 for black; 100 for white),  $a^*$  represents greenness and redness (+100 for red and –80 for green), while  $b^*$  represents yellowness and blueness (+70 for yellow and –80 for blue).  $\Delta E^*$  represents the total color difference

based on  $L^*$ ,  $a^*$ , and  $b^*$  values. All of the samples were analyzed in triplicate, and average values were reported.

## Rheological Measurements

The rheological properties of yogurt-based fermented milk samples were determined as per the slightly modified procedure of Ferragut et al. [19]. In brief, yogurt-based fermented milk samples were investigated with Anton Paar Rheometer (MCR 302, Graz, Austria) using a parallel plate setup with a 2.5 mm gap at 20 °C. A small quantity of the yogurt-based fermented milk sample was placed in the center of the inset plate. First, a frequency of 1 Hz was used to determine the viscoelastic range (0.01–100 Pa), and then a frequency sweep test (0.1–10 Hz at a maximum strain of 0.06) was carried out. The instrument software was used to analyze the rheological measurement results. From the frequency sweep test, the dynamic module  $G'$ ,  $G''$ ,  $G^*$ , and eta ( $\dot{\eta}$ ) values were calculated.

## Texture Analysis

Texture analysis of yogurt-based fermented milk samples was measured using a TA-XT2i texture analyzer (Godalming, UK), equipped with a 25 kg load cell at 22 °C. Profile curves were used to calculate the hardness, cohesiveness, gumminess, adhesiveness, and springiness of the yogurt-based fermented milk samples [20].

## Evaluation of Riboflavin Content in Yogurt-Based Fermented Milk

### Extraction and Estimation of Riboflavin

Riboflavin from the yogurt-based fermented milk was extracted as per the method described by Jaurez et al. [21] and Singh et al. [16]. In brief, equal volumes (1:1) of sample and acetic acid (1%, v/v) were mixed and autoclaved at 121 °C for 20 min. After autoclaving, the mixture was cooled at room temperature and centrifuged at 8000 × g for 20 min. The supernatant was collected and filtered using 0.22 µm cellulose acetate membranes (Merck, Germany) and stored at –20 °C until further use. Extraction of riboflavin was performed in dark to prevent loss due to light.

The amount of riboflavin present in the extracted samples was analyzed using high-performance liquid chromatography (HPLC, Waters 2707, Kolkata, India) equipped with 250 × 4.6 mm (5 µm-100 Å) reverse-phase C<sub>18</sub> column (Kromasil, MO, USA), auto-sampler, binary 515 pumps, and fluorescence detector with excitation wavelength 440 nm and emission wavelength 520 nm. All samples were eluted with isocratic elution at the flow rate of 1 mL/min. Methanol (36): water (65) (v/v) served as a mobile phase, and pure riboflavin was used as a control [14].

## Antimicrobial Activity

The antimicrobial activity of the yogurt-based fermented milk was determined by agar-well diffusion assay. In brief, 100 µL overnight-grown pathogenic cultures (*Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 11229, *Salmonella enterica* ATCC 35664, and *S. enterica* ATCC 13076) were each mixed separately in 25 mL molten semi-soft nutrient agar (0.8%, w/v) and poured into the plates. All the plates were kept at room temperature to solidify, and wells (6 mm) were prepared using a sterile 1 mL tip. An aliquot (100 µL) of supernatant (filtered sterilized) obtained from yogurt-based fermented milk samples was filled into the designated well and incubated at 4 °C for 20 min to diffuse. All the plates were incubated overnight at 37 °C and observed for a zone of inhibition.

## Sensory Evaluation

Yogurt-based fermented milk prepared in this study was evaluated for color, flavor, body texture, and overall acceptability by 30 volunteers (aged 22–45 years) at the NIFTEM campus in India. All the volunteers were instructed to use a 9-point hedonic scale (9-like extremely, 8-like very much, 7-like moderately, 6-like slightly, 5-neither like nor a dislike, 4-dislike slightly, 3-dislike moderately, 2-dislike very much, and 1-dislike extremely) to rate the yogurt-based fermented milk. To clear palates between samples, volunteers were asked to consume a cream cracker and a sip of water. Scores were presented as an average score of the records.

## Statistical Analysis

Experimental results were designed as mean with standard deviation. The statistical analysis was performed using Microsoft Office Excel 2007. The differences between groups were analyzed by one-way analysis of variance (ANOVA) using SPSS base 19.0 statistical software. All tests were performed in triplicates, and differences were observed as statistically significant at  $p < 0.05$ .

## Results and Discussion

### Milk Coagulation Activity

Milk coagulation ability is considered one of the prerequisites for the selection of adjunct cultures for the development of novel dairy products. In this study, we observed that *Lactiplantibacillus plantarum* MTCC 25432, 25433, and 25434 were capable of coagulating milk within 7.0, 7.7, and 8.0 h when supplemented with 1% (w/v) yeast extract. Moreover, when cultivated in the presence of 1% (w/v) glucose, *L. plantarum* MTCC 25432, 25433, and 25434 coagulated milk within 8.0, 8.5, and 18 h, respectively. These results are comparable with the milk coagulation ability showed by *L. plantarum* IMAU10216 (8.0 h), 80106 (8.5 h), and 70095 (9.3 h) in the presence of 1% (w/v) yeast extract [22]. Contrary to our results, *L. plantarum* IMAU strains failed to coagulate milk in the presence of 1% (w/v) glucose. It has been reported that nitrogen, vitamin, and growth factors of yeast extract accelerate the growth of bacteria, thus facilitating the coagulation of milk [23]. The milk coagulation in the presence of glucose could be a strain-dependent property. Moreover, probiotic strains MTCC 25432, 25433, and 25434 used in this study failed to coagulate milk without the supplementation of glucose or yeast extract. These results are corroborated well with the previous finding that *L. plantarum* strains are unable to coagulate milk without additional carbon and nitrogen sources [17].

## Physicochemical Properties of Yogurt-Based Fermented Milk

### Moisture and Total Soluble Solids

Yogurt-based fermented milk prepared with the supplementation of *L. plantarum* MTCC 25434 showed significantly lower moisture levels ( $70.85 \pm 0.04\%$ ) as compared with MTCC 25433 ( $72.82 \pm 0.06\%$ ), MTCC 25432 ( $73.11 \pm 0.04\%$ ), and control ( $76.22 \pm 0.11\%$ ) (Table 1). These results indicated strain-specific moisture level reduction in yogurt-based fermented milk samples. The mechanism by which strains reduce moisture in yogurt-based fermented milk was not explored. However, we hypothesized that the growth of yogurt cultures may ease the growth of *L. plantarum* strains to form better texture. Moreover, low-moisture yogurt has a prolonged shelf life. In this study, the moisture content of prepared yogurt-based fermented milk was within the limit ( $< 84\%$ ) as per the standard to maintain proper texture and mouth feel [24]. Besides this, no change in moisture content was observed after 14 days of storage at  $4\text{ }^{\circ}\text{C}$  (Table 1), and this could be ensuring the maintenance of yogurt-based fermented milk texture and mouth feel.

The total soluble solids (TSS) of different yogurt-based fermented milk samples were in the range of 5.05 to 5.92% ( $^{\circ}\text{Brix}$ ). The yogurt-based fermented milk prepared with *L. plantarum* MTCC 25434 supplementation showed significantly lower levels of TSS ( $5.05 \pm 0.11\%$ ) as compared with MTCC 25433 ( $5.92 \pm 0.17\%$ ), MTCC 25432 ( $5.46 \pm 0.04\%$ ), and control ( $5.32 \pm 0.49\%$ ) (Table 1). The percentage of TSS reflects the mass fraction of substances that remained after the completion of fermentation. The higher TSS is one of the reasons for the better rheology of yogurt-based fermented milk samples [25]. However, in this study, the variations and decrease in TSS (Table 1) observed during the storage

could be due to the strain-specific activities in the presence of yogurt cultures.

### pH and Titratable Acidity

The pH and titratable acidity (TA) are key indicators for assessing the acidity of yogurt [22]. As per the Indian standard of yogurt specification (IS12898:1989), the pH and TA of the yogurt should be between 3.8–4.6 and 0.8–1.2%. In this study, yogurt-based fermented milk prepared with and without supplementation of riboflavin-producing probiotic strains achieved recommended pH and TA (range: 4.1–4.4 and 1.0–1.05%) at 8 h of fermentation and remained stable up to 20 h of incubation (Fig. 2a, b). Besides this, the samples stored at  $4\text{ }^{\circ}\text{C}$  after 8 h of fermentation showed insignificant changes in pH and TA when analyzed at 0, 7, and 14th days (Table 1). These results are corroborated well with IS12898:1989 recommendations and ensure the long-term stability of probiotic-enriched yogurt-based fermented milk.

### Color

Color is one of the factors which often contribute to consumers buying decisions. In this study, we observed that supplementation of riboflavin-producing strains (MTCC 25432, 25433, and 25434) in the fermentation of yogurt-based fermented milk significantly affected color properties (lightness and yellowness) as compared with the control (Table 2). However, the color difference recorded between yogurt-based fermented milk samples prepared with probiotic intervention remained insignificant.

The significantly lowest average lightness ( $L^*$ ) value was recorded with the yogurt-based fermented milk prepared with *L. plantarum* MTCC 25,432 ( $63.21 \pm 0.81$ ) as compared with the control ( $66.80 \pm 0.52$ ). Similarly, lightness

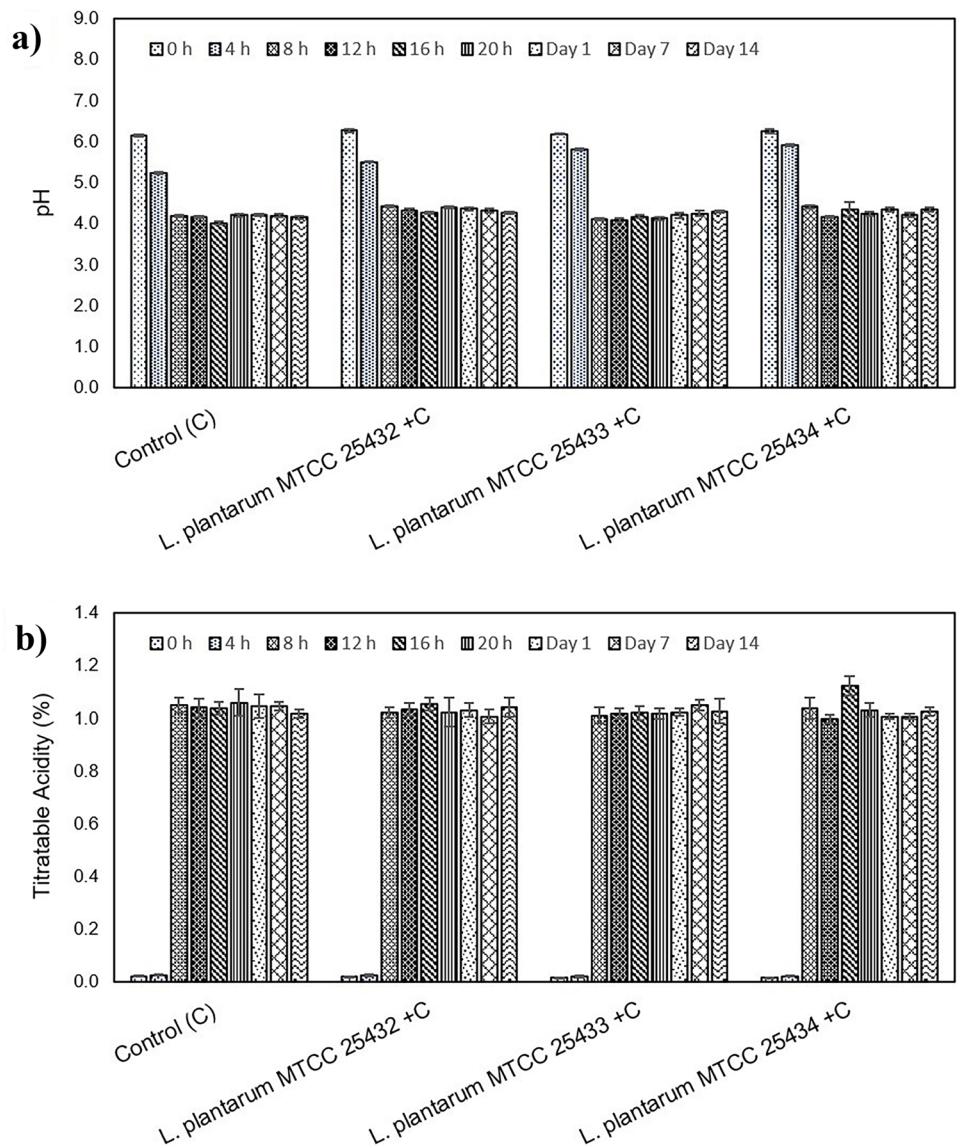
**Table 1** Values of moisture content, pH, titratable acidity, and total soluble solids (TSS) of yogurt-based fermented milk during storage at  $4 \pm 1\text{ }^{\circ}\text{C}$  within 14 days

Yogurt-based fermented milk	Days	Moisture (%)	pH	Acidity (%)	TSS ( $^{\circ}\text{Brix}$ %)
Control (C)	0	$76.22 \pm 0.11$	$4.23 \pm 0.15$	$1.05 \pm 0.05$	$5.32 \pm 0.49$
	7	$75.89 \pm 0.67$	$4.20 \pm 0.10$	$1.03 \pm 0.06$	$5.26 \pm 0.52$
	14	$76.29 \pm 0.16$	$4.16 \pm 0.05$	$1.03 \pm 0.05$	$5.28 \pm 0.52$
<i>L. plantarum</i> MTCC 25432 + C	0	$73.11 \pm 0.04$	$4.36 \pm 0.25$	$1.02 \pm 0.07$	$5.46 \pm 0.04$
	7	$73.14 \pm 0.05$	$4.33 \pm 0.15$	$1.01 \pm 0.04$	$5.45 \pm 0.40$
	14	$73.09 \pm 0.03$	$4.30 \pm 0.26$	$1.02 \pm 0.05$	$5.47 \pm 0.43$
<i>L. plantarum</i> MTCC 25433 + C	0	$72.82 \pm 0.06$	$4.20 \pm 0.10$	$1.01 \pm 0.01$	$5.92 \pm 0.17$
	7	$72.80 \pm 0.04$	$4.23 \pm 0.15$	$1.01 \pm 0.01$	$5.89 \pm 0.14$
	14	$72.78 \pm 0.09$	$4.26 \pm 0.20$	$1.03 \pm 0.02$	$5.71 \pm 0.45$
<i>L. plantarum</i> MTCC 25434 + C	0	$70.85 \pm 0.04$	$4.33 \pm 0.20$	$1.00 \pm 0.01$	$5.05 \pm 0.11$
	7	$70.79 \pm 0.05$	$4.20 \pm 0.10$	$1.00 \pm 0.02$	$5.02 \pm 0.05$
	14	$70.86 \pm 0.03$	$4.30 \pm 0.20$	$1.02 \pm 0.07$	$5.02 \pm 3.03$

C, *Streptococcus thermophilus* NCDC 295 and *L. delbrueckii* subsp. *bulgaricus* NCDC 293



**Fig. 2** pH and titratable acidity (lactic acid) profile of yogurt-based fermented milk prepared with standard starter cultures and in combinations with the different strains of *Lactiplantibacillus plantarum*. C: *Streptococcus thermophilus* NCDC 295 and *L. delbrueckii* subsp. *bulgaricus* NCDC 293



**Table 2** The chromametric analysis of yogurt-based fermented milk samples prepared with standard starter culture and in combinations with different strains of *Lactiplantibacillus plantarum*

Index	Yogurt-based fermented milk samples			
	Control (C)	<i>L. plantarum</i> MTCC 25432 +C	<i>L. plantarum</i> MTCC 25433 +C	<i>L. plantarum</i> MTCC 25434 +C
$L^*$	$66.80 \pm 0.52$	$63.21 \pm 0.81$	$64.86 \pm 0.87$	$64.14 \pm 0.59$
$a^*$	$-2.59 \pm 0.21$	$-2.48 \pm 0.31$	$-2.66 \pm 0.21$	$-2.67 \pm 0.15$
$b^*$	$8.67 \pm 0.13$	$9.64 \pm 0.26$	$9.05 \pm 0.51$	$8.52 \pm 0.69$
$\Delta E^*$	$65.29 \pm 0.86$	$64.71 \pm 0.89$	$65.49 \pm 0.68$	$65.77 \pm 0.92$

C, *Streptococcus thermophilus* NCDC 295 and *L. delbrueckii* subsp. *bulgaricus* NCDC 293.  $L^*$ , lightness index (0 for black; 100 for white).  $a^*$ , greenness and redness (+100 for red and -80 for green).  $b^*$ , yellowness and blueness (+70 for yellow and -80 for blue).  $\Delta E^*$ , total color difference based on  $L^*$ ,  $a^*$ , and  $b^*$  values

recorded for MTCC 25433 ( $64.86 \pm 0.87$ ) and MTCC 25434 ( $64.14 \pm 0.59$ ) was significantly less as compared with the control ( $66.80 \pm 0.52$ ) (Table 2). These results indicated that riboflavin production may responsible for the lightness difference between test and control samples. The significantly highest yellowness ( $b^*$ ) observed with yogurt-based fermented milk prepared with MTCC 25432 ( $9.64 \pm 0.26$ ) as compared with control ( $8.67 \pm 0.13$ ) (Table 2) suggested the influence of the amount of riboflavin present in yogurt-based fermented milk on color properties. The yellowness differences observed between MTCC 25433 ( $9.05 \pm 0.51$ ), MTCC 25434 ( $8.52 \pm 0.69$ ), and control ( $8.67 \pm 0.13$ ) were insignificant. No significant changes were observed in green and redness ( $a^*$ ) properties between tests and control (Table 2). The  $L^*$ ,  $a^*$ , and  $b^*$  values observed in this study are coordinated well with the values ( $L^*$ :  $86.2 \pm 11.0$ ;

**Table 3** Rheological analysis of yogurt-based fermented milk samples by using frequency sweep test

Yogurt-based fermented milk	Storage modulus $G'$ (Pa)	Loss modulus $G''$ (Pa)	Complex modulus $G^*$ [Pa]	Angular frequency (rad/s)	Deflection angle (mrad)
Control (C)	$8.36 \times 10^2$	$4.0 \times 10^2$	$9.30 \times 10^2$	1.26	0.633
<i>L. plantarum</i> MTCC 25432 + C	$2.12 \times 10^2$	$1.32 \times 10^2$	$2.49 \times 10^2$	0.48	1.14
<i>L. plantarum</i> MTCC 25433 + C	$5.41 \times 10^2$	$2.56 \times 10^2$	$5.98 \times 10^2$	1.01	0.626
<i>L. plantarum</i> MTCC 25434 + C	$6.29 \times 10^2$	$2.86 \times 10^2$	$6.90 \times 10^2$	1.05	0.632

C, *Streptococcus thermophilus* NCDC 295 and *L. delbrueckii* subsp. *bulgaricus* NCDC 293

$a^*$ :  $-1.1 \pm 4.9$ ;  $b^*$ :  $9.0 \pm 5.3$ ; a number of studies: 34) given for yogurt in a recent systematic review on the color of milk and milk products [26]. Overall, no significant change in total color difference ( $\Delta E^*$ ) among yogurt-based fermented milk prepared in this study may not negatively affect consumers buying decisions.

### Rheological Analysis

In this study, the yogurt-based fermented milk samples prepared with and without supplementation of probiotics strains were investigated to understand the way yogurt gel flows and deforms when subjected to normal and/or tangential forces. The force delivered perpendicularly or tangentially to the product cross-section is known as shear stress, and the rate at which it is applied is known as shear rate. The apparent viscosity ( $\eta$ ), consistency index, storage modulus ( $G'$ ), loss modulus ( $G''$ ), and loss tangent (or  $G''/G'$ ) are rheological characteristics used to define yogurt [27]. The resistance to flow caused by the internal friction of moving fluid particles under force is known as viscosity. The yogurt is typically non-Newtonian, while the water is Newtonian (shear stress is precisely proportional to shear rate). The normal characteristics of yogurt gels are mild viscoelastic, shear-thinning, or pseudoelastic activity, where a higher shear rate or shear stress causes the viscosity to decrease. The rheological measure of the milk after fermentation is most commonly altered by the *Lactobacillus* and *Bifidobacterium* [28]. In this study, in frequency sweep test, yogurt-based fermented milk prepared with supplementation of *Lactiplantibacillus plantarum* MTCC 25432, MTCC 25433, and MTCC 25434 had lower  $G'$  ( $2.12 \times 10^2$ ,  $5.41 \times 10^2$ , and  $6.29 \times 10^2$  Pa),  $G''$  ( $1.32 \times 10^2$ ,  $2.56 \times 10^2$ , and  $2.86 \times 10^2$  Pa),

and  $G^*$  ( $2.49 \times 10^2$ ,  $5.98 \times 10^2$ , and  $6.90 \times 10^2$  Pa) values as compared with control  $G'$  ( $8.36 \times 10^2$  Pa),  $G''$  ( $4.0 \times 10^2$  Pa), and  $G^*$  ( $9.30 \times 10^2$  Pa) (Table 3). Similarly, the lowest angular frequency was observed for MTCC 25432 (0.48 rad/s) and the highest for control yogurt-based fermented milk (1.26 rad/s). These results indicated that the control had a better semi-solid, unbroken structural network as compared with test (riboflavin-enriched yogurt-based fermented milk) samples. In the stress sweep test, yogurt-based fermented milk prepared with MTCC 25432 showed the highest shear stress (8.61 Pa), shear rate (2.57 1/s), viscosity (3, 56 Pa s), speed (2.44 1/min), and torque (248  $\mu$ Nm) as compared with control and other test yogurt-based fermented milk (Table 4). Overall, these results indicated that the observed rheological variations could be due to exopolysaccharide (EPS) production or interactions between probiotic and traditional yogurt cultures during the fermentation. A similar finding was reported when riboflavin-producing *L. plantarum* strains (MTCC 25432 and 25433) were used to prepare soy curd [16].

### Texture Analysis

The texture is one of the most essential quality parameters of yogurt-based fermented milk. In this study, we observed significantly higher values for hardness (MTCC 25432:  $237.67 \pm 1.08$  g), adhesiveness (MTCC 25433:  $-153.03 \pm 1.06$  g s), springiness (MTCC 25433:  $0.74 \pm 0.65$ ), gumminess (MTCC 25433:  $106.21 \pm 1.05$  g), and chewiness (MTCC 25433:  $79.18 \pm 1.87$ ) with yogurt-based fermented milk samples prepared with riboflavin-producing probiotic strains as compared with control, except cohesiveness (Table 5). These results indicated a

**Table 4** Rheological analysis of yogurt-based fermented milk samples by stress sweep test

Yogurt-based fermented milk	Shear rate [1/s]	Shear stress [Pa]	Viscosity [Pa·s]	Speed [1/min]	Torque [ $\mu$ Nm]
Control (C)	2.42	8.4	3.47	2.42	222
<i>L. plantarum</i> MTCC 25432 + C	2.57	8.61	3.56	2.44	248
<i>L. plantarum</i> MTCC 25433 + C	2.55	8.21	3.53	2.43	246
<i>L. plantarum</i> MTCC 25434 + C	2.53	8.15	3.49	2.42	236

C, *Streptococcus thermophilus* NCDC 295 and *L. delbrueckii* subsp. *bulgaricus* NCDC 293

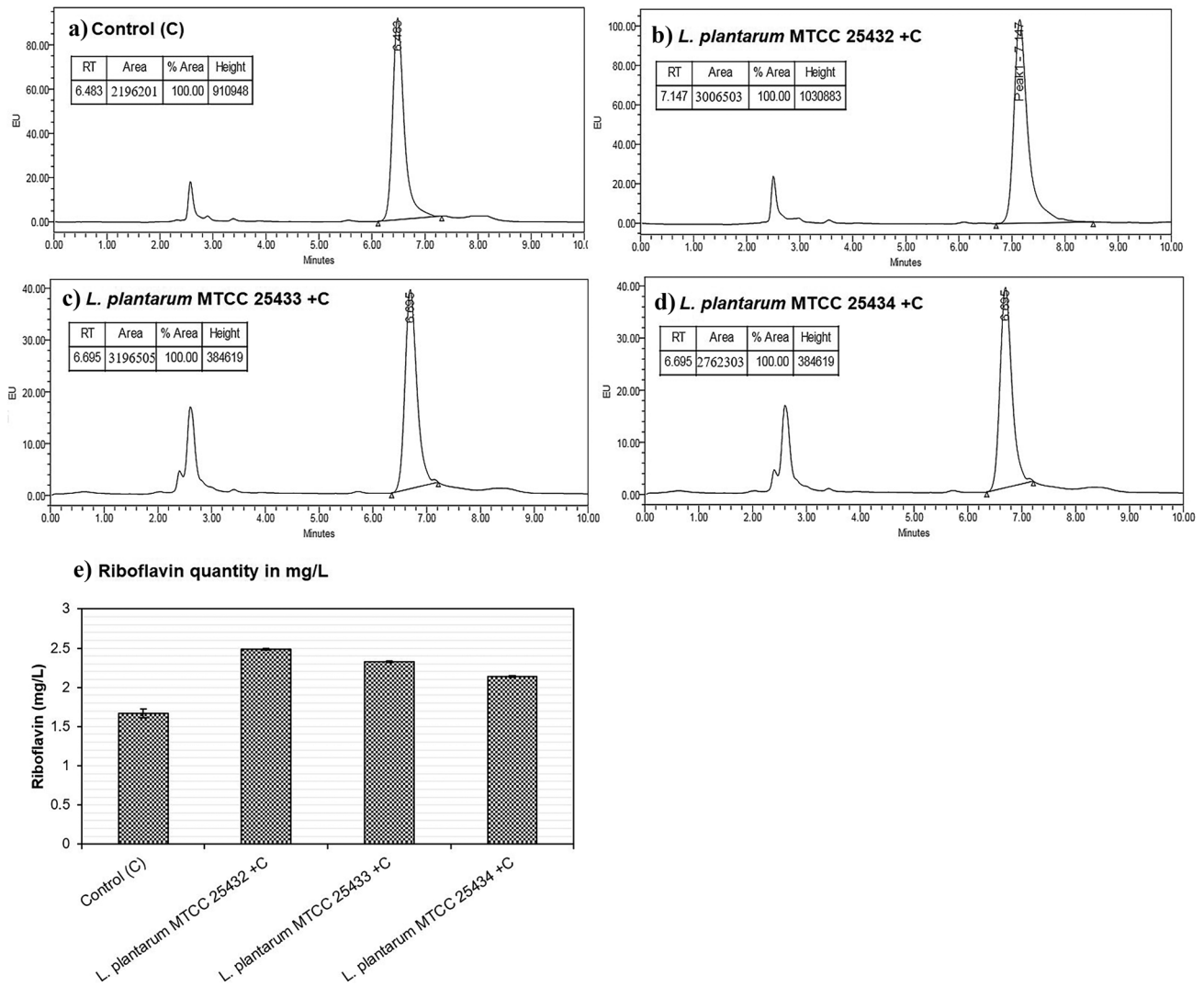
**Table 5** Texture profile analysis of yogurt-based fermented milk samples prepared with standard starter culture and in combinations with different strains of *Lactiplantibacillus plantarum*

Yogurt-based fermented milk	Hardness (g)	Adhesiveness (g s)	Springiness	Cohesiveness	Gumminess (g)	Chewiness
Control (C)	143.42 ± 1.14	- 52.93 ± 1.53	0.39 ± 1.27	0.66 ± 1.41	94.64 ± 1.75	36.9 ± 1.18
<i>L. plantarum</i> MTCC 25432 +C	237.67 ± 1.08	- 126.89 ± 1.25	0.65 ± 0.86	0.39 ± 1.08	93.08 ± 1.45	61.20 ± 1.20
<i>L. plantarum</i> MTCC 25433 +C	230.39 ± 1.92	- 153.03 ± 1.06	0.74 ± 0.65	0.46 ± 1.79	106.21 ± 1.05	79.18 ± 1.87
<i>L. plantarum</i> MTCC 25434 +C	205.91 ± 1.49	- 131.73 ± 1.08	0.67 ± 0.91	0.43 ± 0.98	90.46 ± 1.83	60.77 ± 1.74

*C. Streptococcus thermophilus* NCDC 295 and *L. delbrueckii* subsp. *bulgaricus* NCDC 293

stronger gel structure and greater structural integrity of probiotic yogurt-based fermented milk as compared with the control. Overall, the texture profile of probiotic yogurt-based fermented milk was much improved as compared with the control, which could be due to the rate of acid and

EPS production and the type of strain [29]. Recently, Singh and coworkers [16] reported improved texture quality of soy curd prepared by using *L. plantarum* MTCC 25432 and 25433 strains.



**Fig. 3** HPLC analysis of riboflavin extracted from yogurt-based fermented milk samples prepared with standard starter cultures (a) and in combinations with the different strains of *Lactiplantibacillus plan-*

*tarum* (b, c, d). quantification of riboflavin (e). C: *Streptococcus thermophilus* NCDC 295 and *L. delbrueckii* subsp. *bulgaricus* NCDC 293



**Table 6** Antimicrobial activity of yogurt-based fermented milk samples prepared with standard starter culture and in combinations with different strains of *Lactiplantibacillus plantarum*

Yogurt-based fermented milk	Zone of inhibition in millimeters (mm)			
	<i>Staphylococcus aureus</i> ATCC 6538	<i>Escherichia coli</i> ATCC 11229	<i>Salmonella enterica</i> ATCC 35664	<i>Salmonella enterica</i> ATCC 13076
Control (C)	0	7 ± 1.0	5 ± 0.5	9 ± 1.0
<i>L. plantarum</i> MTCC 25432 + C	16 ± 1.4	19 ± 0.57	15 ± 1.0	13 ± 1.4
<i>L. plantarum</i> MTCC 25433 + C	11 ± 0.5	14 ± 1.4	9 ± 1.0	12 ± 1.0
<i>L. plantarum</i> MTCC 25434 + C	7 ± 1.0	11 ± 0.5	8 ± 1.4	6 ± 1.4

C, *Streptococcus thermophilus* NCDC 295 and *L. delbrueckii* subsp. *bulgaricus* NCDC 293

## Riboflavin Estimation

The yogurt-based fermented milk prepared with supplementation of riboflavin-producing probiotic strains had an enhanced level of riboflavin as compared to the control. HPLC analysis of extracted samples showed characteristic riboflavin peak at 6–8 min (Fig. 3a–d), which was coordinated well with previous studies [16]. The quantitative analysis indicated a significantly highest level of riboflavin in yogurt-based fermented milk prepared with *L. plantarum* MTCC 25432 (2.49 mg/L) supplementation as compared with MTCC 25433 (2.33 mg/L), MTCC 25434 (2.14 mg/L), and control (1.70 mg/L) yogurt (Fig. 3e). In previous studies, *L. plantarum* MTCC 25432 (0.190 mg/L) and 25433 (0.195 mg/L) are reported to enhance riboflavin content in soy curd [16]. Moreover, previous and present studies assert that our riboflavin-producing probiotic *L. plantarum* strains can effectively be employed for the riboflavin bio-enrichment in dairy and nondairy products.

## Antibacterial Activities

Yogurt is known to inhibit pathogenic bacteria, which could be due to antimicrobial compounds and organic acids produced by starter cultures [30]. In this study, we observed improved antimicrobial activity when *L. plantarum* strains were supplemented with standard yogurt cultures (Table 6). The significantly highest antimicrobial activity was observed with yogurt-based fermented milk prepared with supplementation

of MTCC 25432 against tested pathogens (*Staphylococcus aureus* ATCC 6538:16 mm; *Escherichia coli* ATCC 11229: 19 mm; *Salmonella enterica* ATCC 35664: 15 mm; *S. enterica* ATCC 13076: 13 mm) as compared to control and rest of the samples (Table 6). These results corroborated well with the previous study that *L. plantarum* MTCC 25432 supplementation enhanced the antimicrobial activity of soy curd [16]. Moreover, the antimicrobial activity showed by the riboflavin probiotic strains could be due to the acid or antimicrobial peptide production. However, strains were not yet investigated for the production of antimicrobial peptides. Overall, strains antimicrobial activity is the added benefit apart from riboflavin enrichment in yogurt-based fermented milk.

## Sensory Evaluation

Product acceptance of their sensory evolution is very important for the consumer's preferences. The yogurt-based fermented milk prepared in this study had an overall rating in the range of 8.96–6.13 (Table 7), indicating like very much, moderately, and slightly. The sensory evaluation score for body and texture, color, and overall acceptability was similar to the test and control samples, except for flavor, which was significantly higher with MTCC 25432 yogurt-based fermented milk as compared to control and other test samples (Table 7). Enhanced flavor with MTCC 25432 yogurt-based fermented milk could be due to the highest riboflavin or other metabolites produced by the strain. Moreover, all samples with good overall acceptability may positively affect consumers buying decisions.

**Table 7** Sensory score of the yogurt-based fermented milk samples prepared with standard starter cultures and in combinations with the different strains of *Lactiplantibacillus plantarum*

Yogurt-based fermented milk	Body and texture	Flavor	Color	Overall acceptability
Control (C)	7.12 ± 1.08	6.32 ± 0.51	7.06 ± 0.32	7.86 ± 1.05
<i>L. plantarum</i> MTCC 25432 + C	8.92 ± 1.16	8.96 ± 0.24	7.12 ± 0.84	7.06 ± 1.61
<i>L. plantarum</i> MTCC 25433 + C	8.81 ± 1.65	7.89 ± 1.25	7.65 ± 0.27	7.59 ± 1.08
<i>L. plantarum</i> MTCC 25434 + C	7.01 ± 1.59	6.27 ± 1.02	7.01 ± 0.69	6.13 ± 0.29

C, *Streptococcus thermophilus* NCDC 295 and *L. delbrueckii* subsp. *bulgaricus* NCDC 293

## Conclusion

Our study investigated the effect of riboflavin-producing probiotic strains of *Lactiplantibacillus plantarum* on riboflavin content and other physicochemical properties during fermentation and post-fermentation storage of the yogurt-based fermented milk. Among the *L. plantarum*-supplemented yogurts-based fermented milk, the sample prepared with *L. plantarum* MTCC 25432 was found highest riboflavin content (2.49 mg/L). In comparison with plain yogurt-based fermented milk, bio-fortified yogurt-based fermented milk has better riboflavin content, techno-functional properties, and enhanced antimicrobial potential that may increase the value of the product. Moreover, the use of riboflavin-producing *L. plantarum* strains along with standard yogurt cultures could be the best approach to enhancing riboflavin content in yogurt-based fermented milk and fulfilling the daily riboflavin requirement in humans.

**Author Contribution** Taneja NK and Vikram contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by Vikram and Amrutha. The first draft of the manuscript was written by Vikram. Ahire JJ edited the manuscript extensively and wrote the final draft. Taneja NK reviewed and approved the draft. All authors read and approved the final manuscript.

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## Declarations

**Ethics Approval** This study does not contain any work related to the participation of humans and/or animals.

**Competing Interests** Ahire JJ was employed by Advanced Enzyme Technologies Limited. Advanced Enzyme Technologies had no direct and in-direct role in the study design/analysis/writing of this article. Other authors have no conflict of interest to declare.

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