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ENZYMATIC AND ANTIOXIDANT ACTIVITY OF CAMEL MILK FERMENTED WITH DIFFERENT STRAINS OF LACTIC ACID BACTERIA

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ABSTRACT

The aim of the present study was to compare antioxidant activity of different LAB *viz Lactobacillus delbrueckii* sub sp. *Bulgaricus*, *Lactobacillus casei, Lactobacillus plantarum* and *Streptococcus thermophilus*. The camel milk was fermented with *Lactobacillus delbrueckii* sub sp. *Bulgaricus, Lactobacillus casei, Lactobacillus plantarum* and *Streptococcus thermophilus* at 37°C upto 12 hr. The resulting fermented camel milk was evaluated with respect to the changes of pH, acidification) and antioxidant activity using 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), 2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid (ABTS) inhibition assay and enzymatic assay including Catalase, Sod and GST during 0 h to 12 hr fermentation time at 37°C. *L. plantarum* and *S. thermophilus* increases the acidityof fermented milk more rapidly ($p < 0.05$). The *Lactobacillus delbrueckii* showed highest ABTS activity (61.84 ± 0.34) and *S. thermophilus* showed lowest ABTs activity (43.60 ± 0.40), while *Lactobacillus casei* fermented milk had highest DPPH activity (7.52 ± 0.13) at different fermentation time. The *Lactobacillus plantarum* showed highest value of SOD (4.54 ± 0.013), catalase (200.17 ± 0.95) and GST activity (9.13 ± 0.155) at different fermentation time. In conclusion, compared to *Lactobacillus delbrueckii* sub sp. *Bulgaricus*, *Lactobacillus casei* and *Streptococcus thermophilus*, *Lactobacillus plantarum* showed higher antioxidant activity and can be used as a potential probiotics. It also found that the variation in antioxidant activity of fermented camel milk with 4 different LAB could be due to the change in pH, change in fermentation time or formation and breakdown of bio-active peptides during fermentation process.

Key words: Antioxidant activity, camel milk, catalase, fermentation, *Lactobacillus,* lactic acid bacteria, SOD

Lactic acid bacteria (LAB) have long been ingested by people in lot of fermented foods such as dairy products because of their pro-biotic properties which have the ability to produce various antimicrobial compounds and also show anti-tumour activity, alleviation of lactose intolerance stabilisation of gut microflora (Khedid *et al,* 2009). Several studies have been reported that reactive oxygen species (ROS) and free radicals play a crucial role in many diseases like cancer, atherosclerosis and diabetes (Beckman and Ames, 1998). The body has enzymatic antioxidants such as catalase, super oxide dismutase, glutathione S transferase and non-enzymatic antioxidant compounds which defend against ROS but these defense systems are not so efficient to entirely prevent the damage, so therefore, food supplements having antioxidant properties may be used to reduce oxidative damage (Zommara *et al,* 1996; Oxman *et al*, 2000; Terahara *et al*, 2001; Kullisaar *et al*, 2003).

In milk various bioactive peptides are present and the activity of these bio-peptides are further enhanced by fermentation of milk by LAB (Korhonen and Pihlanto, 2006). Fermented camel milk possess antioxidant properties, thus making it a potential candidate for functional and novel foods to improve health through nutrition (Korhonen, 2009). These peptides have various health beneficial effects such as immunomodulatory activities, antioxidative activities, antimicrobial and ACE inhibitory activities (Soleymanzadeh Nazila *et al,* 2016). Therefore, the aim of this study was to use of different strains of LAB for the fermentation of camel milk and to assess their enzymatic and antioxidant activities during the fermentation of the milk at different time intervals.

Materials and Methods

The milk samples collected from National Research Centre on Camel were pooled together and placed into a sterile container.

Sources of cultures

Lyophilised pure culture strains of *Lactobacillus delbrueckii* sub sp. *Bulgaricus* (NCDC- 009), *Lactobacillus casei* (NCDC- 017), *Lactobacillus plantarum*

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(NCDC- 020), *Streptococcus thermophilus* (NCDC- 074) used in this study were obtained from National Dairy Research Institute, Karnal, Haryana (India). Each strain was first activated in sterilised skim milk medium (12.5%), at 37°C for 24 h. These were then cultured and maintain in sterilised litmus milk medium. For fermentation, 1% skim milk culture was used. Each strain was fermented in duplicate for 12 h in skim camel milk and pH, TA, antioxidant (ABTS, DPPH) and enzymatic activity (Catalase, SOD, GST) was determined periodically at 2 h interval (i.e - 0, 2, 4, 6, 8, 10, 12 h) .

Measurement of pH and titratable acidity

The pH was determined by inserting a pH electrode (HANNA) directly fermented camel milk at every 2 h interval. The titratable acidity was determined by titrating 10 ml of homogenised fermented camel milk with 0.1 N NaOH to the phenolphthalein end point at every 2 h interval.

Biochemical analysis

Measurement of ABTS (2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid)) radical scavenging activity

ABTS radical scavenging activity was assayed spectrophotometricaly according to method described by Salami *et al* (2009). This method is based on the ability of antioxidant to blow out ABTS cation radicals ($ABTS^+$), a blue/green chromophore with absorbance at 734 nm, in comparison to standard antioxidants. ABTS assay was carried out by oxidising 7 mM ABTS solution in 2.45 mM potassium persulphate buffer with equal volume (1 : 1) for 12-16 h in the dark to provoke the formation of ABTS radical ($ABTS^+$). Before use, ABTS+ stock solution was diluted with distilled water to obtain absorbance 0.7 ± 0.2 at 734 nm at t_0 (t = 0 min), then 3ml ABTS+ working standard solution was mixed with 60 ul of sample and absorbance was measured after 20 min (t_{20}) at 734 nm in UV spectrophotometer (Biorad Hercules, California, United States).

Measurement of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity

The measurement of the DPPH radical scavenging activity was performed according to methodology described by Brand-Williams *et al* (1994) with slight modification. DPPH make stable free radical in aqueous or ethanol solution. However, fresh DPPH solution was prepared before every measurement. Three ml DPPH reagent (100µM) was mixed with 0.75 ml of 0.1 M Tris-HCL (pH-7.4) and 75ml of hydrolysate sample in test tubes and mixed well. The absorbency in time t = 0 min (t_0) was measured using UV spectrophotometer (Biorad Hercules, California, United States). The sample tubes were also incubated at room temperature under dark for measurement of absorbancy in time t = 20 min (t_{20}). Ethanol was used as a blank. The free radical scavenging activity was calculated as decrease in absorbance.

Enzyme assay

Catalase activity

The catalase activity was performed according to method described by Aebi (1984) and Cuellar-Cruz *et al* (2009) with slight modification. In the ultraviolet range, H_2O_2 shows a continual increase in absorption with decreasing wavelength. The decomposition of H_2O_2 can be followed directly by the decrease in absorbance at 240 nm. The difference in absorbance (ΔA_{240}) per unit time is a measure of the catalase activity. The assay was conducted by taking 1 ml Phosphate buffer 50 mM, pH 7.0 and 1 ml hydrogen peroxide 30 mM and 1 ml diluted sample in phosphate buffer (1:10) in quartz cuvette and absorbance was measured at 240 nm at 60 sec interval for 3 min using UV spectrophotometer (Biorad Hercules, California, United States). Phosphate buffer was used as a blank. The catalase activity was calculated as decrease in absorbance.

SOD (Superoxide dismutase) activity

The SOD activity was performed according to method described by Marklund and Marklund (1974) with slight modification. In a spectrophotometric cuvette, 2.7ml 50 mM Tris Cacodylate buffer (pH - 8.2) containing 1 mM EDTA was added to 300 µl of pyrogallol solution and absorbance was measured at 420 nm at 60 sec interval for 3 min using UV spectrophotometer (Biorad Hercules, California, United States). The resultant absorbance was considered as the experiment pyrogallol blank. The same procedure was carried out using of 100 µl sample (homogenate supernatant) and obtained the ΔA420 nm/minute using the maximum linear rate for both the test and experimental blank. Tris Cacodylate buffer was used as a blank. The SOD activity was calculated as increase in absorbance. The enzyme activity was expressed in terms of units/mg protein in which one unit corresponds to the amount of enzyme that inhibited the autoxidation reaction by 50%.

Table 1. pH of the camel mik milk samples fermented by *L. delbrueckii* sub sp. *Bulgaricus, L. casei, L. plantarum, S. thermophilus*.

S.No.	Strain	0 hr	2 hrs	4 hrs	6 hrs	8 hrs	10 hrs	12 hrs
1	Control	6.55 ± 0.002^{e}	6.54± 0.003^{De}	6.54 ± 0.002^{De}	6.51 ± 0.002^{Ed}	6.05 ± 0.003^{Cc}	5.85 ± 0.003^{Cb}	5.69 ± 0.003^{Ca}
2	*Lactobacillus delbrueckii* sub sp. *Bulgaricus NCDC- 009*	6.53 ± 0.004^{g}	6.48 ± 0.003^{Cf}	6.38 ± 0.002^{Ce}	6.23 ± 0.003^{Dd}	6.21 ± 0.003^{Dc}	6.18 ± 0.004^{Eb}	6.12 ± 0.003^{Ea}
3	*Lactobacillus casei NCDC- 017*	6.53 ± 0.004^{g}	6.48 ± 0.002^{Cf}	6.39 ± 0.002^{Ce}	6.22 ± 0.003^{Cd}	6.21 ± 0.002^{Dc}	6.09 ± 0.003^{Db}	5.91 ± 0.003^{Da}
4	*Lactobacillus plantarum NCDC- 020*	6.53 ± 0.004^{g}	6.28 ± 0.002^{Bf}	5.87 ± 0.004^{Be}	5.36 ± 0.004^{Bd}	5.21 ± 0.003^{Bc}	4.76 ± 0.003^{Bb}	4.58 ± 0.004^{Ba}
5	*Streptococcus thermophilus NCDC- 074*	6.53 ± 0.004^{g}	6.23 ± 0.003^{Af}	5.71 ± 0.003^{Ae}	5.02 ± 0.003^{Ad}	4.67 ± 0.004^{Ac}	4.37 ± 0.004^{Ab}	4.28 ± 0.003^{Aa}

Table 2. Titratable acidity of the camel milk samples fermented by *L. delbrueckii* sub sp. *Bulgaricus, L. casei, L. plantarum, S. thermophilus*.

S.No.	Strain	0 hr	2 hrs	4 hrs	6 hrs	8 hrs	10 hrs	12 hrs
1	Control	0.18 ± 0.003^{a}	0.18 ±0.002^{Aa}	0.19 ± 0.002^{Aab}	0.20 ± 0.002^{Ab}	0.21 ± 0.002^{Ac}	0.25 ± 0.002^{Ad}	0.27 ± 0.002^{Ae}
2	*Lactobacillus delbrueckii* sub sp. *Bulgaricus NCDC- 009*	0.18 ± 0.003^{a}	0.20 ±0.002^{Bb}	0.22 ± 0.004^{Bc}	0.23 ± 0.003^{Bc}	0.24 ± 0.002^{Bd}	0.26 ± 0.003^{Be}	0.28 ± 0.003^{Af}
3	*Lactobacillus casei NCDC- 017*	0.18 ± 0.003a	0.20 ±0.002^{Bb}	0.23 ± 0.003^{Bc}	0.24 ± 0.002^{Cd}	0.26 ± 0.002^{Ce}	0.28 ± 0.003^{Cf}	0.30 ± 0.002^{Bg}
4	*Lactobacillus plantarum NCDC- 020*	0.18 ± 0.003^{a}	0.23 ± 0.003^{Cb}	0.34 ± 0.002Cc	0.45 ± 0.004^{Dd}	0.50 ± 0.003^{De}	0.57 ± 0.002^{Df}	0.64 ± 0.003^{Cg}
5	*Streptococcus thermophilus NCDC- 074*	0.18 ± 0.003^{a}	0.23 ± 0.003^{Cb}	0.34 ± 0.002^{Cc}	0.55 ± 0.003^{Ed}	0.60 ± 0.003^{Ee}	0.65 ± 0.003^{Ef}	0.69 ± 0.003^{Dg}

GST (Glutathione-S-Transferase) activity

The GST activity was performed according to the method described by Habig *et al* (1974), Simons and Vander Jagt (1977) with slight modification. The reaction was measured by observing the conjugation of 1-chloro, 2, 4-dinitrobenzene (CDNB) with reduced glutathione (GSH). This was done by watching an increase in absorbance at 340 nm. One unit of enzyme will conjugate 10.0 nmol of CDNB with reduced glutathione per minute at 25°C. In a spectrophotometric cuvette, 100 mM potassium phosphate buffer containing 1 mM EDTA and 75 mM glutathione, reduced solution (G-SH) and 30 mM 1-chloro-2,4-dinitrobenzene solution (CDNB) were added and absorbance was measured at 340 nm at 60 sec interval for 5 min using UV spectrophotometer (Biorad Hercules, California, United States). The resultant absorbance was considered as the experiment blank. The same procedure was carried out using of 100 µl sample (homogenate supernatant) and obtained the ΔA340nm/minute using the maximum linear rate for both the test and experimental blank. Potassium phosphate buffer was used as a blank. The GST activity was calculated as increase in absorbance. The values were expressed as units/mg protein.

Statistical analysis

The experiments were carried out in 3 times and repeated in duplicate (n = 6). Data were expressed as mean ± S.E.M (standard error). All data were subjected to two-way analysis of variance (ANOVA) using SPSS 20.0 software (SPSS INC., Chicago, IL, USA, 2002) and the individual samples were compared by using Duncan's multiple range test (DMRT), at 95 % confidence level.

Results and Discussion

pH and titratable acidity of camel milk fermented with different cultures

pH and titratable acidity play an important role to determine the quality of fermented milk product. In the present study, the pH of all treatments and control was decreased significantly during the fermentation time i.e 0 to 12 h at 37°C (Table 1). Among all 4 type of bacterial culture *Lactobacillus plantarum* and *Streptococcus thermophilus* reduced the pH of milk more rapidly compared to *Lactobacillus delbrueckii* and *Lactobacillus casei* as shown in Fig 1. Similarly the titratable acidity of fermented camel milk was significantly increased ($P<0.05$) in all the strains during fermentation time (0 h to 12 h) (Table 2). It was found that *L. plantarum* and *S. thermophilus*

increases the acidity more rapidly as compared to *L. delbrueckii* and *L. casei* (Fig 2). It was reported that *L. plantarum* grow better between pH 3 and 4 and more metabolically active and produced different bioactive peptides which may affect the antioxidant property of the fermented product (Abubakr *et al,* 2013). Similarly, decreasing the pH and increasing the titratable acidity more rapidly by *S. thermophilus* suggested that it also produces bioactive peptides during fermentation, which may enhance the antioxidant property of the fermented milk (Julijana *et al,* 2016).

Determination of antioxidant activity:

The ABTS activity deepens on the presence of different amino acids in protein especially Cys, Trp and Met possess the highest antioxidant activity compared to the other amino acids (Salami *et al,* 2009), so during fermentation process various bioactive peptides are formed and degraded, which alter the antioxidant property of the fermented product. The antioxidant activity of camel milk fermented with different bacterial strains was evaluated against 2 different ABTS and DPPH radicals. ABTS assays indicated that the whey fraction fermented with *Streptococcus thermophilus* showed higher antioxidant activity (78.58 ±0.43) at 2 h fermentation time but at the end of the fermentation time (12h) *Lactobacillus delbrueckii* showed highest antioxidant activity (61.84 ± 0.34) and *S. thermophilus* showed lowest activity (43.60 ± 0.40) as shown in Fig 3.

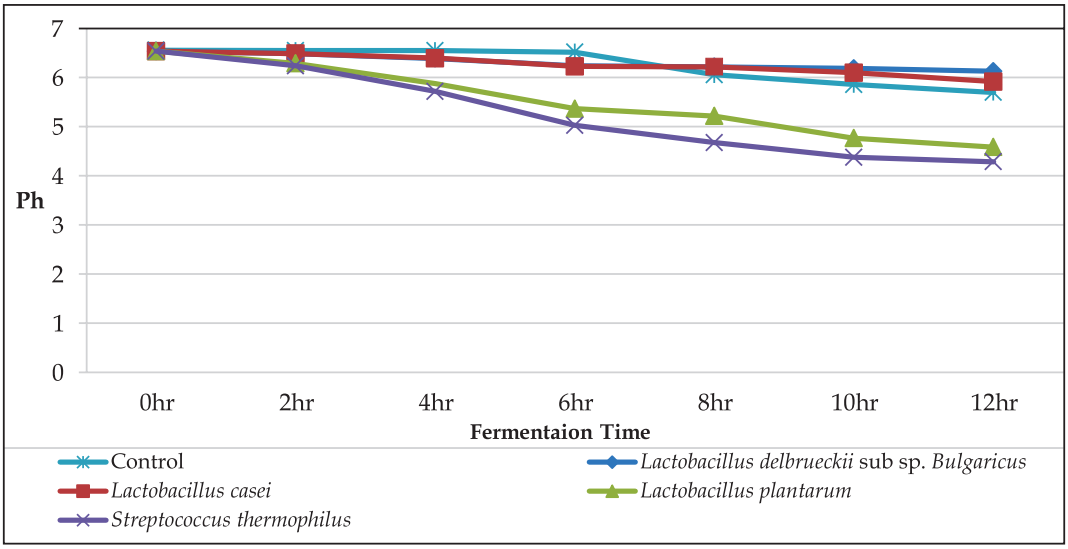

Fig 1. Graphical representation of change in pH of the camel milk samples fermented by 4 different LAB.

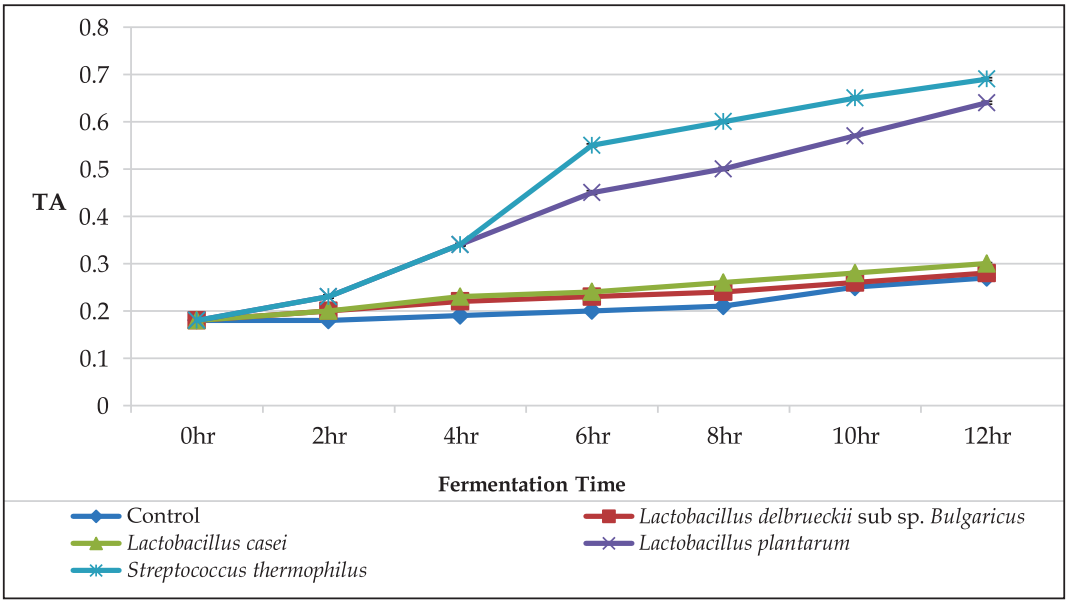

Fig 2. Graphical representation of change in titratable acidity of the camel milk samples fermented by 4 different LAB.

The DPPH activity varies in all the strains and result showed that all 4 strains fermented milk fraction have higher antioxidant activity compared to control at different fermentation time and *Lactobacillus casei* had highest antioxidant activity (7.52 ± 0.13) at 6 h fermentation time compared to all others as shown in Fig 4. The interaction of a potential antioxidant with DPPH depends on its structural conformation. Some compounds react very rapidly with the DPPH and reducing a number of DPPH molecules corresponding to the number of available hydroxyl groups (Brand-Williams *et al*, 1994). The variation in the DPPH activity revealed that all 4 types of bacterial strains produces different type of bioactive peptides according to their metabolic activity during different fermentation time, which may affect the antioxidant activity.

Determination of Enzymatic Antioxidants:

Antioxidant activity of milk and fermented products depends on many components such as sulfur containing amino acids, phosphate, vitamins A, E, carotenoids, zinc, selenium, enzyme like superoxide dismutase, catalase, glutathione

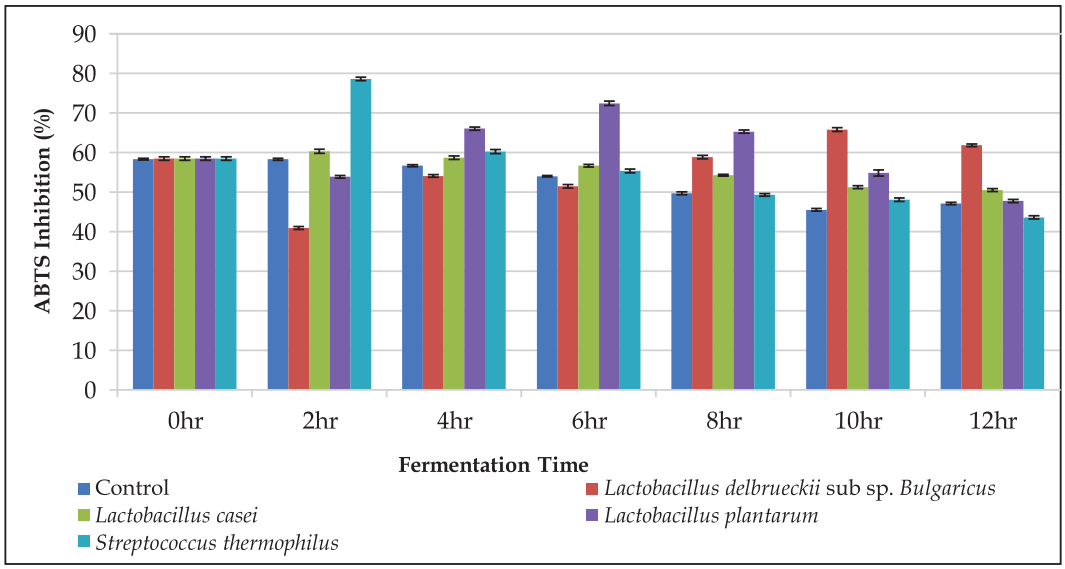

Fig 3. Changes in ABTS activity of fermented camel milk with four different LAB during 0 hr to 12 hr (incubation at 37°C). Values are presented as mean ± SEM (Error bars show standard error).

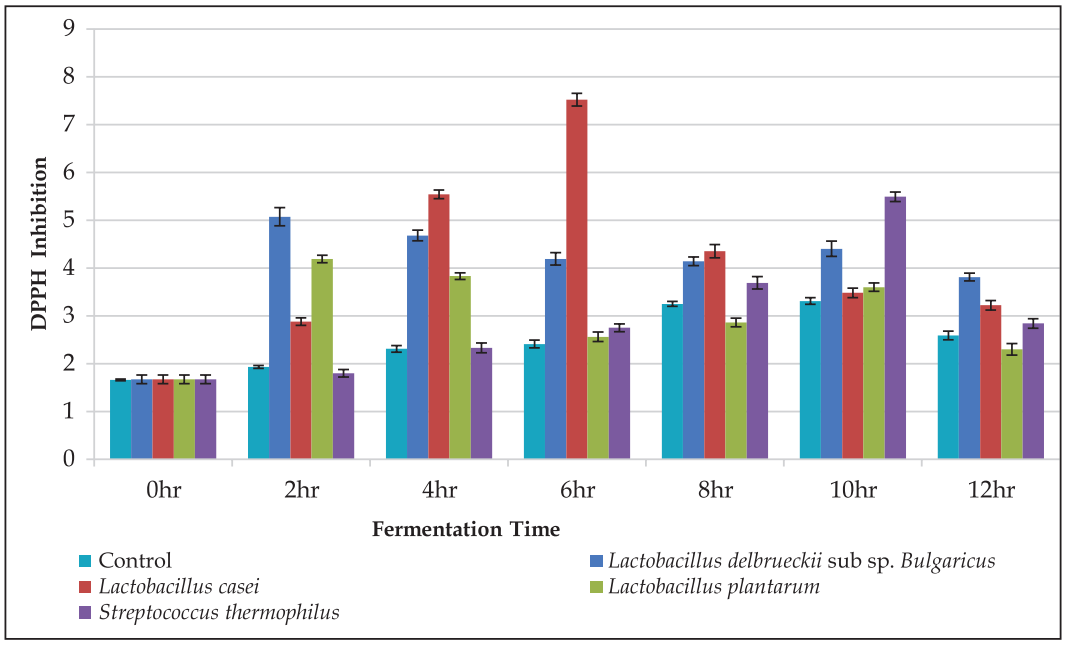

Fig 4. Changes in DPPH activity of fermented camel milk with four different LAB during 0 hr to 12 hr (incubation at 37°C). Values are presented as mean ± SEM (Error bars show standard error).

peroxidase and peptides which are produced during fermentation (Khan *et al,* 2019). In present study, it was found that the SOD activity in camel milk fermented with the strains *viz. L. delbrueckii* sub sp. *Bulgaricus*, *L. casei*, *L. plantarum*, *S. thermophilus* was increased significantly as compared to control during 0 h to 12 h fermentation time and *Lactobacillus plantarum* showed highest SOD activity (4.54 ± 0.013) at 12 h fermentation time (Fig 5).

Similarly, the Catalase and GST activity in all the strains were significantly increased as compared to control (Fig 6 and 7). The results showed that *Lactobacillus plantarum* had highest catalase (200.17 ± 0.95) and GST activity (9.13 ± 0.155) at 10 h and 12 h

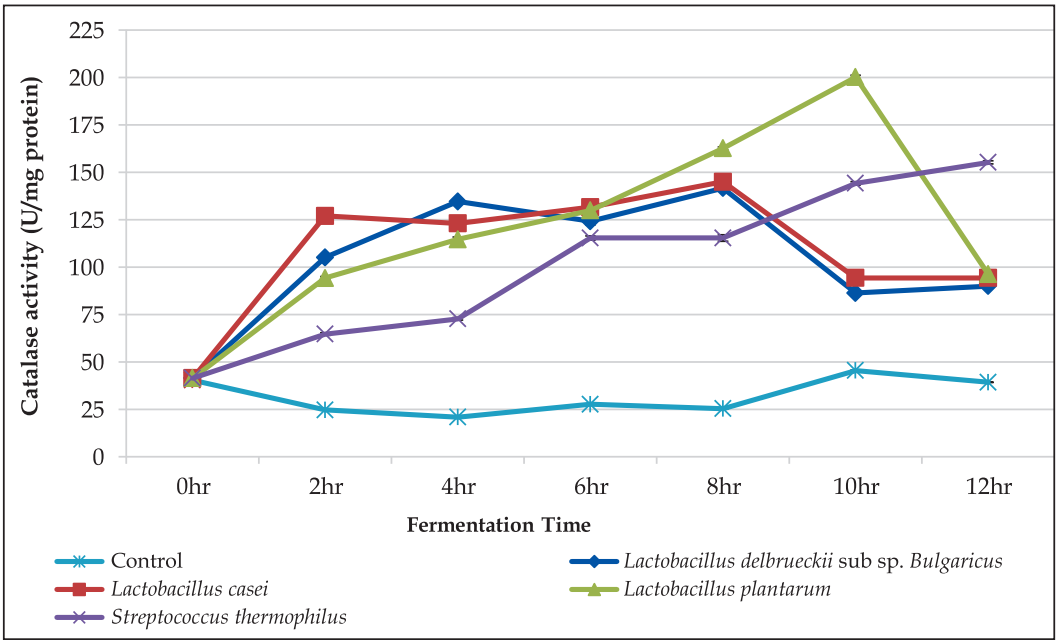

Fig 5. Catalase activity of the camel milk fermented by *L. delbrueckii* sub sp. *Bulgaricus, L. casei, L. plantarum, S. thermophilus* at different fermentation time (incubated at 37°C).

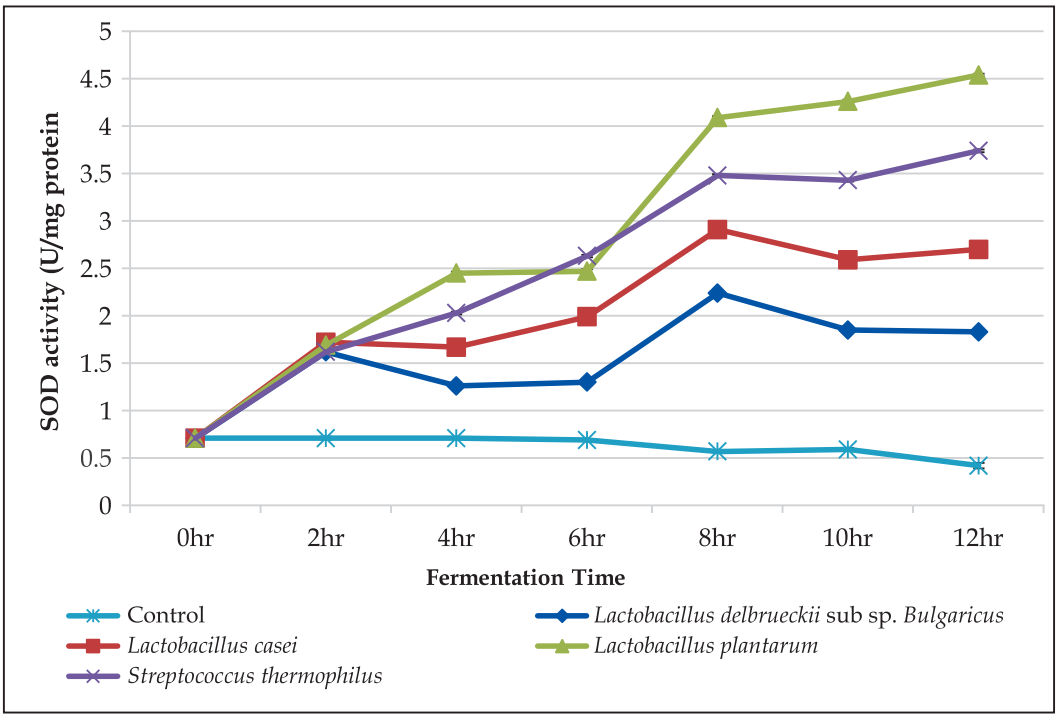

Fig 6. SOD activity of the camel milk fermented by *L. delbrueckii* sub sp. *Bulgaricus, L. casei, L. plantarum, S. thermophilus* at different fermentation time (incubated at 37°C).

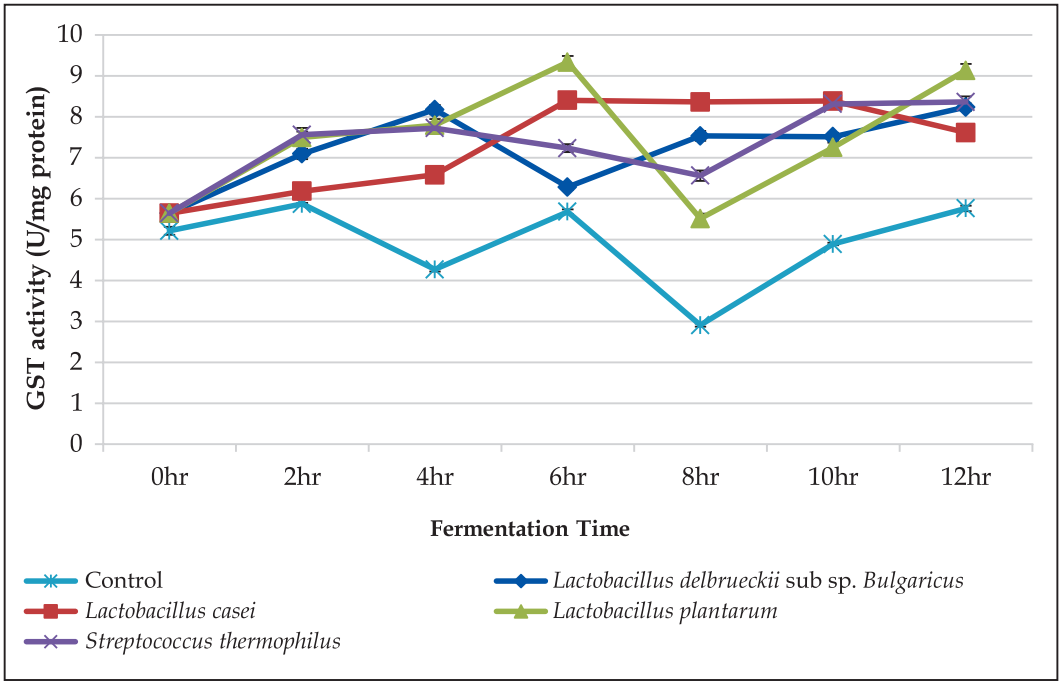

Fig 7. GST activity of the camel milk fermented by *L. delbrueckii* sub sp. *Bulgaricus, L. casei, L. plantarum, S. thermophilus* at different fermentation time (incubated at 37°C).

fermentation time, respectively. Tomusiak-Plebanek *et al* (2018) reported that *L. plantarum* showed highest catalase activity as compared to other *Lactobacillus* spp. These results revealed that the variation in enzymatic antioxidants activity may be due to the change in pH or formation of different bio-peptides or duration of fermentation time. Kleniewska *et al* (2016) noted that exopolysaccharides secreted by probiotic bacteria may play an important role in reducing the oxidative stress. These polysaccharides protect probiotics under starvation conditions or extreme temperature and pH.

Several reports suggested that Lactic acid bacteria (LAB) have antioxidant properties due to presence of enzyme such as catalase, superoxide dismutase and they are usually sensitive to oxidative stress caused by reactive oxygen species (ROS) and the ezymes superoxide dismutase (SOD) and catalase (CAT) can protect against these free radicals by eliminating superoxide and H_2O_2, respectively. In present study, the fermentation of camel milk with different strains of LAB increased the anti-oxidative property of the camel milk. The *Lactobacillus plantarum* showed the highest anti-oxidative activity which suggested that *L. plantarum* can be used as a potential probiotics. During the study we also found that the variation in DPPH, ABTS, Catalase, SOD and GST activity may be due to the change in pH or fermentation time or formation and breakdown of new bio-peptides during fermentation by different strains of LAB.

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