

RESEARCH PAPER

Study of the Anti-oxidant Potential of Buffalo Milk using *Lactobacillus helveticus* and *Lactobacillus fermentum*

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Abstract

The motive of the study was to determine the anti-oxidant potential of Murrah buffalo milk by the process of fermentation using lactic acid bacteria (LAB); *Lactobacillus fermentum* and *Lactobacillus helveticus*. Pooled milk samples of 8 Murrah buffaloes was used for 12 hour fermentation process using two different dairy cultures namely *Lactobacillus helveticus* and *Lactobacillus fermentum*. Anti-oxidant potential was determined by using ABTS (2,2'-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) and DPPH (2, 2'-diphenyl-1-picrylhydrazyl) radical scavenging activity method.

The results showed that Murrah buffaloes had highest anti-oxidant activity at 8 hour of fermentation in both type of milk samples fermented with *Lactobacillus helveticus* and *Lactobacillus fermentum*. The anti-oxidant activity of milk samples fermented with *Lactobacillus helveticus* were higher than milk samples fermented with *Lactobacillus fermentum*. In both type of milk samples, the anti-oxidant activity were first increased up to 8 hour of fermentation than declined in both ABTS and DPPH assay. Thus, it can be helpful to produce a probiotic drink by fermentation of Murrah buffalo milk and gives scope to functional dairy food production.

Keywords: Anti-oxidant potential, fermentation, lactic acid bacteria, milk, Murrah buffalo, *Lactobacillus*

According to 19th Livestock Census-2012, the Female buffalo population has increased by 7.99% over the previous census. The milch buffaloes increased from 48.64 million to 51.05 million with an increase of 4.95% over previous census. Rajasthan have about 12.97 million population of buffalos. As per the nutrient components, buffalo milk contains all the nutrients in higher proportions than cow milk. The compositional differences between buffalo and cow milk are reflected on their physico-chemical properties also. Milk from buffalo is preferred for preparing milk and dairy products of western and traditional (indigenous) type and is nutritionally superior.

Due to high peroxidase activity, buffalo milk can be preserved naturally for a longer period. Buffalo milk contains more calcium, better calcium: phosphorous ratio and less sodium and potassium than in cow milk which makes it a better nutritional supplement for infants. The main buffalo milk proteins show high homology to their cow counterparts, therefore, buffalo milk proteins are potential precursors for diversified functionalities.

Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health (Kitts and Wailer, 2003). These are synthesized in the cell in the form of large prepropeptides,

which are then, cleaved and modified to give active products. Peptides are inactive within the sequence of the parent protein and can be released in three ways: (a) enzymatic hydrolysis by digestive enzymes, (b) food processing and (c) proteolysis by enzymes derived from microorganisms or plants. As signalling molecules, the bioactive peptides play important roles in physiological functions and pathogenesis. Milk-derived bioactive peptides have been identified as potential ingredients of health-promoting functional foods. Many food proteins have peptide sequences with potential multifunctional activities, and there are many reports on antioxidant, antimicrobial, ACE-inhibitory, opioid, antithrombotic, anticancer activities of peptides derived from milk proteins and various food proteins.

Increased public consciousness of diet related health issues has resulted in a consumer's orientation towards healthy foods. Numerous scientific studies have confirmed that many chronic diseases are linked to unbalanced diet. Hence, foods with antioxidant activity are considered useful against these diseases. Fermentations is one of the tools to increase the antioxidant activity of foods like milk.

Lactobacillus fermentum and *Lactobacillus helveticus* both belong to the genus *Lactobacillus*. Species in this genus are used for a wide variety of applications; these applications include food and feed fermentation. It has been found that some strains of *Lactobacillus fermentum* have natural resistance to certain antibiotics and chemotherapeutics. So they are considered potential vectors of antibiotic resistance genes from the environment to humans or animals to humans. *Lactobacillus helveticus*, is considered "good" bacteria that are beneficial to the health of the human body. To determine the anti-oxidant potential of buffalo Milk, an experiment was designed and conducted to produce bioactive peptides from buffalo milk, Using *Lactobacillus fermentum* and *Lactobacillus helveticus*.

MATERIALS AND METHODS

Lyophilized powder containing ampoules of *Lactobacillus helveticus* NCDC 288 and *Lactobacillus fermentum* NCDC 214 were obtained from the National

Collection of Dairy Cultures (Dairy Microbiology Division ICAR-National Dairy Research Institute, Karnal (India)). The organisms were stored at 4°C.

A pre-experimental trial was done by using different starter cultures of lactic acid bacteria procured from NCDC, NDRI Karnal. On the basis of anti-oxidant activity the two cultures viz. *Lactobacillus fermentum* and *Lactobacillus helveticus* were selected for the present investigation.

Chemicals and reagents

Fine chemicals such as ABTS (2,2'-azinobis (3-ethylbenzthiazoline-6-sulphonic acid), DPPH (2,2'-diphenyl-1-picrylhydrazyl), potassium persulphate (K₂S₂O₈), Tris-HCl were obtained from Sisco Research Laboratories (SRL) Pvt. Ltd., India and other chemicals were of analytical grade from reputed companies and used without further purification. All the solutions, prepared with double-distilled water, were kept at 4°C before further use.

Collection of samples

About 2 liter of fresh buffalo milk was collected from buffaloes maintained under the project "Establishment of live demonstration models of diversified livestock production systems for motivating adaption to enhancing agricultural income (RKVY-15)" CVAS, RAJUVAS, Bikaner to perform the different experiments as mentioned under the study.

Following parameters were estimated during this phase:

- ❑ Determination of Physico-chemical properties of Buffalo Milk.
- ❑ Periodical Evaluation of Fermented Buffalo Milk.
- ❑ Characterization of Anti-oxidant Potential of Buffalo Milk during fermentation process.

To determine the physico-chemical properties of buffalo milk, about 10 ml of milk samples from fresh buffalo milk were collected and were analyzed for pH, SNF, fat, specific gravity, water content,

protein, lactose, freezing point depression, salts and conductivity using Milkoscan (Lactoscan milkoanalyser).

For study of periodical evaluation of fermented buffalo milk, about 2 liter of fresh buffalo milk were skimmed to bring the fat contents to below 0.5% using cream separator after determination of its physico-chemical properties.

Fermentation of Milk

Buffalo milk was heated to boil at least for 5 min to inactivate/kill the inherent microbial population present in milk, separately for the process of pasteurization. Then after cooling of milk at room temperature, *Lactobacillus fermentum* NCDC 214 (*L. fermentum*) and *Lactobacillus helveticus* NCDC 288 (*L. helveticus*) cultures were inoculated @ 1% in pasteurized buffalo milk and after proper mixing the samples were drawn at 0, 2, 4, 6, 8, 10, 12 hours and were inoculated at 37°C for different time intermission and subjected to analysis, for change in soluble protein concentration.

The propagation for each strain was performed according to Donker *et al.* (2007), with slight modification in amount of chemicals. In brief, sterile 5 ml aliquots of reconstituted sterile skim milk (RSM) (Himedia Laboratories) were inoculated with each strain individually and incubated at 37°C for 24h. After incubation, the pre-inoculated cultures were prepared by transferring loopfull of activated culture to 10 mL aliquots of litmus milk (Himedia Laboratories) to determine the activation activity of culture by observing change in colour of litmus milk after 24 hour of inoculation. The skim milk and litmus milk was autoclaved following the standard procedure (121°C for 15 min and 15 lbs).

The supernatant/hydrolysate collected by centrifugation at 4°C @ 10000 × g of buffalo milk during fermentation process from experiment was further utilized for antioxidant assay (ABTS and DPPH activity determination).

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

The ability to scavenge 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical by added antioxidants in samples was estimated following the method of Brand-Williams *et al.* (1995), with slight modification in amount of chemicals. Two ml of DPPH reagent (100 µM) was mixed with 0.50 ml of 0.1 M Tris-HCl buffer (pH 7.4) and 50 µl of hydrolysate sample in test tubes. The content was gently mixed and the absorbency in zero minute (At_0) was measured at 517 nanometer (nm) using a spectrophotometer. The sample tubes were also incubated at room temperature under dark for measurement of absorbency in 20 minute (At_{20}). Ethanol was used as blank. The free radical-scavenging activity was calculated as decrease in absorbance from the following equation:

$$\text{DPPH activity (\% inhibition)} = 100 - [(At_{20}/At_0) \times 100]$$

(Where, At_0 = absorbency in zero minute and At_{20} = absorbency in 20 minute)

ABTS (2, 2'-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) radical-scavenging activity

The spectrophotometric analysis of ABTS radical-scavenging activity was determined according to method described by Salami *et al.* (2009). ABTS radical cation (ABTS+) was produced by reacting ABTS+ stock solution with equal volume of 2.45mM potassium persulphate ($K_2S_2O_8$) and allowing the mixture to stand in the dark at room temperature for 16 hours before use. Prior to use, the stock solution was diluted with distilled water to get an absorbance of 0.70 at zero minute. About 4 ml of ABTS+ working standard solution was mixed with 40 µl of hydrolysate and placed in dark to get the absorbance after twenty minutes. The absorbance was measured after 20 min (At_{20}) at 734 nanometer (nm) spectrophotometer. The ABTS+ activity was calculated by using the following formula:

$$\text{ABTS activity (\% inhibition)} = [(0.7 - At_{20}) / 0.7] \times 100$$

(Where, At_{20} = absorbency in twenty minute)

STATISTICAL ANALYSIS

All the experiments of fermentation study were repeated three times and samples were drawn in duplicate. Data collected during the present investigation were subjected to statistical analysis by using F-test and adopting appropriate methods of analysis of variance as described by Snedecor and Cochran (1994). Wherever, the variance ratio was found significant at 5 per cent and highly significant at 1 per cent levels of probability, the significance of mean differences were tested by Duncan's New Multiple Range Test (Duncan's Range Test) as modified by Kramer (1957).

RESULTS AND DISCUSSION

The results obtained in present investigation along with their discussions are presented.

Physico-chemical properties of buffalo milk

The physico-chemical properties of fresh buffalo milk (from 8 buffalos) are shown in Table 1.

Table 1: Physico-chemical properties of buffalo milk

Physico-chemical Property	(Mean ± SE)
Fat (%)	8.53 ± 0.160
SNF (%)	7.73 ± 0.040
Density	1.031 ± 0.410
Protein (%)	3.09 ± 0.020
Lactose (%)	3.85 ± 0.030
Water (%)	6.05 ± 0.650
Salts (%)	0.76 ± 0.007
Freezing Point (°C)	-0.49 ± 0.004
pH	6.71 ± 0.008
Conductivity	2.56 ± 0.030

The results related to physico-chemical properties of buffalo milk are in conformity with Ahmad *et al.* (2008). It may be due to the inherited capabilities of the animals and/or attributed due to various seasonal and environmental factors as well as stage of lactation, age and number of calving. In addition, the feed and water quality and quantity available to the animals also play an important role.

ABTS activity of buffalo milk during fermentation

The data related to ABTS activity (% inhibition) of buffalo milk has been shown in Table 2. The ABTS radical-scavenging activity increased significantly ($P < 0.01$) with the advancement of fermentation time up to 8 hour for buffalo thereafter, decrease in activity was observed. Milk inoculated with *L. helveticus* had the highest antioxidant capacity from mean value of $1.14 \pm 0.00003\%$ at zero hour which increased to $12.28 \pm 0.001\%$ at 8 hour of fermentation after that it decreased appreciable.

Table 2: ABTS activity (Mean ± SE) of buffalo milk during fermentation

Treatment	<i>L. fermentum</i>	<i>L. helveticus</i>	Overall
Fresh	1.14 ± 0.00003	1.14 ± 0.00003	$1.14^a \pm 0.00002$
Hour2	3.72 ± 0.006	4.71 ± 0.001	$4.22^b \pm 0.140$
Hour4	4.94 ± 0.210	5.84 ± 0.010	$5.39^c \pm 0.170$
Hour6	7.71 ± 0.002	7.28 ± 0.002	$7.50^d \pm 0.060$
Hour8	11.14 ± 0.002	12.28 ± 0.001	$11.71^e \pm 0.170$
Hour10	9.57 ± 0.002	11.85 ± 0.004	$10.71^f \pm 0.340$
Hour12	8.28 ± 0.002	8.853 ± 0.001	$8.57^g \pm 0.080$
Overall	$6.64^a \pm 0.500$	$7.42^b \pm 0.570$	7.038 ± 0.380

Note: Means bearing different superscripts differ significantly.

Similar trends were observed with *L. fermentum* during the same incubation time and similar free radical scavenging activity at zero hour in milk samples, which reached to $11.14 \pm 0.002\%$ in milk samples, at 8 hour of fermentation. After that a significant fall in ABTS free radical scavenging activity took place in milk samples. These results are similar to those reported by Ramesh *et al.* (2012). A more apparent increase in the ABTS antioxidant activity was observed in the milk inoculated with *L. helveticus* strains and incubated for 12 hour at 37°C for buffalo milk comparing with results of *L. fermentum*. According to Donkor *et al.* (2007), the variations of biological activities may be attributed to the production of different bioactive peptides, which may or may not have antioxidant properties and it is likely to be strain dependent.

DPPH activity of buffalo milk during fermentation

The DPPH radical scavenging activity of buffalo milk samples increased significantly ($P < 0.01$) with the progress in fermentation time as per data shown in Table 3, but after 8 hour of fermentation, a significant fall in DPPH activity was seen. At 8 hour of fermentation, DPPH activity (% inhibition) in buffalo milk samples was highest (5.83 ± 0.007 and 6.14 ± 0.002 , respectively for *L. fermentum* and *L. helveticus*). Results demonstrated a similar pattern of fermentative potential demonstrated by Ramesh *et al.* (2012). A more apparent increase in the DPPH antioxidant activity was observed in the milk inoculated with *L. helveticus* strains and incubated for 12 hour at 37°C.

Table 3: DPPH activity (Mean \pm SE) of buffalo milk during fermentation

Treatment	<i>L. fermentum</i>	<i>L. helveticus</i>	Overall
Fresh	0.54 ± 0.0002	0.54 ± 0.0002	$0.54^a \pm 0.0001$
Hour2	0.91 ± 0.0010	1.38 ± 0.0010	$1.14^b \pm 0.060$
Hour4	1.41 ± 0.0010	1.70 ± 0.0020	$1.55^c \pm 0.040$
Hour6	2.07 ± 0.0001	3.89 ± 0.0010	$2.98^d \pm 0.270$
Hour8	5.83 ± 0.0070	6.14 ± 0.0020	$5.99^e \pm 0.040$
Hour10	4.13 ± 0.0800	4.81 ± 0.0010	$4.47^f \pm 0.110$
Hour12	3.57 ± 0.0050	3.68 ± 0.0030	$3.62^c \pm 0.010$
Overall	$2.64^a \pm 0.270$	$3.16^b \pm 0.290$	2.90 ± 0.200

Note: Means bearing different superscripts differ significantly.

According to Table 3, the free radical scavenging activity in all samples changed significantly ($P < 0.01$) from zero to 12 hour. Milk inoculated with *L. helveticus* had the highest antioxidant capacity which increased from mean value of $0.54 \pm 0.0002\%$ at zero hour which increased to $6.14 \pm 0.002\%$ at 8 hour of fermentation, after that it decreased significantly. Similar trends were observed with *L. fermentum* during the same incubation time and similar free radical scavenging activity at zero hour in buffalo milk samples, which reached to $5.83 \pm 0.007\%$ in milk samples, at 8 hour of fermentation. After that a significant fall in DPPH radical scavenging activity takes place in both types of milk samples.

On the basis of data the DPPH anti-oxidant activity (% inhibition) of *L. helveticus* was significantly higher, when compared with *L. fermentum* in buffalo milk samples during fermentation process. According to Ramesh *et al.* (2012), the antioxidant activity of the strains of *Lactobacillus* assessed increased with an increase in the level of fermentation at a certain time period, after that it reduced.

CONCLUSION

According to ABTS and DPPH radical scavenging anti-oxidant activity of buffalo milk samples fermentative potential of *L. helveticus* was found more, when it was compared with *L. fermentum*. These milk samples which were fermented with *L. helveticus* at the time period of fermentation, where it show highest anti-oxidant activity (both ABTS and DPPH basis) can be used as probiotic drink. This probiotic drink full of anti-oxidants and can be used in production of functional dairy product and has beneficial effect on human health.

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