DETERMINATION OF THE BIOACTIVE POTENTIAL (ANTIOXIDANT ACTIVITY) OF BUFFALO MILK DURING FERMENTATION PROCESS[#]

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Received on: 16.09.2017
Accepted on: 10.01.2018

ABSTRACT

An experiment was conducted to explore the possibilities of utilization of buffalo milk for production of bioactive peptides which have antioxidant potential by action of fermentation using two dairy cultures: *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis.* Pasteurized buffalo milk was incubated with these two cultures @ 1% at 30ºC for a period of 12 hour fermentation. During this period change antioxidant potential was measured using ABTS and DPPH radical scavenging activity. According to ABTS (2, 22 -azinobis (3-ethylbenzthiazoline-6-sulphonic acid) and DPPH (2, 22 -diphenyl-1-picrylhydrazyl) radical scavenging activity antioxidant activity of buffalo milk samples, the fermentative potential of *Lactococcus lactis* subsp. *cremoris* was found significantly higher (P<0.05), when it was compared with *Lactococcus lactis* subsp. *lactis*. Thus, milk samples fermented with *Lactococcus lactis* subsp. *cremoris* were used for production of fermented buffalo milk products at the time period of fermentation, where it shows highest antioxidant activity (both ABTS and DPPH) (i.e.8 hours of fermentation for buffalo milk).

Key words: Buffalo milk, fermentation, ABTS, DPPH

Introduction

Milk is one of the best food that is given to humans with all essential nutrients (Nema *et al.,* 2015). The highest risk from oxidative metabolism by-prod-ucts is the formation of free radicals. The damage to the organism caused by free radicals is immense and is a major threat for the welfare of the whole organism and is known to cause a variety of potentially fatal diseases. The objective of this research is to determine the anti-oxidative capacity of fermented buffalo milk with different types of microbiological strains. India has highest population of buffalo in the world and murrah buffalo mostly used for upgrading the low milk producing breeds (Malhotra *et al.,* 2015). Buffalo milk contains all the nutrients in higher proportions than cow milk as per the nutrient components.The compositional differences between buffalo and cow milk are reflected on their physico-chemical properties.

Milk from buffalo preferred for preparing dairy products of western and traditional indigenous) type and nutritionally superior. Buffalo milk contains less cholesterol in compared to cow milk and more tocopherol. Due to high peroxidase activity, buffalo milk can be preserved naturally for a longer period. Buffalo milk contains more calcium, better calcium:phosphorus ratio and less sodium and potassium than cow milk which makes it a better nutritional supplement for infants. *Lactococcus lactis* has two subspecies with few phenotype and genotype differences, *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*, where *Lactococcus lactis* subsp. *lactis* is preferred for making soft cheese while *Lactococcus lactis* subsp. *cremoris* is for hard cheese. *Lactococcus lactis* is a gram-positive bacterium used extensively in the production of buttermilk and cheese. *Lactococcus lactis* has crucial importance for manufacturing dairy products, such as buttermilk and cheeses. When *Lactococcus lactis* subsp. *lactis* is added to milk, the bacterium uses enzymes to produce energy molecules (ATP), from lactose. Industrial research on *Lactococcus lactis* deals with the production of L-alanine, which is used as sweetener in dairy products.

Materials and Methods

To study on the fermentative potential of buffalo milk by using *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis*, an experiment was designed and conducted at National Research Centre on Camel, Bikaner, to produce bioactive peptides from buffalo milk. A pre experimental trial was done by using different starter cultures of lactic acid bacteria procured from NCDC (National Collection of Dairy Cultures), NDRI (National Dairy Research Institute) Karnal, Hariyana, India. On the basis of antioxidant activity these two cultures *viz*. *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* were chosen for the present investigation. About 2 litre 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)" College of Veterinary and Animal Science, Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, India, at weekly interval for period of 2 months to perform the different experiments as mentioned below under the study. Fresh buffalo milk was skimmed to bring the fat contents to below 0.5% using cream separator. The samples were heated to boil at least for 5 min to inactivate/ kill the inherent microbial population present in milk.

Then *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* cultures were inoculated @ 1% and after proper mixing the samples were inoculated at 30°C and samples were drawn at 0, 2, 4, 6, 8, 10, 12 hours and were subjected to analysis for change in soluble protein concentration etc.

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Bacterial cultures and their propagation

Glass ampoules containing lyophilized powder of *Lactococcus lactis* subsp. *cremoris* NCDC 81 and *Lactococcus lactis* subsp. *lactis* NCDC 88 were obtained from the NCDC (National Collection of Dairy Cultures) Dairy Microbiology Division ICAR-National Dairy Research Institute, Karnal (INDIA). The organisms were stored at 4°C. The propagation for each strain was performed according to Donker *et al.* (2007) with slight modification. Sterile 5 ml aliquots of reconstituted sterile skim milk (RSM) (Himedia Laboratories) were inoculated with each strain individually and incubated at 30°C for 24 hour in BOD incubator. After incubation, the pre-inoculated cultures were prepared by transferring loop full of activated culture to 10 ml aliquots of litmus milk (Himedia Laboratories) to determine the activation of culture activity by observing change in colour of litmus milk after 24 hours of inoculation. The skim milk and litmus milk were autoclaved by following the standard procedure (121°C for 15 min @15 lbs).

Culture performance during cultivation in milk

Formation of serial dilution of the culture was done and appropriate dilution was selected for enumeration by the pour plate technique. All samples were enumerated on MRS agar at 30°C for 24 hours. Plates containing 30-300 colonies were enumerated and the colony forming units (CFU) per gram of the product was calculated. These cultures were then used for fermentation of fresh pasteurized milk samples for 12 hours at 30°C corresponding to cell count 10^7-10^8 CFU/ml as per suggested by Ramesh *et al.* (2012). The supernatants collected by centrifugation of buffalo milk during fermentation and then utilized for antioxidant assay (ABTS and DPPH etc.).

DPPH (2, 22 -diphenyl-1-picrylhydrazyl) radical-scavenging activity

The ability to scavenge DPPH (2, 22 -diphenyl-1-picrylhydrazyl) radical by added antioxidants in samples was estimated by following the method of Brand-Williams *et al.* (1995) with slight modification. About 2 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 and the content was gently mixed and immediately absorbance was measured at 517 nm (nanometer) by using a spectrophotometer and then the sample tubes were incubated at room temperature under dark for 20 minutes and then again measured the absorbance. Ethanol was used as blank. The free radical-scavenging activity was calculated from the following equation:

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

Where At_{20}= absorbance at 20 minute

At_0= absorbance at zero minute

ABTS+ (2,22-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 2.45 mM potassium persulphate ($K_2S_2O_8$) and allowing the mixture to stand in the dark at room temperature for 16 hours before use. For making working solution of ABTS, the stock solution of ABTS was diluted with distilled water to make its absorbance 0.70 and equilibrated at 30°C exactly 6 min after initial mixing. About 4 ml of ABTS+ working standard solution was mixed with 40µl of hydrolysate/standard and absorbance was measured after 20 minuts @ 734 nm by using spectrophotometer.

The ABTS+ activity was calculated by using the following formula:

ABTS activity (% inhibition) = $[(0.70-At_{20})/0.70] \times 100$

Where At_{20}= absorbance of mixture at 20 minute

0.70=absorbance of working solution of ABTS

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 Chochran (1994). Wherever, the variance ratio were found significant at 5% and highly significant at 1% 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

ABTS activity (% inhibition) of buffalo milk during fermentation

The data related to ABTS activity (% inhibition) of buffalo milk has been shown in Table 1(a) and Table 1(b) and is depicted in Fig. 1. The ABTS radical-scavenging activity increased significantly ($P<0.01$) with the advancement fermentation time up to 8 hours, after that, decrease in activity was observed. Milk inoculated with *Lactococcus lactis* subsp. *cremoris* had the highest antioxidant capacity which increased from mean value of 1.14±0.001% at zero hour (fresh milk) in buffalo milk to 14.50±0.142% at 8 hour, after that it decreased significantly. Similar trends were observed with *Lactococcus lactis* subsp. *lactis* during the same incubation time and similar free radical scavenging activity at zero hour which reached to 10.25±0.054% in buffalo milk samples, at 8 hour of fermentation. After that a significant fall in ABTS free radical scavenging activity takes place. Results were showing similar trend with Ramesh *et al.* (2012). The overall ABTS activity (% inhibition) for buffalo milk was observed 9.33±0.673 and 6.30±0.443, for *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis,* respectively. According to Table 1(b) the free radical scavenging activity in all samples changed significantly ($P<0.01$) from zero to 12 hour. On the data basis shown in Fig. 1, the ABTS anti oxidant activity (% inhibition) of *Lactococcus lactis* subsp. *cremoris* was significantly higher, when compared with *Lactococcus lactis* subsp. *lactis* in buffalo milk samples during fermentation process. 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. These findings of proteolytic activity were in accordance with the findings of Salami *et al.* (2011) and Jrad *et al.* (2014) but the method of production of bioactive peptides from milk sample were different (digestive enzymes v/s LAB fermentation) .

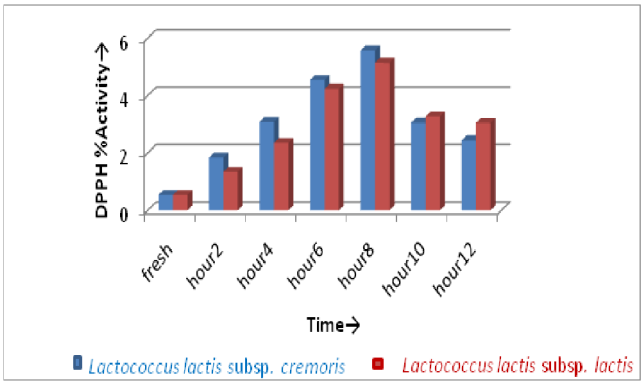

Fig. 1: ABTS activity (% inhibition) of buffalo milk during fermentation

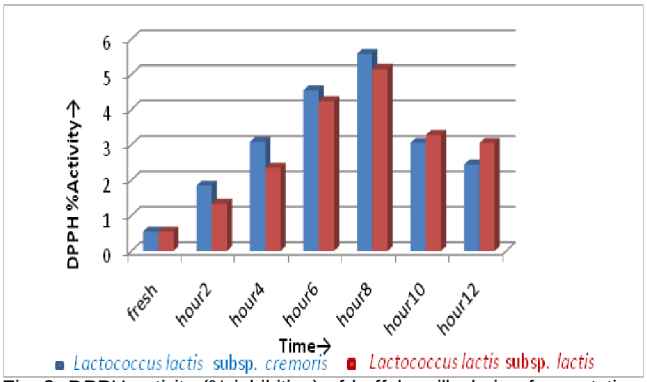

Fig. 2: DPPH activity (% inhibition) of buffalo milk during fermentation

Table 1(a): ABTS (Mean ± SE) activity (% inhibition) of buffalo milk during fermentation

Treatment	*Lactococcus lactis* subsp. *cremoris*	*Lactococcus lactis* subsp. *lactis*	Over all
Fresh	1.14^{a}±0.001	1.14^{a}±0.001	1.14^{a}±0.001
Hour2	5.42^{b}±0.004	4.23^{b}±0.060	4.82^{b}±0.180
Hour4	8.98^{c}±0.004	5.54^{c}±0.025	7.26^{c}±0.518
Hour6	12.23^{e}±0.047	7.44^{e}±0.025	9.84^{e}±0.723
Hour8	14.50^{g}±0.142	10.25^{g}±0.054	12.38^{g}±0.645
Hour10	12.62^{f}±0.144	9.13^{f}±0.033	10.88^{f}±0.530
Hour12	10.41^{d}±0.123	6.36^{d}±0.020	8.38^{d}±0.613
Overall	9.33^{b}±0.673	6.30^{a}±0.443	7.81±0.434

Note: Means bearing different superscripts differ significantly.

DPPH activity (% inhibition) of buffalo milk during fermentation

The mean data related to DPPH activity of buffalo milk has been presented in Table 2(a) and Table 2(b) and depicted in Fig. 2. The DPPH activity of fermented buffalo milk increased significantly (P<0.01) with the progress in fermentation time, and a positive relationship between fermentation time and DPPH activity could be established; however, the higher DPPH-scavenging activity was decreased after 8 hour of fermentation. Data show in Table 2 (a) reveals that, at 8 hours of fermentation, the DPPH activity of buffalo milk samples were highest 5.58 ± 0.007 and 5.15 ± 0.011, respectively for *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis*). Results demonstrated a similar pattern of fermentative potantial with Ramesh *et al.* (2012). According to Table 2(b) the free radical

Table 1(b): Analysis of variance for ABTS activity (% inhibition) of buffalo milk during fermentation

Source	D.F.	Mean Square	Level of sig.
Treated bacteria	1	192.580	**
Hour	6	176.721	**
Reminder	76	0.760	

** = Significant at 1% (P<0.01)

Table 2(a): DPPH (Mean ± SE) activity (% inhibition) of buffalo milk during fermentation

Treatment	*Lactococcus lactis* subsp. *cremoris*	*Lactococcus lactis* subsp. *lactis*	Over all
Fresh	0.54^{a}±0.005	0.54^{a}±0.005	0.54^{a}±0.003
Hour2	1.84^{b}±0.013	1.34^{b}±0.011	1.59^{b}±0.076
Hour4	3.08^{c}±0.012	2.36^{c}±0.009	2.72^{c}±0.110
Hour6	4.55^{f}±0.013	4.23^{f}±0.009	4.39^{f}±0.048
Hour8	5.58^{g}±0.007	5.15^{g}±0.011	5.36^{g}±0.065
Hour10	3.06^{e}±0.016	3.28^{e}±0.008	3.17^{e}±0.035
Hour12	2.45^{d}±0.013	3.05^{d}±0.013	2.75^{d}±0.091
Overall	3.01^{b}±0.241	2.85^{a}±0.230	2.93±0.166

Note: Means bearing different superscripts differ significantly.

Table 2(b): Analysis of variance for DPPH activity (% inhibition) of buffalo milk during fermentation

Source of variation	D.F.	Mean Square	Level of sig.
Treated bacteria	1	0.561	**
Hour	6	31.362	**
Reminder	76	0.052	

** = Significant at 1% (P<0.01)

scavenging activity in all samples changed significantly (P<0.01) from zero to 12 hour. Milk inoculated with *Lactococcus lactis* subsp. *cremoris* had the highest antioxidant capacity which increased from mean value of 0.54±0.005 at zero hour (fresh milk) in buffalo milk to 5.58±0.007 at 8 hour of fermentation after that it decreased significantly. Similar trends were observed with *Lactococcus lactis* subsp. *lactis* during the same incubation time and similar free radical scavenging activity at zero hour in buffalo milk samples, which reached to 5.15 ± 0.011 at 8 hour of fermentation, respectively. Subsequently a significant fall in DPPH radical scavenging activity takes place. On the basis of data shown in figure 2 the DPPH antioxidant activity (% inhibition) of *Lactococcus lactis* subsp. *cremoris* was significantly higher, when compared with *Lactococcus lactis* subsp. *lactis* during fermentation process. Thus the fermentative potential of *Lactococcus lactis* subsp. *cremoris* was more when it was compared with *Lactococcus lactis* subsp. *lactis*, thus milk samples fermented with *Lactococcus lactis* subsp. *cremoris* of buffalo milk canbe used for production of fermented buffalo milk products at the time period of fermentation, where it show highest antioxidant activity (both ABTS and DPPH basis) (i.e. 8 hours of fermentation for buffalo milk).

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