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Research Article

FORTIFICATION AND COMPARATIVE STUDIES ON NUTRIENTS IN FERMENTED MILK PRODUCTS

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A R T I C L E I N F O

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A B S T R A C T

Fermented milk products play important roles in nutritional diet. Historically, fermentation process involved unpredictable and slow souring of milk caused by the organisms inherently present in milk. However, modern microbiological processes have resulted in the production of different fermented milk products of higher nutritional value under controlled conditions. These products represent an important component of functional foods into which probiotic organisms are incorporated to make them more valuable. On the other hand, fortification of milk with micronutrients plays a major role in rendering nutritional deficiency problems in humans. It can improve the palatability and sensory profile of the products. This article provides an overview of fortification especially with calcium citrate and whey protein concentrate for both cow and buffalo milk and comparative studies of nutrients of fermented dairy products, which are consumed by the consumers through their regular intake.

INTRODUCTION

Milk is the most nutritionally complete food containing nearly all the constituents of nutritional importance to humans. Milk is excellent source of most B vitamins [1, 2]. Yoghurt is made by fermentation milk of two types of lactic acid bacteria, namely *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. Yoghurt like a healthful food is a great source of protein and calcium.

Yoghurt is a unique dairy food because the starter cultures actually produce enzyme which degrade a lactose sugar during fermentation. Yoghurt starter cultures are using for their possible role in just about everything from improved digestion and reduced risk of intestinal infection to improved immune function and reduced risk of certain cancers [3].

Probiotics are ingested as fuel for bacteria already present in the gastrointestinal tract. When the normal balance of these bacteria is disturbed by illness or antibiotic treatment, the most common effect is diarrhea. Probiotics work by colonizing the small intestine and crowding out disease-causing bacteria, thereby restoring balance to the intestinal flora [4].

Fortifications of yogurt with calcium is the best choice for calcium enrichment. Yogurt can carry higher calcium density and calcium from yogurt is highly bioavailable, these characteristics make yogurt as ideal vehicle for calcium fortification .

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It is generally agreed that the bioavailability of organic calcium is much higher than inorganic calcium [5]. Calcium makes up about 2% of the body-weight and about 39% of the total body minerals [6]. Ionized calcium in blood performs certain regulatory functions such as, contraction of muscle, coagulation of blood, transmission of nerve impulses, activation of enzyme reactions, stimulation of hormone secretions, and integrity of intracellular cement substance [7-11]. Milk is recognized as "the richest natural source of calcium". Even though, cow milk contains only 100-120 mg / 100 ml of Calcium. Yogurt is being the best choice for calcium enrichment. Yogurt can carry higher calcium density and calcium from yogurt is highly bioavailable, these characteristics make yogurt as ideal vehicle for calcium fortification [5]

Whey proteins have been used for many years as highly nutritious food supplement [12]. Whey proteins have proportionately more sulfur-containing amino acids (cysteine, methionine) than caseins, which contributes to the higher PER of whey proteins (3.2) than of casein (2.6). Further, among all protein sources, whey proteins contain the highest concentration of the branched-chain amino acids L-isoleucine, L-leucine, and L-valine. Virtually every amino acid present in sweet-type whey exceeds Food and Agriculture Organization/World Health Organization (FAO/ WHO) nutritional intake recommendations, both for children aged 2 to 5 years and for adults [13]. The present study has been designed to analyse the effects of calcium citrate and whey protein concentrate for fortification and supplementation of

Yogurt without developing adverse physical or organoleptic characteristics along with the biochemical, microbial and antioxidant properties of cow milk and buffalo milk yogurt.

MATERIALS AND METHODS

Sample collection

Cow milk (Mother Dairy, 100 ml) and buffalo milk (100 ml) was collected from local market of Barrackpore, Kolkata and properly pasteurized.

Materials

- ✓ Calcium citrate (C6H5O7)2Ca3.4H2O food additive E333 (21% Ca) was purchased from Barrackpore, Kolkata local market.
- ✓ Whey protein concentrate 80% protein (WPC 80%) is manufactured by Agri Mark, Inc. (USA), WPC consisting of Protein 83.7%, lactose 5.5%, minerals 1.2%, moisture 5.0% and milk fat 4.6%.
- ✓ *Lactic acid culture* (MAO 16,20u. Texel, France) containing *Streptococcus salivarius ssp. thermophilus* and *Lactobacillus delbruckii ssp. bulgaricus* was supplied from Dairy Pilot Plant. [14]

Fortification with Calcium and whey protein concentrate

Standardized buffalo milk contained 162 mg Ca/100ml. Ca citrate was added to elevate the Ca content of milk by 50 and 100%. Ca citrate contains 21% calcium; therefore, Ca citrate was added in a ratio of 386mg/100g standardized buffalo milk to make calcium content 150% and 771mg/100g standardized buffalo milk to make calcium content 200% [15].

Trials were prepared as follows

- ✓ Control (standardized milk) without addition of neither calcium citrate nor whey protein concentrate (WPC).
- ✓ Tr.1: fortified milk with 50% plus Ca + 0.2% WPC.
- ✓ Tr.2: fortified milk with 100% plus Ca + 0.2% WPC.
- ✓ Tr.3: fortified milk with 50% plus Ca + 0.3% WPC.
- ✓ Tr.4: fortified milk with 100% plus Ca + 0.3% WPC.
- ✓ Tr.5: fortified milk with 50% plus Ca + 0.5% WPC.
- ✓ Tr.6: fortified milk with 100% plus Ca + 0.5% WPC.

Preparation of Yogurt

All treatments were prepared and pasteurized at 85°C for 30 min, then cooled to 42°C and inoculated with 2% lactic acid culture (W/W),) packaged in PVC containers (120g), then incubated at 42±1°C until coagulation. The samples were immediately stored at 6±2^{o}C until loop.

Fresh samples were taken from each treatment for chemical analysis and sensory evaluation.

Microbiological test

Isolation of lactic acid bacteria from curd sample

Both the cow milk and buffalo milk curd sample were taken on separately in sterilized flask in aseptic condition. Serial dilution was made from 10^{-1} to $10^{-}5$ and from which 10^{-2} and 10^{-3} were selected and those were used to spread on MRS agar (Himedia, ref-M641-500G) and incubated for 24-72 h at 30°C. Isolated pure culture was maintained in MRS Broth at 5°C and in Agar slant. The culture was maintained by monthly sub culturing using MRS Agar medium.

Identification of isolates

Isolates were identified according to morphology and biochemical test like Gram test, carbon dioxide production from glucose (Hot loop test) and growth at 15°C,25°C,and 45°C respectively and appearance of colonies [16].

Catalase test

It was performed by adding 1 ml of H_2O_2 (3% v/v) to a loopful of culture on a glass slide and immediate appearance of bubbles was observed. Bubble appearance indicates that the test was positive and the organism was termed as catalase test positive or vice-versa [17].

Hot loop test

Microbial cultures were grown in APT broth (difco) in addition to 1% dextrose and 0.5% sodium acetate (ADAC medium) at 27°C. After 24 h, a heated inoculating loop was plunged immediately into the culture and gas formation was observed [18].

Antibiotic sensitivity assay

Antibiotic susceptibility test was done by kirby-bauer method, also known as paper disk diffusion method with streptomycin (S25) (Ref- SD006-5CT) and chloramphenicol (C30) (Ref-SD091-5CT) disks and afterwards the inhibition zones were measured in mm.

In this method, a lawn of bacteria was spread onto a Nutrient agar plate and the paper docs impregnated with antibiotics were placed onto the bacterial lawn. After incubating the inoculating plates at 35°C for 16-24 h, these were examined for clear zones, called zones of inhibition around the discs. This test was used for determined of effectiveness of *Lactobacillus* isolates against specific antibiotic or drugs. Antibiotic sensitivity test of isolated LAB cultures and all the prepared fermented samples were done by this method. All experiments were carried out in triplicates [19].

Physiochemical analysis of curd sample

Moisture content

The moisture content of the sample was analyzed by Moisture Analyzer (digital) IR-35 (Denver instrument) at 130°C for 10 min.

Total soluble solid (TSS)%

The Total soluble solid percentage was measured by Refractometer M-115.

Determination of Titrable acidity

Reagents

1. Standard Sodium Hydroxide Solution – 0.1 N
2. Phenophthalein Indicator – Dissolve 0.5 g phenolphthalein in100 ml of 50% ethyl alcohol

Procedure

10 gm of the curd samples were measured accurately in a suitable dish or basin. 30 ml of warm water was added along with 1 ml of phenolphthalein indicator. The mixture was shaken well and titrated against standard NaOH solution. The titration process was completed in 20 sec. A blank was prepared by taking 10 g of curd sample diluted with 30 ml of water in another dish for comparison of colour.

Calculation

Titrable acidity as Lactic acid = 9 AN/ W
Where A = Volume of standard NaOH required for titration
N = Normality of Standard NaOH solution
W = weight of the sample taken for test.

pH value

The pH of the sample was measured by pH -meter (Mettler Toledo) at 21.6°c.

Measurement of Protein by Lowry Method

The principle behind the Lowry method of determining protein concentrations lies in the reactivity of the peptide nitrogen[s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteay phosphomolybdic phosphotungstic acid to heteropoly molybdenum blue by the copper-catalyzed oxidation of aromatic acids. The Lowry method is sensitive to pH changes and therefore the pH of assay solution should be maintained at 10 - 10.5 [20].

Reagents

2% Na_2CO_3 in 0.1 N NaOH, 1% NaK Tartrate in H_2O, 0.5% $CuSO_4$.5 H_2O in H_2O, Reagent I: 48 ml of A, 1 ml of B, 1 ml C, 1 part Folin-Phenol [2 N]: 1 part water, BSA Standard- 1 mg/ ml.

Procedure

- ✓ 0.2 ml of BSA working standard in 5 test tubes and make up to 1ml using distilled water.
- ✓ The test tube with 1 ml distilled water serves as blank.
- ✓ Add 4.5 ml of Reagent I and incubate for 10 minutes.
- ✓ After incubation add 0.5 ml of reagent II and incubate for 30 minutes
- ✓ Measure the absorbance at 720nm and plot the standard curve.
- ✓ Estimate the amount of protein present in the given sample from the standard curve.

Measurement of fat unsaturation test

According to AOAC methods of test for Dairy chemistry, for chemical analysis of milk, milk was added with chloroform and bromine water & red coloration was observed. After this, $KMnO_4$ solution was added [20].

Measurement of folic acid

Folic acid content was measured as per procedures of USP XVIII [21]. 1ml of homogenised sample was mixed with 25 ml of distilled water and shaken well. Then kOH solution was added drop wise to the diluted sample, a sticky layer appeared then dissolve completely, a clear solution obtained. After this volume made to 100 ml with distilled water and 1 gm Zn dust was added to it. Allow it for waited 1 hour. Then 1 ml 5N HCL, 1ml NaNo2(0.2%) were mixed well and added to the previous solution. 1ml of ammonium sulfonate was added along with 1ml of 5% EDTA & 0.1% N NED. Mixture was shaken for 2 min after each addition, a purple colour was developed and then O.D is taken at 550nm.Conc of folic acid was determined from a standard curve.

Measurement of riboflavin

Conc. of Riboflavin in sample was measured by spectroflurimeter method described by [22]. 25 gm sample was dissolved in 50 ml of dist water in a 500 ml of volumetric flask. Then 1 ml glacial acetic acid was added to it. The solution was then mixed with further 5oo ml dist water and stirred until completely dissolved & the volume made upto 1 lit with distilled water and OD was taken in spectrofluorimeter at 524 nm.

Determination of antioxidant activity scavenging of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical

The DPPH radical scavenging activity was evaluated using the method of Liasi et. al. [23]. DPPH radical solution (0.004%, w/v) in 95% ethanol was prepared. A volume of 2 ml of DPPH in ethanol was added to 2 ml of sample, well vortexes and incubated for 30 min in dark room at room temperature. Absorbance of each sample at 517 nm was measured using UV-Visible spectrophotometer (Varian Carry 50 Conc.). Ethanol was used as a blank, while DPPH solution in ethanol served as control. The antioxidant activity was expressed as percentage of DPPH activity calculated as following:

[(Absorbance of blank - absorbance of sample) × 100] / (Absorbance of blank).

Sensory evaluation

Sensorial properties were evaluated by ten panelists familiar with the product after 6hr. storage of the samples at 5°C.

Water holding capacity (WHC)

A sample of about 30 g of native yogurt (NY) was centrifuged for 10 min at 700 Rpm and 20°C. The whey expelled (WE) was removed and weighed.

The WHC expressed in % was defined as: WHC (%) = 100 * (NY – WE)/NY.

Table 1 Analysis of cow milk and buffalo milk sample isolates

Samples	Gram Staining	Catalase test	Hot loop test	Morphology
Cow milk	+ ve	-ve	+ ve	Rod
Fortified cow milk	+ ve	-ve	+ ve	Rod
Buffalo milk	+ ve	-ve	+ ve	Rod
Fortified buffalo milk	+ ve	-ve	+ ve	Rod

Table 2 Sensory evaluation of Fortified cow milk and Buffalo milk samples

Sensory Evaluation	Control milk sample	Fortified cow milk sample						Buffalo Milk sample					
		Tr. 1	Tr. 2	Tr. 3	Tr. 4	TR. 5	Tr. 6	Tr. 1	Tr. 2	Tr. 3	Tr. 4	Tr. 5	Tr. 6
Color		8	8	8	8	8	7	8	8	8	8	8	7
Flavor		7.8	6.3	8.1	6.2	8.3	5.9	8.1	6.9	8.2	6.2	8.6	5.0
Texture		8.8	8.5	8.7	8.9	8.3	6	7.5	7.6	7.8	7.3	8.2	6.5
Mouthfeelnes		7.6	6.8	8.2	6.7	8.2	5.6	8.4	6.2	8.3	6.7	8.7	5.4
Overall Acceptability		8	8	8	8	8	6	8	8	8	7	8	6

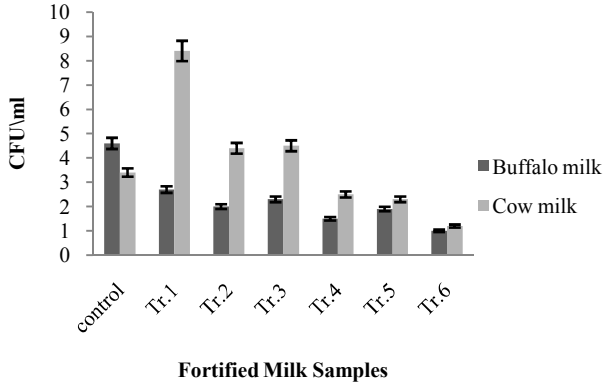

Figure 1 Colony Forming Unit of Fortified Cow milk and Buffalo milk samples

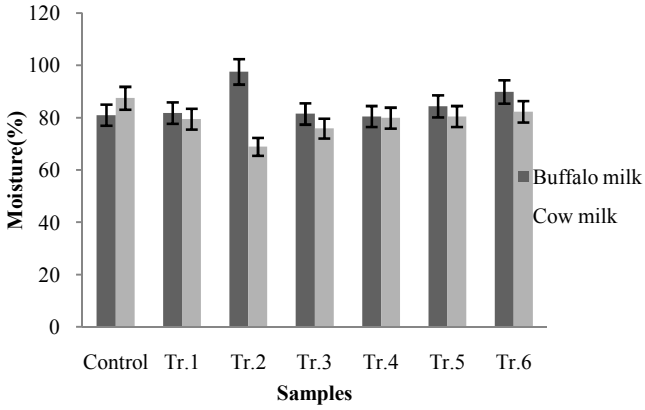

Figure 2 Moisture content of Fortified Cow milk and Buffalo milk samples

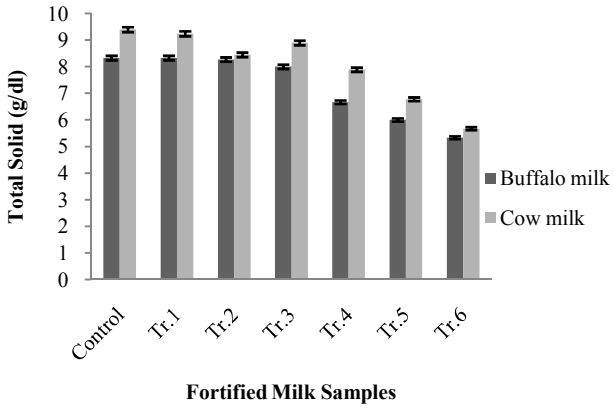

Figure 3 Percentage Total Solid Content of Fortified Cow milk and Buffalo milk samples

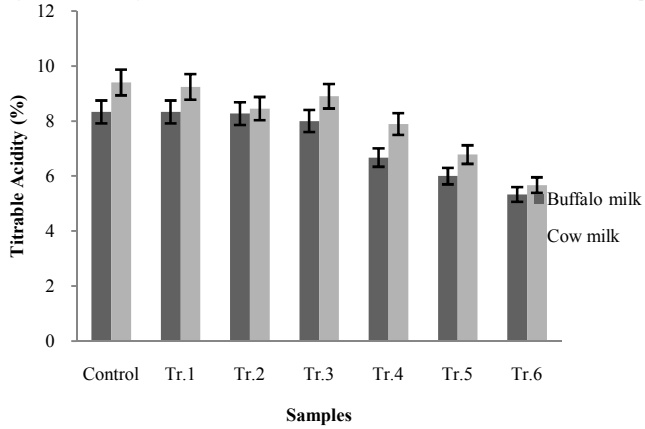

Figure 4 Tritrable Acidity percentage of Fortified Cow milk and Buffalo milk samples

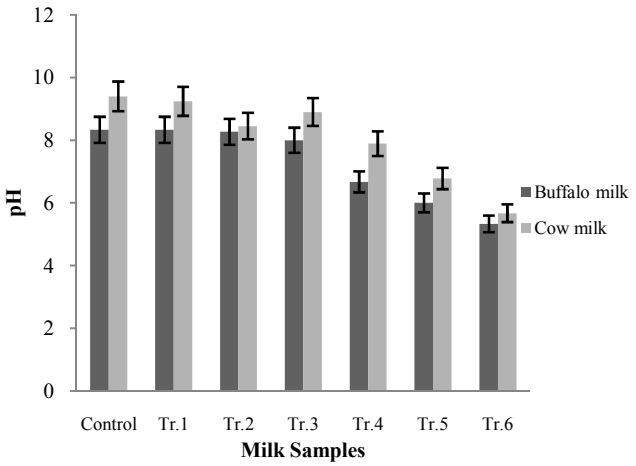

Figure 5 pH content of Fortified Cow milk and Buffalo milk samples

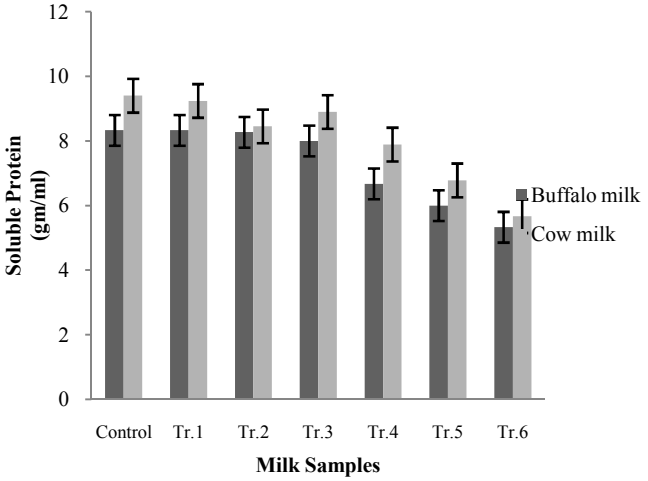

Figure 6 Soluble Protein content of Fortified Cow milk and Buffalo milk samples

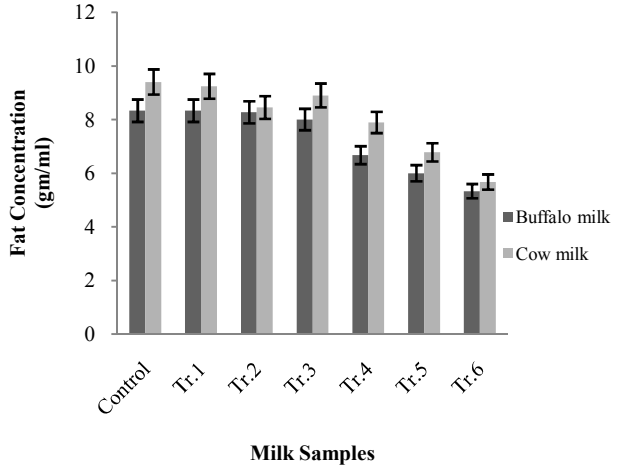

Figure 7 Comparison Percentage of Fat Content between Fortified Cow milk and Buffalo milk samples

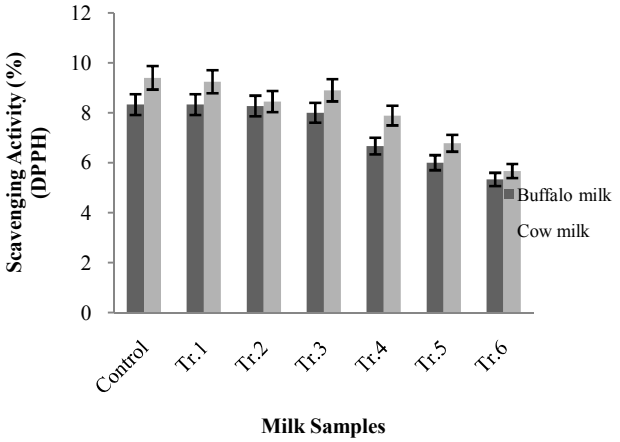

Figure 8 DPPH Scavenging Activity (%) of Fortified Cow milk and Buffalo milk samples

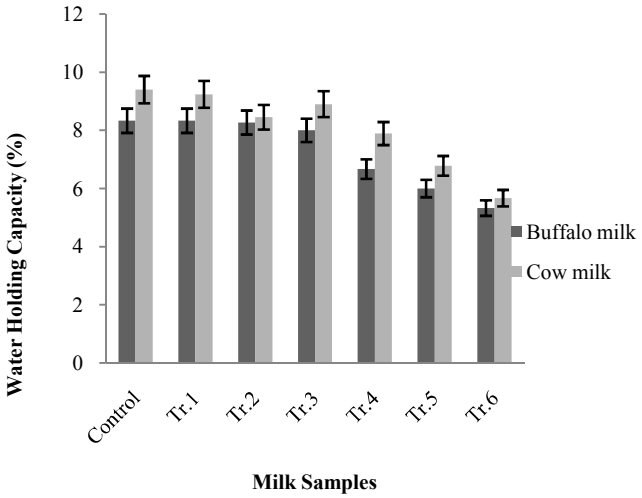

Figure 9 Water Holding Capacity (%) of Fortified Cow milk and Buffalo milk samples

RESULTS AND DISCUSSIONS

Identification of Isolates (Table 1)

The isolates of milk samples were examined under various parameters. All the samples were Gram Positive in character. Catalase is an enzyme, which is produced by microorganisms that live in oxygenated environments to neutralize toxic forms of oxygen metabolites; hydrogen peroxide (H_2O_2). The catalase enzyme neutralizes the bactericidal effects of H_2O_2 and protects them. Anaerobes generally lack the catalase enzyme.

The samples were allowed to hot loop test and all the samples showed positive results.

Morphology of all the microbial samples was in rod shaped. Cow and buffalo milk both samples were serially diluted and were inoculated with MRS Agar medium and the colonies formed were calculated by colony forming unit (CFU) (Table1).

Anaerobic habitats often have low pH and high concentration of organic acids, these conditions can inhibit the growth of many bacteria. So, after fortification of both the cow milk and buffalo milk, growth of microorganisms can be decreased because presence of both citric acid and lactic acid in the samples (Figure 1).

Antibiotic sensitivity assay

In this analysis, buffalo milk showed inhibition zones having diameter of 17 m and 24 m towards Streptomycin discs (S25) and Chloramphenicol discs (C30) respectively that means buffalo milk was antibiotic resistant.

In Cow milk samples no inhibition zone was observed in case of Antibiotic sensitivity assay with Streptomycin discs (C25) and Chloramphenicol discs (C3).

Moisture Content

Moisture content of cow millk was slightly higher than the buffalo milk and it was not changed even after fortification with whay protein isolate (WPC), but with increased concentration of WPC, moisture content was slightly higher in buffalo milk (Figure 2).

Total solid content

The total solids content (TS) of buffalo and cow milk was analyzed, and results are shown in Figure 3. The results of present study revealed that TS content of Buffalo milk was slightly higher than that of Cow milk. The inherent properties of buffalo milk like total solid content, superior whiteness and viscosity render it eminently suitable for the manufacture of traditional (indigenous) milk products like khoa, dahi, paneer etc. Cow milk on the other hand yields a soft coagulum, making it suitable for preparing channa and other channa based products like sandesh, rasagolla, chumchum and rasamalai. Total solids were increased due to addition of calcium citrate and WPC. The amount of increment was relative to the amount of calcium citrate or WPC have been added to each treatment.

Tritrable acidity(%)

Tritrable acidity was found to be higher in cow milk than buffalo milk samples after fortification of calcium citrate and WPC, because after fortification both citric acid and lactic acid was predominantly present in milk , so the tritrable activity was increased. The result was observed from a comparative study between ow milk and buffalo mklk samples (Figure 4).

pH

pH values were slightly varied among fortified samples due to the buffering effect of both Ca citrate and WPC which were added to the samples, however, control had significantly ($P \leq 0.005$) lower pH. The milk sample was acidic due to presence of lactic acid fortified with calcium (Figure 5).

Protein content

Buffalo milk is rich in proteins when compared to cow milk, and the difference is about 10–11% which is more heat resistant. The proteins present in buffalo milk make it difficult for infants and older people to digest it. Based solely on the protein content, the obvious choice would be cow's milk (Figure 6).

Fat content

The most prominent difference between cow's and buffalo's milk is the fat. It makes the consistency of milk differ. We know that a fatty foodstuff takes time for digestion and absorption and it stays in the stomach for a longer time giving us a feeling of heavy stomach or stomach fullness similarly buffalo milk has high fat and therefore it takes much more

time for digestion. As a result cow's milk is suggested over buffalo's milk particularly for infants and elders.

Buffalo milk contains cholesterol therefore several individuals who are suffering from obesity, kidney diseases, diabetes, PCOD, dys-lipidaemia, hypertension etc. are recommended to have buffalo's milk. Due to high peroxidase activity, buffalo milk can be well-preserved naturally for a longer period of time. Buffalo milk comprises more calcium to phosphorous ratio and less sodium and potassium than in cow milk which makes it a better nutritional supplement for infants (Figure 7).

Antioxidant activity

DPPH scavenging activity

The DPPH assay method is the most sensitive method giving the highest antioxidant activities that would indicate milk samples had more hydrogen donating ability of antioxidant to radicals or the sample contained peptides acting as electron donors and could react with free radicals to form more stable products. The ABTS assay gives the lowest antioxidant activities probably because milk had less antioxidant compounds to reduce ABTS. In general, different results of antioxidant assays were observed probably because of relative differences in the ability of antioxidant compounds in the milk extract to quench aqueous peroxyl radicals and to reduce ABTS+ (2,2-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid)), the DPPH free radical and ferric iron in the in vitro system. After Fortifications antioxidant activity was increased (Figure 8).

Water holding capacity (WHC)

Buffalo milk had higher WHC than cow milk. WHC significantly ($P \leq 0.005$) increased from control buffalo milk sample to fortified milk samples. Treatments 2, 4 and 6 had higher WHC than treatments 1, 2 and 3. As the added amount of Ca citrate or WPC increased, the WHC increased. The water-binding and emulsification properties of WPC are useful in adding lubricity and good mouth feel. In addition, Ca of Ca citrate play a principle role in forming a colloidal network that can trapping the water in the matrix of Yogurt body. It was found that fortification of pasteurized yogurt mixed with 50 mg Ca/100 ml of calcium lactate, significantly ($P \leq 0.005$) increased the water holding capacity (WHC) by 7.76 % on 1st day of storage (Figure 9).

Sensory Evalution

Sensory evaluation of the milk samples were done by Hedonic rating scale.

Here, high score for appearance and texture have been gained by most of the prepared Yogurt samples. Control sample and fortified milk samples i.e., 1, 2, 3, 4 and 5 gained 80-90% of full score, while treatment 6 had comparatively lower score. These results led to the conclusion that the added amounts of Ca citrate or WPC have no definite effect on appearance or texture as it evaluated organoleptically. Flavor and mouth feel of prepared yogurt showed clear differences between treatments. Control sample and fortified samples 1, 2 and 3 gained 80% or more from the full score of flavor. Then it was gradually decreased from samples 4 to 6 where it had the minimum score (5.33 points). Treatments 2, 4 and 6 got lower flavor score than those got by treatments 1, 3 and 5. Worthwhile to report here that Ca citrate is odorless and it has a slight sour taste. The added amounts of Ca citrate to treatments 2, 4 and 6 were the duplicate amounts added to treatments 1, 3 and 5. It seems that the sour taste of Ca citrate made the tender acid taste of Yogurt got sharpen, consequently inversely affected the perception, flavor and mouth feel of treatments 2, 4 and 6. The other factor that strongly affected flavor and mouth feel of prepared Yogurt is the addition of WPC. It is well known that supplementation of some dairy products such as ice cream, Yogurt and milk-based beverages smoothen the texture and improve mouthfeelness.

CONCLUSION

Milk and milk products provide a convenient and useful vehicle for fortification with micronutrients. The risks associated with fortification are minimal except if good manufacturing practices are not followed and only isolated incidents of this type have ever been reported.

Water-holding properties of a protein are the results of a broad array of factors governing protein-water interaction in food systems. Although there are several theories describing water-holding characteristics of proteins, it has not been possible to define exactly the term 'water-holding'. Thus, it is useful to use terms such as 'bound', 'free', or 'structural' water specifically in context with the measuring technique and the environmental conditions employed. There are many methods available that pro-vide information on the mechanisms of hydration and water holding.

In this study we have concluded that all the samples were Gram positive in character. Buffalo milk showed antioxidant resistant activity. Buffalo milk content higher concentration of all the nutrients samples than Cow milk. After Fortification both the milk samples were more beneficial for human health because of their increasing nutrient content, flavor, aroma, and increasing bioavailability.

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