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Effect of calcium and vitamin D_2 fortification on physical, microbial, rheological and sensory characteristics of yoghurt

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Abstract

The effect of calcium and vitamin D_2 fortification on yoghurt was evaluated. Two levels (500 and 600 ppm) of calcium (Calcium phosphate and calcium citrate) along with vitamin D¬2 (600IU/L) were used to fortify milk for yoghurt preparation. Acidity, pH, water holding capacity and syneresis of fortified yoghurt were not affected significantly ($p>0.05$) in comparison to control yoghurt. However, acetaldehyde content decreased and setting time increased upon fortification of yoghurt. Microbial growth significantly ($p<0.05$) decreased in calcium phosphate fortified yoghurt, whereas calcium citrate fortified yoghurt showed no difference to control yoghurt. Firmness and viscosity decreased in calcium phosphate fortified yoghurt, whereas it increased in calcium citrate fortified yoghurt as compared to control. Rheological characteristics reveled that calcium phosphate fortified yoghurt showed higher shear thinning effect, whereas calcium citrate fortified showed less shear thinning effect in comparison to control.

Introduction

Yoghurt is a cultured dairy product with excellent sensory properties that is widely consumed as a healthy and nutritious food. Chemically, yoghurt is a complex gel system incorporating milk constituents in its structure with no signs of syneresis. Estrada *et al*. (2011) defined yoghurt as a coagulated milk product made from heat treated milk and fermented using lactic acid bacteria (LAB) containing *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. In yoghurt, the LAB must be alive and in substantial amounts. Yoghurt has gained wide popularity especially among women, children and teenagers who consume yoghurt as a part of their daily diet. The healthy image associated with yoghurt and other dairy products has led to increase in its consumption.

Calcium and vitamin D both are recognized as key nutrients in promoting bone health. Dietary calcium deficiency has been linked epidemiologically to several chronic diseases including osteoporosis, osteomalacia, hypertension, colon cancer and obesity (Kaushik *et al*., 2014). Vitamin D is a group of sterol compounds that has major role in the matrix of cartilage and bone. Vitamin D has long been known to play an important role in bone development by promoting calcium absorption in the gut and bone mineralization (Bilodeau *et al*., 2011).

Yoghurt represents the excellent source of calcium, therefore, it is believed that fortification of yoghurt with calcium would meet the daily intake of individuals at risk for calcium deficiency related diseases and provide extra calcium to meet their daily requirements in one or more serving (Pirkul *et al*., 1997). Till date, studies on single micronutrient fortification of yoghurt have been reported. No work has been reported on feasibility and technological aspects of multiple nutrient i.e. vitamin D and calcium fortification. Supplementation studies that have increased dietary vitamin D intakes have been found to reduce rates of fracture when the vitamin D is complemented with calcium supplements (Boonen *et al*., 2007).

Multiple micronutrient fortification can be more effective in improving nutritional status than fortification with a single key micronutrient. The multiple micronutrient fortification of appropriate food vectors, including milk and milk products, is of interest from the nutritional standpoint. Therefore, objective of present study was to develop calcium and vitamin D fortified yoghurt and determine effect of fortification on sensory, physico-chemical properties, microbiological, flavor production, textural and rheological properties of yoghurt.

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Materials and Methods

Materials

Cow and buffalo milk were collected from cattle yard of National Dairy Research Institute (Karnal, India). Bacterial starter culture [NCDC 074 (Streptococcus thermophilus) and NCDC 009 (Lactobacillus delbrueckii subsp. bulgaricus)] used for preparation of yoghurt were obtained from National Collection of Dairy Cultures (NCDC), National Dairy Research Institute, Karnal, Haryana, India. The viable count in the inoculum was 367 and 329 log CFU/g for NCDC 074 and NCDC 009, respectively. Encapsulated vitamin D_2 (100,000 IU/g) was procured from DSM Nutritional Products (Singapore). Salts used for calcium fortification of milk were calcium phosphate dibasic and calcium citrate tetrahydrate, procured from Himedia Chemicals Limited, India

Fortification of milk

Cow milk and buffalo milk were mixed in 1:1 ratio and toned milk was prepared by mixing whole milk, skim milk and water. The fat and solid non-fat (SNF) were adjusted to 3.0 and 8.5%, respectively using Pearson square method. Calcium content was calculated based on their respective molecular weight of the salts used and were added to milk at levels of 500 and 600 ppm in milk (45°C) accompanied by thorough mixing for complete dispersion of salts. Vitamin D_2 concentrated solution was prepared in water and added to milk for attaining the level of 600 IU/L to milk. Fortified raw milk samples were evaluated for heat stability. The control and multiple fortified milk samples were used for preparation of yoghurt.

Yoghurt preparation

Yoghurt was prepared from unfortified and fortified milk samples by the method of Rasic and Kurman (1978). Calcium salts and vitamin D_2 were added to milk and it was heated to 90°C for 20 minutes. Milk was then cooled at 45ºC and 1.25% by weight Streptococcus thermophilus and 1.25% by weight *Lactobacillus bulgaricus* starter culture were added and incubated at 42ºC for 5-6 h. After the formation of a firm coagulum, yoghurt was cooled to 2-4ºC and stored under refrigeration conditions (4-7ºC).

Setting time

Setting time of sample was recorded from the time of inoculation to the coagulum formation. The time of setting for the yoghurt samples was recorded in hours.

pH and titratable acidity

pH of yoghurt samples was estimated using digital pH meter (LabIndia, India). Titratable acidity of the yoghurt samples was estimated by the method as described in Kaushik *et al*. (2016). Approximately 20 g yoghurt sample was weighed in a conical flask and diluted with twice its volume with double distilled water. 2 ml of phenolphthalein indicator was added and the contents were titrated with 0.1 N NaOH to persistent pink color. Acidity was reported as % lactic acid by weight.

Acidity (% lactic acid) = Volume of NaOH consume, Whereas,

1 ml 0.1 N NaOH = 0.009 g lactic acid

Spontaneous syneresis and Water holding capacity

Spontaneous syneresis and water holding capacity of set yoghurt was determined using method reported by Remeuf *et al*. (2003).

Viscosity

Viscosity of yoghurt samples (20°C) was determined using rotational viscometer (Visco star plus L Model, Fungilab Spain) which is an open, concentric measurement system and allows measurement by immersion. The measuring head and measuring tube were rigidly coupled; the measuring unit was driven by a DC motor. Viscometer was fitted with spindle TL-7 and viscosity was determined at three rpm 10, 20 and 30. A built-in microprocessor calculated the values for the viscosity (centipoise) with the aid of the measured torque, the set shear rate and the measurement system was used.

Firmness of Yoghurt

The firmness of yoghurt was determined by using TA-X T2 (Stable Micro System, UK). Texture expert exceed fitted with 5 Kg load cell. For this Yoghurt samples were prepared in of glass beaker of 100 ml and samples were then transferred to immersion chamber maintained at 25ºC before the analysis. The samples were subjected to mono-axial compression of 25 mm distance on the texture expert exceed by the crosshead speed of 1 mm/sec. After the completion of analysis a graph was obtained with force experienced by probe on Y-axis and time on X-axis. The firmness of yoghurt sample was estimated as the height of positive peak force up to rupture point.

Viable cell count by standard plate count method

This method is used for determining the total

Table 1. Effect of milk fortification on physicochemical and microbial properties of yoghurt

Yoghurt samples	pH	Acidity (% lactic acid)	Water Holding Capacity (%)	Syneresis (%)	Acetaldehyde (mg/g)	Setting time (h)	*Strep. thermophilus* (10^8cfu/ml)	*Lb. bulgaricus* (10^8cfu/ml)
Unfortified (control)	4.40±0.02^A	0.88±0.012^A	77.99±0.54^A	2.17±1.11^A	1.375±0.11^A	4.00±0.00^A	65.67±1.20^A	54.00±1.15^{AB}
CaP500+VD 600	4.39±0.02^A	0.88±0.013^A	77.76±0.62^A	2.17±1.10^A	1.356±0.08^A	4.50±0.00^A	63.33±2.03^{AB}	52.67±0.88^B
CaP600+VD 600	4.39±0.02^A	0.88±0.014^A	77.63±0.68^A	2.18±1.11^A	1.350±0.14^A	4.50±0.00^A	62.67±1.20^B	52.33±0.88^B
CaC500+VD 600	4.40±0.01^A	0.87±0.012^A	77.86±0.60^A	2.17±1.13^A	1.365±0.17^A	4.00±0.00^A	65.33±0.88^{AB}	54.33±0.67^{AB}
CaC600+VD 600	4.41±0.01^A	0.87±0.012^A	77.93±0.55^A	2.17±1.11^A	1.367± 0.09^A	4.00±0.00^A	64.00±1.53^{AB}	55.67±0.88^A

Data are presented as means±SEM (n=3). ^{AB}Means within columns with different uppercase superscript are significantly different ($p<0.05$) from each other. CaP500+VD600: calcium phosphate (@500 ppm calcium) and Vitamin D2 (600 IU/L) fortified yoghurt. CaP600+VD600: calcium phosphate (@600 ppm calcium) and Vitamin D2 (600 IU/L) fortified yoghurt. CaC500+VD600: calcium citrate (@500 ppm calcium) and Vitamin D2 (600 IU/L) fortified yoghurt. CaC600+VD600: calcium citrate (@600 ppm calcium) and Vitamin D2 (600 IU/L) fortified yoghurt

number of viable bacteria in yoghurt and consists of mixing appropriate quantity of yoghurt with a suitable nutrient agar medium in a petri dish and counting the bacterial colonies developed after incubation at a specified temperature for a definite period of time (BIS, 1981).

Acetaldehyde content

Acetaldehyde is the principal flavour component in yoghurt produced by the microbial action on milk components. Acetaldehyde content was determined in yoghurt made from control and fortified milks using Megazyme assay kit (Wicklow, Ireland).

Flow behavior

Flow behavior property of yoghurt was reporting using rheometer (Anton paar, Modular compact rheometer, MCR 52, India) with a plate and plate geometry (CP 75-1-SN23957) having 0.149 mm gap setting and at 25ºC constant temperature. Flow curves were prepared at variable shear rate (0 to 60 s^{-1}). The delay time and integration were both set at 6 sec. The data obtained were fitted to power law equation

$$\text{Shear stress} = K \times (\text{Shear rate})^n$$

Where,

K = Consistency index

n = Power law index. The value of n expresses the flow behavior as Newtonian (n is close to 1) or non-Newtonian (n is far from 1).

Apparent viscosity was measured at a constant shear rate of 100 s^{-1} for about 100 s. This shear rate lies in the linear portion of flow curve. The difference in initial apparent viscosity (η_0) and final apparent viscosity (η_e) was calculated using the following formula:

$$\% \text{ Lost structure} = [(\eta_0 - \eta_e)/ \eta_0] \times 100$$

The % lost structure is a measure of the rate of thixotropic breakdown, meanwhile the ratio of the initial to final apparent viscosity, (η_0/η_e), can be considered as relative measure of the extent of thixotropic.

Sensory analysis

Sensory evaluation was carried out using the composite sensory score card (Kaushik *et al*., 2015). Parameters were color and appearance, odor, taste and mouthfeel. Ten trained sensory panelists (Scientists, National Dairy Research Institute, Karnal), judged the fortified milk on the basis of color and appearance having maximum score 10, odor having maximum score of 20, taste having maximum score of 40 and mouthfeel having maximum score of 30. According to defects, if any, the scores were reduced.

Statistical analysis

Means (n=3), standard error mean (SEM), linear regression analysis and 95% confidence intervals were calculated using Microsoft Excel 2007 (Microsoft Corp., Redmond, WA). Data were subjected to a single way analysis of variance (ANOVA) to calculate CD value.

Table 2. Effect of milk fortification on firmness and viscosity of yoghurt

Yoghurt samples	Textural characteristics: Firmness (N)	Work of adhesion (N.s)	Work of Shear (N.s)	Stickiness (N)	Viscosity (cP): 10 rpm	20 rpm	30 rpm
Unfortified (control)	1.942±0.03^{AB}	-1.772±0.047^{A}	15.565±0.524^{A}	-0.281±0.016^{AB}	3196.43±97.62^{A}	1483.80±40.14^{A}	1187.03±8.92^{A}
CaP500+VD 600	1.921±0.02^{A}	-1.766±0.052^{AB}	15.508±0.602^{A}	-0.292±0.016^{A}	3138.53±86.40^{A}	1455.33±30.96^{A}	1171.80±6.61^{A}
CaP600+VD 600	1.919±0.02^{A}	-1.753±0.024^{AB}	15.486±0.478^{A}	-0.298±0.016^{A}	3115.67±86.52^{A}	1441.37±27.54^{A}	1150.70±22.80^{A}
CaC500+VD 600	1.999±0.03^{B}	-1.747±0.038^{AB}	15.649±0.345^{A}	-0.273±0.016^{B}	3271.80±120.91^{A}	1504.13±35.89^{A}	1225.77±26.37^{A}
CaC600+VD 600	2.032±0.04^{B}	-1.731±0.062^{B}	15.682±0.297^{A}	-0.261±0.016^{B}	3341.00±121.56^{A}	1556.33±34.47^{A}	1215.67±19.72^{A}

Data are presented as means±SEM (n=3). ^{AB}Means within columns with different uppercase superscript are significantly different (p<0.05) from each other.

Table 3. Rheological characteristics of yoghurt samples

Rheological parameters	Power law model		Apparent viscosity/time (Pa/s)			
Sample	K	n	η_0	η_r	η_0/η_r	% Loss structure
Unfortified (control)	243.58±23.4^{A}	0.28±0.04^{A}	24.83±1.21^{A}	1.28±0.09^{A}	23.55	94.84
CaP500+VD 600	271.62±28.3^{A}	0.36±0.02^{A}	23.67±0.74^{A}	1.09±0.14^{A}	22.58	95.40
CaP600+VD 600	271.49±18.7^{A}	0.37±0.05^{A}	23.50±0.96^{A}	1.06±0.11^{A}	22.44	95.48
CaC500+VD 600	253.50±19.4^{A}	0.30±0.02^{A}	24.83±1.34^{A}	1.28±0.07^{A}	23.55	94.84
CaC600+VD 600	269.20±15.8^{A}	0.30±0.03^{A}	25.83±1.42^{A}	1.31±0.12^{A}	24.52	94.93

Data are presented as means±SEM (n=3). ^{AB}Means within columns with different uppercase superscript are significantly different (p<0.05) from each other.

Results and Discussion

Effect of calcium and vitamin D_2 fortification on yoghurt quality parameters were determined. Yoghurt was prepared using both calcium phosphate and calcium citrate at two different concentrations viz. 500 and 600 ppm calcium and vitamin D_2 (600 IU/L).

Effect of milk fortification on pH, acidity, water holding capacity and syneresis of yoghurt

Appropriate acid concentration is one of the important factors ensuring quality of fermented milk. There was slight but statistically non-significant (p>0.05) difference in pH, acidity, water holding capacity and syneresis in yoghurt made from control and fortified milk (Table 1). Yazici *et al*. (1997) reported that initial pH values of calcium-fortified soy milk was higher than control soy milk due to pH change during calcium fortification. He further reported that the pH reduction was considerably slower in calcium-fortified soy milk yoghurts than in control yoghurts. However, calcium-fortified soy milk yoghurts exhibited significantly higher titratable acidity values than control soy milk yoghurts at a given pH, indicated substantial buffering capacity of the added calcium salts. Calcium fortified yoghurt inoculated at 2.5% level required 8 h to reduce pH to the 4.4–4.8 range. Therefore, inoculation rate for the calcium fortified yoghurt was increased to 5%. Control yoghurt (2.5% inoculation rate) and calcium fortified yoghurt samples (5% inoculation rate) required 4 to 5 h to reach the pH 4.4–4.8 range, respectively. Cavallini and Rossi (2009) reported that initial titratable acidity of control soy yoghurt (0.87±0.01% lactic acid) was higher than the fortified product (0.85±0.01% lactic acid). He further reported that calcium citrate fortified soy yoghurt takes slightly longer time to reduce pH to the 4.4 to 4.5 range, respectively, indicating that starter culture were capable of producing enough acid in a suitable time period and that fortification does not make the fermentation process unviable. Umeda and Aoki (2002) and Tsioulpas *et al*. (2007) reported distribution of these calcium forms has a major influence on the structural stability and functionality of the milk proteins. Heat treatment of milk, changes in pH, and addition of calcium salts and chelating agents alter the distribution of calcium and thereby affecting the stability of the casein system during milk processing.

Caskun and Senoglu (2011) reported that lactic acid content of yoghurt decreased with an increase in amount of calcium carbonate in yoghurt. He suggested that the calcium carbonate affected the growth of lactic acid bacteria. Yousef and Rusli (1995) reported that calcium fortification extended the incubation period of yoghurt as the growth of *S. thermophilus* slowed down in presence of calcium. Accordingly, growth of *S. thermophilus* is inhibited at a certain level by the effect of gluconic acid released during yoghurt formation in the media. However, Lb. bulgaricus was not affected by calcium enrichment. Singh *et al*. (2005); Singh and Muthukumarappan (2008) observed higher water holding capacity for fortified yoghurt in comparison to control yoghurt samples. However, Flinger *et al*. (1988) observed that yoghurt, enriched with calcium salts of gluconate, saccharate and citrate produced a weak body and the mix required longer time to set. According to him inhibition of starter culture might have contributed to these defects. Ranjan *et al*. (2006); Singh *et al*. (2005) also reported that firmness of yoghurt decreased due to enrichment with calcium salts. Increase or decrease in gel strength of calcium fortified yoghurt depends upon the intrinsic properties of calcium salts used for enrichment. Calcium fortified soy yoghurt showed more syneresis in comparison to control soy yoghurt (Yousef and Rusli, 1995).

Effect of milk fortification on acetaldehyde content, setting time and Streptococcus thermophilus and Lactobacillus bulgaricus count of yoghurt

The compounds which impart distinctive flavor to yoghurt are lactic acid and a variety of volatile organic aroma compounds, produced by Streptococcus thermophilus and *Lactobacillus bulgaricus* (Beshkova *et al*., 1998). Gallardo-Escamilla *et al*. (2005) reported that the volatile compounds such as acetaldehyde and diacetyl were the key compounds for typical yoghurt aroma. Acetaldehyde is recognized as a major flavor component in yoghurt (Law and Kolstad, 1983).

There was a slight but statistically non-significant difference ($p>0.05$) in acetaldehyde content and setting time of yoghurt samples made from control and fortified milk (Table 1). Acetaldehyde content of fortified samples was lower than control samples. Control sample took slightly less time to set and possessed a firm coagulum as compared to fortified samples. The results revealed that there was an increase in setting time on addition of calcium salt to milk, however these differences were non-significant ($p>0.05$).

S. thermophilus count of yoghurt prepared from calcium phosphate (600 ppm calcium) differed significantly ($p<0.05$) from control yoghurt, whereas, calcium phosphate (500 ppm calcium) and calcium citrate (500 and 600 ppm calcium) fortified yoghurt samples showed no significant difference ($p>0.05$) from control yoghurt. Lb. bulgaricus count of yoghurt prepared from calcium phosphate (500 and 600 ppm calcium) fortified yoghurt samples showed significant difference ($p<0.05$) with control yoghurt, whereas, calcium citrate (500 and 600 ppm calcium) fortified yoghurt samples had no significant difference ($p>0.05$) to control yoghurt.

The acetaldehyde content of yoghurt prepared using different strains was in the range of 1 to 13 ppm (Lees and Jago, 1978). The acetaldehyde content of yoghurt prepared using two strains of yoghurt was estimated and found that the acetaldehyde content ranged between 0.410 to 1.7342 and 0.285 to 1.506 mg/g of yoghurt, respectively. Microbial counts lowered significantly when the pH was 4.3 or lower (Lankaputhra *et al*. 1996). In our case product pH was around 4.4, possibly explaining the high microbial counts between 52 to 66 × 106 CFU/g. Pirkul *et al*. (1997) reported that calcium lactate fortification increased the counts of lactobacilli. Statistically significant differences in the ratios of cocci to rods were observed only between calcium lactate and calcium gluconate fortified yoghurt during storage. Fortification with calcium gluconate increased the ratios of cocci to rods in yoghurt. Cueva and Aryana (2008) determine the microbial count of yoghurt during storage. The storage time significantly affected the microbial counts, at day 7 microbial counts were significantly higher than the microbial counts at the 3rd and 5th weeks.

Greater decrease in viable cell counts, mainly *S. thermophilus* was found in fortified soy yoghurt during storage, which indicated that calcium addition may change the viability of this culture. The viable population of *S. thermophilus* reduced by 0.13 and 0.38 log CFU/g in control and calcium fortified soy yoghurt, respectively, after 28 days of storage at 10°C. A population reduction of Lb. bulgaricus in the control and fortified yoghurt was 0.29 and 0.33 log CFU/g, respectively (Cavallini and Rossi, 2009). Yousef and Rusli (1995) demonstrated that *S. thermophilus* growth was inhibited more noticeably than Lb. bulgaricus during production of calcium fortified yoghurt. They further reported that control soy yoghurt showed higher viable cell counts in comparison to calcium fortified yoghurt.

Bringe and Kinsella (1993) reported that calcium fortification retard acid aggregation of protein particles in casein systems. Gluconic acid, which can be liberated during fermentation of the calcium fortified yoghurt, inhibited the growth of *S. thermophilus* but not Lb. bulgaricus. Extent of inhibition depended on the initial pH of the medium. In calcium fortified yoghurt made with 1.5% culture, growth of Lb. bulgaricus was not affected noticeably; however, the growth of *S. thermophilus* was delayed. When the calcium fortified mix was inoculated with starter culture at a 4% rather than 1.5% level, rate of lactic acid production increased whereas the incubation time decreased. A starter culture, modified to contain *S. thermophilus* and Lb. bulgaricus at 100:1 cell ratio produced a calcium fortified yoghurt which compared favorably (in physical and organoleptic properties) with the non-fortified yoghurt. El-Shenawy and Marth (1990) reported that gluconic acid showed a bacteriostatic effect on Listeria monocytogenes. Gluconic and other organic acids may be liberated from calcium salts when pH of milk decreased during yoghurt manufacture.

Pirkul *et al*. (1997) reported setting time of yoghurt at 42±1°C as 3-4 h, whereas 4-5 h was reported as setting time by Fiszman *et al*. (1999) and Cueva and Aryana (2008). Hashim, *et al*. (2009) reported 4 h setting time at 43°C for yoghurt. Yazici *et al*. (1997) reported that the pH reduction was

Table 4. Sensory analysis scores of yoghurt made from unfortified (control) and calcium (500 and 600 ppm) + vitamin D_2 (600IU/L) fortified milk

Characteristics	Maximum scores	Unfortified (Control)	CaP500+ VD600	CaP600+ VD600	CaC500+ VD600	CaC600+ VD600
Flavour	45	40.33±0.88^A	38.33±0.88^A	37.66±0.67^A	40.00±0.58^A	39.00±0.58^A
Body and texture	30	27.50±0.28^A	27.67±0.33^A	27.33±0.67^A	28.00±0.58^A	27.83±0.60^A
Acidity	10	8.33±0.33^A	8.67±0.33^A	8.00±0.58^A	7.83±0.17^A	7.66±0.17^A
Colour and appearance	10	9.00±0.00^A	9.00±0.00^A	9.00±0.00^A	9.00±0.00^A	9.00±0.00^A
Container and closure	5	5.00±0.00^A	5.00±0.00^A	5.00±0.00^A	5.00±0.00^A	5.00±0.00^A
Total score	100	90.16±1.01^A	88.67±1.45^A	87.00±1.52^A	89.83±0.44^A	88.5±0.76^A

Data are presented as means±SEM (n=30). ^{AB}Means within rows with different uppercase superscript are significantly different (p<0.05) from each other.

considerably slower in calcium-fortified soy milk yoghurts than in control yoghurts. Yousef and Rusli (1995) demonstrated that *S. thermophilus* growth was inhibited more noticeably than Lb. bulgaricus during production of calcium fortified yoghurt. Calcium fortification reduced the microbial growth in yoghurt, therefore, longer time was required for gel setting.

Effect of milk fortification on texture profile of yoghurt

It is evident from Table 2 that control and fortified yoghurt samples showed non-significant difference (p>0.05) in firmness and stickiness, whereas a significant difference (p<0.05) was observed between calcium phosphate and calcium citrate fortified yoghurt. The firmness of yoghurt decreased with the addition of calcium phosphate and increased with the addition of calcium citrate. Increase or decrease in gel strength of calcium fortified yoghurt depends upon the intrinsic properties of calcium salts used for enrichment. In terms of work of adhesion, all fortified samples showed non-significant difference (p>0.05) from control yoghurt except yoghurt fortified with CaC600+VD600.

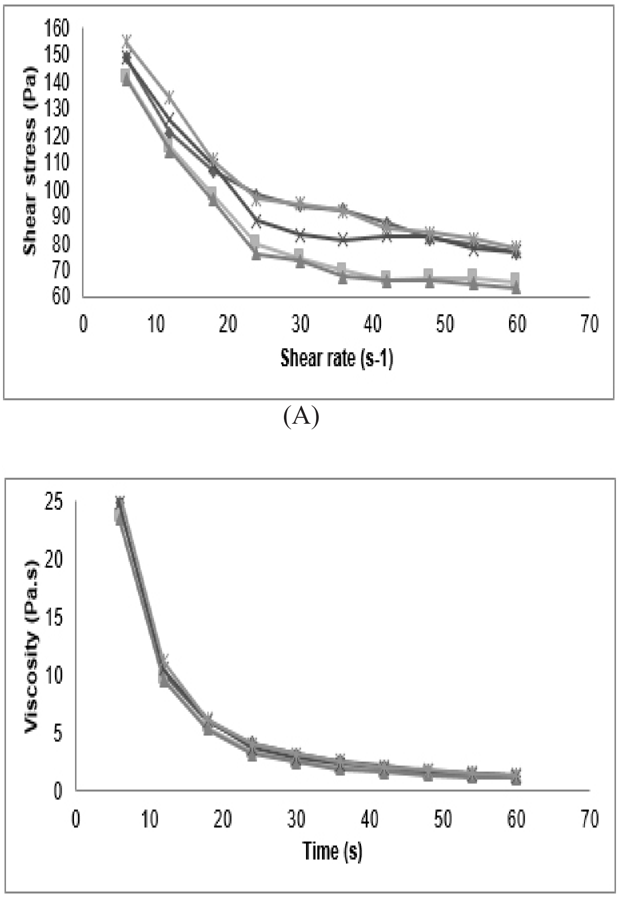
Soy yoghurt fortified with calcium reduced the gel strength but not springiness or cohesiveness. Reduction in gel strength may be a result of the higher ionic strength in the calcium-fortified soy milk which may shield electrostatic interactions at the isoelectric point. Bringe and Kinsella (1993) reported retardation by calcium in acid aggregation of protein particles in casein systems. Gluconic acid, which is liberated during fermentation of the calcium fortified yoghurt, inhibited the growth of *S. thermophilus* but not Lb. bulgaricus. Extent of inhibition depended on the initial pH of the medium. In calcium fortified yoghurt made with 1.5% inoculums, the growth of Lb. bulgaricus was not affected noticeably; however, the growth of *S. thermophilus* was delayed. When the calcium fortified mix was inoculated with starter culture at a 4% rather than 1.5% level, rate of lactic acid production increased and the incubation time decreased. A starter culture, modified to contain *S. thermophilus* and Lb. bulgaricus at 100:1 cell ratio produced a calcium fortified yoghurt which compared favorably (in physical and organoleptic properties) with the non-fortified yoghurt. El-Shenawy and Marth (1990) reported that gluconic and other organic acids may be liberated from calcium salts when pH of milk decreased during yoghurt manufacture. Cavallini and Rossi (2009) reported that addition of calcium citrate (600 ppm) to soy yoghurt did not have any notable effect on consistency.

Calcium ions favor protein–protein interactions of both whey protein and casein, through electrostatic shielding and ion-specific hydrophobic interactions (Cayot and Lorient, 1998; O'Kennedy and Kelly, 2000). In gels obtained from calcium caseinate enriched milk base, calcium bridges could further contribute to a notable increase of the cross linking density of the network. They could also sterically restrain coalescence of the protein particles, leading to a fine stranded gel structure (Remeuf *et al*., 2003).

Flinger *et al*. (1988) observed that in yoghurt enriched with calcium gluconate, calcium saccharate and calcium citrate produced a weak body and the mix required a longer time to set. According to him inhibition of starter culture might have contributed to these defects. Firmness intensities increased significantly with an increase in calcium levels (Hashim *et al*., 2009). The smaller number of junction points and much more open structure in the samples of plain yoghurt contributes to lower firmness and proneness to syneresis. Penetration curve of the plain yoghurts indicated a very low value for resistance to penetration without any measurable value for strain prior to penetration (Fiszman *et al*., 1999).

Apparent viscosity

The yoghurt gel structure is a network of the milk proteins, caseins, and whey proteins formed during



(A)

(B)

Figure 1A and B. Flow curve of control and fortified yoghurt. Parallelograms indicate unfortified (control), rectangles indicate calcium phosphate (500 ppm calcium), triangles indicate calcium phosphate (600 ppm calcium), double cross indicates calcium citrate (500 ppm) and triple cross indicates calcium citrate (600 ppm) fortified yoghurt samples.

acid gelation. The gel formation is driven by changes in the calcium equilibrium in the milk and resulting hydrophobic interactions between the milk proteins (Lucey, 2002). Viscosity of yoghurt prepared from calcium phosphate fortified milk sample was slightly lower than the control yoghurt, whereas, viscosity of yoghurt prepared from calcium citrate fortified milk sample was slightly higher than the control yoghurt. At 10, 20 and 30 rpm, viscosity of all samples showed statistically non-significant difference ($p>0.05$) from each other (Table 4). From the Tables 4, it can be inferred that calcium phosphate decreased microbial growth, whereas calcium citrate increased microbial growth, which in terms affected the gel formation and viscosity in yoghurt. Increased microbial growth observed in calcium citrate fortified yoghurt might be due to utilization of citrate by lactic acid bacteria for energy metabolism. Narvhus and Gadaga (2003) reported that lactic acid bacteria utilize citrate for their metabolism. Bartowsky and Henschke (2004) reported that citrate increased microbial growth and with the addition of milk with citrate, the synthesis of diacetyl enhanced. In lactic acid bacteria, theoretically, 1 mole of citrate produces 1 mole of acetic acid, 2 mole of carbon dioxide and 0.5 mole of (diacetyl + acetoin and 2,3-butanediol) and 12 mole of ATP (Ramos and Santos, 1996).

Addition of ions (as sulfates or chlorides) into aqueous gelatin solutions (1–10%) at 0.15–0.50 mole/L increased viscosity of the solutions, gel strength and decreased the time of gelation (Kozlov *et al*., 1984). Coskun and Senoglu (2011) reported that as the level of calcium carbonate, increased water holding capacity of yoghurt increased and found that calcium fortified yoghurt showed higher viscosity than control yoghurt. This is due to the fact that an increased $CaCO_3$ content increased formation of calcium phosphate cross-links within casein micelles. Singh and Muthukumarappan (2008) prepared calcium lactate enriched fruit yoghurt after fortification of pasteurized yoghurt mix with 500 ppm calcium. Measurements performed on slowly stirred samples (flow curves and final apparent viscosity) showed that calcium enriched fruit yoghurt had stronger structures. Firmness of the calcium fortified fruit yoghurt was attributed to higher extent of colloidal calcium phosphate cross-linking between casein micelles due to increased calcium content by fortification. Ramasubramanian *et al*. (2008) reported that viscosity of yoghurt decreased with addition of calcium.

Dynamic rheology

Rheological properties of yoghurt samples after stirring were determined using rheometer. Rheometer is the best instrument to perform viscometry or oscillation tests on yoghurt. Manual slow stirring appears to be the most effective way to preserve yoghurt structure and at the same time allows preparation of yoghurt sample that can be measured in a rheometer equipped with parallel plate geometry (Vercet *et al*., 2002).

Characterization of flow curves

All yoghurt samples viz. control and fortified yoghurt showed shear thinning behavior (Figure 1). Calcium phosphate fortified yoghurt showed higher shear thinning behavior as compared to control, whereas calcium citrate fortified yoghurt samples showed less shear thinning. Again this is related to intrinsic properties of calcium salts used for enrichment which increase or decrease gel strength of calcium fortified yoghurt. Table 5 shows the consistency index and power law index for control and fortified yoghurt samples. Consistency index of control and fortified yoghurt samples showed non-significant difference ($p>0.05$) and values ranged between "243.58 to 271.62". Also non-significant differences ($p>0.05$) were observed in Power law index for control and fortified yoghurt samples. Singh and Muthukumarappan (2008) found a similar shear thinning behavior with calcium lactate fortified yoghurt. The flow behavior were comparable with some reported values (n: 0.11–0.31, K: 19.2–66.2) from Vercet *et al*. (2002).

Viscosity values showed that all yoghurt samples showed non Newtonian behavior after shear was applied. Unfortified control and fortified yoghurt samples showed thixotropic behavior. Lower viscosity in calcium phosphate and higher viscosity in calcium citrate fortified yoghurt was observed, however, non-significant difference ($p>0.05$) was observed at different shear rates. Almost similar reduction in viscosity was observed in all yoghurt samples. Vercet *et al*. (2002); Singh and Muthukumarappan (2008) also showed thixotropic behavior of yoghurt samples.

Sensory analysis score of yoghurt

Yoghurt was prepared from both control and fortified milk. Yoghurt made from fortified milk scored lower than control. However, these differences in sensory attributes were non-significant ($p>0.05$). All yoghurt samples were comparable to each other and control in all sensory aspects (Table 4). Vetez-Ruiz and Rivas (2001) studied the sensory properties of calcium lactate and calcium chloride fortified yoghurt and found similar sensory scores for unfortified (control and fortified yoghurt. Singh *et al*. (2005) and Singh and Muthukumarappan (2008) fortified yoghurt with 500 ppm calcium as calcium lactate and observed no significant difference between control and calcium fortified yoghurt in flavor, body and texture, appearance and overall acceptability. Pirkul *et al*. (1997) observed similar results for plain yoghurt fortified with calcium lactate, calcium gluconate and both calcium gluconate + lactate. Cavallini and Rossi (2009) reported that addition of calcium citrate (600 ppm) to soy yoghurt did not show any notable effect on sensory attributes in comparison to unfortified yoghurt and observed similar sensory scores for all evaluated attributes (aroma, color, flavor and overall acceptability).

Conclusion

Calcium and vitamin D_2 fortification of yoghurt samples showed similar sensory and physico-chemical properties when compared with unfortified yoghurt (control). Growth of yoghurt starter cultures was slightly effect by fortification. Firmness and viscosity values were also showed no significant difference ($p>0.05$) between control and fortified yoghurt samples. In rheological characteristics, all yoghurt samples showed shear thinning and thixotropic behavior. Hence, it can be concluded that calcium and vitamin D_2 multiple fortification was feasible in yoghurt with good sensory, physico-chemical and textural properties.

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