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Characteristics of reduced fat milks as influenced by the incorporation of folic acid

Kamalesh Achanta

Louisiana State University and Agricultural and Mechanical College

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**CHARACTERISTICS OF REDUCED FAT MILKS AS INFLUENCED BY THE
INCORPORATION OF FOLIC ACID**

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Interdepartmental Program in Animal and Dairy Sciences

by
Kamalesh Achanta
B.Tech (Dairying), ANGRAU, 2002
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ABSTRACT

Folic acid plays an important role in the prevention of neural tube defects such as spina bifida and anencephaly, heart defects, facial clefts, urinary abnormalities and limb deficiencies. Milk and milk products serve as a potential source for folic acid fortification due to the presence of folate binding proteins which seem to be involved in the folate bioavailability. Though milk is not a good source of folic acid, fortification could help in the prevention of the above mentioned defects. The objective of this study was to examine the physico-chemical characteristics of reduced fat milks fortified with folic acid. Reduced fat milks were prepared using 25, 50, 75 and 100% of the recommended dietary allowance of 400 micrograms of folic acid. Treatments included addition of folic acid at these levels before and after pasteurization. Color, pH, fat, protein, viscosity, folic acid concentration, folate binding protein concentration, folate binding protein profile, standard plate count and coliform counts were determined on days 1, 7, 14 and 21. A consumer acceptance test was conducted on day 7. Data from the consumer panel was analyzed using analysis of variance (PROC GLM) with means separation to determine the differences among treatments. Data obtained from the color, pH, fat, protein, viscosity, folic acid concentration, folate binding protein concentration, standard plate count and coliform counts were analyzed using the General Linear Model with a repeated measure in time by the Statistical Analysis System. Significant differences were determined at $P < 0.05$ using Tukey's Studentized Range Test. There were no differences in the electrophoretic mobility of folate binding protein in the samples. The concentration of folic acid was significantly higher in reduced fat milks fortified with folic acid after pasteurization compared to the treatments to which folic acid was added before

pasteurization. The consumer panelists did not find any significant differences in flavor, appearance and texture of folic acid fortified reduced fat milks compared to that of the control. Fortification of reduced fat milks with folic acid can be accomplished with out adversely affecting the product characteristics.

CHAPTER 1: INTRODUCTION

Consumer demand exists for healthier products which is evidenced by the growth of the functional food industry from \$ 31.6 billion in 1999 (Anonymous, 2000) to \$ 55 billion in 2001 (National Business Journal, 2002). Competition in the dairy industry is driving the fluid milk sector to improve the nutritional value as well to decrease the fat content of the dairy products, which enables them to compete with innovative new products. Substantial increase in the production and consumption of low fat but, nutritionally rich products focuses on the importance of improving these products. Consumers expect fluid milk products to be healthy and nutritious. Low fat and non fat dairy products are targeted towards people who are trying to lose weight and people with cardiovascular problems. There has been a tremendous increase in the sales of reduced and low fat milk from 15,918 million pounds in 1980 to 23,559 million pounds in 2003 (Milk Facts, 2004). Of the total fluid milk product sales in 2003, reduced and low fat milk accounted for 43.5 % compared to that of whole milk (32.9%) and non fat milk (14.41%) (Milk Facts, 2004).

Vitamins are essential for human health. Folic acid is one of the important vitamins which when deficient can cause severe negative health effects. Folic acid deficiency is responsible for neural tube defects (NTDs) such as anencephaly and spina bifida in humans (Czeizel, 1995) and may cause several different age-related diseases, including coronary artery disease (Swain and St. Clair, 1997), stroke (Elkind and Sacco, 1998), and cancers (Duthie, 1999). Folate deficiency is responsible for impaired cell division due to reduced purine and pyrimidine synthesis, resulting in megaloblastic anemia (Scott, 1999). Folic acid is important in the nervous system at all ages, but in elderly people deficiency leads to aging brain processes, increases the

risk of Alzheimer's disease and vascular dementia and, if critically severe, can lead to a reversible dementia (Reynolds, 2002).

Folic acid is a water soluble vitamin and is known by several different names such as folate, folacin, folacid, folbal, PGA, Pterol-L-glutamic acid, Pterol-L-monoglutamic acid and vitamin B₉ (American Chemical Society, 2002). The term “folate” refers to the class of compounds having a chemical structure and nutritional activity similar to that of folic acid (pteroyl-L-glutamic acid) (Blakley, 1988). In nature, folic acid exists primarily as reduced one-carbon-substituted pteroylglutamates, varying in the presence of substituents and the number of glutamyl residues attached to the pteroyl group (Bailey, 1988).

Folic acid is found in broccoli, spinach, romaine lettuce, dried beans and liver (Wardlaw, 1999). Milk and milk products are not a good source of folic acid. Cow's milk contains 5-7 µg of folic acid (Renner, 1983, and Scott, 1989). The naturally occurring form of folate in unprocessed milk is 5-methyltetrahydrofolate (Verwei et al, 2003). 5-methyltetrahydrofolate is considered to be the predominant dietary folate which is moderately stable compared to folic acid itself which is quite stable (Jones and Nixon, 2002). In bovine milk, 65 % of the folate is present as 5-methyltetrahydrofolate monoglutamate and around 3% is pteroylmonoglutamate (Selhub, 1989).

Bioavailability is defined as the process of absorption and metabolic utilization of a nutrient, involving processes of intestinal absorption, transport, metabolism and excretion (Gregory, 1995). Prior to absorption, deconjugation of folate polyglutamates to monoglutamates by a γ -glutamyl hydrolase (conjugase) is required (Halsted, 1990). Two separate γ -glutamyl hydrolase activities take place in human jejunal mucosa: one soluble and intracellular; the other membrane-bound and concentrated in the brush border cells. Human brush border γ - glutamyl which has been shown to play a principal role in the digestion of dietary folate hydrolase is zinc-

dependent with optimum activity at pH 6.5, (Reisenauer et al, 1977). The monoglutamate folate compounds are absorbed by an energy dependent, carrier-mediated process at physiological concentrations and by passive absorption at higher concentrations (Selhub et al, 1984). Absorption takes place mainly in the jejunum and is markedly influenced by pH with a maximum absorption at pH 6.3. During transport of physiological concentrations of folate over the intestinal mucosa absorbed folate derivatives are converted to 5-methyltetrahydrofolate via one carbon substitution and thereafter transported to the liver, which stores folate in the form of polyglutamates (Steinberg et al, 1979). Once absorbed, folate acts as a cofactor for many essential cellular reactions including the transfer of single-carbon units; it is required for cell division because of its role in DNA synthesis (Rosenblatt and Mudd et.al, 1995).

In the fetus, the relation between maternal folate status and the risk of neural tube defects is well established: clinical trials have shown that periconceptual preventive treatment with 400 µg or higher of folic acid significantly reduces the risks of neural tube defects (Reynolds, 2002 II). Hall and Solehdin (1997) reported that folic acid taken by the mother during the initial stages of pregnancy can prevent neural tube defects such as spina bifida and anencephaly, heart defects, facial clefts, urinary abnormalities and limb deficiencies. The U.S. Center for Disease Control recommends women of child bearing age to consume 400 µg of folic acid daily to prevent neural tube defects (CDC 1993). An additional 200 µg is added when pregnant and 100 µg when lactating (Yates et al, 1998). The current RDA for children 1-3 years of age is 150ug/day; 300ug/day for children 4-8 years; 300ug/day for children 9-13 years; and 400ug/day for ages 14 to over 70 years (Food and Nutrition Board, 2002).

A relationship between folate intake and several types of cancers, including colorectal, breast, cervical, pancreatic, brain and lung cancers, has been observed in several population

based studies (Choi and Mason, 2000). This relationship is most clearly defined for colorectal cancer and its precancerous condition, colorectal adenomas. Lower risks for disease are commonly observed in individuals with higher folate intake; therefore, folic acid can be an important contributor to overall folate intake in the diet and may play an active role in contributing to cancer risk reduction, a 30% lower risk was associated with consumption of ≥ 400 $\mu\text{g}/\text{d}$ of total folate compared with women in the lowest folate intake groups (≤ 200 $\mu\text{g}/\text{d}$) (Giovannucci et al, 1998). The hypothesized relation between folate and stroke is believed to be mediated by homocysteine levels (Institute of Medicine, 2002 and De La Vega, 2002), primarily because folate supplementation lowers homocysteine levels (Health Canada, 1997, De Walle and de Jong-van den Berg, 2002). The risk of stroke might be reduced by 19% to 24% if serum homocysteine levels were lowered by 3 $\mu\text{mol}/\text{L}$ (Miller et al, 2000, and Stevenson et al 2000). For the levels of homocysteine to be lowered, vitamin supplementation of at least 5 mg folic acid per day is needed (Maxwell, 2000).

Folate binding protein exists as a minor whey protein in milk (Salter et al., 1981) that is crucial to the assimilation, distribution and retention of folic acid (Davis and Nichol, 1988). Almost all naturally occurring folate in milk is bound to FBP (Ghitis et al, 1969). The majority of FBP in bovine milk exist as the soluble form, although a small amount of particulate FBP are found. The soluble form of FBP has been suggested to play a role in folate transport in serum and milk, whereas the particulate form is anchored in the intestinal cell membrane and is associated with membrane transport of folate (reviewed by Wagner, 1985 and Anthony, 1996). The FBP in bovine milk exists in concentrations of about 10 mg/L (Salter et al, 1972), and binds approximately 1 mol of folate per mol protein at pH 7.2 (Salter et al, 1981). The existence of a 'milk folate binder' was originally suggested by Ghitis and associates (1969). The complete

amino acid sequence of this minor whey protein was determined by Svendsen et al, (1984). The FBP contains a single polypeptide chain of 222 amino acid residues with eight disulphide bridges. The FBP from cow's milk has a molecular weight of 35000 ± 1500 Daltons with a high proportion of half-cystine (18 residues/molecule) and 10.3 % of carbohydrate (Fucose, Mannose and Galactose) (Salter et al, 1981). The FBP's are present in large quantities in commercial whey protein concentrate preparations (Waxman and Schreiber, 1975). The FBP is temperature stable. The FBP isolated from whey powder obtained from milk vacuum evaporated at 68°C and spray dried at 180°C was able to bind 0.5 mol folate/mol (Salter et al, 1981). Pasteurization of milk (at 72°C for 15 s) is suggested to result in heat-induced alterations of the FBP molecule (Gregory, 1982). In contrast, FBP is shown to be relatively resistant to acidic environments.

Folate binding proteins in milk are interesting because they seem to be involved in bioavailability of folate (Verwei et al, 2003). FBP may protect folate from bacterial uptake and degradation (Ford, 1974; Tani and Iwai 1984) and enhances absorption of folate by mucosal cells (Colman et al, 1981; Salter and Blakeborough, 1988). When rats were fed with folate bound to FBP, the excretion of folate was greatly reduced (Tani and Iwai, 1984). Bound folate was absorbed at a slower rate in the small intestine of rats when compared to free folate, but the total amount of bound folate absorbed was almost the same as that of free folate (Tani et al, 1983). Folate bound to FBP was not taken up by folate requiring intestinal bacteria like *Lactobacillus* and *Streptococcus*. Since milk contains FBP, benefits of fortifying milk with folic acid would be enhanced. Consumption of reduced fat milk regularly lowered the risks of cancers of oral cavity, stomach, rectum, lung and cervix (Mettlin, 1990). Adding folic acid to reduced fat milk may have additional health benefits. It is a common practice to add vitamins A and D during fluid

milk processing. The effects of direct addition of a water soluble vitamin, folic acid, to reduced fat milk on the physico-chemical and sensory characteristics are not known.

The objectives of this study were:

1. To determine the effect of different concentrations of folic acid on the physicochemical and sensory characteristics of reduced fat milks over a three week storage period.
2. To elucidate the effect of the stage of addition of folic acid on the physicochemical and sensory characteristics of reduced fat milks over a three week storage period.

CHAPTER 2: MATERIALS AND METHODS

2.1 Experimental Design

Reduced fat milks were fortified with folic acid at 25, 50, 75, or 100% of the RDA of 400 μg folic acid per 236 ml serving. Folic acid was added before or after HTST (high temperature short time pasteurization). Color (L^* , a^* , b^* , C^* , h^*), pH, fat, protein, viscosity, folic acid concentration, folate binding protein concentration, folate binding protein profile, standard plate count and coliforms count were determined on day 1, 7, 14 and 21. A consumer acceptance test was conducted on day 7. The experiment was conducted and analyzed as a randomized complete block with repeated measures. The replications were the blocks. Three replications were conducted.

2.2 Reduced Fat Milks Preparation

Raw whole milk (Approx. 360 liters) was obtained from the LSU dairy farm and held at 4° C until use (< 1 h). Cream and skim milk at 4° C were separated using a De Laval 392 Airtight Cream separator (Alfa Laval Inc., Richmond, VA). Reduced fat milk (2% total fat) was prepared by adding an appropriate amount of cream to skim milk. Prior to pasteurization, USP grade folic acid (Ampak Co., New York) at 25, 50, 75 or 100% of the RDA of 400 μg was added separately to four equal volumes of the reduced fat milk. They were then homogenized using a two stage homogenizer (APV Americas, Lake Mills, WI) at 1500 psi and 500 psi followed by pasteurization for 16 s at 72.5 °C (163 °F). Folic acid at 25, 50, 75 or 100% of the RDA of 400 μg was also added separately to four equal volumes of unfortified reduced fat milks after homogenization and pasteurization. Control was homogenized and pasteurized reduced fat milk.

2.3 Analytical Procedures

2.3.1. Color

Color was determined by L*, a*, b*, C*, h* values using a Hunter MiniScan XE Plus, portable color spectrophotometer (Model No. 45 / 0-L, Hunter Associates Laboratory Inc. Reston, VA). Standardization was done using Miniscan XE Plus Reflectance Standard (D 65 / 10°). An average of five readings per sample was recorded. Chroma is defined as the Hue is the wavelength of light reflected from or transmitted through an object, seen as the visible spectrum. Hue angle = $[\tan^{-1}(b^* / a^*)]$. Chroma is the intensity of the color, $C^* = [(a^{*2} + b^{*2})^{1/2}]$.

2.3.2. pH

The pH was measured at 25° C using an Ultra Basic Bench top pH meter (Denver Instruments Company, Arvada, CO) which was calibrated prior to use by commercial pH 4.00 and 7.00 buffers (Fisher Scientific).

2.3.3. Fat

Fat content was determined using Babcock's method according to (Richardson, 1985).

2.3.4. Protein

Protein content was determined using Bentley 2000 Fat and Protein Analyzer (Bentley Instruments Inc., Chaska, MN) at the LSU Dairy Improvement Center, Baton Rouge, LA 70820.

2.3.5. Viscosity

Viscosity was measured at 21° C using a Brookfield DVII+ viscometer with helipath stand. The spindle used was a RV spindle disc1 set at 10 rpm. Data points (100

per sample) were collected using Wingather® software (Brookfield Engineering Lab, Stoughton, MA). Viscosity readings were expressed in centipoise (cp).

2.3.6. Folic Acid Concentration

Folic acid concentration was determined by using high performance liquid chromatography (HPLC) with methods modified from Albala-Hurtado et al, (1997). The HPLC system was comprised of a Waters (Waters Corp., Milford, MA) 501 pump, Waters 717 Plus auto-sampler, and Waters 486 tunable UV detector set at 282 nm. Peak areas were calculated using the Waters Millennium® software. The separation was carried out isocratically using a Waters Spherisorb 5um ODS2 4.6x250 mm column with guard cartridge. Samples were prepared as follows: 10.5 g of the sample was weighed into 50 ml centrifuge tubes with screw on caps, 1g of crystalline trichloroacetic acid (TCA) was added and the mixture was shaken for 10 minutes on a mechanical shaker. The mixture was centrifuged at 1250 g for 10 minutes. The supernatant was decanted to a 10 ml volumetric flask and 3ml of 4% w/v TCA was added to the solid phase. The mixture was shaken for 10 minutes and centrifuged again at 1250g for 10 minutes. The supernatant was then added to volume in the 10 ml volumetric flask, wrapped in aluminum foil to protect from light. Samples were filtered through a 45 micron filter (Sigma Aldrich, St. Louis, MO) and placed in clear glass HPLC vials protected from light with aluminum foil. Eluent was prepared as described in Albala- Hurtado, et al, (1997). A standard curve (Figure 1) was prepared by quantifying 25, 50, 75 and 100 % RDA standards of folic acid with HPLC grade double distilled water. 100% RDA standard was prepared by adding 1.69 mg of folic acid to 1 L HPLC grade double distilled water. 75, 50 and 25% RDA solutions were prepared by adding 75 ml of the 100% solution to 25 ml

HPLC grade double distilled water; 50 ml of the 100% solution to 50 ml HPLC grade double distilled water; 25 ml of the 100% solution to 75 ml HPLC grade double distilled water respectively. A sample volume of 10 μ l was injected using an auto sampler (Waters Corp., Milford, MA). Run time for samples was 20 minutes using a flow rate of 1 ml per minute. These known concentrations of folic acid solutions were filtered through a 45 micron filter (Sigma Aldrich, St. Louis, MO) and injected using an auto sampler. Under the conditions used in this experiment, folic acid is known to elute out of the column and be detected by the UV detector at 11-14 minutes (Albala-Hurtado, et al, 1997). Folic acid peak areas corresponding to its known concentrations were used to construct a standard curve. Peak areas of folic acid in the milk samples were fitted to the standard curve and corresponding values in mg/L were recorded.

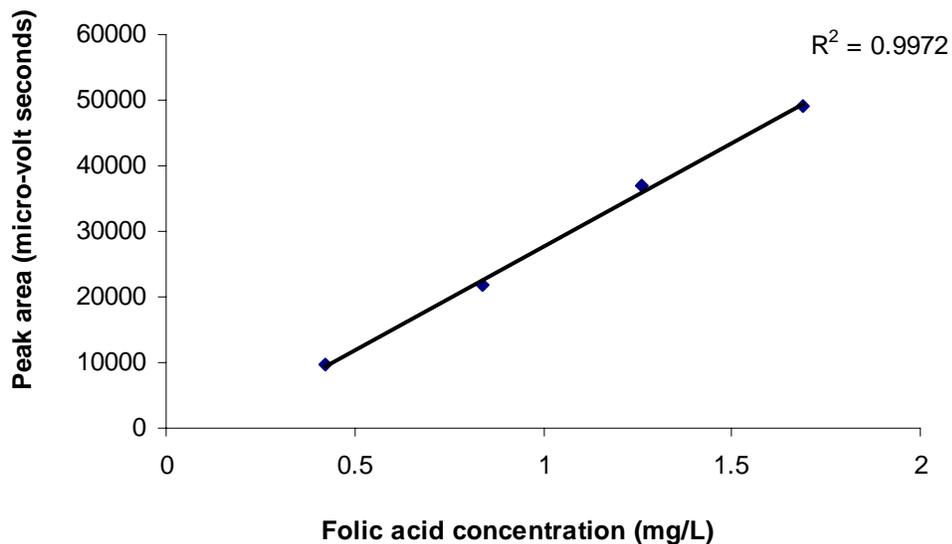


Figure 1. Standard curve of folic acid concentrations

2.3.7. Folate Binding Protein (FBP) Concentration

Quantification of total folate binding protein was performed using Enzyme-Linked Immunosorbent Assay (ELISA), according to Hoier-Madsen et al. (1986) with slight modifications. FBP in reduced fat milk samples was extracted in Triton X- 100 (~ 30 ml/L). Antibody solution (10 mg anti-FBP/ L carbonate buffer, pH-9.6) was coated to a microtiter plate with 96 wells (Sigma Aldrich, Milwaukee, WI). 100 µl of the extracted and diluted sample was added. After 30 min incubation at 37°C, the solution was discarded and the plate was washed four times with NET buffer (0.15 M NaCl, 0.05 M TRIS and 0.001 M EDTA, pH-7.4). 100 µl of goat anti bovine FBP-HRP (US biological, Swampscott, MA) diluted 1: 5000 with assay buffer (NET+ Fish gelatin) was added and incubated for 1 h at 37°C, well protected from light by wrapping the microtiter plate in an aluminum foil. The solution was discarded and the plate was washed four times with NET. After washing, 100 µl of ABTS Stable Liquid Substrate (US biological, Swampscott, MA) was added and the reaction was stopped 10 min later with 2M-H₂SO₄. A standard curve (Figure 2) was obtained by using known concentrations (2.5, 5, 10 and 20 µg/100 µl) of bovine milk FBP antigen (Fitzgerald Industries International, Concord, MA). Sample FBP concentrations were derived from the standard curve of the purified bovine milk FBP plotted against the ratio of the relative absorption measured at 405 nm using a MRX Revelation Microplate reader (Dynex Technologies, Inc. Chantilly, VA). A blank was included in the assay.

2.3.8. Folate Binding Protein Profile

The folate binding protein profile was studied by polyacrylamide gel electrophoresis (PAGE) on a Novex XCell Mini Cell (Novex, San Diego, CA) using 4-

12% NuPage gels (Novex, San Diego, CA). Samples were prepared by dissolving 0.2g reduced fat milk in 1ml distilled water, 27.9µl of mixture was added to 31.1µl distilled water and 25µl of 4X buffer containing 2% lithium dodecyl sulfate (Novex, San Diego, CA). Samples were heated in a 70° C water bath for 10 minutes. Ten µl of 10X reducing agent containing 500mM dithiothreitol (Novex, San Diego, CA) was added to each sample. Samples were vortexed for 5 sec. Twenty µl of each sample was loaded into each well of the gel. Twenty µl of the broad range prestained standards (Bio-rad Laboratories, Richmond, CA) and twenty µl FBP were also loaded to the wells separately. Running buffers were prepared by adding 50 ml of 3-N-morpholino propane sulfonic acid (MOPS) to 950 ml of distilled water; 600 ml was put in the lower, outer chamber while 200 ml was placed in the upper chamber. Before the upper chamber was filled, 500µl of antioxidant (Novex, San Diego, CA) was added to the 200 ml buffer solution. Two gels were run simultaneously at 400 volts for 1 hour. Gels were taken out of carriage and placed in staining trays containing 110ml distilled water, 40ml methanol, and 40ml stain A containing ammonium sulfate and phosphoric acid (Novex, San Diego, CA) for 10 minutes, followed by a 10ml addition of stain B (Novex, San Diego, CA). Gels were stained for 12-14 hrs followed by destaining in distilled water for one week. Images were recorded using a Kodak Easy Share CX4300 digital camera.

2.3.9. Standard Plate Counts

Standard plate counts (cfu / ml) were determined according to (Richardson, 1985). Aerobic Plate Count Petrifilms (3M, St. Paul, MN) were used to enumerate the bacteria in reduced fat milks. The SPC estimates the total number of aerobic type microorganisms.

2.3.10. Coliform Counts

Coliform counts (cfu / ml) were determined according to (Richardson, 1985). Coliform Count Plate Petrifilms (3M, St. Paul, MN) were used to enumerate the coliforms in the reduced fat milks.

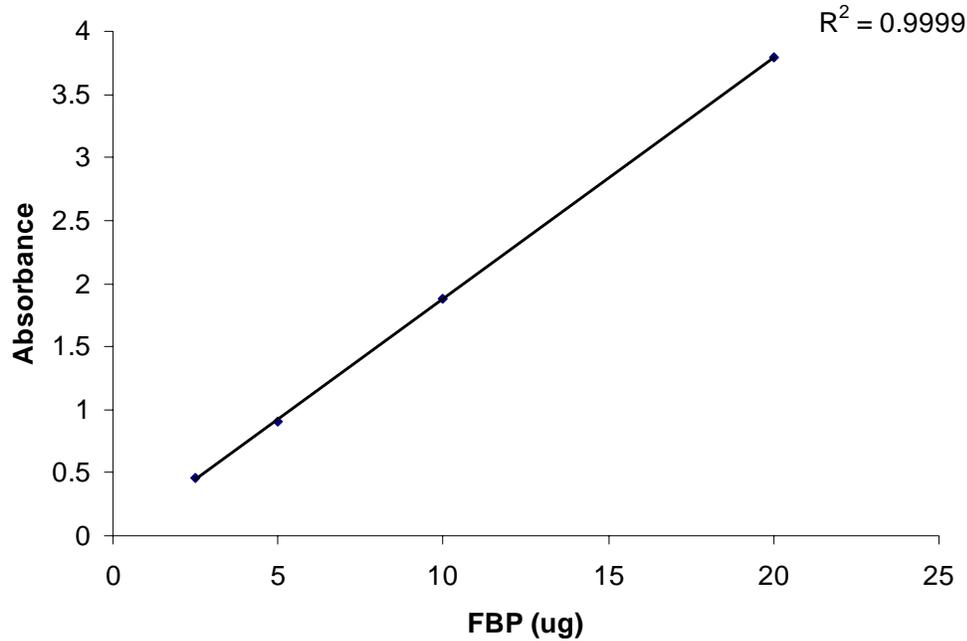


Figure 2. Standard curve of FBP concentrations

2.4. Consumer Acceptability Testing

Consumer acceptability testing was conducted in the sensory evaluation room in the LSU Creamery on the seventh day and eight days of preparing the reduced fat milks. Consumers (n = 180) evaluated overall acceptability, appearance / color, flavor, texture / mouthfeel and overall acceptance of reduced fat milks fortified with folic acid using a 9-point hedonic scale (1= dislike extremely, 5= neither dislike nor like, and 9= like extremely).). A balanced incomplete block design (Plan 11.3 a, Treatments (t) = 9, Units per block (k) = 2, Replications (r) = 8, Total number of blocks (b) = 36, Number of times that two treatments appear together in a block (λ) = 1, (Efficiency) E = .56, type II

(designs arranged in groups of replications) described by Cochran and Cox (1957) was used. This design allowed consumers to evaluate two out of nine samples and provided the means to obtain consistent, reliable data.

2.5. Statistical Analysis

Data from the consumer panel was analyzed using analysis of variance (PROC GLM) with means separation to determine the differences among treatments (SAS version 9.0, Cary, NC). Significance was established at $p < 0.05$ using Tukey's Studentized Range Test. Data obtained from the color, pH, fat, protein, viscosity, folic acid concentration, folate binding protein concentration, standard plate count and coliform counts were analyzed using the General Linear Model with a repeated measure in time.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Standard Plate Counts

The mean values for standard plate count (SPC) values are reported in Figure 3. SPC values were not different among the treatments and a similar increase was seen during storage. The SPC values were higher on days 14 and 21 compared to day 1. A significant increase in the SPC during refrigerated storage is evidence of psychrotrophic growth and suggests that post-pasteurization contamination has occurred. Psychrotrophic microorganisms in milk cause spoilage by altering the constituents of milk (Cousin, 1982). During growth at low temperature, lipases and proteases are produced that may adversely influence the quality and shelf life of most dairy products (Griffiths et al., 1981; Fox and Stepaniak, 1983; Stepaniak and Fox, 1985; Fairbairn and Law, 1986).

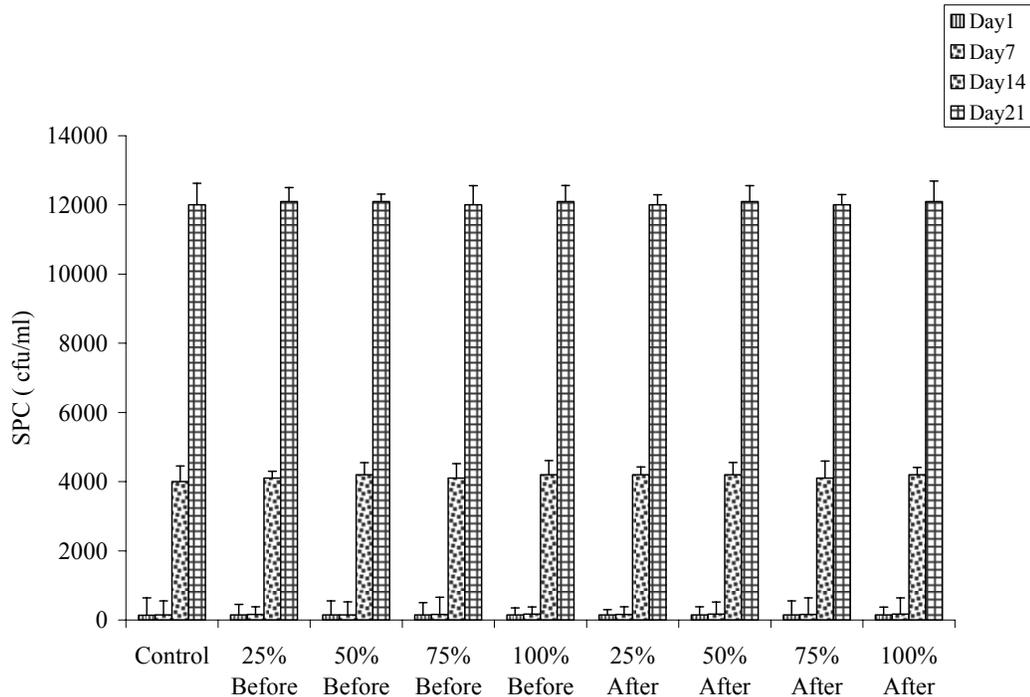


Figure 3. Mean (\pm SE) SPC values of reduced fat milks fortified with folic acid at 25, 50, 75 and 100% of RDA added before or after pasteurization

3.2 Color

The L* (lightness) values are reported in Figure 4. There was no significant ($p>0.05$) difference in the concentration of folic acid added and the mean L* (lightness) values of the samples. There was no treatment and time interaction nor did pasteurization effect the mean L* (lightness) values. Time overall was found to be significant ($p<0.05$). The L* (lightness) values were higher at day 21 compared to day 1. The probable reason for the increase in the L* (lightness) on day 21 compared to day 1 can be attributed to the increase in the bacterial counts (Figure 3). Campbell et al, 2003 have reported a similar increase in the L* (lightness) of milks fortified with conjugated linoleic acid over a 3 week storage period. Rowe et al, (1990) have reported that lipases are produced during the late log and early stationary phases of growth. Agglutination of fat globules into cream flakes or flecks takes place due to the production of lipases (Labots and Galesloot, 1959).

The mean a* (green to red) values are reported in Figure 5. Addition of folic acid did not affect the a* values. There was no treatment and time interaction nor did pasteurization affect the mean a* values. Time overall was found to be significant ($p<0.05$). The mean a* values were higher at day 21 compared to day 1. The probable reason for the increase in the mean a* values on day 21 compared to day 1 can be attributed to the increase in the bacterial counts (Figure 3). No overall significant ($p>0.05$) differences were observed in the concentration of folic acid added and the mean a* values between samples.

The mean b* (yellowness) values are reported in Figure 6. Pasteurization did not have any effect on the mean b* values. There was no treatment and time interaction. No significant ($p>0.05$) differences were found overall on days 1, 7, 14 and 21. There were significant ($p<0.05$) differences in concentration of folic acid added and the mean b*

(yellowness) values. The mean b^* (yellowness) values increased with increasing concentration of folic acid. The increase in the mean b^* (yellowness) values can be attributed to the yellow color of the folic acid itself.

The mean C^* (chroma) values are reported in Figure 7. Overall, there were no significant ($p>0.05$) differences for pasteurization or for different concentrations of folic acid added. No significant ($p>0.05$) differences were found overall on day 1, 7, 14 and 21. There was no treatment and time interaction.

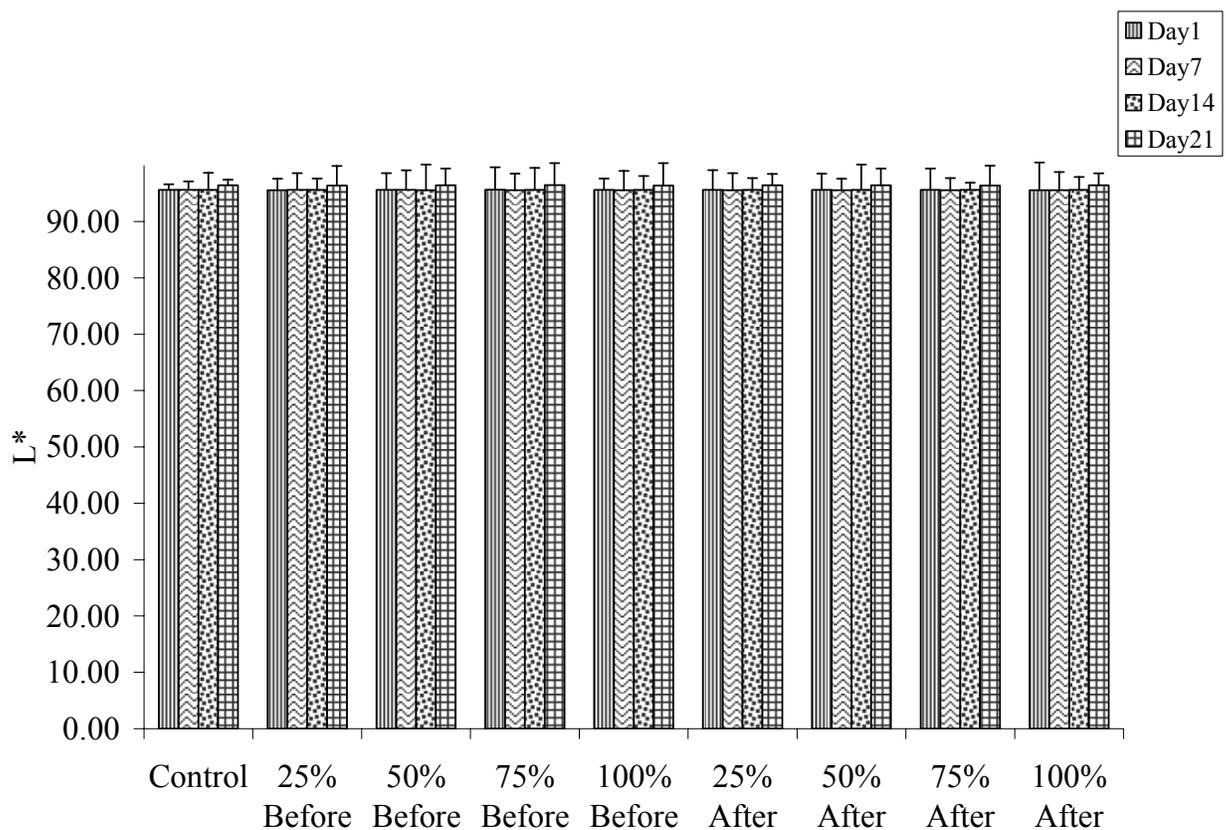


Figure 4. Mean (\pm SE) L^* values of reduced fat milks fortified with folic acid at 25, 50, 75 and 100% of RDA added before or after pasteurization

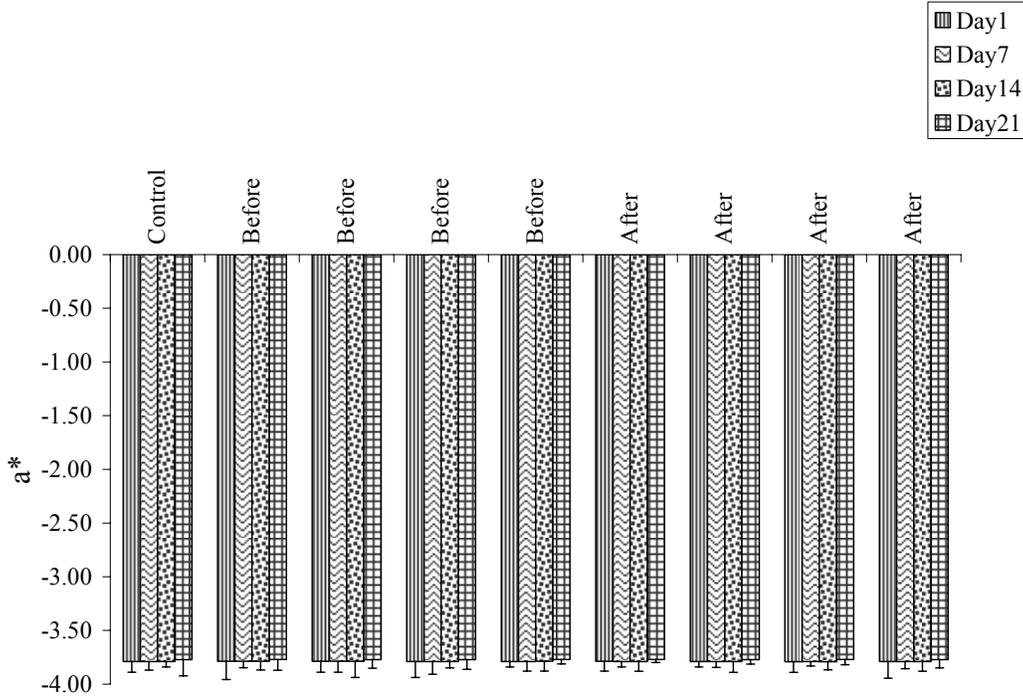


Figure 5. Mean (\pm SE) a^* values of reduced fat milks fortified with folic acid at 25, 50, 75 and 100% of RDA added before or after pasteurization

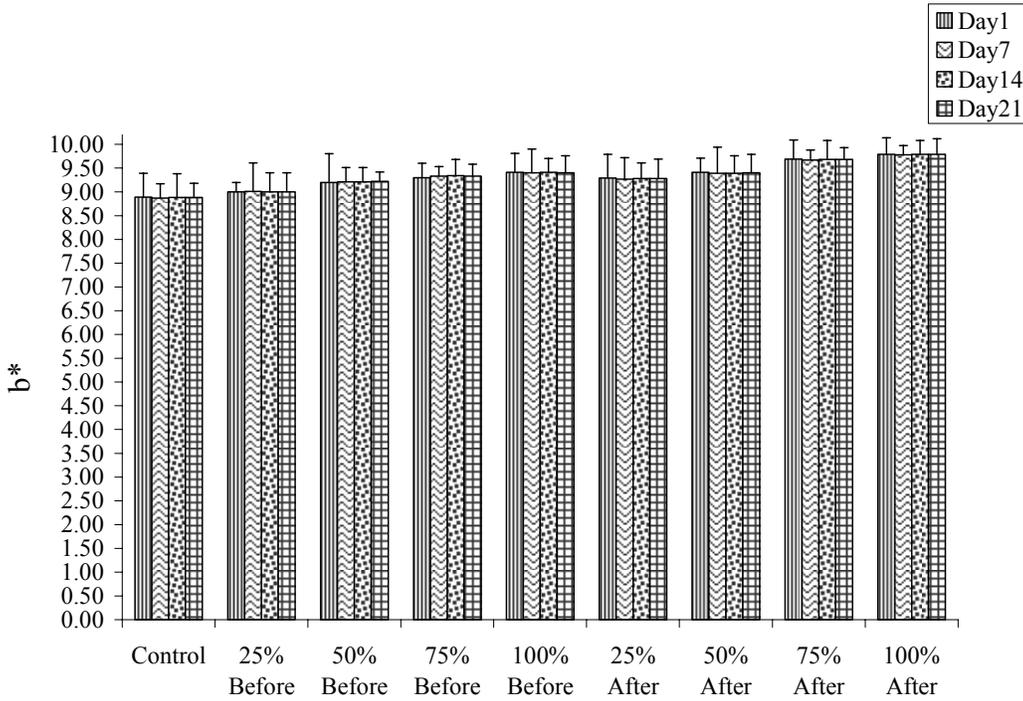


Figure 6. Mean (\pm SE) b^* values of reduced fat milks fortified with folic acid at 25, 50, 75 and 100% of RDA added before or after pasteurization

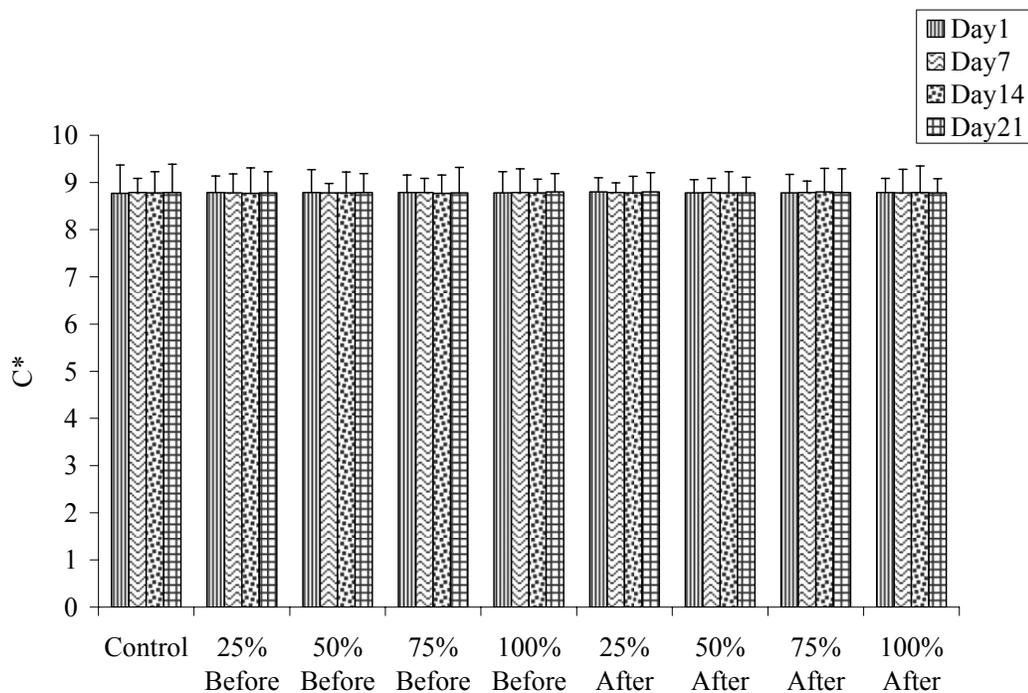


Figure 7. Mean (\pm SE) C* values of reduced fat milks fortified with folic acid at 25, 50, 75 and 100% of RDA added before or after pasteurization

The mean h^* (hue angle) values are reported in Figure 8. An h^* value of 0° is red, 90° is yellow, 180° is green, and 270° is blue. Pasteurization did not have any effect on the mean h^* values. No significant ($p > 0.05$) differences were found overall on day 1, 7, 14 and 21. There were significant ($p < 0.05$) differences in concentration of folic acid added and the mean h^* values between samples. Values for fortified samples were close to the 90° indicating a yellow color.

3.3 pH

The mean pH values are reported in Figure 9. There were no significant ($p > 0.05$) differences in the pH values and concentration folic acid added before versus after pasteurization. It was expected that folic acid addition might lower pH values; however, no such effect was observed. Time overall was found to be significant ($p < 0.05$). This may be due to the bacterial growth over the period of time (Figure 3).

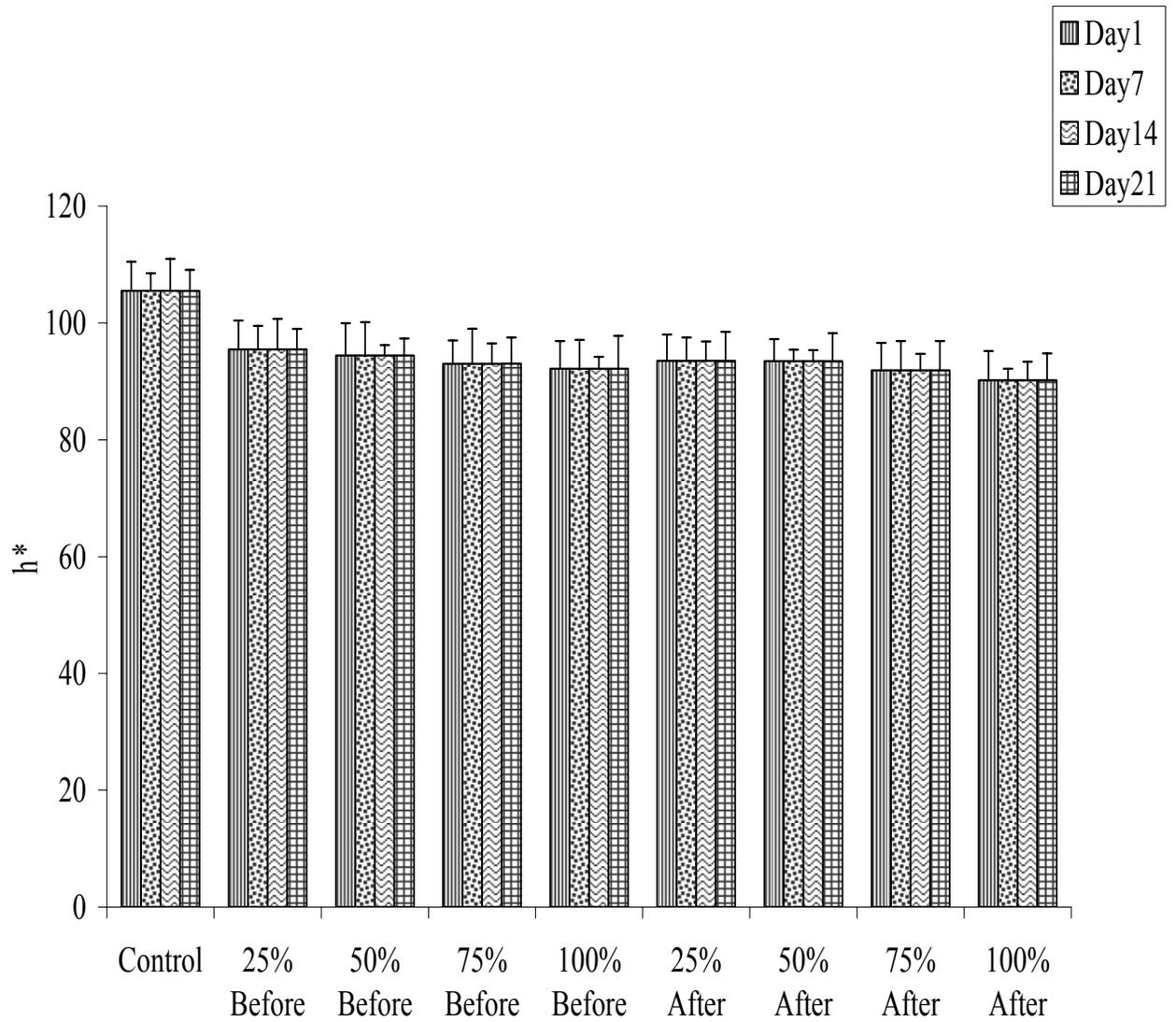


Figure 8. Mean (\pm SE) h^* values of reduced fat milks fortified with folic acid at 25, 50, 75 and 100% of RDA added before or after pasteurization

3.4 Fat, Protein and Viscosity

The mean fat (%), protein (%) and viscosity (cp) values are reported in Figures 10, 11 and 12 respectively. Overall, there were no significant ($p>0.05$) differences for pasteurization or for the concentration of folic acid addition. No significant ($p>0.05$) differences were found overall on day 1, 7, 14 and 21. The different concentrations of folic acid added had no effect on the fat, protein and viscosity values of the samples. Neither was there any treatment and time interaction.

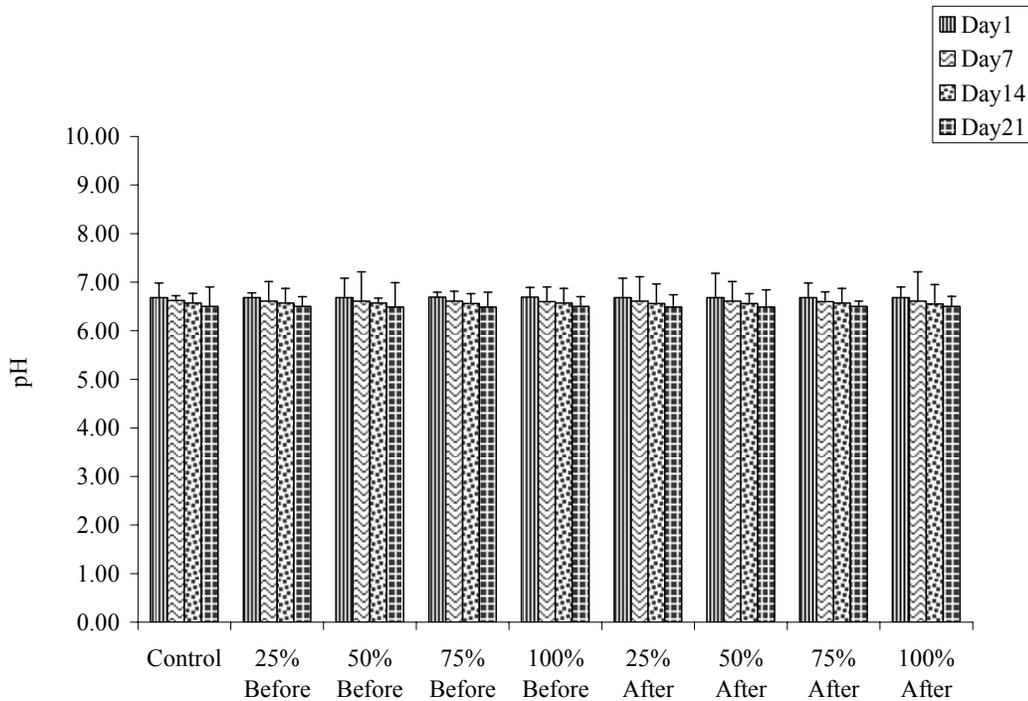


Figure 9. Mean (\pm SE) pH values of reduced fat milks fortified with folic acid at 25, 50, 75 and 100% of RDA added before or after pasteurization

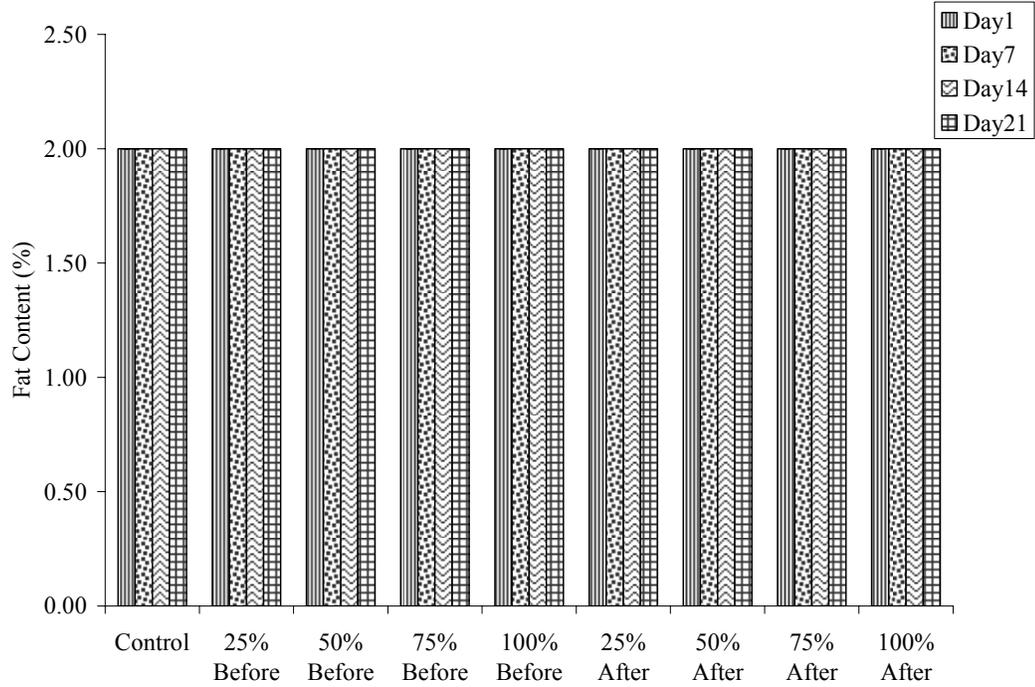


Figure 10. Mean fat content of reduced fat milks fortified with folic acid at 25, 50, 75 and 100% of RDA added before or after pasteurization

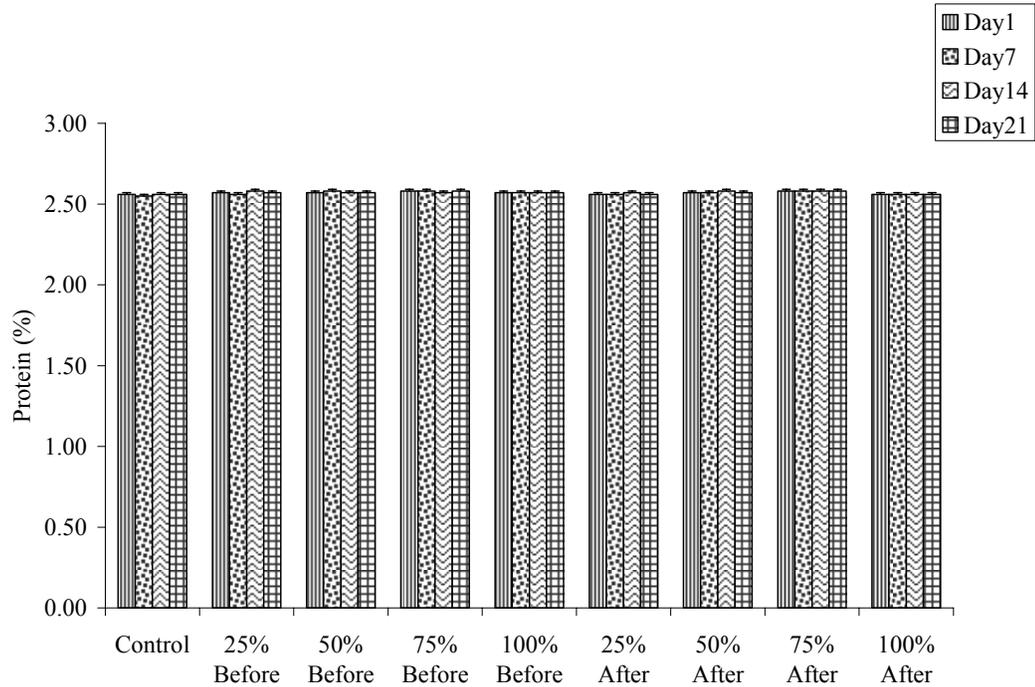


Figure 11. Mean (\pm SE) protein content of reduced fat milks fortified with folic acid at 25, 50, 75 and 100% of RDA added before or after pasteurization

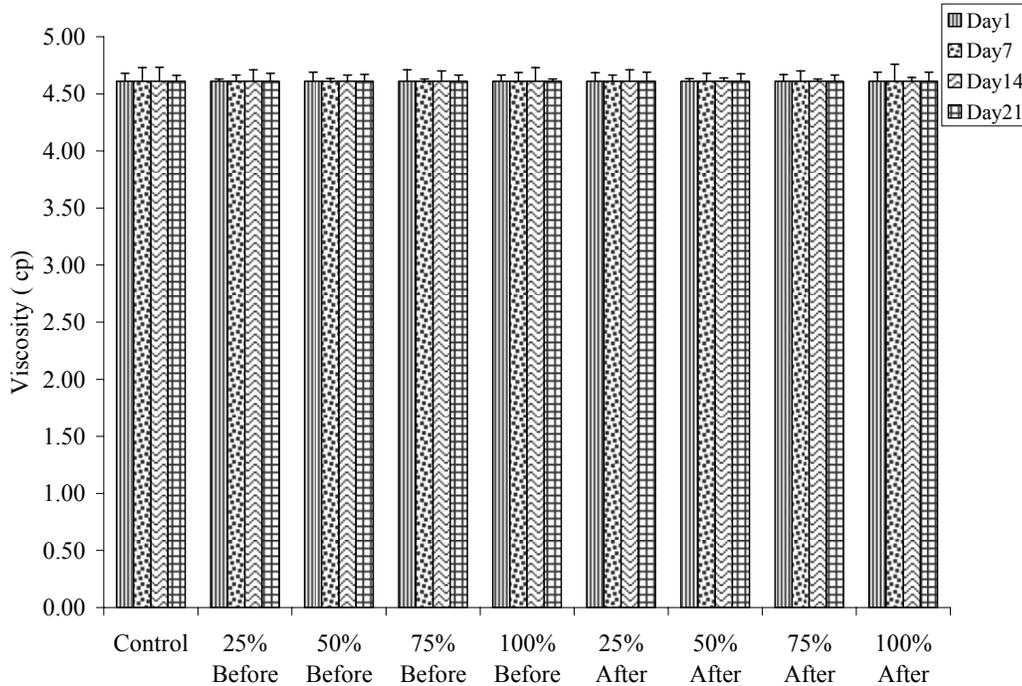


Figure 12. Mean (\pm SE) viscosity values of reduced fat milks fortified with folic acid at 25, 50, 75 and 100% of RDA added before or after pasteurization

3.5 Folic Acid Concentration

Folic acid concentrations as peak areas are reported in Figure 13. Amount of folic acid added before versus after pasteurization affected mean peak area values. Mean values appeared lower for folic acid added before pasteurization for the samples indicating that pasteurization has an effect on the folic acid. Reports in Groff and Grooper (1998) indicated that there are folic acid losses on cooking. Forssen et al (2000) reported that the loss of folates due to pasteurization is less than 10%. Significant differences ($p < 0.05$) were found in overall level of folic acid addition. Peak areas increased proportionally as folic acid levels increased. There were no significant ($p > 0.05$) differences among overall mean peak areas on days 1, 7, 14 and 21 indicating no significant ($p > 0.05$) losses of folic acid concentration over the storage period. Anderson

and Oste (1994) reported that folate levels in pasteurized milk were not reduced during storage beyond the expiration date.

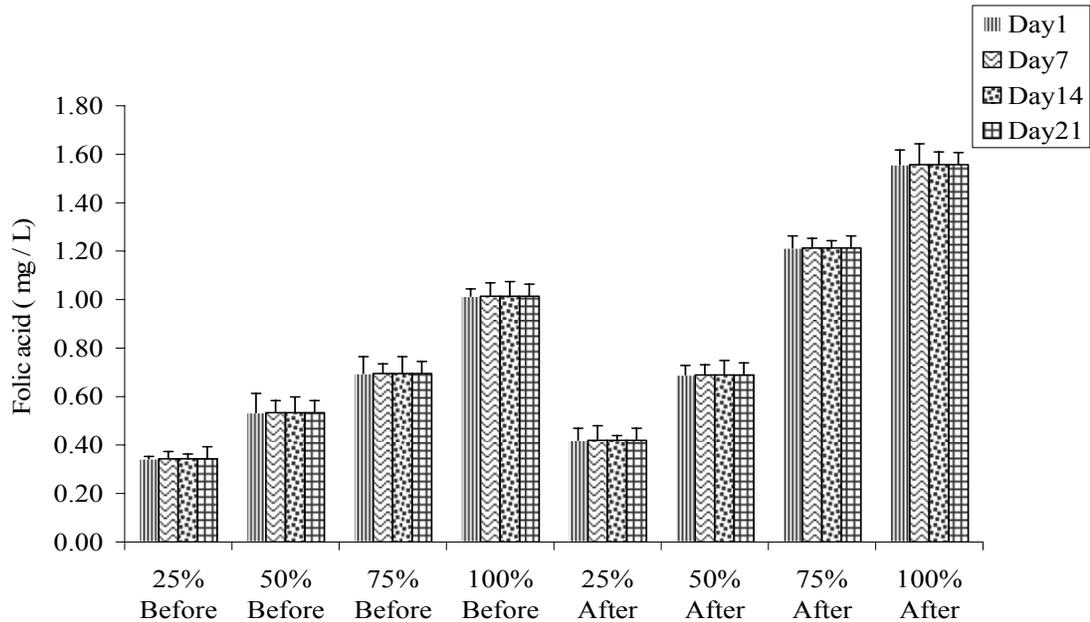


Figure 13. Mean (\pm SE) concentration (mg/L) of folic acid in reduced fat milks at 25, 50, 75 and 100% of RDA added before or after pasteurization

3.6 Folate Binding Protein Concentration

Folate binding protein (FBP) concentrations obtained from ELISA are reported in Figure 14. Pasteurization had no significant ($p > 0.05$) effect on the FBP content. Wigertz et al, (1996) have reported that most of the FBP was retained in spray dried skim milk powder, where the temperature applied is very high compared to that of pasteurization. There was a significant ($p < 0.05$) difference in the level of folic acid addition and the mean FBP values of the samples. The FBP values decreased significantly ($p < 0.05$) as the concentration of folic acid increased. The probable reason for the decrease in the values of FBP with the increase in the folic acid concentration may be attributed to the binding of

folic acid to FBP, which may not be permitting it to react with the antibody to produce color during ELISA. Ghitis et al, (1969) reported that binding of pteroylglutamate (Folic acid) is quantitative, rapid, irreversible and is neither dependent on temperature or pH. Salter et al. (1981) reported that the soluble FBP of cow's milk bind 1 mol folate / mol FBP. Verwei et al. (2003) reported that folate binding concentration of the milk is inversely proportional to the free folic acid fractions, which means that FBP concentration decreases as the concentration of free folic acid increases. There was no treatment and time interaction. Time overall was found to have no significant ($p>0.05$) effect on the FBP concentration of the samples.

3.7 Folate Binding Protein Profile

The electrophoretic migration patterns of folate binding protein are shown in Figures 15, 16, 17 and 18. There were no differences in protein/peptide migration patterns observed. Folate binding protein bands in all samples showed separations at around 35kDa. The FBP from cow's milk has a molecular weight of 35000 ± 1500 with a high proportion of half-cystine (18 residues/molecule) and about 10.3 % of carbohydrate (Fucose, Mannose and Galactose) (Salter et al, 1981). Gels indicated no differences in folate binding protein migration patterns over the storage period with addition of all four levels of folic acid before and after pasteurization of the reduced fat milks. Presence of FBP in the samples is clearly evident from the gels but the concentration of FBP cannot be determined using gel electrophoresis. Results from ELISA indicated that the FBP concentration decreased with the increase in folic acid concentration. But the gels were not showing the variation in the FBP concentration. In all the gels there was a clear band broadening. Wagner (1981) reported that analyses of soluble FBP's by gel electrophoresis are complicated by band

broadening due to the micro heterogeneity in charge and carbohydrate content. Salter et al. (1981) reported that FBP showed heterogeneity during polyacrylamide gel electrophoresis.

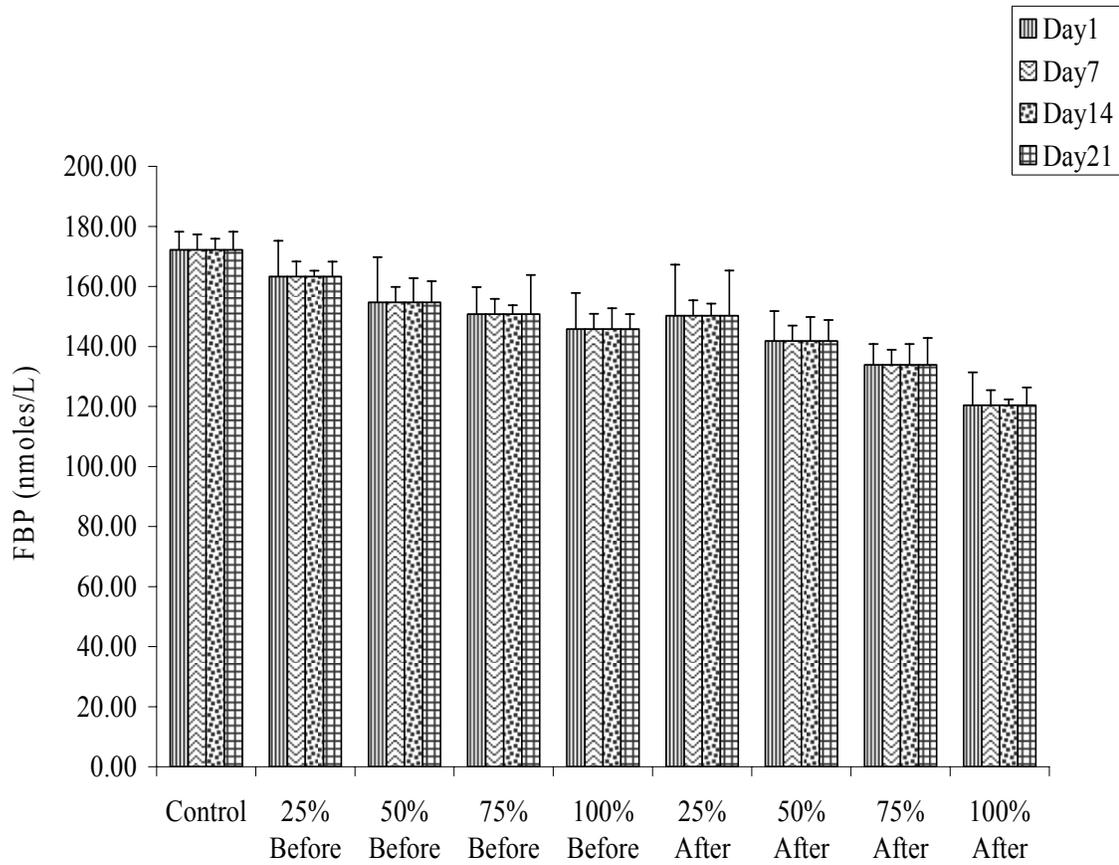
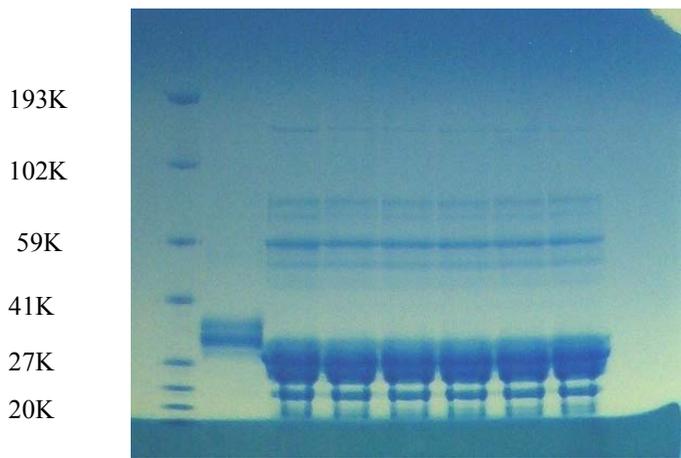


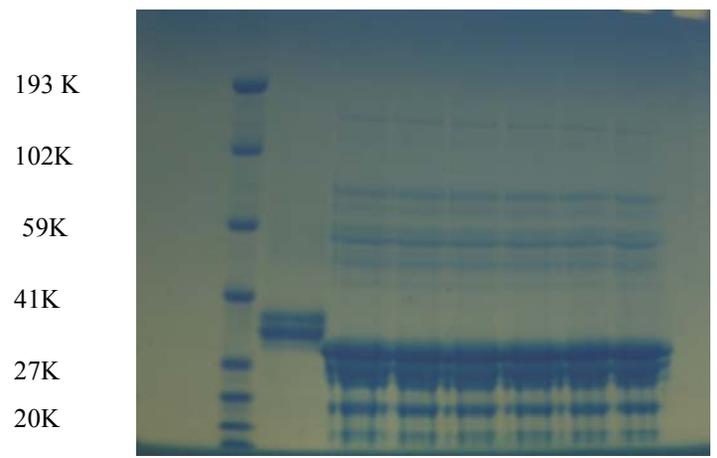
Figure 14. Mean (\pm SE) concentration (nmol/L) of folate binding protein in reduced fat milks at 25, 50, 75 and 100% of RDA added before or after pasteurization



Std 1 2 3 4 5 6 7

(A)

Std= Molecular weight standards;
 1= FBP standard,
 2= Raw milk,
 3=Control,
 4=25% RDA before pasteurization,
 5=25%RDA after pasteurization,
 6=50%RDA before pasteurization,
 7=50%RDA after pasteurization

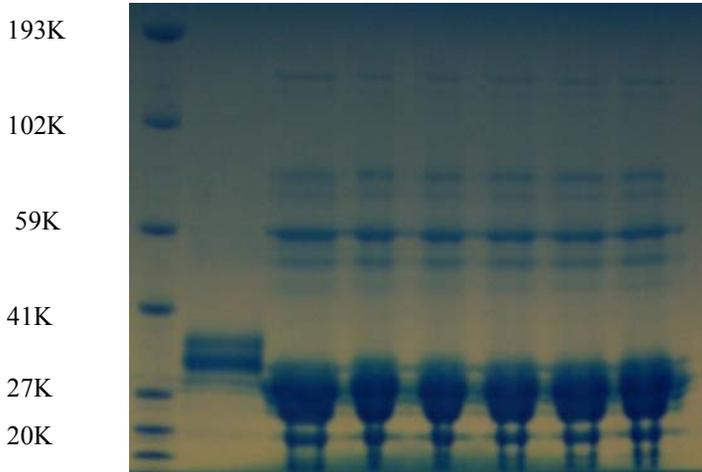


Std 1 2 3 4 5 6 7

(B)

Std= Molecular weight standards;
 1= FBP standard,
 2= Raw milk,
 3=Control,
 4=75% RDA before pasteurization,
 5=75%RDA after pasteurization,
 6=100%RDA before pasteurization,
 7=100%RDA after pasteurization

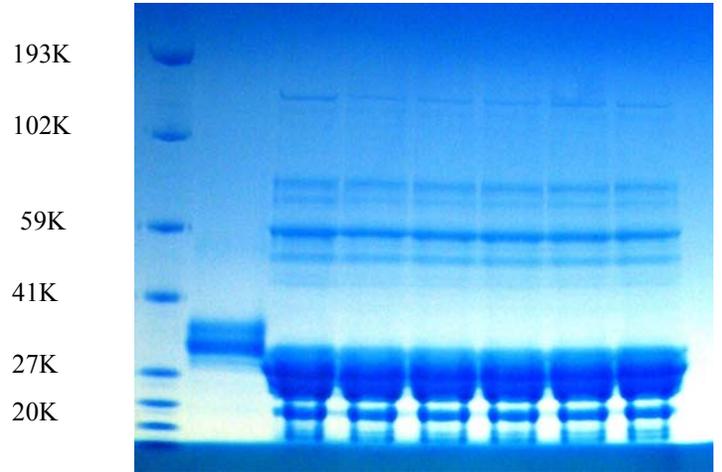
Figure 15. Polyacrylamide gel electrophoresis of reduced fat milks on day 1.



Std 1 2 3 4 5 6 7

(C)

Std= Molecular weight standards;
 1= FBP standard,
 2= Raw milk,
 3=Control,
 4=25% RDA before pasteurization,
 5=25%RDA after pasteurization,
 6=50%RDA before pasteurization,
 7=50%RDA after pasteurization

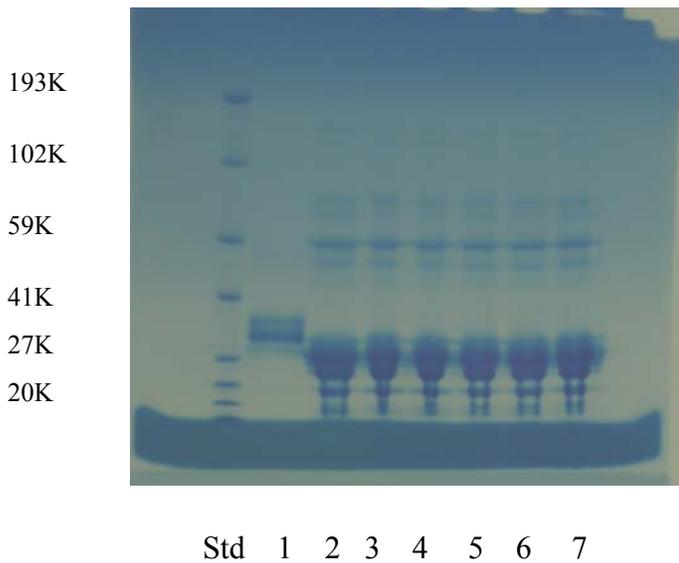


Std 1 2 3 4 5 6 7

(D)

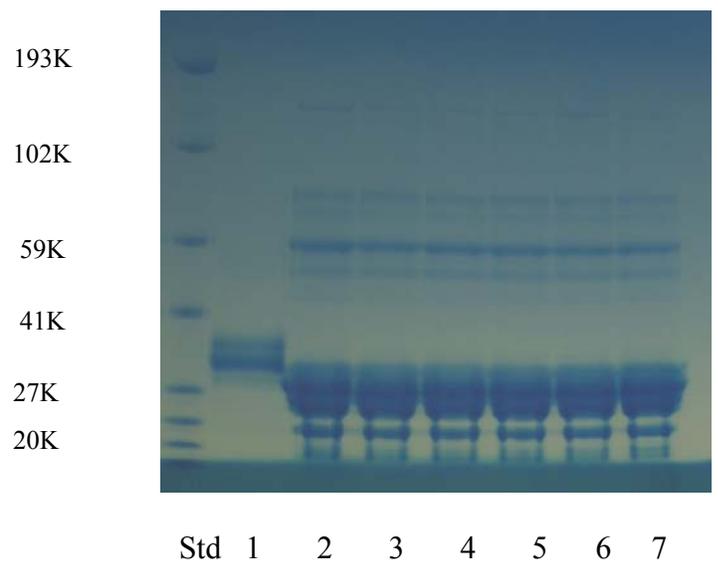
Std= Molecular weight standards;
 1= FBP standard,
 2= Raw milk,
 3=Control,
 4=75% RDA before pasteurization,
 5=75%RDA after pasteurization,
 6=100%RDA before pasteurization,
 7=100%RDA after pasteurization

Figure 16. Polyacrylamide gel electrophoresis of reduced fat milks on day 7.



(E)

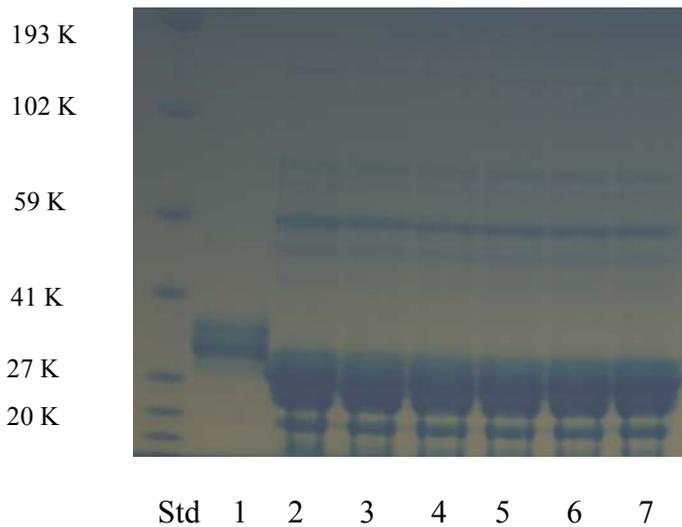
Std= Molecular weight standards;
1= FBP standard,
2= Raw milk,
3=Control,
4=25% RDA before pasteurization,
5=25%RDA after pasteurization,
6=50%RDA before pasteurization,
7=50%RDA after pasteurization.



(F)

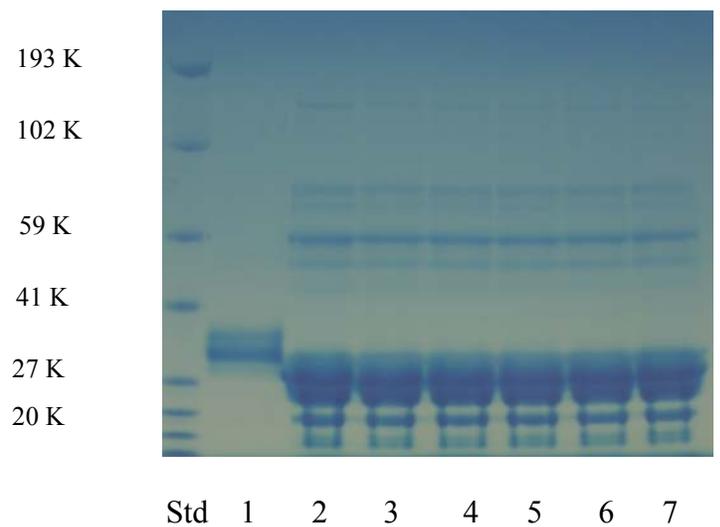
Std= Molecular weight standards;
1= FBP standard,
2= Raw milk,
3=Control,
4=75% RDA before pasteurization,
5=75%RDA after pasteurization,
6=100%RDA before pasteurization,
7=100%RDA after pasteurization.

Figure 17. Polyacrylamide gel electrophoresis of reduced fat milks on day 14.



(G)

Std= Molecular weight standards;
 1= FBP standard,
 2= Raw milk,
 3=Control,
 4=25% RDA before pasteurization,
 5=25%RDA after pasteurization,
 6=50%RDA before pasteurization,
 7=50%RDA after pasteurization,



(H)

Std= Molecular weight standards;
 1= FBP standard,
 2= Raw milk,
 3=Control,
 4=75% RDA before pasteurization,
 5=75%RDA after pasteurization,
 6=100%RDA before pasteurization,
 7=100%RDA after pasteurization

Figure 18. Polyacrylamide gel electrophoresis of reduced fat milks on day 21.

3.8 Coliform Counts

The coliform counts were < 10 for 10^{-1} dilution. No coliforms were detected during the storage period. This indicates that the heat treatment was effective and there was no post pasteurization contamination. The presence of coliforms in pasteurized milk can be attributed to two events: an insufficient heat treatment (Raju and Nambutripad, 1987; Salji et al, 1988); or a contamination of pasteurized product during packaging (Barnard, 1981 and Burden et al, 1995).

3.9 Consumer Acceptability

The mean flavor scores are reported in Figure 19. Flavor scores are from 1 to 9 (1= dislike extremely, 5= neither dislike nor like, and 9= like extremely). No significant differences were found in folic acid addition before vs. after pasteurization. Level of folic acid addition had no overall effect on flavor scores. The mean flavor score was 7.75, which indicates that the consumer panelists liked the flavor of the samples moderately.

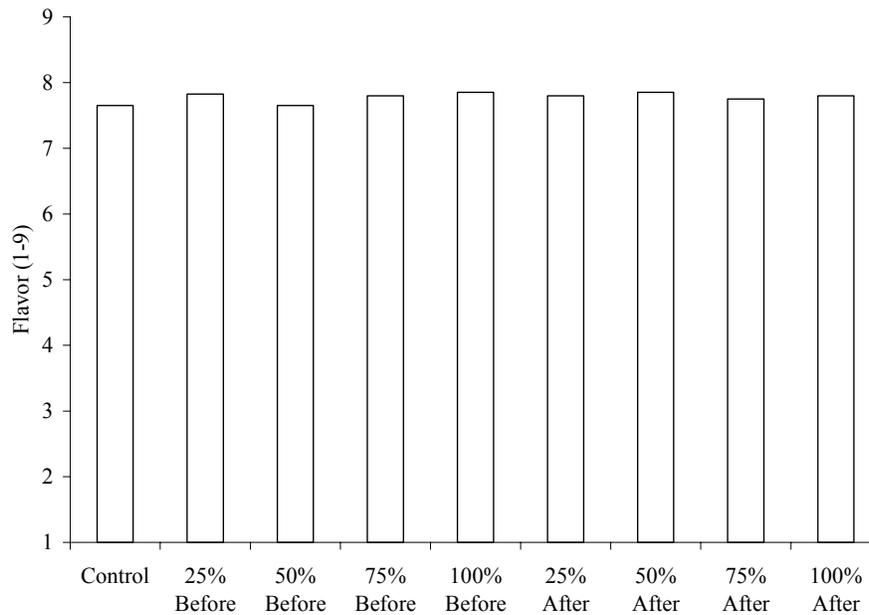


Figure 19. Mean flavor scores of reduced fat milks fortified with folic acid at 25, 50, 75 and 100% of RDA added before or after pasteurization

The mean appearance scores are reported in Figure 20. Appearance scores are from 1 to 9 (1= dislike extremely, 5= neither dislike nor like, and 9= like extremely). No significant differences were found in folic acid addition before vs. after pasteurization. Level of folic acid addition had no overall effect on appearance scores. The mean appearance score was 8.15, which indicates that the appearance of the samples was very much liked by the consumer panelists. Appearance is the most important sensory characteristic which determines the initial consumer acceptance of products (Hutchings, 1977).

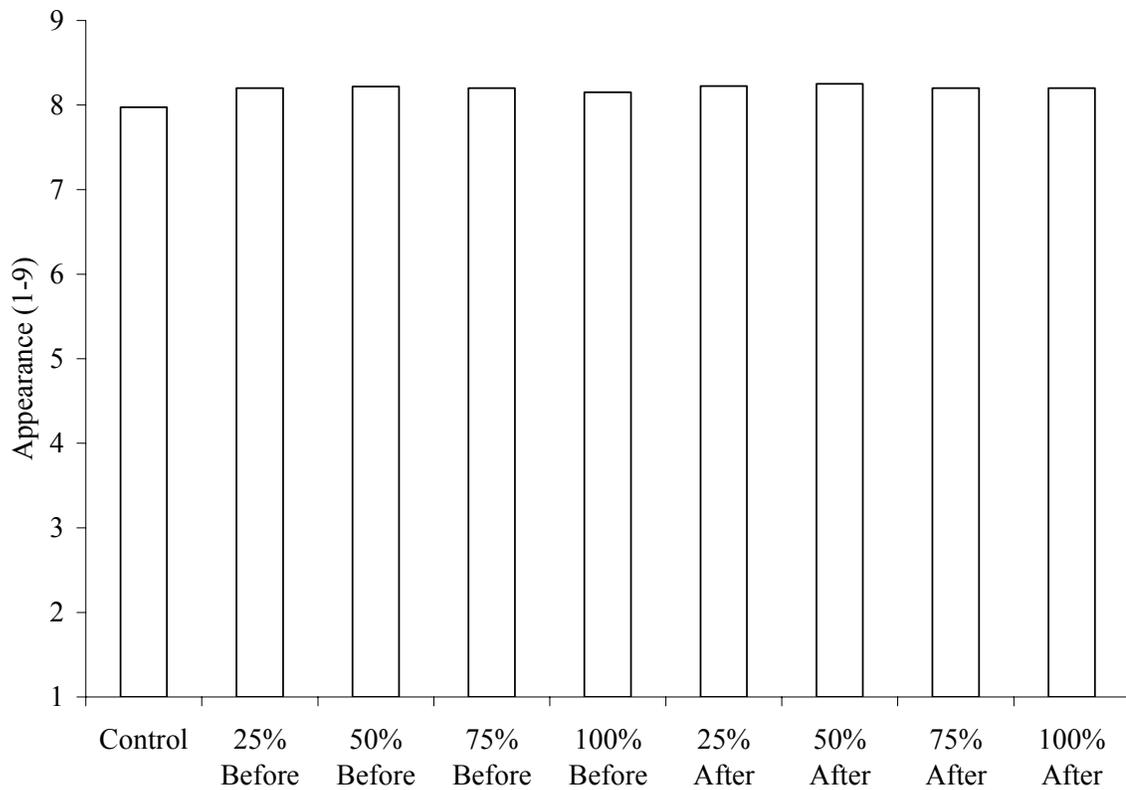


Figure 20. Mean appearance scores of reduced fat milks fortified with folic acid at 25, 50, 75 and 100% of RDA added before or after pasteurization

The mean texture / mouthfeel scores are reported in Figure 21. Texture / mouth feel scores are from 1 to 9 (1= dislike extremely, 5= neither dislike nor like, and 9= like extremely). No significant differences were found in folic acid addition before vs. after pasteurization. Level of folic acid addition had no overall effect on texture / mouth feel scores. The mean texture / mouth feel score was 7.77, which indicates that the texture / mouth feel of the samples was liked moderately by the consumer panelists.

The overall product acceptability was 100 % for all the samples. Indicating that the consumer panelists liked all the samples irrespective of the addition of folic acid before or after pasteurization.

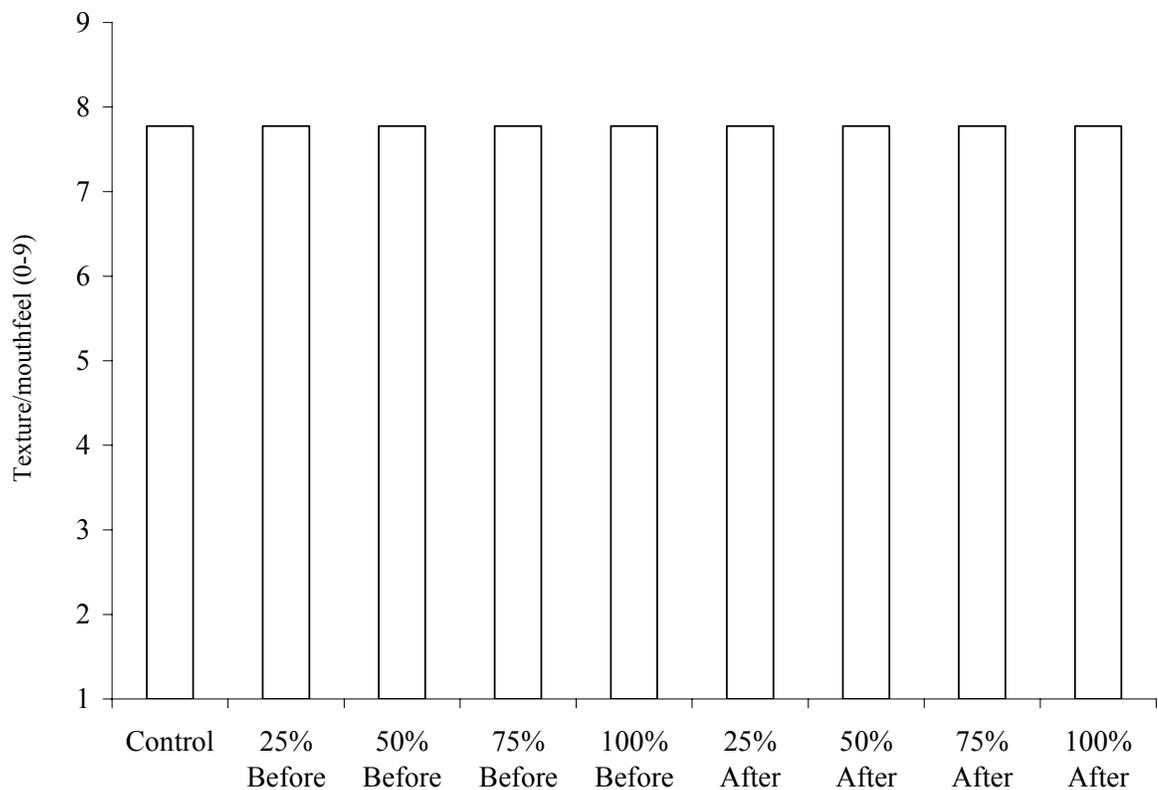


Figure 21. Mean texture/mouthfeel scores of reduced fat milks fortified with folic acid at 25, 50, 75 and 100% of RDA added before or after pasteurization

CHAPTER 4: CONCLUSIONS

Addition of folic acid before or after pasteurization at 25, 50, 75 and 100% of the RDA of 400 µg had no effect on the fat content, protein content of reduced fat milks. Viscosity of the reduced fat milks was not affected by the addition of folic acid before or after pasteurization. Peak areas obtained from the HPLC increased proportionally with the increase in the folic acid content of reduced fat milks. However the samples to which folic acid was added before pasteurization had a lower overall mean folic acid levels than that of samples with folic acid added after pasteurization. Indicating that pasteurization has an effect of the folic acid content. Results from ELISA indicated that the mean concentration of folate binding protein in the samples decreased with the increase in the folic acid content. The decrease in the values of folate binding protein may be attributed to the binding of folic acid to folate binding protein, which may not be permitting folate binding protein to react with the antibody to produce color during ELISA. Folate binding protein profile obtained from PAGE indicated no differences in folate binding protein migration patterns over the storage period with addition of all four levels of folic acid before and after pasteurization of the reduced fat milks. However there was a clear band broadening of folate binding protein due to the micro heterogeneity in charge and carbohydrate content. Addition of folic acid to reduced fat milks before or after pasteurization did not impact flavor, appearance and texture / mouthfeel scores. Fortification of reduced fat milks with folic acid can be accomplished with out adversely affecting the product characteristics.

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VITA

Kamalesh Achanta was born on January 8, 1980, in the town of Rajahmundry in India. He grew up in the city of Hyderabad, the capital city of the state Andhra Pradesh. He graduated from high School in the year 1998. In fall of 1998, he joined the Acharya N G Ranga Agricultural University, Tirupathi, and obtained his bachelor of technology degree in the Department of Dairy Technology in fall 2002. He joined Louisiana State University in the spring of year 2004 and is set to obtain the degree of Master of Science in the Department of Dairy Science with dairy foods technology as the specialization in December 2005.