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Effects of Iron Fortification on Microbiological, Physical, Chemical, and Organoleptic Properties of Yogurt

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EFFECTS OF IRON FORTIFICATION ON MICROBIOLOGICAL, CHEMICAL, PHYSICAL, AND ORGANOLEPTIC PROPERTIES OF YOGURT

by

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ABSTRACT

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It has been shown that iron binds strongly to the proteins in milk, and our aim was to determine whether or not this binding was affected by lowering pH in the manufacture of yogurt. Iron-protein complexing was studied using two different techniques. 1) Skim milk was fortified with 10 mg iron/100 ml and the pH of the milk was adjusted to 6.7, 6.2, 5.8, 5.3, 4.5, and 4.0. The milk was fractionated by ultracentrifugation at 52,000 x g for 60 minutes. The pellets and serum were then analyzed for iron, calcium, and phosphorus content by inductively coupled plasma spectroscopy. SDS-PAGE gels were used to determine protein profiles in the pellets and serum. 2) Yogurt was made from milk fortified with $FeCl_3$, iron complexed with casein, and iron complexed with whey proteins. Small samples of the yogurt were then freeze-dried on carbon-

coated grids and examined by transmission electron microscopy at 80 KV.

Affinity of iron for milk proteins was independent of pH. Iron fortification of milk did not cause loss of calcium or phosphorus from casein micelles. Electron spectroscopic imaging (ESI) showed that iron was bound to casein when yogurt was fortified with $FeCl_3$ or iron-casein complex. When fortified with iron-whey protein complex, the iron was distributed throughout the non-micellar portion of the yogurt.

To determine effects of iron on yogurt quality, low-fat (2%) and nonfat iron fortified yogurt was made with three sources of iron: $FeCl_3$, iron complexed with casein, and iron complexed with whey protein, at three levels (10, 20, 40 mg/kg). Iron content and lipid oxidation were determined over one month of storage at 4°C.

Iron fortification had no effect on the rate of fermentation by the lactic cultures. There was no significant increase in oxidation levels between iron-fortified yogurt and unfortified yogurt ($P > .05$). No differences in the appearance, mouth feel, flavor, and overall quality between iron-fortified yogurt and unfortified yogurt were detected in consumer sensory analysis. Our study showed that high quality iron-fortified yogurt could be manufactured without added food safety risks. (214 pages)

I dedicate this work to my husband, Nader Soltani, and to my children, Hoda and Mohammad, for their many sacrifices, support, encouragement, and patience throughout my education.

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PART 1. LITERATURE REVIEW

INTRODUCTION

The most important deficiency diseases in developing countries are protein-energy malnutrition (PEM), nutritional anemia, and iodine deficiency disorders (52). Nutritional iron deficiency continues to be a major global health problem (81). According to Tomkins (86), possible strategies to prevent diarrhoea in children living in developing countries include dietary supplementation with vitamin A1, zinc, and iron, promotion of breastfeeding, and improvement in the standard of personal hygiene.

Two ways to increase iron intake are by providing supplemental medicinal iron or by fortification of food products (10). The advantages of iron supplementation are that it produces rapid changes in iron status and directs iron to the specific populations that are at risk for iron deficiency. The disadvantages of iron supplementation are gastrointestinal side effects of oral iron, difficulty in maintaining motivation of the participants, providing an effective system of health delivery, and high cost (22).

An alternative, more effective long-term approach of increasing dietary intake of iron to the general population would be to add iron directly to the diet by iron fortification of food products. However, selection of appropriate iron sources poses several technical difficulties in the fortification process because many of

the iron sources may alter appearance or taste of the food products or are poorly absorbed. For example, soluble ferrous salts usually cause color changes by complexing with sulfur compounds, tannins, polyphenols, and other food ingredients (22). In addition, chemically reactive forms of iron catalyze oxidation reactions, which would result in the development of unpleasant odors and flavors.

Another important factor in developing a successful fortification process is the selection of an appropriate food vehicle. The two important considerations in selecting food vehicles are their consumption pattern and technical feasibility (22). They should reach the vulnerable segment of the population, be unrelated to socioeconomic status, have a low potential for excessive intake, have good defect-masking qualities, and have low consumer cost (22). Some of the potential food vehicles for iron are wheat flour, salt, sugar, rice, condiments, maize, milk products, and processed cereals.

Iron Fortification

Iron Sources. Ferrous sulfate and ferrous gluconate are water soluble iron sources that have the highest relative bioavailability values in rats but cause rapid fat oxidation and unwanted color changes in food products (42). Hurrel et al. (43) studied potential iron sources for fortification of infant cereals. They selected ferrous

fumarate, ferrous succinate, and ferric saccharate as the most suitable sources for infant cereal fortification. Infant absorption of ferrous fumarate was identical to ferrous sulfate, whereas the absorption values for ferrous succinate, ferric saccharate, and ferric pyrophosphate were 92, 74, and 39% of the ferrous sulfate. They concluded that ferrous fumarate and ferrous succinate were the most feasible sources for infant cereal fortification because these sources were highly bioavailable and did not cause fat oxidation or discoloration.

Milk. Milk is an excellent source of nutrients such as calcium, protein, and vitamins, but contains less than one milligram of iron per liter (5). It is also consumed in substantial amounts by most people, and its iron fortification could provide an effective means to alleviate iron deficiency. Stekel et al. (81) investigated the efficiency of a fortified, acidified milk in preventing iron deficiency in infants. Infants from age 3 to 15 mo received acidified milk fortified with 15 mg of iron as ferrous sulfate and 100 mg of ascorbic acid/100 g of powdered milk. The control group received unfortified milk, and 25.7% of them showed anemia compared to only 2.5% of those infants who received fortified milk. Therefore, iron fortification of milk or other dairy products could be used to reduce iron deficiency in the infants. Pizarro et al. (69) also found

that anemia was present in 25.7% of the infants who received unfortified milk but in only .8% of the infants who consumed fortified milk.

Iron fortification of milk has always been difficult because of production of undesirable color and flavor changes due to iron-stimulated autoxidation of milk fat. It has been proposed (49) that cocoa and chocolate contain natural antioxidants which would prevent development of oxidative rancidity in milk. Douglas et al. (28) fortified chocolate milks with nine commonly used iron sources and with ferripolyphosphate and ferripolyphosphate-whey protein complex. They found that sodium ferric pyrophosphate, ferripolyphosphate, and ferripolyphosphate-whey protein complex caused little or no off-color even after 2 wk of storage. The other iron sources caused off-colors. In general, ferric compounds produced little or no off-flavors in chocolate milks, and ferrous compounds caused off-flavors initially, but flavor scores improved after 14 d of storage at 4°C.

Baldwin et al. (5) reported that fortification of milk with reduced iron, complexed with citric and phosphoric acids, lowered the intensity of cooked flavor and exerted little influence on oxidized flavor in milk pasteurized at 80°C for 25 s. They also concluded that the likelihood of

oxidized off-flavors would be greater if the iron sources were added after pasteurization at 72°C for 17 s.

The effect of pasteurization temperature has also been documented by other investigators. When whole milk was fortified with ferric iron compounds and pasteurized at minimum to moderate temperatures (below about 79°C), it had a uniform lipolytic rancid flavor. This off-flavor was reduced or completely diminished simply by pasteurization at 81°C (31).

It has been shown that homogenized iron fortified milk is more susceptible to oxidized off-flavor because it provides a strong reducing system which causes conversion of ferric iron to the stronger pro-oxidant ferrous form (77). Kurtz et al. (50) reported that ferric ammonium citrate and ferric chloride (20 mg Fe/L) could be used for fortification of skim milk and nonfat dry milk without causing adverse flavor effects.

Direct addition of iron to milk might have detrimental effects on its quality and acceptability due to development of oxidized off-flavor, color changes, and metallic flavors (89). Microencapsulation has been used for many years to protect sensitive food components, preserve desirable flavors and aroma, inhibit nutritional loss, and mask undesirable flavors (30, 44).

Cheese. Cheddar cheese and process Cheddar cheese are considered to be appropriate vehicles for delivering iron to consumers. Zhang and Mahoney (94) investigated the effects of iron fortification on quality of process Cheddar cheese fortified with iron-casein, iron-whey protein complex, and $FeCl_3$. They indicated expert panelists could not detect any significant differences in oxidized off-flavor or cheese flavor between fortified and unfortified cheeses that were aged as long as 3 mo. Zhang and Mahoney concluded (93) that ferripolyphosphate whey protein complex, iron-casein, and $FeCl_3$ were potential iron sources for fortification of cheddar cheese.

Iron Microencapsulation. Iron microencapsules can be used to fortify cereals and flour. Hurrel (42) found fortification of wheat flour with encapsulated $FeSO_4$ resulted in minimal oxidation and favorable taste panel scores. Jackson and Lee (44) used the microencapsulation techniques to fortify cheese and other high-fat and high-moisture foods with iron. They reported commercial iron microcapsules were not suitable for cheese fortification because they released iron during cheese making. Jackson and Lee (45) found when Havarti-style cheese was fortified with stearine-coated microcapsules containing iron as $FeSO_4$, $FeSO_4$ with ascorbic acid, or $FeCl_3$, it had lower levels of malonaldehyde, indicating less lipid oxidation. Cotton seed

stearine (m.p. 62.8°C) has shown a good oxidation stability and retention capability under rapid stirring at 39°C.

Bakery Products. Iron fortification of bread and bakery products has been successful. In the United States, iron fortified wheat flour accounts for about 20% of the population's iron intake (22). Burri (13) reported wheat fortification or malted milk with $FeSO_4$ shows no oxidation off-flavor, whereas ferric pyrophosphate stimulated oxidation.

Salt. The utilization of salt as an iron vehicle has been studied in India for several years. The overriding difficulty associated with salt fortification is the bioavailability of the iron sources. Salt fortification with sodium iron pyrophosphate, ferric orthophosphate, or ferric pyrophosphate is not feasible because of low bioavailability of the iron sources (64). Some of the iron sources suitable for salt fortification are combinations of ferric orthophosphate, starch, and ascorbic acid (75, 76), or combinations of ferrous sulfate, sodium hexametasulfate (stabilizer), and sodium acid sulfate (enhancer) (65, 83).

Sugar. Sugar fortification has been studied in Guatamala. One of the highly bioavailable iron sources used for sugar fortification is sodium ferric EDTA (88). It gave a slight yellowish tinge to the refined sugar, which was not apparent when the fortified sugar was added to tea (88).

Rice. More than half the world's population consume rice on a regular basis, and these are in the countries where nutritional anemia is prevalent (22). Therefore, it appears rice is an appropriate vehicle to deliver iron to these populations. However, there are several obstacles for rice fortification such as the poor color-masking property of rice and low bioavailability of iron sources (22). Peil et al. (68) suggested polymer coating of iron could mask color changes in fortified rice, resist washing and cooking, and yet quickly dissociate in the intestinal tract.

Fish Sauce. In East Asia countries, fish sauce was proposed to be an appropriate vehicle for iron fortification (36) because problems with off-flavor, odor, and color are reduced in a highly flavored and colored fish sauce.

Iron Deficiency

Iron deficiency is one of the worldwide deficiency diseases. Insufficient dietary intake of iron or poor utilization of iron usually result in iron deficiency (89). Iron deficiency is considered an important public health problem because of its consequences on health. There is a high iron need during pregnancy and growth. Also individuals with excessive or frequent menstrual losses, low iron diet, and marked hemorrhage require a high iron diet (62).

Dallman et al. (26) estimated the prevalence of iron deficiency anemia in the United States was at 5.7% for infants, 5.9% for teenage girls, 5.8% for young women, and 4.4% for elderly men.

The most extensively studied impacts of iron deficiency are reduced work performance and immune response (23).

Physical Performance. Some of the adverse effects associated with iron deficiency are impaired cognitive function and noncognitive disturbances which limit activity and work capacity. Infants with iron deficiency anemia have lower mental and motor developmental test scores (55).

There is also evidence that iron deficiency anemia causes limitations in maximal physical performance, submaximal endurance, and spontaneous activity in adults (56). In male and female distance runners, depletion of iron stores is frequently seen. This is because of inadequate iron intake and increased iron excretion through sweating and gastrointestinal blood loss. McDonald and Keen (58) suggested athletic performance is improved by dietary trace element supplementation. It has been shown that iron and magnesium deficiency could cause a significant reduction in exercise performance. Iron supplementation could also be helpful in reducing blood lactate concentrations following heavy exercise (40).

Shachar et al. (78) investigated the effect of iron deficiency in rats on the blood-brain barrier and insulin

transport. They found the brain uptake index for L-glucose and insulin increased by 70 and 100%, respectively, in iron deficient rats. They concluded iron-deficiency anemia selectively affected the integrity of the blood-brain barrier and brain function.

Immune Function. Another problem associated with iron deficiency is abnormalities in immune function. Several studies have shown the total number of T cells decreased in iron deficient individuals, and the level of depression was proportional to the severity of iron deficiency (3, 16, 23, 70, 80). Under experimental conditions, iron deficient patients showed abnormalities in cell-mediated immunity and ability of neutrophils to kill different kinds of bacteria (23). Blakley and Hamilton (9) studied the effects of iron deficiency on the immune response in mice. They reported reduction of antibody production (T-lymphocyte dependent response) in iron deficient mice.

Other immunological alterations associated with iron deficiencies are impairment of lymphocyte transformation (48, 80), decreased production of migration inhibition factor (48), and impaired cutaneous delayed hypersensitivity (16, 57). Some of the biochemical abnormalities associated with iron deficiency include decreased activity of iron containing enzymes such as Cytochrome "C" and cytochrome oxidase (25, 46).

Effects of Diet on Iron Status. Impaired absorption of iron could be attributed to high intakes of dietary fiber, phytate, tannins (8), and low intakes of flesh foods (63). In addition, diets high in soy protein decrease absorption of nonheme iron (21). There are several studies indicating potential problems in iron status of vegetarians. Dwyer et al. (29) investigated nutritional status of vegetarian children and found that 25% of preschool vegetarian children showed mild iron deficiency despite adequate dietary iron intakes. Bindra and Gibson (8) reported a high incidence of iron deficiency among adult lacto-ovo-vegetarians. Anderson et al. (1) suggested iron status of vegetarians could be improved by exceptionally high intakes of ascorbic acid.

Iron Requirements. Adolescent females are more vulnerable to iron deficiency than are adolescent males. Males have a highly favorable position concerning iron requirements during adolescence (32). Males require only 1 mg of iron per day to replace physiological blood loss (37) while adult females require an additional .5 mg/day because of menstruation (7). The iron requirements for pregnant women could increase to as much as 5 to 6 mg/day in the last trimester (10, 33). Severe iron deficiency anemia during pregnancy could result in increased risk of premature delivery and increased maternal and fetal morbidity and mortality (4).

Preschool children between ages 1 to 5 are also in danger for iron deficiency. In developed countries, the availability of dietary iron is usually limited because milk constitutes a large portion of the diet among preschool children and thus it displaces other iron-rich foods from the diet. In developing countries where cereals are excessively used, economic factors usually limit the intake of meat, poultry, or fish that would increase iron absorption from cereals (24).

Iron Bioavailability

The nutrient density of a food or the amount of a nutrient per unit energy, depends on bioavailability of each individual nutrient (39). Two factors that influence iron absorption are iron status of the individuals and composition of their diet (39).

Different studies report considerable variation in bioavailability of iron from ferrous sulfate (33 to 80%). This variability could be due to the age of ferrous sulfate salt used in the study. Park et al. (66) reported that storage of a fresh ferrous sulfate salt for 3 mo reduced its bioavailability from 84 to 65%.

Dietary iron can be classified as either heme or non-heme iron.

Heme Iron. Heme iron (a ferroprotoporphyrin), a component of hemoglobin and myoglobin, has a higher

bioavailability than nonheme iron. Based on an individual's iron stores, 15 to 35% of heme iron is absorbed (62). However, the majority of dietary iron intake is non-heme iron from cereals, vegetables, fruits, eggs, and fortified foods (39). So despite lower absorption of non-heme iron (2 to 20%), it contributes more to the body's iron pool because foods contain more nonheme iron (62).

Nonheme Iron. Nonheme iron absorption is markedly influenced by the iron status of the subjects and interaction of the promoters and the inhibitors of iron absorption present in individual diets (38). Some studies suggest animal foods may enhance nonheme iron bioavailability. Factors that increase nonheme iron absorption include consumption of high levels of ascorbic acid and meat products such as beef, pork, chicken and fish. Monsen (62) reported consumption of ascorbic acid and meat/fish/poultry increases nonheme iron bioavailability four-fold. Egg yolk, phytates, and tea decreased non-heme iron absorption (39).

Layrisse et al. (53) reported nonheme iron absorption is 10-fold greater when consumed with veal muscle than when taken with a meal of maize. The importance of animal protein in the human diet is such that some investigators recommend as much as 28 mg iron/day for adult women when less than 10% of their calories are derived from animal protein, whereas 14 mg iron/day is recommended for those in

which 25% of calories are supplied by animal proteins (20). However, other studies indicate not all animal foods enhance iron absorption. For example, eggs inhibit dietary iron absorption (14).

Effects of Food Processing on Iron Bioavailability. The effect of food processing to alter iron bioavailability is well established (47, 85, 91). Jansuittivechakul et al. (47) studied the effect of autoclaving on meat enhancement of dietary iron bioavailability and found heat treatments improved iron bioavailability of meat and meat/hemoglobin mixtures, despite the fact that the heme iron contents were decreased in the cooked products. Sterilization enhances the relative bioavailability of ferrous sulfate, sodium ferric pyrophosphate, ferric orthophosphate, and ferric pyrophosphate supplemented in milk-based infant formula (85), soy isolate infant formula (84), and basal diets (91). Wood et al. (91) reported heat and pressure processing significantly enhanced the relative biological values for sodium ferric pyrophosphate and ferric pyrophosphate. Also typical retort conditions increase relative biological value of electrolytic iron and carbonyl iron by solubilization and oxidation of the iron sources to the ferrous form (18). Theuer et al. (85) also showed sterilization of liquid milk-based infant formulas increased the relative iron availability of ferric pyrophosphate from 75 to 125% and of sodium iron pyrophosphate from 40 to 60%.

Effects of Trace Elements on Iron Absorption. Fortification of food products with one trace element may impair utilization of another trace element in the body because many trace elements interact with each other and with other nutrients (60). Momcilovic et al. (61) reported that a high dietary iron/zinc ratio may cause low zinc availability from infant cereals. However, it has been shown that if milk were fortified with a physiological dose of iron, it would not interfere with the absorption and metabolism of zinc (59).

Cook et al. (19) investigated the effects of calcium supplements (calcium carbonate, calcium citrate, and calcium phosphate) on absorption of dietary nonheme iron and iron supplements. They reported that when calcium carbonate was taken without food, it did not prevent absorption of ferrous sulfate with doses of either 300 mg calcium and 37 mg iron, or 600 mg calcium and 18 mg iron. However, at the latter dose, calcium citrate and calcium phosphate significantly reduced absorption of iron by 49 and 62%, respectively. They concluded that regular calcium supplements when taken with food would inhibit iron absorption. Studies by Kwock et al. (51) showed iron may be supplied by different vehicles to the body, but once absorbed, it is metabolized in a similar manner.

Iron Bioavailability of Dairy Products. Dairy products are good sources of minerals, vitamins, and high quality proteins but contain almost no dietary iron. Therefore, dairy products could be used as a logical vehicle for iron fortification. The bioavailability of iron in dairy products depends on the iron sources (2, 54, 92) and on processing (85).

Milk. The wide consumption of milk by infants and children and its high nutrient density make it an attractive vehicle for iron fortification. Carmichael et al. (15) studied the effect of milk and caseins on the absorption of supplemental iron in mice and chicks. They found nonfat cow's milk and its constituent phosphoproteins did not inhibit iron absorption. In fact, in the chicks, milk significantly increased the absorption of iron from ferric nitrilotriacetate chelate. Park et al. (67) compared the bioavailability of iron in goat milk with cow milk fed to anemic rats and found iron bioavailability of goat milk was greater than cow milk.

Tsuchita et al. (87) investigated the iron bioavailability of ferric pyrophosphate by hemoglobin repletion assay. The relative bioavailability of ferric pyrophosphate, mixed with skim milk and dehydrated, was 100% that of $FeSO_4$ by slope ratio analysis. They concluded

bioavailability of ferric pyrophosphate was improved by mixing with skim milk and heat treating.

Ranhotra et al. (71) studied bioavailability of a water soluble citrate phosphate iron complex in milk. They found bioavailability of iron was as high as ferrous sulfate (99% vs 100%), and it was not affected by milk or milk components.

Cheese. Zhang and Mahoney (92) found that bioavailability of iron in Cheddar cheese fortified with ferric chloride, iron-casein, ferripolyphosphate-whey protein, and iron-whey protein complex was high (5, 8, 6, and 7%, respectively) and similar to ferrous sulfate (5%). They concluded that iron-fortified cheese was a good source to increase human dietary iron intake.

Studies on cottage cheese fortified with ferric ammonium citrate showed that bioavailability of iron was not affected relative to the time of iron addition during the manufacturing procedure (90).

Infant Formula. Stekel et al. (82) investigated the bioavailability of iron added to infant formula. They found a higher range of mean absorption (5.9 to 11.3%) when ferrous sulfate was added in conjunction with ascorbic acid (100 mg/L). They also reported that the amount of milk fat, the addition of carbohydrates, or acidification would not affect iron absorption.

Infant Cereals. Infants grow rapidly and require a significant source of dietary iron. Rios et al. (73) reported that utilization of sodium iron pyrophosphate and ferric orthophosphate as iron sources in infant cereals were not suitable because they were poorly absorbed (mean < 1.0%) and did not meet the nutritional needs of infants. When reduced iron of very small particle size and ferrous sulfate were added to infant cereals, they were absorbed to a greater extent (mean 4% and 2.7%, respectively).

The problems of discoloration, distribution of iron, and reduced shelf life limit utilization of reduced iron and ferrous sulfate in infant cereals.

Iron-Binding Milk Proteins

Most iron added to milk binds to protein molecules. Basch et al. (6) investigated the distribution of added iron and polyphosphate phosphorus in cow's milk and found casein showed a greater binding affinity for iron than for phosphorus; about 85 to 95% of iron and 50 to 55% of phosphorus are bound to acid precipitated casein. Iron binding by casein is attributed to clustered phosphorylserine residues (41).

Ferrous salts are not usually recommended for fortification of milk and milk products because their incomplete binding to the casein fraction allows some of the Iron (II) to bind to milk fat and cause organoleptic

deterioration of the supplemented products (41). Ferric iron binds rapidly to the casein phosphoserines and forms Iron (III)-di-O-phosphorylserine (41). Therefore, ferric iron has been suggested as a more suitable source for fortification of milk and milk products.

Most of the total iron in infant formulas (casein or soy based) is bound to soluble proteins (35). Saltman and Hegenauer (74) reported that in cow milk, fat contained 3% of fortified iron while casein bound 75% of added iron. Human milk fat chelated significantly more iron than cow milk fat; 23% of iron was present in milk fat with 36% in casein.

Lactoferrin and transferrin are also iron-binding proteins (17). Lactoferrin (LF) occurs in three isomers: LF-alpha binds iron; however, LF-beta and LF-gamma do not bind iron but show RNase activity (34). These isoforms are very similar in isoelectric point, partial proteolytic peptide patterns, and N-terminal amino acid sequence (34).

Shimazaki (79) compared structure and iron-binding capacity of lactoferrin isolated from cow colostrum and cheese whey. He reported no differences in secondary and tertiary structures between the lactoferrins. However, the iron-binding capacity of cheese whey lactoferrin was about 70% of the native lactoferrin.

In a comparison of human milk lactoferrin and bovine colostrum lactoferrin, it was shown that lactoferrin from

human milk was resistant to trypsin digestion, whereas lactoferrin from bovine colostrum lost its iron-binding and antimicrobial activities after being exposed to trypsin. Iron saturation of purified lactoferrin protects both human and bovine proteins from inactivation by protease (11, 12).

Davidson and Lonnerdal (27) studied the effects of glycan chain of lactoferrin for iron absorption. They found that fusocyclated glycans, which are part of the carbohydrate chain of lactoferrin, were necessary for receptor recognition in small intestine.

SUMMARY

One of the world-wide deficiency diseases is iron deficiency, which is usually caused by inadequent dietary intake of iron or poor utilization of iron. Iron deficiency could result in decreased work performance and improper functioning of the immune system.

The most vulnerable segments of population that are at risk for iron deficiency are infants, teenage girls, young women, and elderly men.

An effective means to increase dietary intake of iron is iron fortification of food products. The two most important considerations for iron fortification of food products are 1) functionality (product compatibility) and 2) bioavailability (absorption and utilization of a nutrient by man and animals) (72). Therefore, an optimal iron source

should have high bioavailability and desirable physical-chemical and organoleptic properties. An appropriate food vehicle should reach the populations that are at risk of iron deficiency, mask low grade off-flavors, have a low potential for excessive intake, and be low cost.

Iron fortification of milk, cheese, cereals, bakery products, salt, sugar, rice, and fish sauce has been studied. Iron-fortified Cheddar cheese fortified with $FeCl_3$, iron-casein, and iron whey protein complex is considered an appropriate vehicle to increase human dietary iron intake because these iron sources have high bioavailability and do not increase oxidized off-flavor during the storage period.

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PART 2. BINDING OF IRON TO CASEIN AND WHEY PROTEIN IN SKIM MILK AND IRON-FORTIFIED YOGURT

ABSTRACT

Iron-binding affinity of casein and whey protein was studied by fortifying skim milk with 10 mg iron per 100 ml and adjusting its pH to 6.7, 6.2, 5.8, 5.3, 4.5, and 4.0.

Samples were fractionated by ultracentrifugation at 52,000 *g* for 60 min. The pellets and serum were collected, digested with nitric acid, and analyzed for iron, calcium, and phosphorus by inductively coupled plasma spectroscopy. Protein profiles were also obtained by SDS-PAGE.

SDS-PAGE of serum showed distinct bands for α_{s1}-casein (α_{S1}-CN), β-casein (β-CN), and κ-casein (κ-CN) at pH 6.7, 6.2, 5.8 and 5.3. These bands were missing at pH 4.5 and 4.0, indicating that at higher pH, some casein was retained in the supernatant after ultracentrifuging. The iron measured in the serum was most likely associated to casein. SDS-PAGE of pellets showed more intense bands for β-lactoglobulin (β-LG), bovine serum albumin (BSA), and κ-CN at pH 4.5 and 4.0.

More iron was present in pellets at pH 4.0 and 4.5 because more protein (casein and denatured whey proteins) is precipating at these pH. When the results were expressed in terms of micrograms of iron per gram of protein, pellets at pH 4.0 and 4.5 showed the lowest amount. This is probably due to presence of denatured proteins such as β-LG, BSA, and κ-CN with low iron-binding affinity, which

contributed to higher protein content of pellets at pH 4.0 and 4.5.

As pH decreased, calcium and phosphorus dissociate from casein micelles. Their behavior was independent of iron, which indicates that perhaps iron has different binding sites than colloidal calcium phosphorus in the casein micelles.

To be able to predict chemical and microbiological shelf stability, it is important to know where iron is located in the yogurt because iron can accelerate fat oxidation and is a required nutrient for some microorganisms. Therefore, skim yogurt was fortified with $FeCl_3$, iron complexed with casein, and iron complexed with whey protein. Small samples of the yogurt were then freeze-dried on 600 H mesh carbon coated grids and examined (without heavy metal staining) by transmission electron microscopy at 80 KV. Elemental maps for iron were obtained using electron spectroscopic imaging (ESI).

Using ESI it was observed that when yogurt was fortified with iron-casein complex, the iron remained bound to the casein and was distributed throughout the micelles; with iron-whey protein complex, it was distributed throughout the non-micellar portion of the yogurt; with fortification by $FeCl_3$, the iron was observed to be bound

preferentially to the casein and was located within the casein micelles.

INTRODUCTION

Iron deficiency continues to be one of the major nutritional deficiencies in the world, as well as in the United States (2, 6, 17). It is more common in infants, young children, and women of child-bearing age in the United States (7, 14). Dairy products such as milk, cheese, and yogurt are excellent sources of calcium (27). However, they contain almost no dietary iron (4, 26, 16). Therefore, iron fortification of dairy products would provide an excellent source of both nutrients.

Demand for high quality and healthful dairy products has increased the consumption of yogurt in recent years. High protein and calcium with low fat content of yogurt make it an ideal dairy product for health conscious consumers.

Consideration of iron fortification of yogurt leads to the question of iron location in high-acid foods. This information is important for predicting shelf stability of yogurt from microbiological and chemical oxidation perspectives. It is important to study iron-protein complexing to understand oxidative deterioration which often is a problem of iron fortified products. Iron fortification usually accelerates fat oxidation. Understanding where iron

is bound in yogurt is essential to developing high quality products with superior iron bioavailability.

It was first thought that binding of added or natural iron was nonionic and that iron was bound to fat globule membranes (1). King et al. (15) studied the distribution of natural and added iron in milk. They reported most of the natural iron was bound to the fat glouble, but none of the added iron was associated with the fat globules. Later workers (3, 11, 29) found iron was mostly bound to the milk proteins, primarly casein, a phosphoprotein.

Reddy and Mahoney (22) studied binding of iron to different milk proteins at pH 6.6 and found iron binding of these proteins increased with an increase in free iron concentration but at a fixed free iron concentration, the amount of iron bound to different proteins was different, which indicated that these proteins have different binding affinities for iron. They also studied binding of Fe(III) to α_{s1}-CN at different pH (5.6, 6.1, 6.6, 7.2, and 7.8) and found that the free energy change (ΔG) for binding of Fe (III) to α_{s1}-CN is small and negative, indicating iron binding is instantaneous and thermodynamically favorable.

Because the pH of yogurt is relatively low (4.0-4.5), our aim was to determine the fate of iron under low pH conditions. The purpose of this study was to study iron-protein complexes and understand whether iron is bound or

exists in soluble form in iron-fortified yogurt. Iron that is bound to proteins may not be available to culture or spoilage organisms and would not affect their growth. However, the bioavailability of this iron to humans would be high due to action of proteolytic digestive enzymes unless it is bound to proteins that have poor gastrointestinal digestibility.

MATERIALS AND METHODS

Pasteurized (79°C for 28 s) skim milk was obtained from the Gary H. Richardson Dairy Products Laboratory at Utah State University and was skimmed by centrifugation (20°C) (Sorvall Instruments, RC5C Du Pont) at 8,000 *g* for 1 h. Glass fiber filter paper (Whatman GF/A) was then used to remove excess fat. Half of this milk was used as control and the other half was fortified with $FeCl_3$ at the rate of 10 mg iron per 100 ml of milk. They were stirred for 1 h at room temperature and then transferred into ultracentrifuge tubes (Sorvall Instruments, Du Pont).

Acidification of Milks

The pH of each milk type was adjusted to 6.7, 6.2, 5.8, 5.3, 4.5, and 4.0 with .1N NaOH, .1N HCl, or 1N HCl. Samples were then centrifuged (20°C)(Sorvall Instruments, RC70, Du Pont) at 52,000 *g* for 1 h. Pellets and serum were

collected. Pellets were then freeze-dried (DURA-DRY, FTS Systems, Inc. Stone Ridge, NY) overnight.

Mineral Analysis

Samples of milk, serum, and dried pellets were digested with concentrated (16 *M*) nitric acid by heating them below their boiling points. Hydrogen peroxide (30%) was added dropwise at the end of digestion until a white ash was formed. This ash was then dissolved with .5 ml of 6 N HCl and diluted 10-fold with distilled deionized water. Total iron, calcium, and inorganic phosphate were then determined using inductively coupled plasma spectroscopy (ICP) (Thermo Jarrel Ash, ICAP 9000, Franklin, MA).

Protein Analysis

Protein content of the milk, serum, and dried pellets was estimated by a semi-micro Kjeldahl procedure for nitrogen (9) using automatic Kjeltec equipment (Kjeltec Auto 1030 Analyzer, Fisher Scientific Co.). Duplicate samples were used for each pH. Protein content of these samples was then calculated by multiplying the nitrogen content of the sample by 6.38.

SDS-PAGE

The SDS-PAGE of serum and pellets at pH 6.7, 6.2, 5.8, 5.3, 4.5, and 4.0 was performed using a Phast System

(Pharmacia, Uppsala, Sweden) with a Phast Gel homogenous 20 gel (Pharmacia, Uppsala, Sweden) (20).

Pellets were dissolved in 4.0 ml of SDS-PAGE sample buffer (20 mM Tris, 2 mM EDTA, 5% SDS, pH = 8). Then, .2 and .4 ml of this solution were diluted with .25 ml of distilled deionized water and .5 ml of SDS-PAGE sample buffer. Serum from each pH was diluted (1 : 1) with SDS-PAGE sample buffer, adjusted to pH 8.0 with 1 N and .1 N NaOH, followed by 50 μl and 20 μl addition of β-mercapto ethanol to pellets and serum, respectively. These samples were placed in a boiling water bath for 5 min and then cooled in water bath to room temperature.

To each sample was then added 2.0 μl of bromophenol blue dye (4.5% wt/vol). Samples were loaded automatically at the anodic end of the gel (250 V, 1.0 mA, 3.0 W, and 15°C at 0 Vh). The gels were run for 95 Vh at 10.0 mA with the final condition being 250 V, 3.0 W, and 15°C.

The gels were stained with .1% Coomassie blue and 10% acetic acid solution. A solution of 30% methanol and 10% acetic acid was used to destain gels in the development unit of the Phast System (20).

The gels were kept in a fresh destaining solution overnight and then transferred into a preservative solution (10% glycerol and 10% acetic acid). After 2 h, they were air dried at room temperature and photographed.

Electron Microscopy

To further study iron binding of casein at low pH, skim yogurt was fortified (40 mg iron/kg yogurt) with $FeCl_3$, iron complexed with casein, and iron complexed with whey protein (30).

The McManus and McMahon (1994, Personal Communication) procedure for mineral analysis of milk by transmission electron microscopy was used to determine iron distribution in iron fortified yogurt. Carbon-coated grids (600 H mesh) were soaked in poly-L-lysine solution and then air dried. Yogurt was diluted in double distilled deionized water (1:1). Carbon grids were then placed on yogurt samples for 5 min, rinsed with double distilled deionized water, and frozen instantly by immersion in liquid nitrogen. These grids were then transferred inside an Ion Beam Sputter Turbo Molecular Pump (IBSTM 200S, VCR GROUP) and freeze-dried overnight. These samples, without heavy metal staining, were examined by transmission electron microscopy (Zeiss 902 CEM TEM) at 80 KV.

Electron spectroscopic imaging (ESI) was used to produce a map of iron distribution in the samples. Images were acquired at electron energies equivalent to the iron edge and the adjacent background. Subtracting these images provides an iron map of the sample. This map can then be overlaid on the sample image. Computer-enhanced

color images were printed on a Mitsubishi color video printer.

Statistical Analysis

The experiments to determine the amount of calcium and phosphorus in pellets and serum at different pH were conducted using a split plot design, and analysis of variance was by Minitab. The whole plot effect was iron fortification, and the split plot effect was pH. Interactions among these main effects were also determined. The complete randomized design was used to analyze the effect of pH on the amount of iron in pellets and serum. Least significant differences (LSD) were used to assess significant differences in iron, calcium, and phosphorus content of pellets and serum at different pH levels.

RESULTS AND DISCUSSION

Iron Analysis

The amount of iron present in pellets increased with decreasing pH. This increase was gradual between pH 6.7 and 5.3 with a sudden increase at pH 4.5. The iron content in pellet at pH 6.7 was not significantly different ($P > .05$) from those at pH 6.2, 5.8, and 5.3. However, the iron content at pH 4.0 and 4.5 was significantly higher ($P < .05$) from those at pH 6.7, 6.2, 5.8, and 5.3. Pellets at pH 4.0 and 4.5 showed the largest amount of iron,

and the values were not significantly different ($P > .05$) from each other (Table 9-10, Appendix A).

At pH 6.7, about 82% of the iron was partitioned in the pellet while 13% was in the serum. At pH 4.0, the iron content of the pellet and serum changed markedly to 93% and 3.5%, respectively (Table 1).

TABLE 1. Distribution of iron between pellets and serum of milk fortified with 10 mg iron/100 ml. Milk at various pH was centrifuged at 52,000 g for 1 h at 20°C.

pH	Pellets (%)	Serum (%)
6.7	82.5	12.9
6.2	83.6	12.7
5.8	83.8	12.0
5.3	84.3	10.7
4.5	91.7	4.8
4.0	92.8	3.5

However, pellets at pH 4.0 and 4.5 contained the smallest amount of iron per milligram of protein (Figure 1) and were significantly different ($P < .05$) from pellets at the other pH. β-Lactoglobulin and BSA have lower iron-binding affinity and significant amounts were sedimented at these pH as shown by SDS-PAGE. The protein content and dry weight of pellets increased with reduction of pH because at pH 4.5, any denatured whey proteins become insoluble and sediment with the micelles. Likewise, the small casein micelles that were observed to be non-sedimentable at pH $\geq$

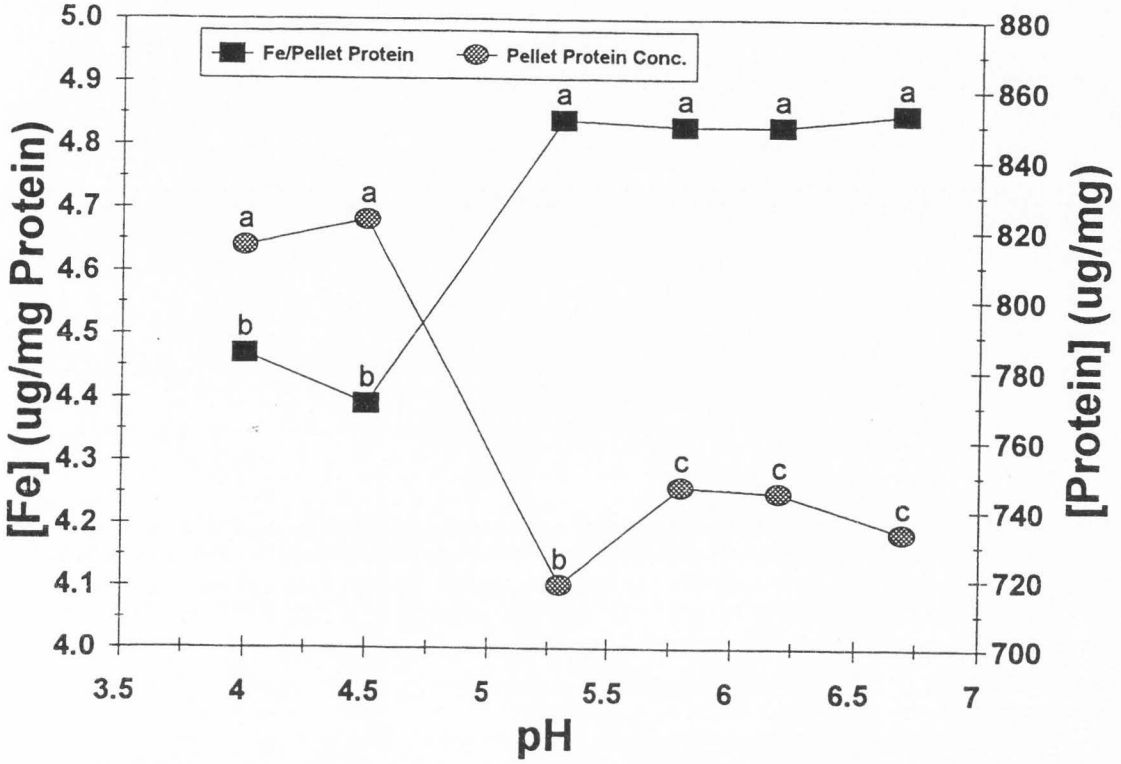

Figure 1. Effect of pH on iron and protein content of ultracentrifuged pellets of milk fortified with 10 mg iron/100 ml. Data points within each line with the same letter are not significantly different (α = .05, iron/pellet protein $LSD_{.05}$ = .18).

5.3, sedimented at pH ≤ 4.5. Such small micelles contain a higher proportion of κ-casein than β-casein and α_{s1}- casein (22) and would thus bind less iron.

The protein content of pellets increased with decreasing pH (Table 2), whereas serum protein concentration decreased.

TABLE 2. Protein content of pellets and serum of milk fortified with 10 mg iron/100 ml. Milk at various pH was centrifuged at 52,000 g for 1 h at 20°C.

pH	Pellets (mg protein/g dry pellet)	Serum (mg protein/g serum)
6.7	733.6 ± 5.9	7.5 ± .09
6.2	745.0 ± 9.0	7.5 ± .01
5.8	746.8 ± 13.3	7.5 ± .03
5.3	718.5 ± 10.7	7.9 ± .00
4.5	822.7 ± .3	5.9 ± .02
4.0	815.3 ± 3.0	5.8 ± .00

Alteration of the pH of milk affects the integrity of casein micelles (5, 18, 21, 28). As the pH is decreased from the native pH of milk, some caseins dissociate from micelles (9, 13, 23, 28) with maximum dissociation being observed at about pH 5.3. Then as the pH is further lowered, these caseins reassociate with the micelles. Also at pH 4.5 denatured whey proteins will become insoluble and sediment with the micelles. Thus at pH ≤ 4.5, the pellets contain a higher protein content. There will be virtually no non-sedimentable casein (very small micelles and casein

dissociated from micelles) at this pH. Also, there will be a small amount of whey proteins that sediment. With the pasteurization conditions used for processing the milk (79°C for 28 s), it would be expected that about 10% of the whey proteins would have been denatured.

The iron content in the serum phase significantly decreased ($P < .0005$) as pH decreased to ≤ 4.5 (Table 11-12, Appendix A). When the results were expressed as ratio of iron to protein, the amount of iron present also decreased as pH was reduced from 6.7 to 4.0 (Figure 2). In all cases, the reduction was gradual between pH 6.7 and 5.3 and then a sharp decrease at pH 4.5. There was a significant difference ($P < .05$) in the amount of iron present in serum protein at different pH.

The SDS-PAGE electrophoretic patterns of serum at pH 4.0 and 4.5 were different from other pH values (Figure 3). α_{s1}-Casein (α_{s1}-CN), β-casein (β-CN), and κ-casein (κ-CN) were present at pH's 6.7, 6.2, 5.8, and 5.3. However, these proteins were missing at pH 4.5 and 4.0, indicating that at higher pH, some caseins were not precipitated during ultracentrifuging. Some of the iron measured in the serum was most likely bound to these caseins.

The electrophoretic patterns of pellets at pH 4.0 and 4.5 were also slightly different from those at other pH. At pH 4.0 and 4.5, more β-lactoglobulin (β-LG) had sedimented.

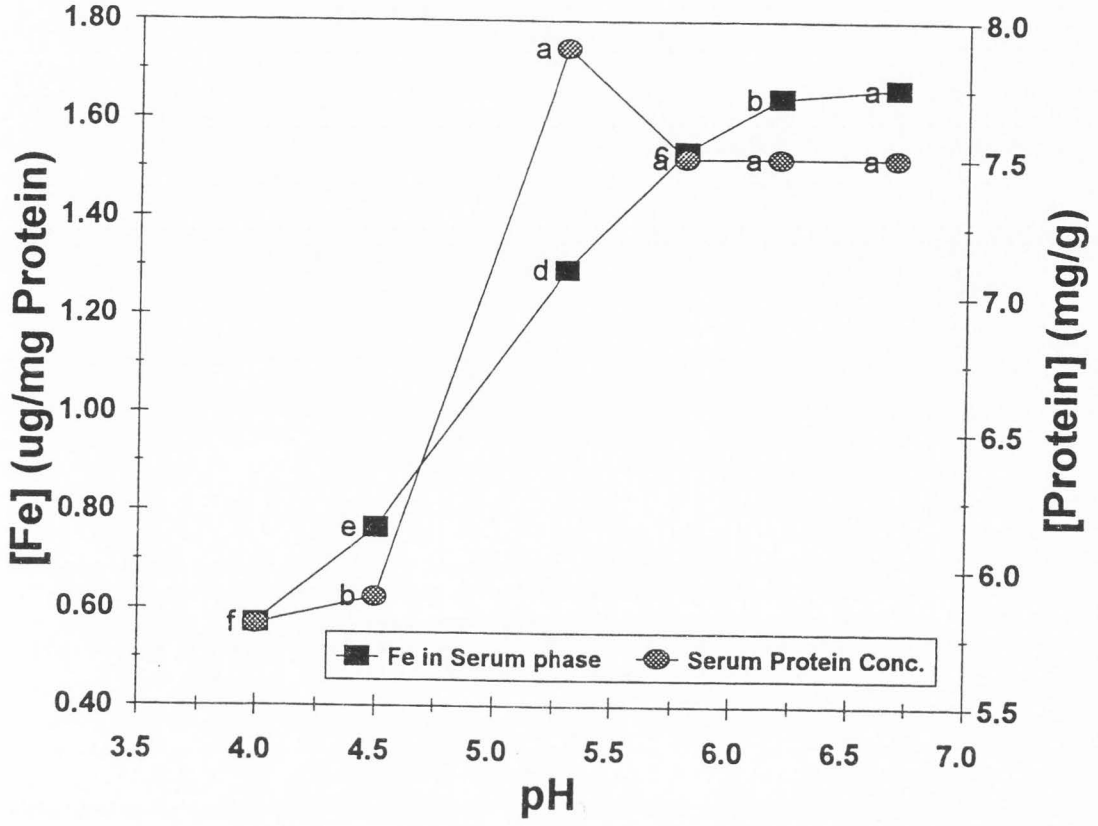

Figure 2. Effect of pH on iron and protein content of ultracentrifuged serum of milk fortified with 10 mg iron/100 ml. Data points within each line with the same letter are not significantly different (α = .05, iron/serum protein $LSD_{.05}$ = .07).

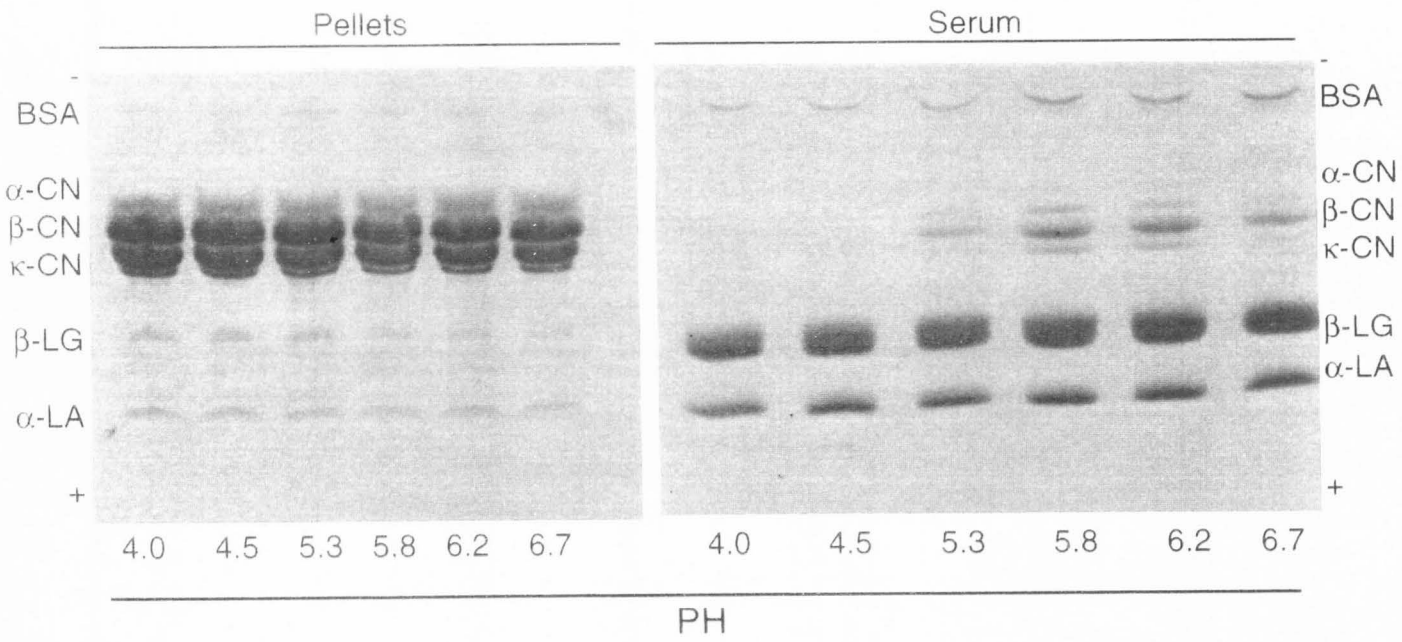

Figure 3. The SDS-PAGE electrophoretogram of serum and pellets at different pH (CN = casein; α-LA = α-lactalbumin; β-LG = β-lactoglobulin).

This was a result of the milk having been pasteurized at 79°C for 28 s, denaturing some whey proteins that were not soluble at these pH.

All pellets contained some whey proteins. Micelles contain about 4 ml serum per gram of casein, and milk serum contains about 6.5 mg whey protein per gram. Therefore, the pellets are expected to contain about 26 mg whey protein per gram of casein (1.0-2.6%) (19).

Pellets were first overloaded onto the gel to show small quantities of whey protein present. When sample concentration was reduced to one half of original dilution, the β-LG band was only present at pH 4.0 and 4.5. Additional bands, which are most likely due to bovine serum albumin (BSA), also appeared only at pH 4.0 and 4.5.

Reddy and Mahoney (22) pointed out that α_{s1}-CN, β-CN, κ-CN, β-LG, α-lactalbumin (α-LA), and BSA have different iron-binding affinities. Those milk proteins (α_{s1}-CN, βCN, κ-CN) that contain phosphoryl serine groups as well as carboxyl groups have greater iron-binding affinity than those (α-LA, βLG) that do not have phosphoryl serine groups. The relative binding of Fe(III) to these proteins is as follows: α_{s1}-CN > βCN > BSA > κ-CN > βLG > α-LA. They studied which amino acid side chain groups are involved in the binding of iron to milk proteins and found iron does not always bind to the phosphoryl serine groups. In α_s1-CN and β-CN,

phosphoryl serines and carboxyl groups (Asp and Glu) bind iron while in κ-CN and BSA, only the carboxyl groups are involved in the iron binding.

There was more iron present in pellets at lower pH (4.5 and 4.0) but less iron in proportion to protein. However, at the same time more proteins (both casein and whey proteins) were precipitated at these pH, and the whey proteins have lower binding affinity for iron. Taking this into account, it appears that the iron binding to milk proteins is independent of change in pH from pH 6.7 down to pH 4.0, which in general is in agreement with Reddy and Mahoney's (22) work. They found that the number of iron-binding sites on the protein were independent of change in pH. Also, free energy change (ΔG) for binding of iron to different milk proteins was small and negative, indicating that such binding is instantaneous and thermodynamically favorable.

Demott and Dincer (10) also studied binding of added iron to various milk proteins and found about 85% of the added iron in skim milk was bound to casein. This was in the proportion of 72 : 21 : 4 for α_{s1}-CN, β-CN, and κCN, respectively. Other proteins in milk that also bind iron include lactoferrin, catalase, peroxidase, and xanthine oxidase (3). These proteins are present in low concentrations in milk, and some iron recovered in the serum

could be bound to these proteins. King et al. (15) reported 90 to 110% of iron in the micellar fraction of milk and 21 to 23% in the centrifuged whey. Our values are smaller than theirs perhaps because their total iron recovery was 111 to 133% compared to our iron recovery of 95 to 96%.

Calcium and Phosphorus Analysis

The dissociation behavior of calcium and phosphorus from the casein micelles in iron fortified milk and control was sigmoid in shape as pH decreased, which is similar to previously published papers (8, 28). The amount of calcium in pellets decreased as the pH decreased in both iron fortified milk and control. Micellar calcium phosphate is solubilized as the pH decreased (Table 13, Appendix A). The pellet at pH 6.7 contained the highest amount of calcium, which was significantly different ($P < .05$) from those at other pH. The least amount of calcium was present in the pellets at pH 4.0 (Figure 4).

The concentration of calcium in serum of iron fortified milk and control increased as pH decreased from 6.7 to 4.0 (Figure 5). Serum at pH 4.0 and 4.5 contained the highest amount of calcium, which was significantly higher ($P < .05$) than those at other pH (Table 14, Appendix A).

Phosphorus behaved similarly to calcium as pH was decreased in iron fortified milk and control. However,

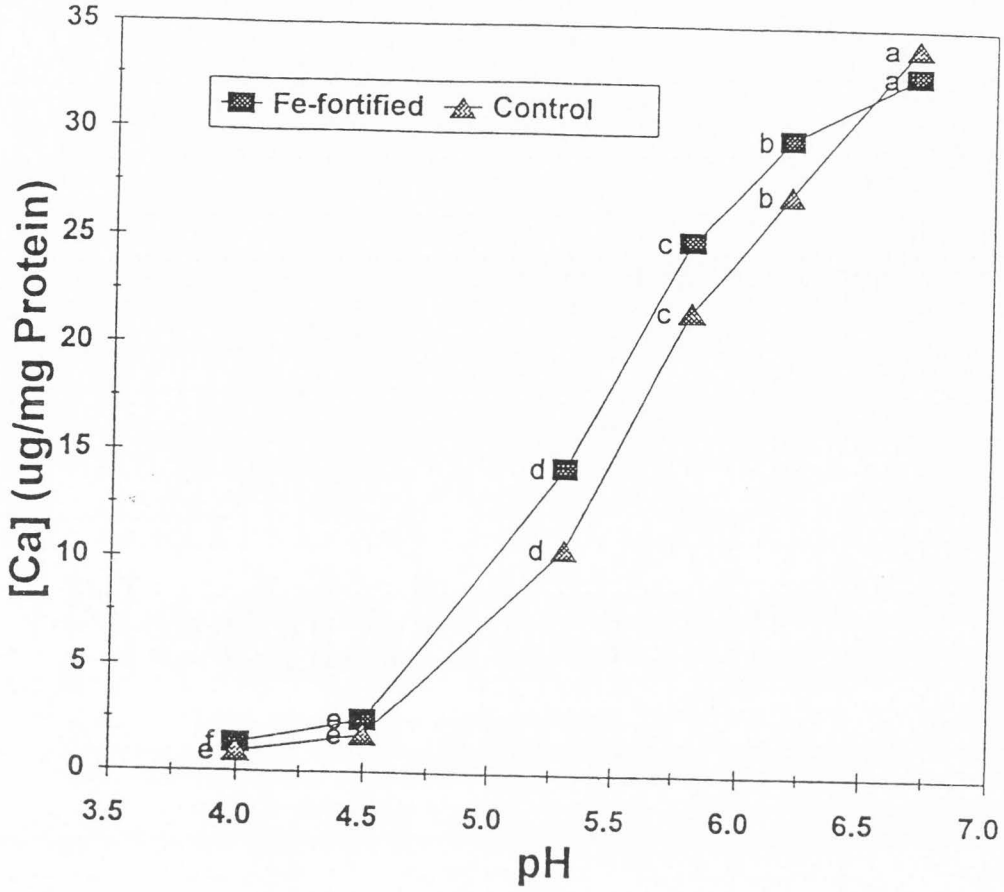

Figure 4. Effect of pH and iron fortification on calcium content of ultracentrifuged milk pellets. Data points within each line with the same letter are not significantly different (α = .05, $LSD_{.05}$ = .61).

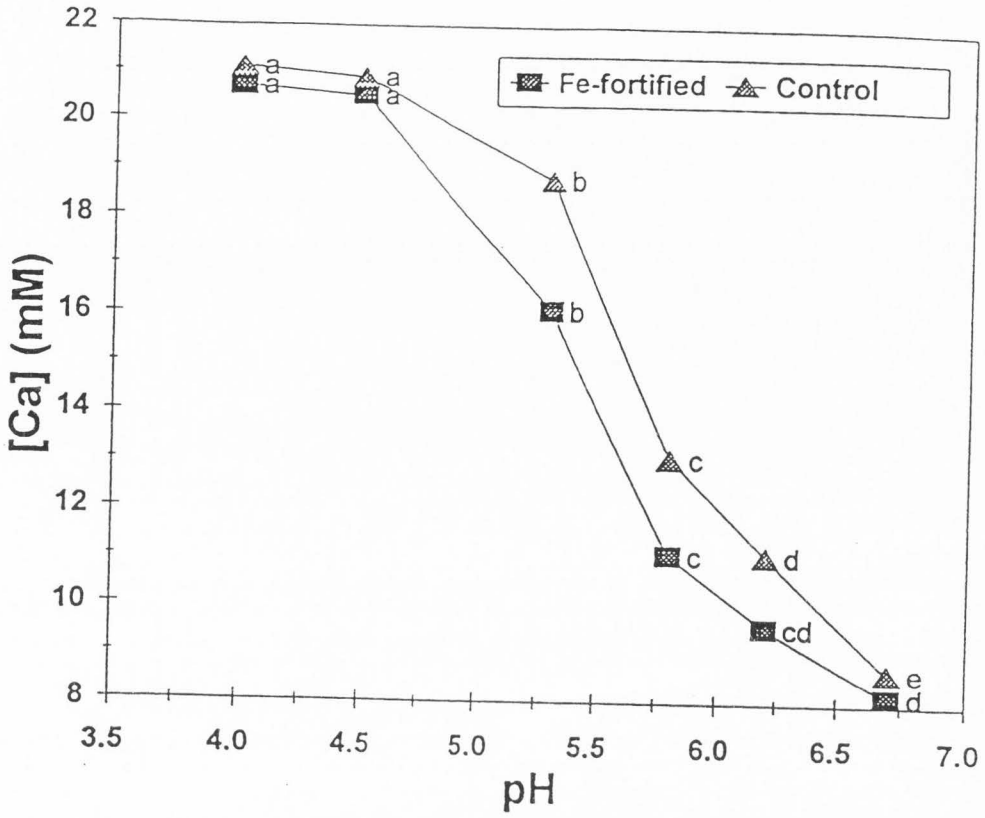

Figure 5. Effect of pH and iron fortification on calcium content of ultracentrifuged milk serum. Data points within each line with the same letter are not significantly different (α = .05, $LSD_{.05}$ = 1.5).

there was more variation in calcium content of the pellets (Table 3) than in phosphorus content (Table 4).

TABLE 3. Analysis of variance for the calcium to protein ratio in ultracentrifuged pellets of iron-fortified milk at different pH.

Source	df	MS	*P*
Replication	1	.83	.2775
Fortification (F)	1	14.38	.0709
Error (a)	1	.18	
pH	5	747.50	.0000
F x pH	5	3.88	.0000
Error (b)	10	.08	
Total	23		

TABLE 4. Analysis of variance for phosphorus to protein ratio in ultracentrifuged pellets of iron-fortified milk at different pH.

Source	df	MS	*P*
Replication	1	.002	.3918
Fortification (F)	1	13.696	.0054
Error (a)	1	.001	
pH	5	145.906	.0000
F x pH	5	.758	.0293
Error (b)	10	.189	
Total	23		

Also the interaction between the fortification and pH effect was significant for both calcium and phosphorus content of ultracentrifuged milk pellets ($P < .00005$ and $P = .029$, respectively). There was an average of 12 and 28% more calcium and phosphorus in iron-fortified pellets than in the control. The increased calcium and phosphorus

content of iron-fortified pellets suggests either iron has different binding sites (most likely carboxyl groups), which can then bind to more calcium and phosphorus, or it forms coordination complexes with colloidal calcium phosphate, again allowing more phosphorus and calcium complexing. At pH 4.5, 3 μg calcium/mg protein remained in the pellet of the iron fortified milk, whereas 8 μg phosphorus/mg protein remained. In comparison, the control milk pellet had only 2 μg calcium and 7 μg phosphorus/mg protein.

Pellets showed the highest amount of phosphorus at pH 6.7 and the least amount at pH of 4.0. Also, the amount of phosphorus in protein of pellets decreased gradually between pH 6.7 and 5.8, with a sharp decrease at pH 4.5. The phosphorus content in protein of pellets at pH 4.5 and 4.0 was significantly lower ($P < .05$) than those at other pH (Figure 6).

The phosphorus content of serum in iron fortified milk and control at different pH is shown in Figure 7. The amount of phosphorus in serum and serum protein increased with decreasing pH in both iron fortified milk and control. There was no significant difference ($P > .05$) in phosphorus content in serum at pH 6.7, 6.2, and 5.8 in iron-fortified milk. However, the phosphorus content of serum at pH 4.0, 4.5, and 5.3 was significantly different ($P < .05$) from those at higher pH (Table 15, Appendix A).

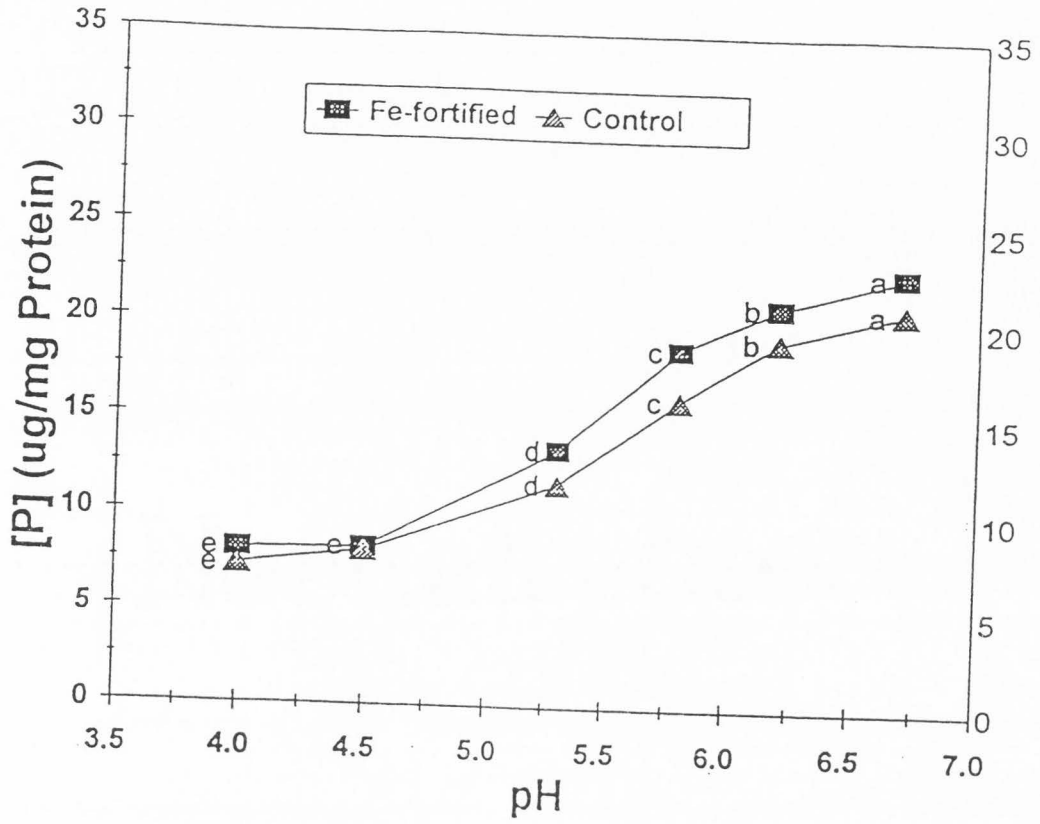

Figure 6. Effect of pH and iron fortification on phosphorus content of ultracentrifuged milk pellets. Data points within each line with the same letter are not significantly different (α = .05, $LSD_{.05}$ = 1.0).

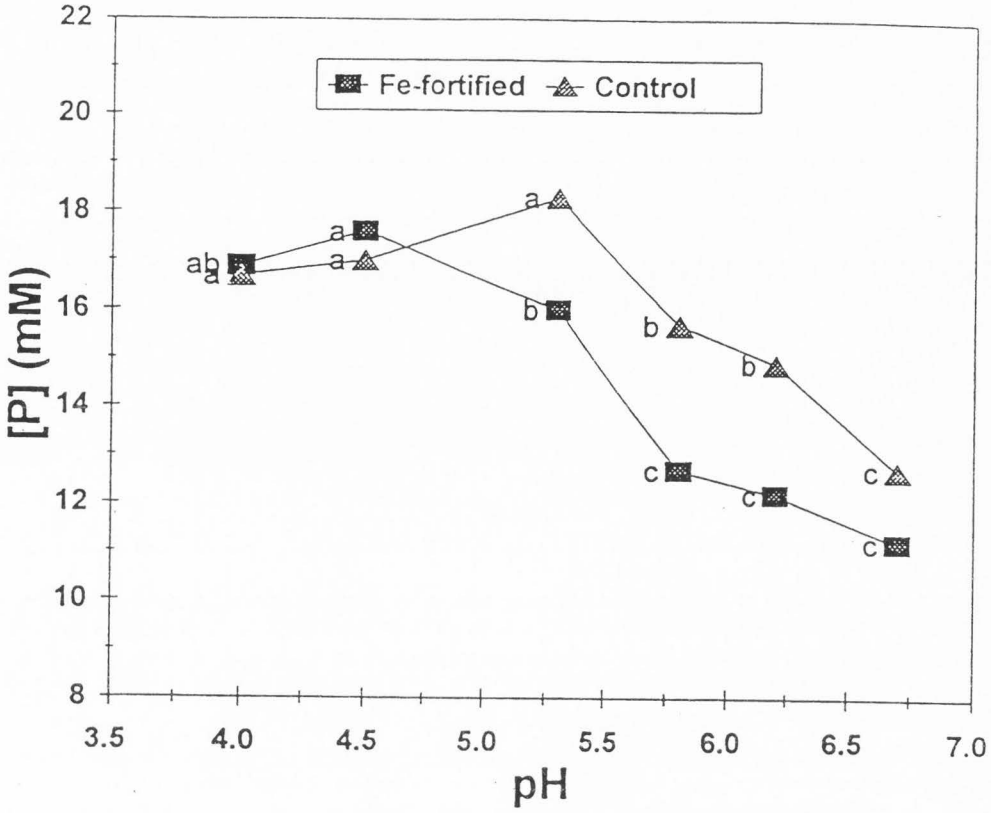

Figure 7. Effect of pH and iron fortification on phosphorus content of ultracentrifuged milk serum. Data points within each line with the same letter are not significantly different (α = .05, $LSD_{.05}$ = 1.55).

Calcium and phosphorus showed the same trend with reduction of pH in both iron-fortified milk and control. Therefore, one can postulate that iron probably has different binding sites than calcium and phosphorus, and presence of iron would not interfere with normal behavior of calcium and phosphorus in milk as pH is reduced.

Therefore, iron fortification of milk did not affect the overall expected behavior (5, 18, 21, 28) of calcium and phosphorus as a function of pH except to delay their release from the micelles. At pH 6.7, most of the calcium (70%) and inorganic phosphate of milk are associated with casein micelles (12, 28). Micellar or colloidal calcium phosphate maintains the micellar structure by acting as a binding agent between micellar subunits (24, 25). The casein structure and composition is altered as the pH of milk decreases. To obtain an equivalent amount of micellar calcium phosphate solublization, iron-fortified milk would need to be acidified to a slightly lower pH.

Electron Microscopy

Electron spectroscopic imaging utilizes the loss of energy from electrons that are defracted from atoms transmitted through a sample when using a transmission electron microscope. These defracted electrons will have energies based upon the element with which they interact, allowing them to be separated based on their energy. By

incorporating ESI into a Zeiss 902 CEM, it was possible to map for the presence of iron in the yogurt.

Figure 8 shows iron distribution in unfortified yogurt (a) and yogurts that were fortified with iron complexed with casein (b), $FeCl_3$ (c), and iron complexed with whey protein (d). The concentration of iron in the micelles of unfortified yogurt did not differ from the intermicellar regions, and so no iron gradient was observed. This is not suprising considering the fact that the average iron content of milk is very small (.52 ± .06 ppm) (11).

However, in yogurt fortified with iron-casein complex or $FeCl_3$, there was more iron distributed throughout the casein micelles than in the intermicellar regions. This was expected for yogurt fortified with iron-casein complex because the iron had been prepared as a complex bound to casein, and this iron-casein would join into the casein micelle network when the milk is acidified. When yogurt was fortified with $FeCl_3$, the iron was also observed to be predominantly located in the casein micelles. These results further confirm our earlier findings, and are in general agreement with Reddy and Mahoney (22), that iron preferentially binds to casein rather than to the whey proteins. We found most of the added iron was associated with the casein fraction at different pH.

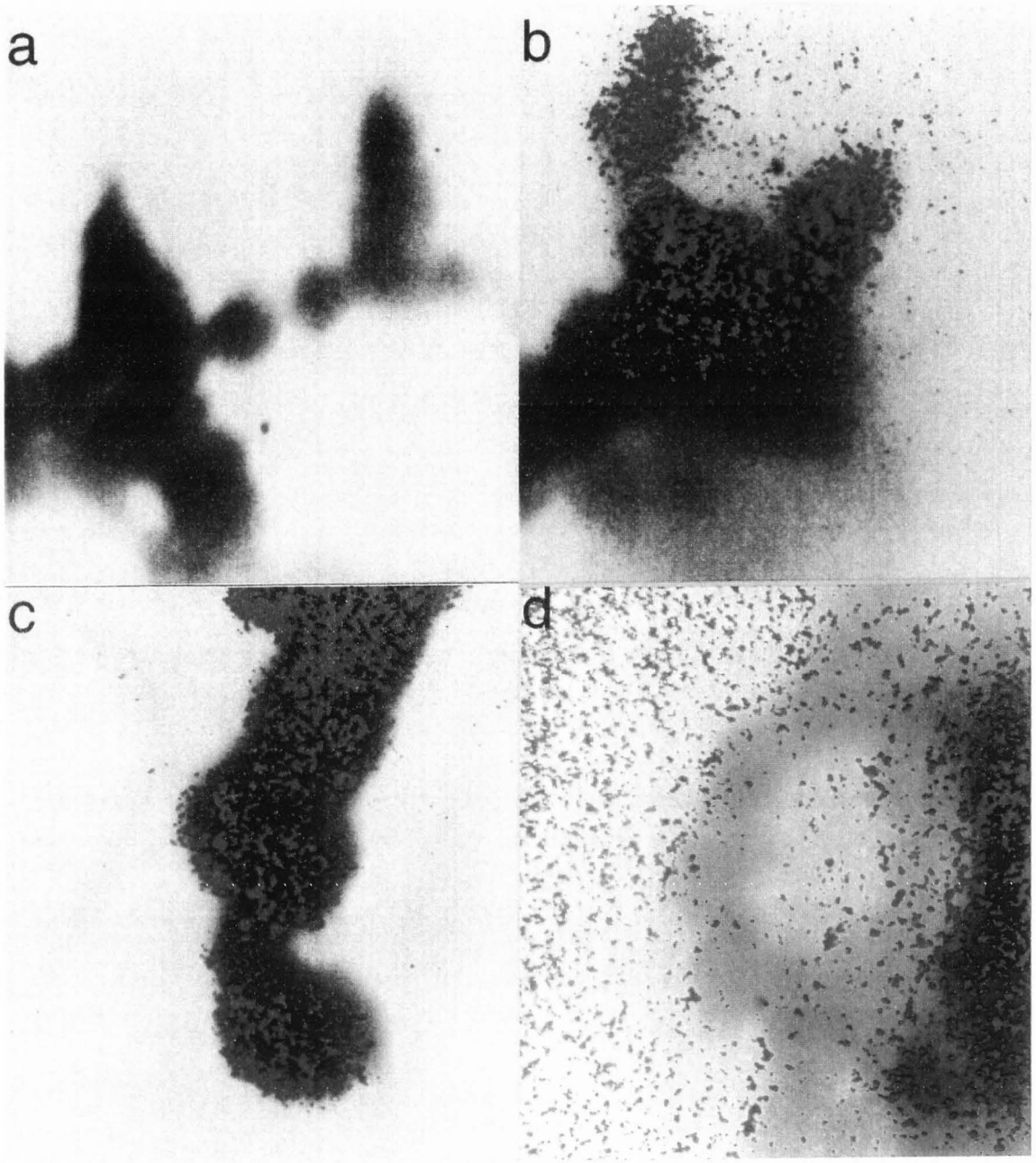

Figure 8. Transmission electron micrographs of iron distribution (red) in (a) unfortified yogurt and yogurts fortified with (b) iron complexed with casein, (c) $FeCl_3$, and (d) iron complexed with whey protein. Dark areas are the casein micelle network; Magnification is 50,000 X.

When milk was fortified using iron complexed to whey proteins, it appears that the iron stays bound to the whey proteins rather than exchanging to the caseins. As shown in Figure 8d, there was more iron observed throughout the non-micellar region than in the casein micelles.

CONCLUSIONS

Iron binding to milk proteins was independent of changes in pH. There was more β-lactoglobulin, bovine serum albumin, and κ-casein present in the casein pellets at pH 4.0 and 4.5. These proteins, while having low iron-binding affinity, increase protein content of pellets at low pH, which in turn decrease the iron-to-protein ratio of the pellets at these pH.

Fortification of milk with iron did not cause loss of calcium or phosphorus from casein micelles. In fact, iron fortification caused greater retention of calcium and phosphorus in the micelles as milk was acidified to pH 5.3. At pH 4.5, there was no difference in calcium and phosphorus in casein micelles.

Iron was preferentially bound to casein over whey proteins when yogurt was fortified with $FeCl_3$ and iron-casein complex. When fortified with iron-whey protein complex, the iron remained bound to the whey proteins and was distributed throughout the non-micellar portion of the

yogurt. At low pH, iron appears to remain bound to the milk proteins.

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PART 3. MANUFACTURING AND MICROBIAL ANALYSIS OF YOGURT FORTIFIED WITH FERRIC CHLORIDE, IRON-CASEIN, AND IRON-WHEY PROTEIN COMPLEX

ABSTRACT

Low fat (2%) and nonfat yogurt fortified with three iron sources (ferric chloride, iron-casein and iron-whey protein complex) at three levels (10, 20, and 40 mg iron/kg yogurt) were made. The starter cultures were able to ferment milk to yogurt and grow to high numbers.

Survival of *Lactobacillus delbruekii* ssp. *bulgaricus* and *Streptococcus thermophilus* were monitored during 30 d of storage at 4°C. The mean bacterial counts after one day of storage in iron fortified skim yogurts were 6.9 x 10^8 CFU/ml for *L. delbruekii* ssp. *bulgaricus* and 7.0 X 10^8 CFU/ml for *S. thermophilus* which were not significantly different ($P > .05$) from bacterial counts in unfortified yogurts. These counts decreased to 2.5 x 10^8 and 1.9 x 10^8 CFU/ml respectively in iron fortified yogurt, after 30 d of storage.

To determine whether the presence of excess iron enhances growth of pathogenic and spoilage microorganisms, *Pseudomonas fluorescens* ATCC 31732 and *Escherichia coli* (Dairy Isolate, Nordica) were inoculated separately at the rate of 10^3 and 10^5 CFU/ml of yogurt into iron-fortified yogurt mix (20 mg iron/kg yogurt) at the time of packaging. These samples were tested to determine changes in pH and the number of *E. coli, P. fluorescens*, and starter cultures after 1, 7, and 14 d of storage.

After one day of refrigerated storage, the viable numbers of *E. coli* in iron fortified yogurt that was inoculated with 10^5 and 10^3 CFU/ml, were 2.5 x 10^5 and 3.2 x 10^3 CFU/ml, respectively, and in unfortified yogurt were 1.8 x 10^5 and 1.4 x 10^3 CFU/ml. However, they decreased substantially after 7 d of storage to < 1 CFU/ml in all samples. *Pseudomonas fluorescens* showed no viability after 1, 7, or 14 d of refrigerated storage. Therefore, the biochemical properties of yogurt did not support growth of spoilage and pathogenic microbes such as *P. flurescens* or *E. coli*.

INTRODUCTION

Yogurt has gained widespread consumer acceptance in the United States. Consumption of yogurt has increased from .12 kg per person in 1960 to 1.9 kg in 1986 (an increase of 1500%) (18). Yogurt is primarily consumed by women, children, and teenagers as a luncheon or snack food. However, it is these very populations that while having high calcium requirements are frequently deficient in iron (6, 10). Yogurt is an excellent source of calcium and protein (24), but typical of all dairy products, it contains very little iron (3, 23, 13).

Iron fortification of dairy products such as cheese and yogurt could increase their nutritive values and

consumer appeal. Zhang and Mahoney successfully fortified Cheddar cheese (28) and process Cheddar cheese (30) with ferric chloride and iron-milk-protein complexes. They found that iron fortification had no or minor effects on cheese quality. No differences in oxidized off-flavor or cheese flavor was detected among the iron fortified cheeses and un-fortified cheese. It has also been shown that iron fortified cheese has high iron bioavailability sufficient to meet human needs (27).

Iron is also a very important element in microbial physiology (26). Although lactic acid bacteria do not require iron for growth (17), they are nutritionally fastidious. Nutrients must be in a form that can be degraded and utilized by these microorganisms. It is possible that iron forms complexes with some nutrients, making them unavailable to the bacteria for their growth and survival. It has been shown that addition of hemin to cultures of some strains (*Streptococcus*, *Pneumococcus*, and *Leuconostoc*) results in formation of some pigments resembling Cytochrome a, b, and development of cyanide sensitive respiration and oxidative phosphorylation (17, 25). Therefore, it is important to determine whether the presence of excess iron enhances or retards growth and survival of starter cultures.

Many important enzyme systems require iron for their proper functioning. Therefore, most microorganisms possess

mechanisms for obtaining iron to sustain their growth (14, 26). However, these mechanisms are not known to exist in lactic acid bacteria. The addition of iron to yogurt may allow for such organisms (e.g., *Pseudomonas*) to grow.

Fluorescent *Pseudomonas* ssp. are the predominant lipolytic psychrotrophs in raw and ultra-heat-treated milk (12). In the storage of refrigerated milk and dairy products, these gram negative psychrotrophic bacilli represent a major problem. Their high-affinity iron uptake systems are mediated by the action of siderophores, which they produce under iron limited conditions (1, 9). These siderophores are low molecular weight, high-affinity, iron-chelating agents that bind iron and return it to the cell. *Escherichia coli* strains also show a wide distribution in food environment in low numbers (11), and siderophores are involved in their virulence (1).

Therefore, iron-fortified food products are potential candidates for supporting growth of spoilage and pathogenic microorganisms. It is essential to characterize the growth or destruction of these spoilage and pathogenic micro-organisms to ensure iron fortification of yogurt does not present safety problems.

Our objective was to apply the techniques of iron-fortifying cheese to making an iron-fortified yogurt. We determined the effect of iron fortification on the fermentation of milk by *Lactobacillus delbruekii* ssp.

bulgaricus, and *Streptococcus thermophilus*, their survival during storage, and survival of spoilage bacteria *P. fluorescens and E. coli* during storage of yogurt.

MATERIALS AND METHODS

Iron Sources

The iron sources used to manufacture iron-fortified yogurt were ferric chloride ($FeCl_3$), iron complexed with casein (iron-casein complex), and iron complexed with whey protein (iron-whey protein complex) (27, 30).

Ferric chloride (Catalog Number F-2877) was obtained from Sigma Chemical Company (St. Louis, MO). The iron-casein complex was prepared by adding 50 ml of .5 M $FeCl_3$ into 600 ml of skim milk and then precipating the iron-casein complex at pH 4.6 (28). Iron-whey protein complex was made by mixing 50 ml of .5 M $FeCl_3$ with 600 ml of acid (cottage cheese) whey and adjusting its pH to 3.5 (29).

The iron-casein and iron-whey protein solutions were kept at room temperature for 1 h until a clear precipitate was formed. They were then centrifuged (Sorvall Instruments, RC5C DuPont) at 8,000 *g* for 5 min. The pellets were washed once with .25% lactic acid solution and twice with double distilled deionized water. They were freeze-dried for 48 h, ground, and sieved until a very fine powder was formed.

The ferrozine method (5, 22) was used to determine iron content of the iron-casein and iron-whey protein complexes. Iron-casein complex contained 56.0 mg iron/g of powder with 46.8% recovery, while the iron content of iron-whey protein complex was 136.5 mg/g of powder with 88.6% recovery.

Preparation of Cultures

Frozen cultures of *L. delbruekii* ssp. *bulgaricus* and *S. thermophilus* were obtained from Heart to Heart Foods, Inc. (Richmond, UT). To prepare a yogurt mother culture, Sterilized MRS broth (Difco Laboratories, Detroit, MI) (4, 21) and Elliker broth (Difco Laboratories, Detroit, MI) (8, 23, 24) were inoculated with *L. delbruekii* ssp. *bulgaricus* and *S. thermophilus*, respectively, at the rate of 1.0% and incubated anaerobically (BBL Gas Pak, Becton Dickinson Microbiology Systems) at 41°C for 15 h. Reconstituted non-fat dry milk (NDM) (11% total solids) was prepared, autoclaved at 121°C for 15 min, then cooled to room temperature. Two volumes of reconstituted NDM were then mixed with one volume of sterilized glycerol. Ten percent of each culture was added to this mixture, mixed, and stored at -70°C until used.

The day prior to making yogurt, 1.0% of each starter culture was added separately to sterilized reconstituted NDM and incubated anaerobically at 41°C for 15 h. These cultures were used to inoculate milk for yogurt production.

Manufacturing Iron-Fortified Yogurt

Nonfat and 2% fat milk were obtained from the Gary H. Richardson Dairy Products Laboratory at Utah State University and to each of these was added 6% sugar, 5.8% NDM, and .7% stabilizer.

Hydrated iron sources ($FeCl_3$, iron-casein and iron-whey protein complex) were added separately to the yogurt mix to give 10, 20, and 40 mg iron/kg yogurt for each source. Regular nonfat and 2% fat yogurt were also made using the same procedure without adding any iron. The experiment was duplicated.

The yogurt mixes were stirred and heated to 82°C for 30 min. They were then cooled to 41°C and inoculated (1%) with each starter culture, mixed well, and packaged. The yogurt mixes were fermented for approximately 5 ± .5 h at 42°C. When pH of 4.2 was achieved, the individual cups were transferred to a cold room at 4°C. Viable numbers of each starter bacteria were determined after 1, 15, and 30 d of storage.

Enumeration of Starter Bacteria

At pH 5.4, *S. thermophilus* do not grow on MRS media, while *L. delbruekii* ssp. *bulgaricus* produce small star-shaped white colonies. On M17-lactose media *S. thermophilus* produce small creamy colonies, and *L. delbruekii* ssp. *bulgaricus* are inhibited.

S. thermophilus. Sterile M17-lactose (Difco Laboratories, Detroit, MI) media were used to enumerate *S. thermophilus* in yogurts using the spread plate method. Yogurt was diluted 10^5, 10^6, and 10^7 in .85% saline, and then .1 ml was spread over the M17-lactose plates (duplicate plates for each dilution) and incubated anaerobically at 41°C for 48 h. Identification of the colonies was confirmed by Gram reaction and microscopic examinations.

L. delbruekii ssp. *bulgaricus*. MRS media (Difco Laboratories, Detroit, MI) were used to enumerate *L. delbruekii* ssp. *bulgaricus* in yogurts. The powder was mixed according to manufacturing instructions, and the pH was adjusted to 5.4 with lactic acid, autoclaved, and poured into sterile petri dishes.

Yogurt was diluted 10^5, 10^6, and 10^7 in sterile .85% saline, and .1 ml of each dilution was spread over the plates and incubated anaerobically at 41°C for 48 h. Gram reaction and microscopic examinations were then used to confirm their identity.

Propagation of *E. coli* and *P. fluorescens*

Escherichia coli (EC) broth was prepared by adding 2% pancreatic digest of casein, .5% lactose, .5% NaCl, .4% K_2HPO_4, .15% bile salt mixture, and .15% KH_2PO_4 to double distilled deionized water (2).

Pseudomonas F (PF) broth was made by adding 2% proteose peptone No.3 (Difco Laboratories, Detroit, MI), 1% glycerol, 1% pancreatic digest of casein, .15% K_2HPO4, and .073% $MgSO_4$ to double distilled deionized water (2).

EC broth and PF broth were then autoclaved at 121°C for 15 min. EC broth was cooled to 37°C and inoculated (.1%) with *E. coli* (Dairy Isolate, Nordica) and incubated aerobically at 37°C overnight. PF broth was cooled to 30°C, inoculated (.1%) with *P. fluorescens* ATCC 31732, and incubated aerobically at 30°C overnight.

These bacteria were then centrifuged at 10,000 *g* (Sorvall Instruments, RC5C, DuPont) for 10 min and the cells were resuspended separately in autoclaved .85% saline to an OD_{590} of .3. These suspensions were added to the yogurt during packaging.

Iron-Fortified Yogurt Inoculated with *P. fluorescens* and *E.coli*

Nonfat yogurt mix from skim milk containing 6% sugar, 5.8% NDM, and .7% yogurt stabilizer was made. Half of the mix was used as control, and the other half was fortified with 20 mg iron/kg yogurt mix. They were heat treated to 82°C for 30 min and cooled to 41°C before inoculating with 1% of each yogurt starter culture separately. The yogurt mixes were then inoculated separately with *E. coli* and *P. fluorescens* ATCC 31732 to 0, 10^3, and 10^5 CFU/ml of yogurt.

They were incubated at 42°C for approximately 5 h until pH = 4.2 was reached and then placed in cold room at 4°C. The yogurt was analyzed after 1, 7, and 14 d storage. Two replications of these treatments were done.

Enumeration of *P. fluorescens* and *E. coli*

Pseudomonas fluorescens was enumerated using a spread plate method on PF agar and incubated for 10 d at 10°C. *Escherichia coli* was enumerated using a pour plate method on violet red bile agar (VRBA) (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD) (16, 19). Yogurt samples were added to approximately 15 ml of warmed sterile VRBA and mixed thoroughly by tilting and rotating each plate. The mixture was then solidified, and an additional 3 to 4 ml of VRBA was distributed over the solidified medium, completely covering the surface to prevent surface colony formation. Solid plates were inverted and incubated aerobically at 37°C for 48 h. The total numbers of *E. coli* were determined by counting dark red colonies on each VRBA plate.

Statistical Analysis

The experiments were conducted using a complete split plot design and analysis of variance was done using Minitab. We evaluated the effects of fortification of yogurt with $FeCl_3$, iron-casein, and iron-whey protein

complex at various levels (10, 20, and 40 mg iron/kg yogurt) on the survival of *L. delbruekii* ssp. *bulgaricus* and *S. thermophilus* during storage. The main effects were milk fat, iron sources, levels of each iron source, and storage time. Interactions (2-, 3-, and 4-way) among these main effects were also determined. Least significant differences (LSD) were used to assess significant difference in colony counts during the storage period for fixed iron source, level of each iron source, and milk fat.

RESULTS AND DISCUSSION

Fermentation Process

All yogurts had smooth texture, strong gels, high viscosity upon stirring, and uniform body with no wheying off during syneresis. After approximately 5 h of incubation, the mean pH of all samples, including the unfortified yogurt, was 4.3 ± .1. The rate of acid production was the same for all samples during storage period. Addition of iron to milk had no effect on either starter culture in fermenting the milk. Both *L. delbruekii* ssp. *bulgaricus* and *S. thermophilus* grew to high numbers and produced acid in all iron-fortified yogurts. Their growth was independent of iron sources and iron concentration.

After 1 d of storage, the mean pH of unfortified nonfat yogurt and iron-fortified nonfat yogurt were 4.20 ± .05 and 4.24 ± .01, respectively. These values decreased to

4.00 ± .07 and 4.03 ± .03, respectively, at d 30. A similar pattern of acid production was observed for 2% fat yogurt.

Lactic Acid Bacterial Counts During Storage

The total viable numbers for *L. delbruekii* ssp. *bulgaricus* during storage in nonfat yogurt fortified with ferric chloride are shown in Figure 9. There were no significant differences ($P = .18$) in *L. delbruekii* ssp. *bulgaricus* counts between unfortified yogurt and fortified yogurts as shown in Table 5.

Bacterial survival was independent of both iron source and quantity of iron added. Similar patterns of bacterial survival were observed among yogurts that were fortified with the protein complexed (iron-casein or iron-whey protein complex) iron sources at various levels during storage period. After 1 d of storage, the mean *L. delbruekii* ssp. *bulgaricus* counts for unfortified yogurt and iron fortified yogurt were 6.1×10^8 CFU/ml and 6.9×10^8 CFU/ml. Their viable numbers showed a slight (but not statistically significant ($P > .05$)) decrease after 15 d of storage. By 30 d, the decrease was significant ($P < .05$) with mean counts of 5.1×10^8 CFU/ml and 2.5×10^8 CFU/ml for unfortified and iron-fortified yogurts.

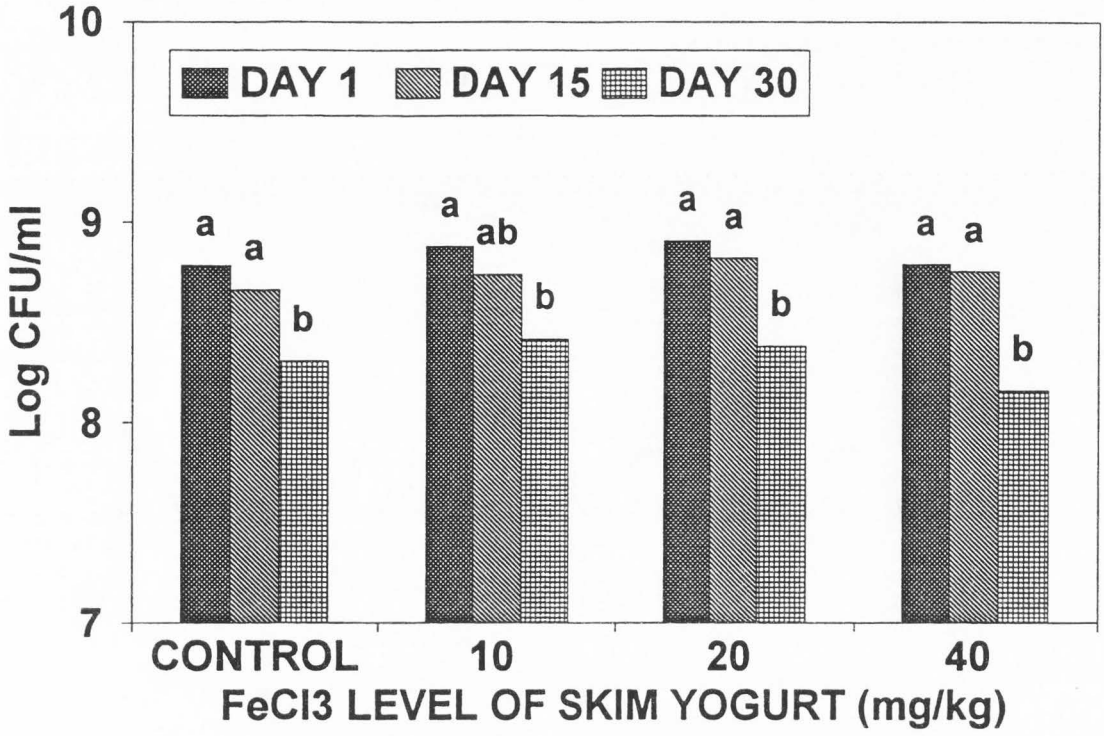

Figure 9. Mean survival of *L. delbruekii* ssp. *bulgaricus* in nonfat yogurt fortified with $FeCl_3$ (0, 10, 20, and 40 mg iron/kg yogurt) over 30 d of storage. Values for the same iron level with the same letter superscript are not significantly different ($LSD_{.05}$ = .34).

TABLE 5. Analysis of variance for *L. delbruekii* ssp. *bulgaricus* counts of nonfat and 2% iron-fortified yogurt over 1 mo of storage at 4°C.

SV	df	MS	*P*
Rep	1	.08269	.2125
Milk (M)	1	.10290	.1919
Error (a)	1	.00995	
Treatment (T)	9	.03363	.6112
Cont. VS Rest	1	.08132	.1779
Among Rest	8	.02767	.7121
Source (S)	2	.00805	.8248
Level (L)	2	.01288	.7363
S x L	4	.04488	.3933
M x S	2	.11434	.0897
M x L	2	.06918	.2156
M x S x L	4	.05200	.3229
Error (b)	18	.04137	
Day (D)	2	3.18973	.0081
Error (c)	2	.02622	
M x D	2	.01083	.6831
T x D	18	.02079	.7512
S x D	4	.01059	.8240
L x D	4	.01467	.7205
S x L x D	8	.02877	.4362
M x T x D	18	.01821	.8381
M x S x D	4	.00229	.9876
M x L x D	4	.01200	.7885
M x S x L x D	8	.03037	.3980
Error (d)	38	.02814	
Total	119		

Streptococcus thermophilus showed the same survival behavior as *L. delbruekii* ssp. *bulgaricus* in iron-fortified yogurt during 30 d of storage (Figure 10). Its survival was independent from presence of iron, type of iron source, and level of each source. In general there were no significant differences ($P = .64$) in total *S. thermophilus* counts between unfortified yogurt and iron-fortified yogurt. Total colony counts were also not significantly different

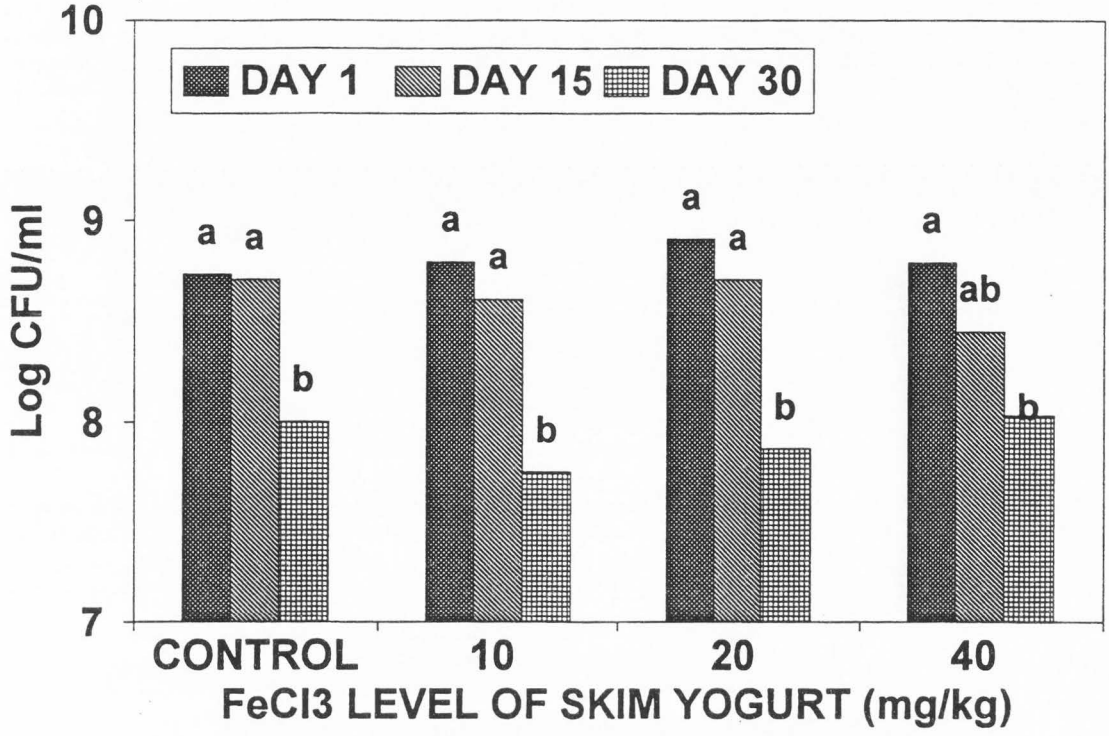

Figure 10. Mean survival of *S. thermophilus* in nonfat yogurt fortified with $FeCl_3$ (0, 10, 20, and 40 mg iron/kg yogurt) over 30 d of storage. Values for the same iron level with the same letter superscript are not significantly different ($LSD_{.05}$ = .49).

($P = .99$) among different iron sources and at various levels.

The bacterial populations in the experimental iron fortified yogurts were similar to those yogurts currently being produced commercially. Matalon and Sandine (15) studied viability of yogurt starter bacteria in six American commercial yogurts. They reported the mean bacterial populations for *L. delbruekii* ssp. *bulgaricus* and *S. thermophilus* were 2.7×10^8 CFU/ml and 6.5×10^8 CFU/ml. Davis (7) studied the lactic acid bacteria counts in 12 British yogurts and reported that the average viable number of *L. delbruekii* ssp. *bulgaricus* was 2.4×10^8 CFU/ml and of *S. thermophilus* was 3.3×10^8 CFU/ml.

The mean viable numbers of *S. thermophilus* in unfortified yogurt and iron fortified yogurt produced in this study were 5.4×10^8 CFU/ml and 7.0×10^8 CFU/ml after 1 d of storage. Their number decreased significantly ($P = .09$) after 30 d in all treatments (Table 6). At the end of storage period the average bacterial counts for unfortified nonfat yogurt and iron-fortified nonfat yogurt were 2.4×10^8 CFU/ml and 1.9×10^8 CFU/ml. When the entire experiment was repeated for 2% fat yogurt, *L. delbruekii* ssp. *bulgaricus* and *S. thermophilus* plate counts showed the same pattern as in nonfat yogurt.

TABLE 6. Analysis of variance for S. *thermophilus* counts of nonfat and 2% iron-fortified yogurt over 1 mo of storage at 4°C.

SV	df	MS	*P*
Rep	1	.00236	.9772
Milk (M)	1	2.7008	.4395
Error (a)	1	1.8432	
Treatment (T)	9	.00938	.9744
Cont. VS Rest	1	.00769	.6423
Among Rest	8	.00960	.9647
Source (S)	2	.01329	.6855
Level (L)	2	.00034	.9901
S x L	4	.01238	.8343
M x S	2	.00552	.8532
M x L	2	.01078	.7353
M x S x L	4	.02755	.5412
Error (b)	18	.03447	
Day (D)	2	4.93144	.0909
Error (c)	2	.49331	
M x D	2	1.94947	.0000
T x D	18	.01338	.9993
S x D	4	.01100	.9461
L x D	4	.01484	.9102
S x L x D	8	.01176	.9899
M x T x D	18	.01061	.9998
M x S x D	4	.01055	.9499
M x L x D	4	.02036	.8509
M x S x L x D	8	.00775	.9975
Error (d)	38	.06031	
Total	119		

Our results indicate iron fortification of yogurt does not affect acid production nor growth and survival of starter bacteria.

Fate of *P. fluorescens* and *E. coli* in Iron-Fortified Yogurt

Contamination of yogurt could occur during culturing or packaging by improper sanitation or handling procedures. We chose to inoculate iron-fortified yogurt with strains of *P.*

fluorescens and *E. coli* because they most frequently are the contaminants of other dairy products. They can also utilize iron to enhance their growth.

The viable numbers of *E. coli* did not increase during either incubation or manufacture of yogurts. Yogurt made from milk inoculated with *E. coli* at the rates of 10^3 and 10^5 CFU/ml had the same *E. coli* population after culturing the milk to pH 4.2 and cooling overnight to 4°C. After 1 d of storage, there were significantly more (p = .01) viable *E. coli* in iron-fortified yogurts (3.2 x 10^3 CFU/ml and 2.5 x 10^5 CFU/ml) than in unfortified yogurts (1.4 x 10^3 CFU/ml and 1.8 x 10^5 CFU/ml. However, they did not survive during storage and after 7 d at 4°C. The *E. coli* populations declined to less than 1 CFU/ml for both unfortified and iron-fortified yogurts.

Pseudomonas fluorescens was not viable at d 1, 7, or 14 of storage in iron-fortified and unfortified yogurt. Two probable factors contributing to their destruction were the incubation temperature of yogurt (42°C for 5 h) and continuous liberation of lactic acid by starter bacteria. *Pseudomonas fluorescens* are psychrotrophs that grow well at or below 7°C, and their optimum temperature is between 20°C and 30°C.

The starter cultures were able to ferment milk and grow to high numbers even in the presence of high levels of *E.*

coli and *P. fluorescens*. There was no significant difference in lactic acid bacteria counts between yogurts that were inoculated with different levels of *E. coli* and *P. fluorescens* and uninoculated controls. Presence of iron had no significant effect on lactic acid bacterial counts. After 1 d of storage, the mean *S. thermophilus* and *L. delbruekii* ssp. *bulgaricus* counts were 1.0×10^9 CFU/ml and 8.3×10^8 CFU/ml, respectively. Their viable numbers decreased only slightly after 14 d of storage (Table 21-22, Appendix B).

Most bacteria grow best at neutral pH (6.6-7.5) and show fastidious behavior in their relationship to pH (11). After 1 d of storage, the mean pH value of yogurts was 4.26 ± .04 and it decreased significantly ($P = .02$) to 4.06 ± .05 after 14 d (Table 23, Appendix B). Adding iron also had a significant effect ($P = .02$) on pH. Lactic acid bacteria produce lactic acid, which reduces pH and inhibits growth of many bacteria, especially gram-negative species (20).

In iron-fortified yogurt, most of the iron is bound to milk proteins and is thus unavailable for other microorganisms unless they produce high iron-affinity siderophores. However, biochemical characteristics of yogurt apparently do not provide a suitable medium for growth of spoilage and pathogenic microbes such as *P. fluorescens* or *E. coli*. The low pH of yogurt along with

high viable numbers of lactic acid bacteria apparently inhibits their bacterial growth. Because of this, our results indicate that yogurt is an excellent candidate for iron fortification of dairy products, which will not represent a significant food safety hazard.

CONCLUSIONS

Iron-fortified yogurt can be manufactured using ferric chloride, iron-casein, or iron-whey protein complex as iron sources. The iron-fortified yogurts contain high levels of viable lactic acid organisms even after 30 d of storage. Iron fortification had no influence on acid production during yogurt production.

The liberation of acid by lactic acid bacteria along with their high viable numbers and the low pH of yogurt apparently prevents spoilage of the iron-fortified yogurt by *E. coli* and *P. fluorescens*. Therefore, it appears that yogurt could be used as a safe vehicle for delivering iron to consumers.

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PART 4. EFFECTS OF IRON FORTIFICATION OF YOGURT ON ITS OXIDATION DETERIORATION AND ORGANOLEPTIC PROPERTIES

ABSTRACT

Iron-fortified yogurts (nonfat and 2%) were manufactured from milk fortified with $FeCl_3$, iron-casein, and iron-whey protein complex at levels of 10, 20, and 40 mg iron/kg yogurt. Unfortified yogurts (nonfat and 2%) served as negative controls. Lipid oxidation and iron concentration were monitored over 30 d storage of the yogurts at 4°C using ferrozine assay and thiobarbituric acid test (TBA). The organoleptic characteristics of yogurt were determined by a panel of trained judges and consumer panels.

The respective iron concentrations and recoveries of iron-casein complex were 56.0 mg iron/g protein and 46.8%, and for iron-whey protein complex were 136.5 mg iron/g protein and 88.7%. The iron recovery in iron-fortified yogurts was within the expected target iron concentration. No significant increase ($P > .05$) in chemical oxidation levels between iron-fortified yogurt and unfortified yogurt was detected.

Trained panelists scored oxidized, metallic, bitter, and off-flavor in the range of "not perceptible" or "very slightly perceptible" for both iron-fortified and control yogurts. A panel of 75 lay judges did not detect any significant differences ($P > .05$) in the appearance, mouth feel, flavor, or overall quality between flavored yogurts fortified with different iron sources. They also gave

similar hedonic scores to all of the selected attributes of iron-fortified and control yogurts. All yogurt samples were liked by the panelists. Our results indicate yogurt is a suitable vehicle for delivering iron, an important micronutrient, to the populations that are at risk of iron deficiency.

INTRODUCTION

Iron deficiency is one of the major nutritional concerns in developing countries. Even in the United States some segments of the population (such as infants, children, adolescents, pregnant women, women at child-bearing age, and elderly) are at risk for iron deficiency (9, 16, 24). Two ways to increase iron intake are either with supplemental medicinal iron or by fortification of food products (21). Dairy products provide high quality proteins, vitamins, and minerals but contain almost no dietary iron. Therefore, dairy products are logical vehicles for iron fortification because they have high nutritive values, reach target populations, and are widely consumed (2, 8, 17).

The quality of iron-fortified dairy products depends on the iron sources used, levels of iron, and the properties of dairy products utilized for iron fortification (22, 27). The two major off-flavors associated with fortified dairy products are oxidized flavor due to lipid oxidation and

metallic taste due to iron salts (17). Zhang and Mahoney (27, 29) manufactured Cheddar cheese and process Cheddar cheese fortified with iron-casein complex, iron-whey protein complex, and $FeCl_3$ and saw no difference in oxidized off-flavors of 3-mo-old iron-fortified Cheddar cheese compared to unfortified cheeses.

Fortification of milk with ferrous sulfate and ferric or ferrous ammonium sulfate causes oxidative deterioration of milk fat and, subsequently, high thiobarbituric acid (TBA) numbers (14, 26). Using an unchelated form of iron showed greater potential for fat oxidation than using chelated iron. Hegenauer et al. (15) found chelated iron inhibited oxidation of milk lipids. Fortification of cottage cheese with ferric ammonium citrate did not produce off-flavors over two months of storage (22). Douglas et al. (11) studied color, flavor, and iron bioavailability in iron fortified chocolate milk. They reported that chocolate milk fortified with ferripolyphosphate-whey protein complex showed good flavor properties. However, fortification of skim milk with ferric chloride or ferrous gluconate caused oxidized off-flavor (11).

To our knowledge, there is no report on iron fortification of yogurt. Yogurt has the characteristics of high acidity and distinct flavor, which may make it suitable for iron fortification. Its acidity and strong flavor will

mask many low-grade off-flavors that may be produced by iron. Furthermore, much of the yogurt consumed contains fruits, the ascorbic acid of which can enhance absorption of the fortified iron. Also, most yogurt being marketed today is nonfat yogurt, which should also reduce the likelihood of off-flavors developing from oxidation of fat.

The purpose of this study was to fortify yogurt with ferric chloride, iron-casein, and iron-whey protein complex and to evaluate yogurt quality chemically by TBA assay and organoleptically by expert and consumer sensory panels.

MATERIALS AND METHODS

Iron Sources

In this study three different iron sources were used. Ferric chloride (Catalog Number F-2877) was obtained from Sigma Chemical Company (St. Louis, MO). The iron-casein and iron-whey protein complex were prepared as described earlier (13, 28) by fortification of skim milk and cottage cheese whey with $FeCl_3$ and then adjusting pH to their isoelectric points to precipitate casein and whey protein.

Iron-Fortified Yogurt

Iron solutions ($FeCl_3$, iron-casein and iron-whey protein complex) were used to fortify standardized (6% sugar, 5.8% NDM, and .7% stabilizer) nonfat and lowfat yogurt mixes with 10, 20, and 40 mg iron/kg yogurt for each

source (13). Negative controls with no iron were also made. The yogurt mixes were heat-treated to 82°C for 30 min, cooled to 41°C, and inoculated with 1% yogurt cultures. They were incubated at 42°C until pH of 4.2 was achieved and then stored in a cool room at 4°C.

Iron Analysis

The ferrozine method (7, 23) was used to quantitate iron content of iron-casein, iron-whey protein complex, and iron-fortified yogurts and controls. The glassware used for iron analysis was soaked in 6 N HCl for 48 h in order to solubilize any iron contaminant and then rinsed with double distilled deionized water. Samples of iron protein complexes (.1 g) and yogurts (1.0 g) were wet ashed with concentrated nitric acid by heating them below boiling temperature until they were dry. At the end of the ashing process, drops of 30% H_2O_2 were added until a white ash was formed. The ashes were then dissolved in .5 ml of 6 N HCl and diluted with double distilled deionized water to the concentration necessary for colorimetric assay based on their original iron content.

To reduce all of the iron to the ferrous form, 1 ml of this solution was mixed with 1 ml of 1% ascorbic acid solution in .2 N HCl. The mixture was kept at room temperature for 15 min. Then 1 ml of 10% ammonium acetate buffer and 1 ml of 1 mM ferrozine coloring agent was added,

mixed well, and kept at 20°C for 30 min to complete color formation. The absorbance at 562 nm was recorded against a reagent blank using a dual beam spectrophotometer (UV.VIS Recording Spectrophotometer, Shimadzu, Japan). Iron standards (1, 2, 4, 8, and 10 μg iron/ml) were also prepared in duplicate and a wheat flour standrd (U.S. Department of Commerce, National Bureau of Standards, Washington, DC.) was used to determine the accuracy of ferrozine assay. The iron content of each sample was calculated using the standard curves and appropriate dilution factors.

Thiobarbituric Acid Test

Oxidized materials were analyzed spectrophotometrically using a TBA test (6). A stock solution of 15% trichloroacetic acid, .375% 4,6-dihydroxypyrimidine-2-thiol, and .25 N HCl was prepared. One gram of yogurt was weighed into a glass screw-top test tube and 9 ml of stock solution was added, mixed well, and heated in a boiling water bath for 15 min. They were then cooled to room temperature and centrifuged (Sorvall Instruments, RC5C, DuPont Products, Hoffman Estates, IL) at 7000 *g* for 15 min at 20°C.

The absorbance of the samples was determined at 532 nm using a dual beam spectrophotometer (UV.VIS Recording Spectrophotometer, Shimadzu, Japan). Samples were evaluated after 1, 15, and 30 d of storage at 4°C.

Organoleptic Analysis

Selecting and Training Judges. A pool of 21 potential panelists was recruited from graduate students, faculty, and staff at the Department of Nutrition and Food Sciences, Utah State University to become familiarized with oxidized, metallic, and off-flavors in yogurts that were fortified with 40 and 100 mg iron/kg yogurt with $FeSO_4$ compared to unfortified yogurt.

The trainees tasted samples of known oxidized and metallic off-flavor yogurt and of negative control yogurt. They were exposed to a set of extreme contrast flavors between yogurt fortified with 100 mg iron/kg yogurt and unfortified yogurt to ensure their recognition of metallic and oxidized flavor and also to discriminate between those who could detect off-flavors and those who lack detection ability. They were also given a sample of yogurt fortified with 40 mg iron/kg yogurt, which had a very mild off-flavor. The trainees were then allowed to discuss their perception of each off-flavor and repeat tasting until they could recognize oxidized and metallic off-flavors in yogurts. Testing sessions were held on three different days. The same set of samples was presented as unknowns to the potential judges. They evaluated bitter, oxidized, metallic, off-flavor, and acid flavor on a rating scale of 1 to 9 (1 = not perceptible and 9 = extremely strong). Eleven

panelists were selected based on their sensitivity and ability to detect oxidized and metallic off-flavors in the iron-fortified yogurts.

Sample Preparation and Serving. Nonfat and lowfat (2%) iron-fortified yogurt with three sources ($FeCl_3$, iron-casein and iron-whey protein complex) at three levels (10, 20, and 40 mg iron/kg yogurt) for each source was made and packaged as plain yogurt. Negative controls were also prepared. The yogurt samples were scooped into plastic cups coded with three-digit random numbers. The samples were then capped and refrigerated at 4°C until tested. Various orders of tasting were selected for each judge to avoid positional bias. Judges were provided with individual booths in an air-conditioned taste panel room (Sensory Analysis Laboratory, Utah State University, Logan, UT), score sheets, and drinking water. They were asked to rinse thoroughly between tasting the samples to avoid flavor overlapping between samples.

Trained Panel. The 11 trained judges evaluated yogurt samples after 1, 15, and 30 d of storage at 4°C. Each judge was given four samples and asked to evaluate each sample for presence of any bitter, oxidized, metallic, off-flavor, and acid flavor on a rating scale of 1 to 9 (1 = not perceptible and 9 = extremely strong). Judges were asked to evaluate

these five attributes to prevent them from making an expectation error.

Consumer Panel. Nonfat and lowfat (2%) yogurts were fortified with $FeCl_3$, iron-casein, and iron-whey protein complex at a rate of 20 mg iron/kg yogurt. The unfortified yogurt served as a negative control. All samples were strawberry flavored. Seventy-five volunteer lay panelists evaluated appearance, mouth feel, flavor, and overall quality of the yogurts on a 9-point hedonic scale (1). The hedonic scale, which measures the level of acceptance for foods, has nine categories, as follows: like extremely, like very much, like moderately, like slightly, neither like nor dislike, dislike slightly, dislike moderately, dislike very much, and dislike extremely. The judges were served with four samples and requested to rinse their mouths between samples.

Statistical Analysis

The TBA experiments were conducted using a complete split plot design, and analysis of variance was done using Minitab. We determined the effects of fortification of yogurt with $FeCl_3$, iron-casein, and iron-whey protein complex at various levels (10, 20, and 40 mg iron/kg yogurt) on the optical density (532 nm) of TBA assay samples of nonfat and lowfat yogurts over 1 mo of storage. The main effects were milk fat, iron sources, level of each source,

and storage time. The interactions between main effects were also determined.

To analyze the data from the trained taste panel, a split-plot design was used (Minitab, Inc). The main effects were judges, fat level, iron sources, level of each source, and storage time. Interactions (2-, 3-, and 4-way) among the main effects were also determined. A completely randomized design was used to analyze the consumer taste panel data (Minitab, Inc).

RESULTS AND DISCUSSION

Iron Recovery in Iron-Protein-Complexes and Yogurt

The iron concentration and recoveries in iron-casein complex were 56.0 mg iron/g protein and 47%, and in iron-whey protein complex were 137 mg iron/g protein and 89%. Zhang and Mahoney (28) reported iron contents and recoveries in iron-casein complex and iron-whey protein complex as 23 and 99 mg iron/g protein and 92 and 98%, respectively. This difference could be due to slight variations in preparation procedures for iron sources. Table 7 shows the iron concentration in yogurt fortified with different sources in comparison to the expected concentration.

All samples were within the approximate target iron concentration, and the calculated iron contents were close to the expected iron concentration. The iron recovery in

TABLE 7. Iron concentration of nonfat and 2% fat yogurts fortified with $FeCl_3$, iron-casein, and iron-whey protein complex at 10, 20, and 40 mg iron/kg yogurt.

Iron Sources	Expected iron level (mg iron/kg yogurt)	Measured iron level (mg iron/kg yogurt)	
		Nonfat	2% Fat
$FeCl_3$	10	11.3 ± .5	10.3 ± .8
	20	20.2 ± 1.0	18.7 ± 1.4
	40	38.0 ± 3.5	35.6 ± 2.6
Iron-casein	10	10.8 ± 1.0	10.7 ± .6
	20	20.6 ± .8	19.2 ± 1.0
	40	39.1 ± 1.8	37.8 ± 1.5
Iron-whey	10	10.6 ± .2	10.9 ± .4
	20	20.2 ± .8	21.3 ± 1.9
	40	39.2 ± 1.3	38.4 ± 1.8

yogurt was similar for fortification with $FeCl_3$, iron-casein, and iron-whey protein complex. We expected 100% iron retentation in yogurt because, unlike cheese, none of the iron is lost during manufacturing. In contrast, almost one third of the iron (19-29%) was lost during processing when Cheddar cheese was fortified with $FeCl_3$ (28), although this can be improved by using microencapsulated iron (17).

Yogurt could be used as a good vehicle to deliver iron to consumers. For example, an 8-oz cup of yogurt fortified with 20 mg iron/kg yogurt would provide approximately 20% of the recommended daily allowance for women. According to the new FDA nutritional labeling requirement, a claim could be made on iron content if the product contains at least 10% of

RDA. Therefore, iron-fortified yogurt can have a health claim not only for calcium but also for iron and protein.

It has been shown that people who consume low iron-density diets consume more dairy products, whereas those with high iron-density diets consume the least dairy products (12). This is because dairy products contain almost no dietary iron (4, 18, 25). Iron-fortified yogurt could be a major contributor of dietary iron for those with low-iron diets and eventually decrease the incidence of iron deficiency.

Yogurt Quality

Oxidation. Fortification of yogurt with $FeCl_3$, iron-casein, and iron-whey protein complex did not significantly ($P = .23$) increase oxidation (as measured by the TBA test) in comparison to unfortified yogurt (Table 8 and Figure 11). During 30 d of storage, there was a slight increase in oxidation, but statistically it was not significant ($P = .56$). This is not surprising considering the fat levels (nonfat and 2%) and pH (4.2) of yogurt. The low fat and high acidity of yogurt prevent or greatly reduce oxidation potency and formation of iron hydroxides. In addition, the iron is bound to milk proteins (casein and whey proteins) that probably reduce its ability to participate in iron-catalyzed hydroxyl radical formation and peroxidation (3, 10). For lipid peroxidation to take place,

iron must freely change its oxidation state from Fe^{+2} to Fe^{+3} (5, 19, 20). However, the binding of iron to milk proteins may affect its ability to change oxidation state. Another requirement for lipid peroxidation is presence of polyunsaturated fats. Milk fat contains 70% saturated acids and 30% unsaturated fatty acids. Of these unsaturated fatty acids, only 3% are polyunsaturated (4), so butter fat is inherently slow to oxidize.

TABLE 8. Analysis of variance for chemical oxidation (measured using the TBA assay) of nonfat and 2% unfortified and iron-fortified yogurts over 1 mo of storage at 4°C.

SV	df	MS	*P*
Rep	1	.00204	.3325
Milk (M)	1	.05246	.0720
Error (a)	1	.00068	
Treatment (T)	9	.00069	.2500
Cont. VS Rest	1	.00076	.2283
Among Rest	8	.00069	.2600
Source (S)	2	.00031	.5400
Level (L)	2	.000009	.9815
S x L	4	.00122	.0804
M x S	2	.00030	.5521
M x L	2	.00044	.4279
M x S x L	4	.00049	.4351
Error (b)	18	.00049	
Day (D)	2	.00238	.5646
Error (c)	2	.00309	
M x D	2	.00536	.0000
T x D	18	.00037	.1662
S x D	4	.00010	.7987
L x D	4	.00038	.2255
S x L x D	8	.00057	.0440
M x T x D	18	.00029	.3462
M x S x D	4	.00026	.4034
M x L x D	4	.00027	.3828
M x S x L x D	8	.00037	.2059
Error (d)	38	.00025	
Total	119		

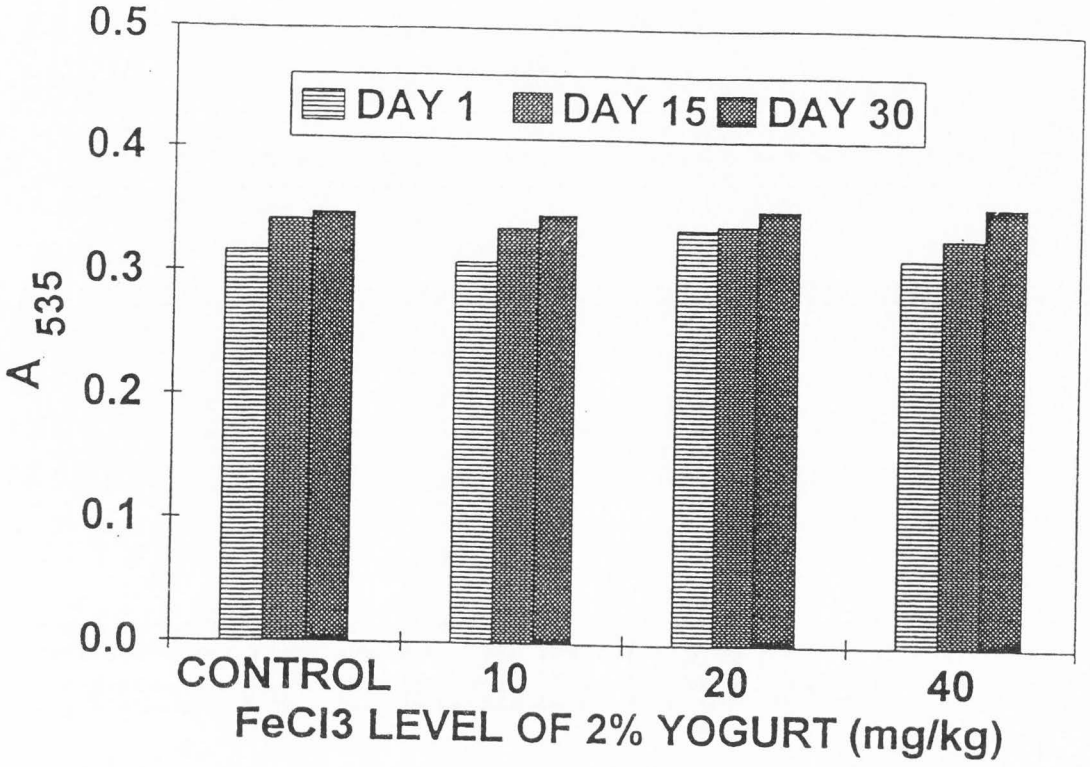

Figure 11. Comparison of chemical oxidation between unfortified yogurt and yogurts fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of $FeCl_3$ during storage.

Zhang and Mahoney (29) found low lipid peroxidation in iron fortified process Cheddar cheese. They suggested that high protein content of cheese may act as an iron chelator. They also found a slight increase in oxidation (measured by TBA assay) of iron-fortified Cheddar cheese, but it was within the range for unfortified cheese. We expect higher lipid peroxidation in cheese because of higher fat content and lower acidity of cheese in comparison to yogurt. Jackson and Lee (17) reported oxidation in Havarti-style cheese made with stearine-encapsulated iron solutions was lower than in free-iron solutions. They found encapsulation method does not completely protect cheese from oxidation because iron may release from the microcapsules during cheese making and storage time, leading to higher TBA values in fortified cheese in comparison to control cheese.

Sensory Evaluations. Since iron fortification affects certain flavor properties of food products, the sensory evaluation included two main parts. The first part involved assessing the negative impacts of iron fortification such as presence of metallic, bitter, and oxidized off-flavor using trained panelists over 30 d storage period. We found a significant difference ($P < .00005$) among trained judges for all selected attributes which indicates variation in the sensity threshold of the judges. Also all of the judges were aware of the presence of iron in the yogurt and had been selected for their sensitivity to detecting off-flavors

in yogurt, which may contribute to an error of expectation among judges. The second part dealt with how well iron-fortified yogurt was liked or disliked by lay panelists.

Far more effects were shown for acid flavor (Appendix c, Table 28). There were significant differences between nonfat and lowfat yogurts ($P = .023$), type of iron sources ($P = .072$), fat level by iron source interaction ($P < .00005$), and storage time ($P < .00005$), as well as the storage time by fat-level interaction ($P < .00005$) and the three-way interaction fat level by iron source by storage time ($P < .00005$). Level of iron fortification was not significant ($P = .65$).

When the data from the sensory evaluations conducted by the trained panel were examined using analysis of variance (Table 24-27, Appendix C), it was observed that bitter, oxidized, metallic, and off-flavors were affected differently. The level of fat (nonfat versus 2% fat) affected bitter flavor ($P = .066$), off-flavor ($P = .079$), and metallic flavor ($P = .082$) but did not affect oxidized flavor ($P = .35$). While there was some effect of using different iron sources on bitter flavor ($P = .049$) and oxidized flavor ($P = .036$), there was none on metallic flavor ($P = .51$). However, when comparing the fortified yogurt, there were no significant differences between 10, 20, and 40 mg iron/kg yogurt.

While the overall effect of storage time was not significant for bitter, metallic, or oxidized flavors (P = .54, .89, and .33), there was a significant interaction between fat level and storage time (P = .018, .052, and .102), and between iron source and storage time (P = .039, .024, and .032). Even though these flavor scores were all between 1 (not detectable) and 3 (slightly perceptible), as shown in Figures 12-15, there were some differences in the way in which these scores changed during the 30 d storage.

After one d of storage, the mean bitter, oxidized, and metallic scores for unfortified non-fat yogurt were 1.45, 1.64, and 2.09, respectively, and the average for non-fat yogurt fortified with different levels of $FeCl_3$ were 1.27, 2.18, and 2.0. These scores were in the range of not detectable (1) or very slightly perceptible (3). After 30 d of storage, the panelists could not detect any significant increase in bitter (P = .54), oxidized (P = .33) and metallic flavor (P = .89). Although the probability values from the analysis of variance for bitter and oxidized flavor indicated significant difference ($P < .05$) between iron sources, the scores for all samples were rated as "not perceptible" or "very slightly perceptible."

The lay panel of judges did not detect significant differences in the appearance, mouth feel, flavor, and overall quality (P = .96, .52, .91, and .72) between yogurt

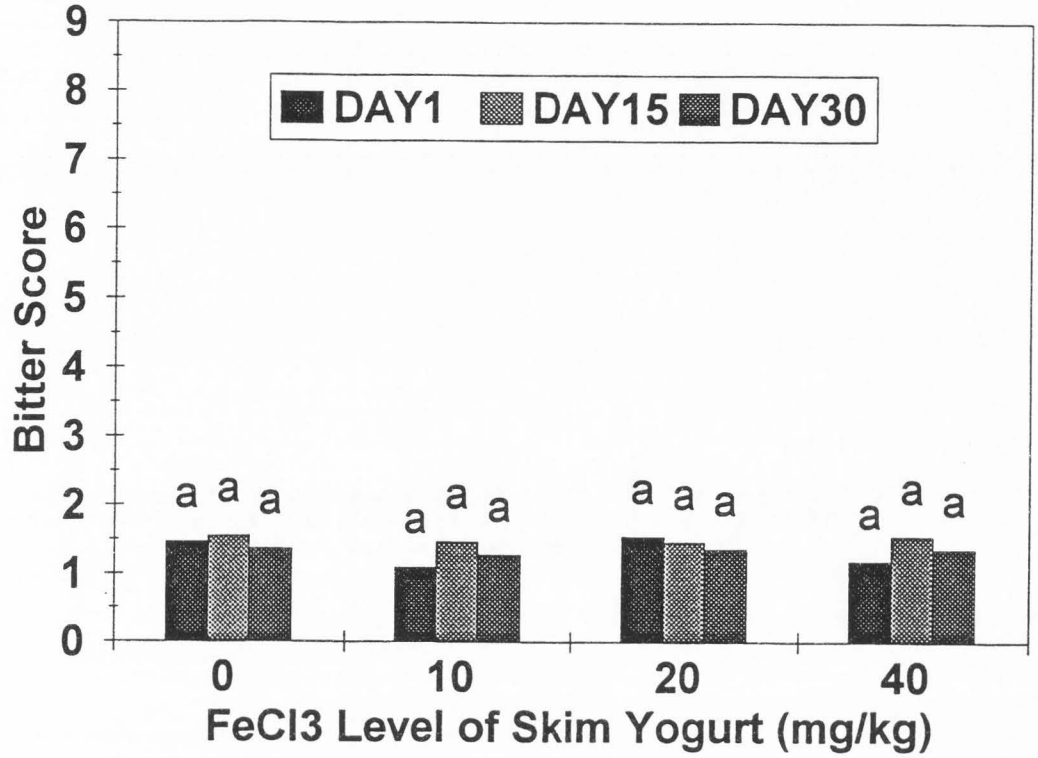

Figure 12. Bitterness scores of skim unfortified yogurt and skim yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of $FeCl_3$ during storage. (Data points with the same letter are not significantly different.)

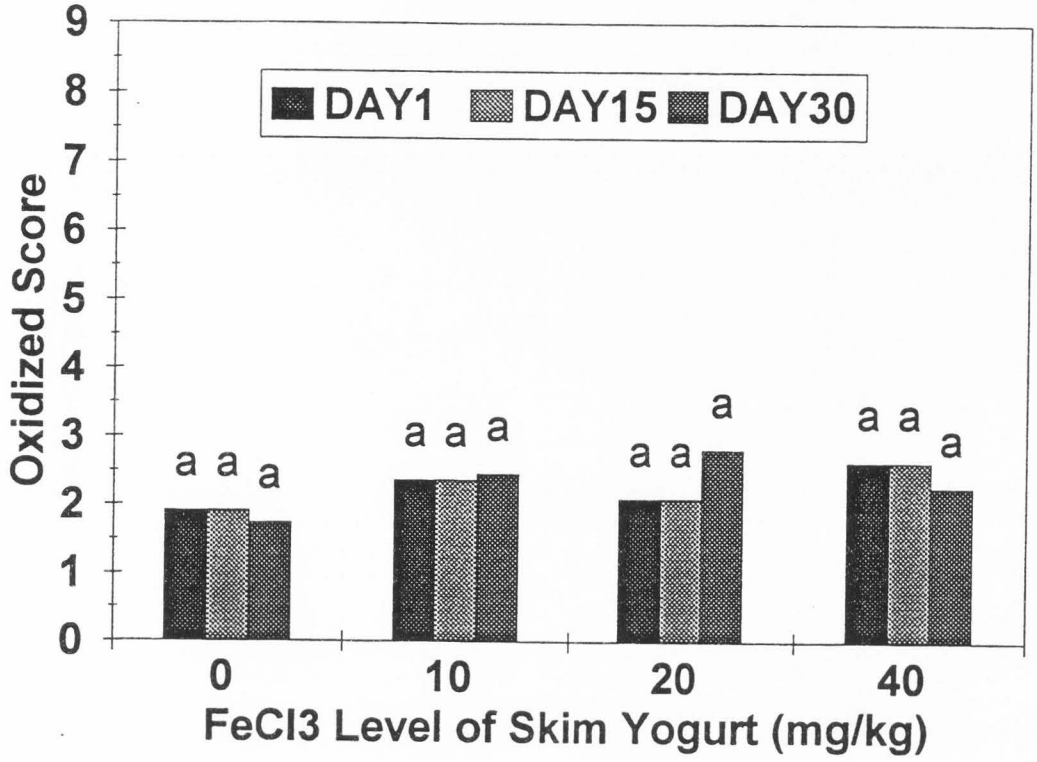

Figure 13. Oxidized flavor scores of skim unfortified yogurt and skim yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of $FeCl_3$ during storage. (Data points with the same letter are not significantly different.)

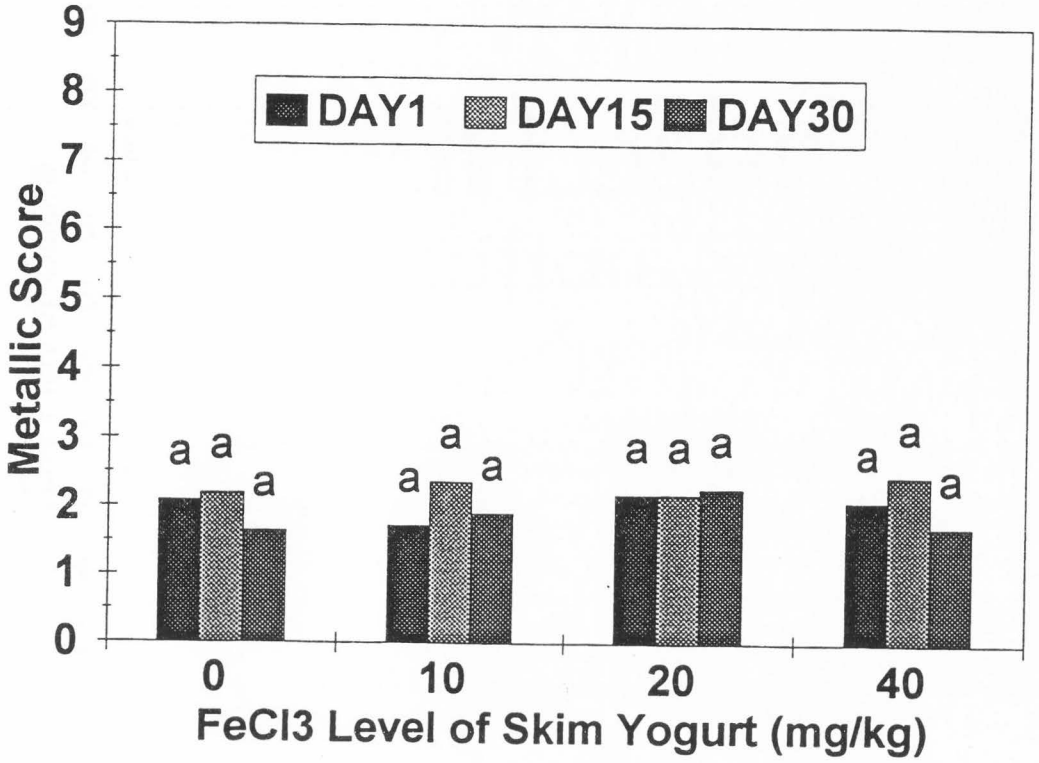

Figure 14. Metallic flavor scores of skim unfortified yogurt and skim yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of $FeCl_3$ during storage. (Data points with the same letter are not significantly different.)

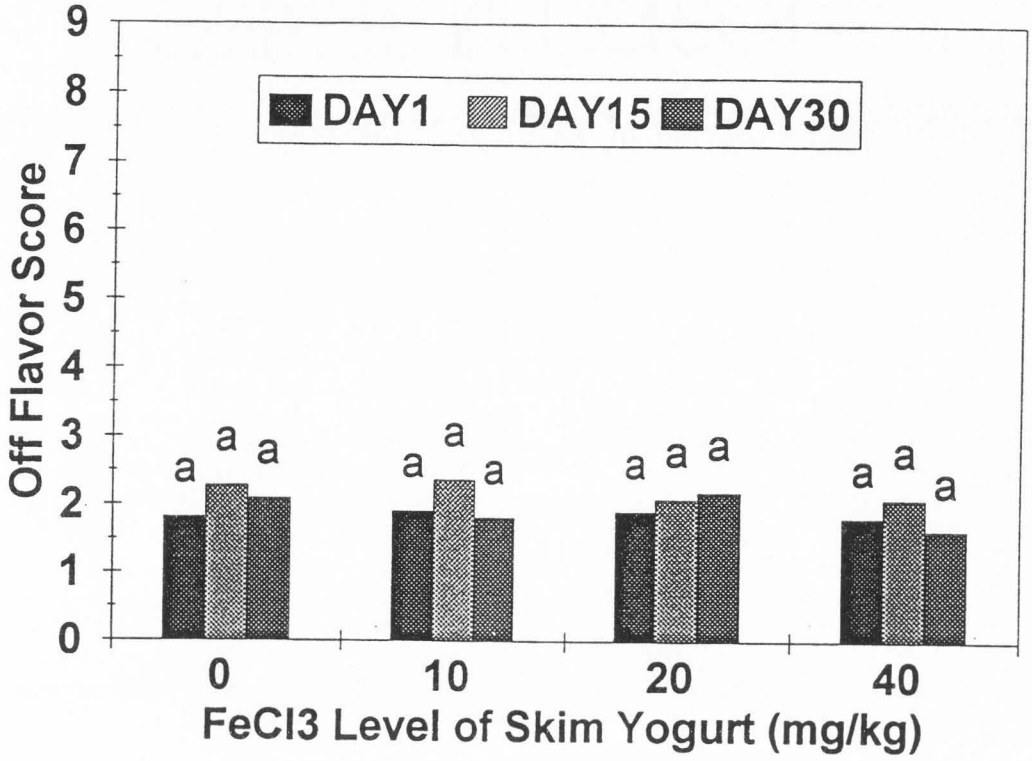

Figure 15. Off-flavor scores of skim unfortified yogurt and skim yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of $FeCl_3$ during storage. (Data points with the same letter are not significantly different.)

fortified with $FeCl_3$, iron-casein, and iron-whey protein complex (Appendix C and Tables 29-32). All of the scores for the fortified yogurt were comparable to the control unfortified yogurts (Figures 16-19). The appearance score for 2% fat yogurt was significantly higher (P = .043) than non-fat yogurt. However, there was no significant difference in the mouth feel, flavor, and overall acceptability (P = .51, .96, and .48) between 2% and nonfat yogurt.

All yogurt samples were rated above average on the hedonic scale and were liked by the panelists. The mean appearance, mouth feel, flavor, and overall scores for unfortified skim yogurt were 7.41, 6.99, 6.52, and 6.83, respectively, and for iron-fortified yogurt were 7.19, 7.13, 6.7, and 6.85, respectively. Zhang and Mahoney (27, 29) also conducted sensory evaluations to determine the effect of iron fortification on Cheddar cheese and process cheese quality. They reported that expert trained panelists could not detect any differences in oxidized off flavor or cheese flavor among iron fortified process Cheddar cheeses that were stored for 3 mo. Also they found similar hedonic scores for the flavor, texture, and overall quality of the iron fortified and unfortified cheeses.

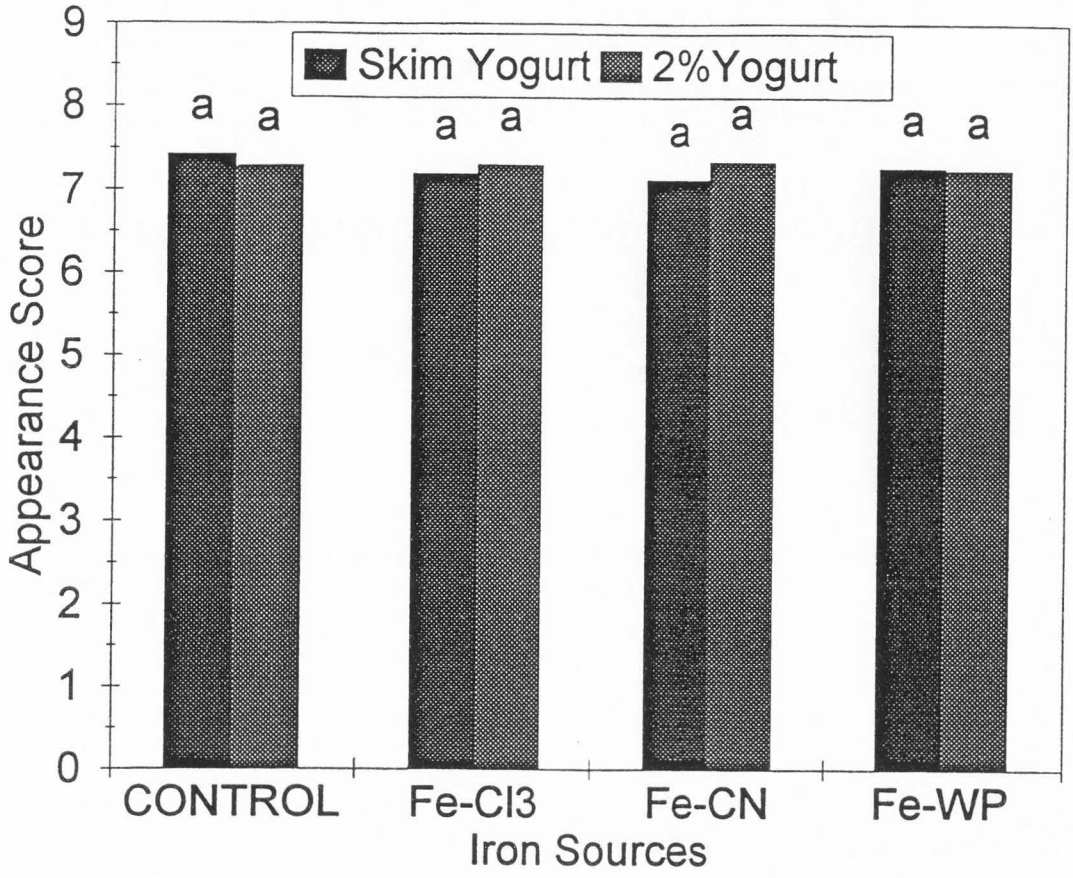

Figure 16. Appearance scores of strawberry flavored unfortified yogurt and yogurts fortified with $FeCl_3$, iron-casein (Fe-CN), and iron-whey protein complex (Fe-WP). (Data points with the same letter are not significantly different.)

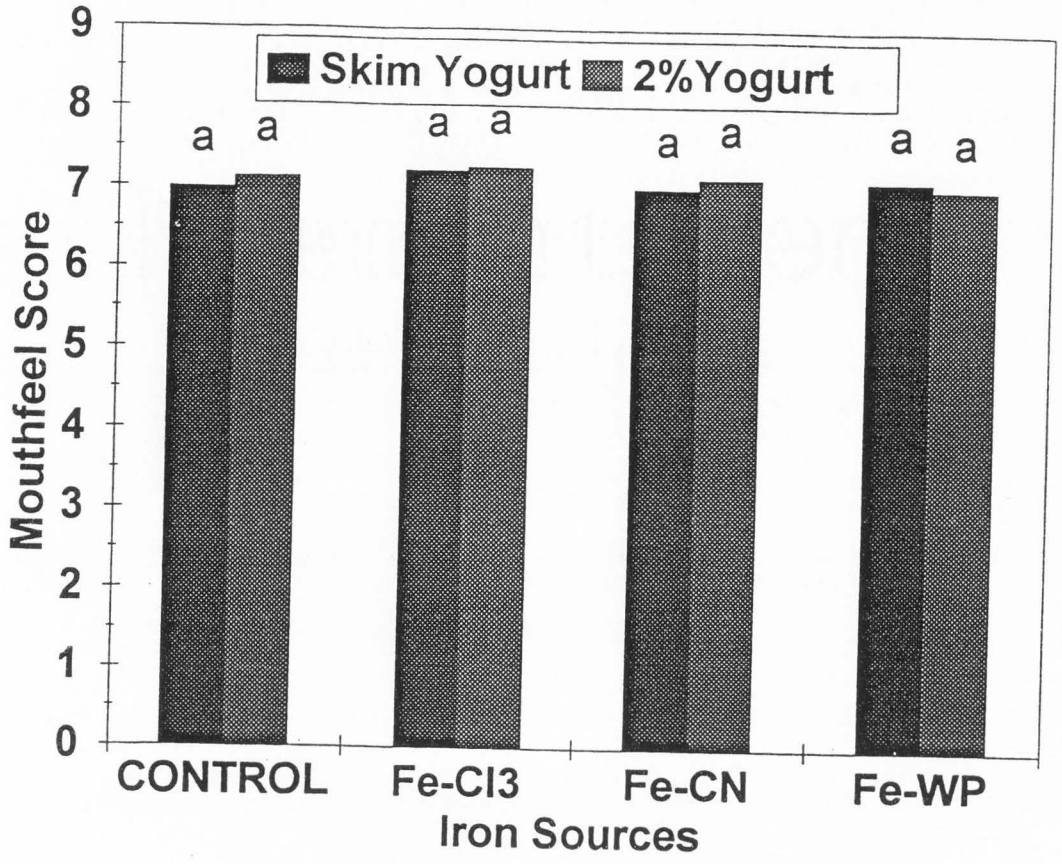

Figure 17. Mouth feel scores of strawberry flavored unfortified yogurt and yogurts fortified with $FeCl_3$, iron-casein (Fe-CN), and iron-whey protein complex (Fe-WP). (Data points with the same letter are not significantly different.)

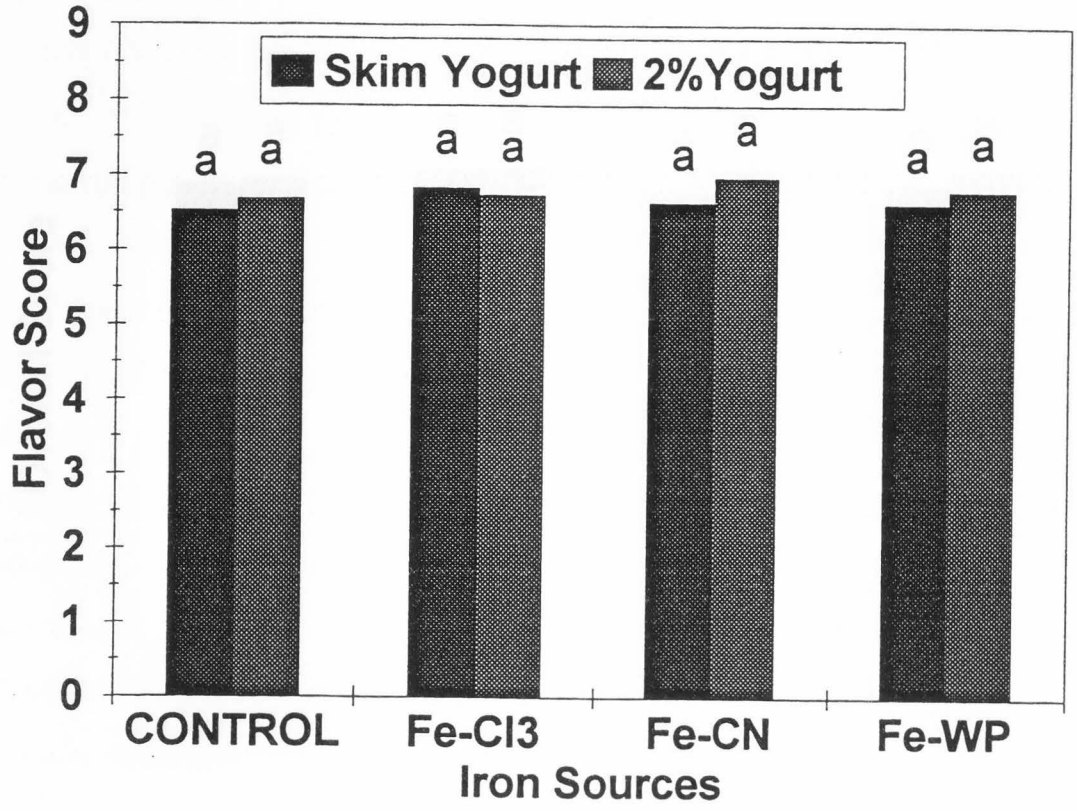

Figure 18. Flavor scores of strawberry flavored unfortified yogurt and yogurts fortified with $FeCl_3$, iron-casein (Fe-CN), and iron-whey protein complex (Fe-WP). (Data points with the same letter are not significantly different.)

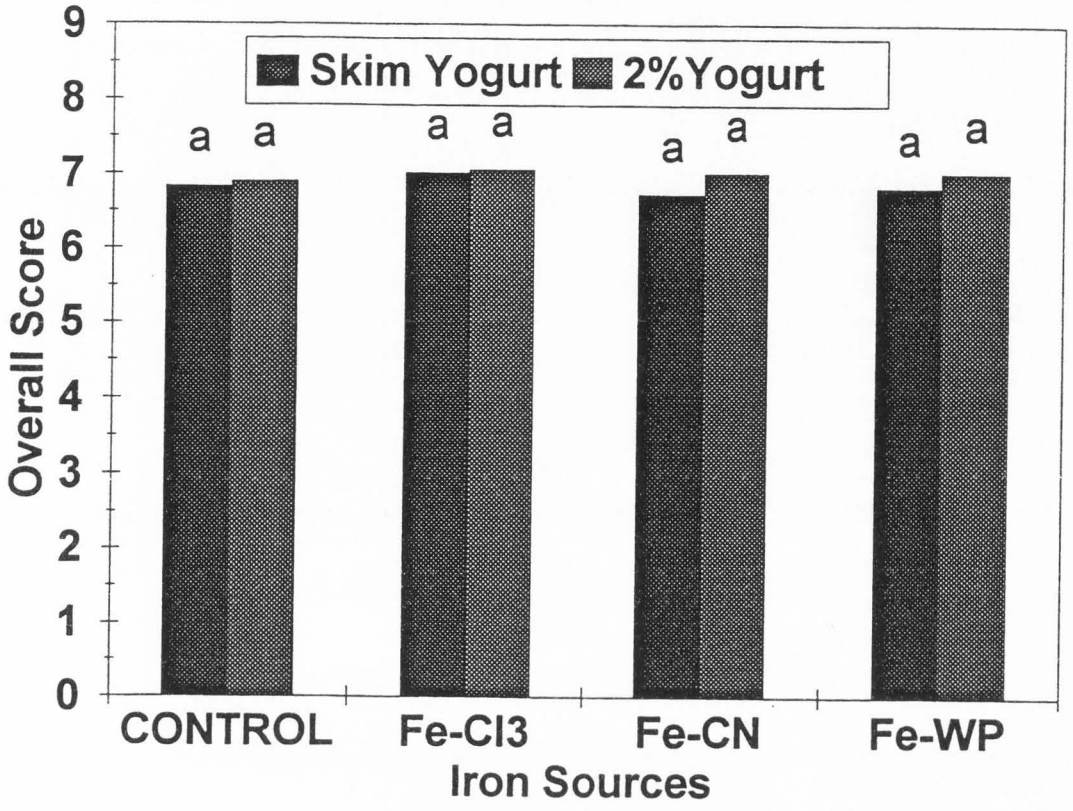

Figure 19. Overall scores of strawberry flavored unfortified yogurt and yogurts fortified with $FeCl_3$, iron-casein (Fe-CN), and iron-whey protein complex (Fe-WP). (Data points with the same letter are not significantly different.)

CONCLUSIONS

The quality of iron-fortified yogurt was not significantly affected when measured either by chemical assay or sensory analysis. We have shown that fortification of yogurt with different iron sources is not only technically feasible, but that iron fortification does not cause bitter, metallic, oxidized, and off-flavor in yogurt, and it does not change appearance, mouth feel, flavor, and overall quality of yogurt. Therefore, yogurt could be considered as an appropriate vehicle for delivering iron, calcium, and protein to the consumers. Ferric chloride, iron-casein, and iron-whey protein complex are potential iron sources for fortification of yogurt.

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GENERAL SUMMARY

1. Iron binding to casein and whey proteins was independent of change in pH.

2. Fortification of milk with iron did not cause loss of calcium or phosphorus from casein micelles. It resulted in greater retention of calcium and phosphorus in the micelles as milk was acidified to pH 5.3.

3. Iron was bound preferentially to casein when yogurt was fortified with $FeCl_3$ or iron-casein complex. When fortified with iron-whey protein complex, iron was distributed throughout the non-micellar portion of the yogurt.

4. Ferric chloride, iron-casein complex, and iron-whey protein complex are suitable iron sources for fortification of yogurt.

5. Iron-fortified yogurt contained high levels of viable lactic acid bacteria. Growth and survival of *E. coli* and *P. fluorescens* were inhibited in iron-fortified yogurt because of liberation of acid by lactic acid bacteria and their high viable numbers.

6. The quality of iron-fortified yogurt was not affected when measured either by TBA assay or sensory analysis. Iron fortification did not cause bitter, metallic, oxidized, and off-flavor in yogurt, and it did not change appearance, mouth feel, and flavor of yogurt.

APPENDICES

APPENDIX A

TABLE 9. Analysis of variance for the amount of iron in pellets of iron-fortified milk and control at different pH.

Source	df	MS	F	*P*
Replication	1	.82	.03	.8693
pH	5	663.03	24.08	.0016
Error	5	27.54		
Total	11			

TABLE 10. Analysis of variance for the amount of iron of pellets protein in iron-fortified milk and control at different pH.

Source	df	MS	F	*P*
Replication	1	.00007	.01467	.9083
pH	5	.09206	19.3	.0028
Error	5	.00477		
Total	11			

TABLE 11. Analysis of variance for the amount of iron in serum of iron-fortified milk and control at different pH.

Source	df	MS	F	*P*
Replication	1	1.01	.871	.3936
pH	5	556.84	480.03	.0000
Error	5	1.16		
Total	11			

TABLE 12. Analysis of variance for the amount of iron in serum protein of iron-fortified milk and control at different pH.

Source	df	MS	F	*P*
Replication	1	.003008	4.021	.1013
pH	5	.441828	590.679	.0000
Error	5	.000748		
Total	11			

TABLE 13. Analysis of variance for the amount of calcium in pellets of iron-fortified milk and control at different pH.

Source	df	MS	F	*P*
Replication	1	3837	5.022	.2672
Fortification (F)	1	66513	87.058	.0679
Error (a)	1	764		
pH	5	3504030	9870.507	.0000
F x pH	5	36333	102.346	.0000
Error (b)	10	355		
Total	23			

TABLE 14. Analysis of variance for the amount of calcium in serum of iron-fortified milk and control at different pH.

Source	df	MS	F	*P*
Replication	1	7	.233	.7137
Fortification (F)	1	212540	7084.66	.0076
Error (a)	1	30		
pH	5	3440754	268.266	.0000
F x pH	5	21908	1.708	.2203
Error (b)	10	12826		
Total	23			

TABLE 15. Analysis of variance for the amount of phosphorus in serum of iron-fortified milk and control at different pH.

Source	df	MS	F	*P*
Replication	1	104	.764	.5427
Fortification (F)	1	178205	1310.331	.0176
Error (a)	1	136		
pH	5	360987	38.204	.0000
F x pH	5	34655	3.668	.0381
Error (b)	10	9449		
Total	23			

TABLE 16. Iron and protein content of serum and iron content in serum protein at different pH.

pH	Fe in serum (μg)	Protein in serum (mg/g)	Fe in serum protein (μg)
6.7	51.5a ±0.8	7.5 ±0.09	1.7a ±0.0
6.2	51.0a ±0.0	7.5 ±0.01	1.6b ±0.0
5.8	47.9b ±0.2	7.5 ±0.03	1.5c ±0.0
5.3	42.7c ±2.3	7.9 ±0.00	1.3d ±0.1
4.5	19.4d ±0.8	5.9 ±0.02	0.8e ±0.0
4.0	14.1e ±0.1	5.8 ±0.00	0.6f ±0.0

$LSD_{.05}$ for iron in serum = 2.8.
$LSD_{.05}$ for iron in serum protein = .07.
Values in the same column with the same letter are not significantly different.

TABLE 17. Calcium content of pellet and of pellet protein in iron-fortified milk and control at different pH.

	Iron fortified milk		Contol	
pH	Ca in casn. pellet (μg)	Ca in protein of casn. pellet (μg/mg)	Ca in casn. pellet (μg)	Ca in protein of casn. pellet (μg/mg)
6.7	2226.6a ±14.1	32.7a ±0.2	2444.6a ±25.5	34.1a ±0.4
6.2	2058.9b ±23.0	29.7b ±0.3	1833.1b ±57.3	27.2b ±0.8
5.8	1734.1c ±12.0	25.0c ±0.2	1477.1c ±47.4	21.7c ±0.7
5.3	997.1d ±0.7	14.3d ±0.0	714.6d ±31.1	10.6d ±0.5
4.5	206.1e ±9.2	2.5e ±0.1	152.3e ±1.0	1.8e ±0.0
4.0	113.4f ±0.4	1.4f ±0.0	82.7f ±7.1	1.0f ±0.1

$LSD_{.05}$ of differences among pH at a fixed fortification for calcium in casein pellet = 42.0.
$LSD_{.05}$ of differences among pH at a fixed fortification for calcium in protein of casein pellet = .61.
Values in the same column with the same letter are not significantly different.

TABLE 18. Calcium content of serum and of serum protein in iron-fortified milk and control at different pH.

	Iron fortified milk		Contol	
pH	Ca in serum (μg)	Ca in serum protein (μg/mg)	Ca in serum (μg)	Ca in serum protein (μg/mg)
6.7	1359.2a ±26.3	43.9a ±0.8	1440.4a ±20.8	40.9a ±0.6
6.2	1603.8ab±14.5	51.7ab±0.5	1837.9b ±41.8	52.9b ±1.2
5.8	1850.5b ±18.1	59.3b ±0.6	2157.0c ±162.1	62.6c ±4.7
5.3	2678.4c ±12.3	81.1c ±0.4	3079.3d ±293.4	95.9d ±9.1
4.5	3487.5d ±28.0	137.7d ±1.1	3528.1e ±10.1	138.5e ±0.4
4.0	3505.5d ±14.3	142.1d ±0.6	3571.4e ±106.4	143.3e ±4.3

$LSD_{.05}$ of differences among pH at a fixed fortification for calcium in serum = 252.3.
$LSD_{.05}$ of differences among pH at a fixed fortification for calcium in serum protein = 8.0.
Values in the same column with the same letter are not significantly different.

TABLE 19. Phosphorus content of pellets and of pellets protein in iron-fortified milk and control at different pH.

	Iron fortified milk		Contol	
pH	P in casn. pellet (μg)	P in protein of casn. pellet (μg/mg)	P in casn. pellet (μg)	P in protein of casn. pellet (μg/mg)
6.7	1542.5a ±0.7	22.7a ±0.0	1490.5a ±10.6	20.8a ±0.1
6.2	1450.0b ±11.3	20.9b ±0.2	1292.5b ±26.2	19.2b ±0.4
5.8	1298.7c ±1.8	18.7c ±0.0	1094.5c ±10.6	16.1c ±0.2
5.3	934.7d ±6.0	13.4d ±0.1	785.5d ±21.9	11.6d ±0.3
4.5	687.5e ±9.2	8.2e ±0.1	692.8e ±62.6	8.1e ±0.7
4.0	673.7e ±13.8	8.1e ±0.2	623.8e ±85.2	7.3e ±1.0

$LSD_{.05}$ of differences among pH at a fixed fortification for phosphorus in casein pellet = 80.3.
$LSD_{.05}$ of differences among pH at a fixed fortification for phosphorus in protein of casein pellet = 1.0.
Values in the same column with the same letter are not significantly different.

TABLE 20. Phosphorus content of serum and of serum protein in iron-fortified milk and control at different pH.

pH	Iron fortified milk		Contol	
	P in serum (μg)	P in serum protein (μg/mg)	P in serum (μg)	P in serum protein (μg/mg)
6.7	1441.2a ±1.1	46.6a ±0.0	1639.3a ±40.5	46.5a ±1.1
6.2	1567.7a ±13.8	50.5a ±0.4	1911.5b ±26.3	54.9b ±0.8
5.8	1649.9a ±11.8	52.8a ±0.4	2002.9b ±123.6	58.2b ±3.6
5.3	2074.7b ±1.4	62.8b ±0.0	2323.7c ±251.6	72.4c ±7.8
4.5	2305.1c ±31.7	91.0c ±1.2	2223.9c ±7.0	87.3d ±0.3
4.0	2216.7bc±5.9	89.8c ±0.2	2188.0c ±111.3	87.8d ±4.5

$LSD_{.05}$ of differences among pH at a fixed fortification for phosphorus in serum = 216.6.
$LSD_{.05}$ of differences among pH at a fixed fortification for phosphorus in serum protein = 7.0.
Values in the same column with the same letter are not significantly different.

APPENDIX B

TABLE 21. Analysis of variance for *S. thermophilus* counts of nonfat yogurt inoculated with 10^3 and 10^5 CFU/ml of *E. coli* and *P. fluorescens* over 14 d of storage at 4°C.

Source	df	MS	F	*P*
Replication	1	.01568		
Fortification (F)	1	.01094	1.313	.2814
Level (L)	4	.03233	3.881	.0422
F x L	4	.01292	1.551	.2679
Error (a)	9	.00833		
Day (D)	2	.03656	1.903	.3444
Error (b)	2	.01921		
F x D	2	.01238	1.383	.2762
L x D	8	.00244	.273	.9667
F x L x D	8	.00217	.242	.9767
Error (c)	18	.00895		
Total	59			

TABLE 22. Analysis of variance for *L. delbruekii* ssp. *bulgaricus* counts of nonfat yogurt inoculated with 10^3 and 10^5 CFU/ml of *E. coli* and *P. fluorescens* over 14 d of storage at 4°C.

Source	df	MS	F	*P*
Replication	1	.029040		
Fortification (F)	1	.000167	.012	.9151
Level (L)	4	.038144	2.700	.0994
F x L	4	.005454	.386	.8135
Error (a)	9	.014128		
Day (D)	2	.056047	1.761	.3621
Error (b)	2	.031820		
F x D	2	.000187	.021	.9792
L x D	8	.005534	.615	.7542
F x L x D	8	.004612	.513	.8312
Error (c)	18	.008992		
Total	59			

TABLE 23. Analysis of variance for pH values of nonfat yogurt inoculated with 10^3 and 10^5 CFU/ml of *E. coli* and *P. fluorescens* over 14 d of storage at 4°C.

Source	df	MS	F	*P*
Replication	1	.0164672		
Fortification (F)	1	.0213571	8.043	.0195
Level (L)	4	.0016229	.611	.6652
F x L	4	.0004588	.173	.9467
Error (a)	9	.0026553		
Day (D)	2	.2025857	60.238	.0163
Error (b)	2	.0033631		
F x D	2	.0003740	1.115	.3495
L x D	8	.0004639	1.383	.2687
F x L x D	8	.0005389	1.607	.1916
Error (c)	18	.0003354		
Total	59			

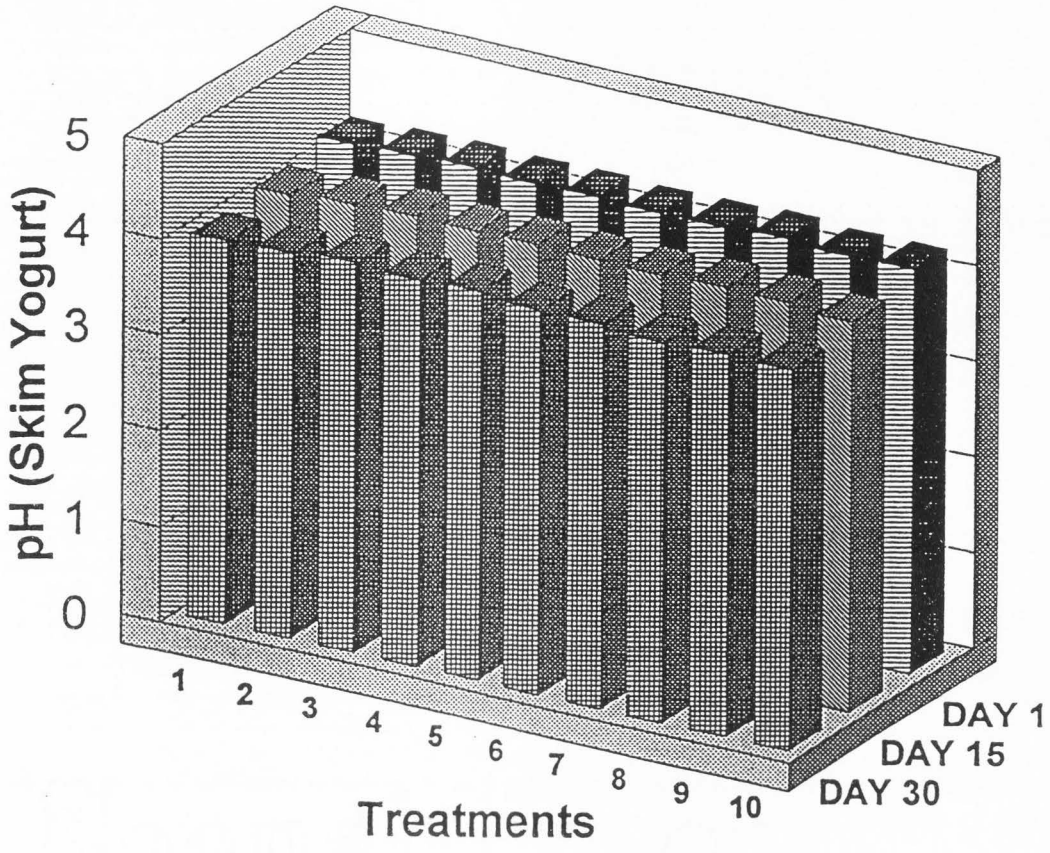

Figure 20. Comparison of acid production in regular and iron-fortified skim yogurt during 30 d of storage. Treatments; 1 = control, 2-4 = yogurt fortified with $FeCl_3$ at the rate of 10, 20, and 40 mg iron/kg yogurt respectively, 5-7 = yogurt fortified with iron-casein complex at the rate of 10, 20, and 40 mg iron/kg yogurt respectively, 8-10 = yogurt fortified with iron-whey protein complex at the rate of 10, 20 and 40 mg iron/kg yogurt.

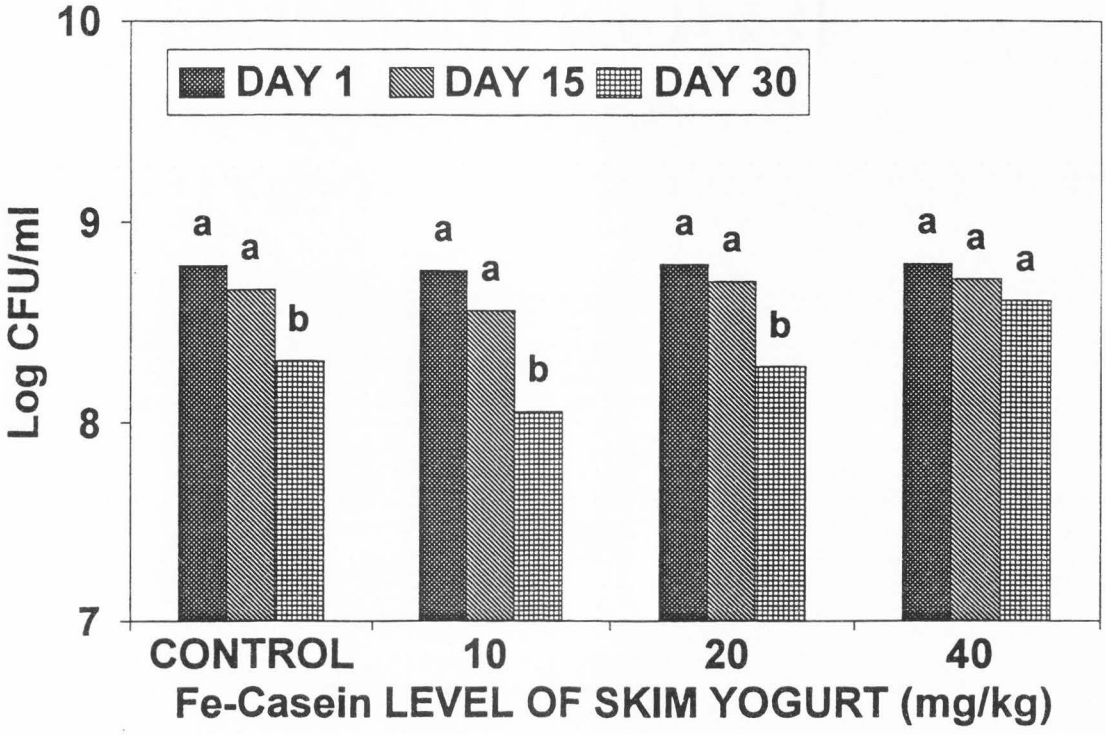

Figure 21. Mean survival of *L. delbruekii* ssp. *bulgaricus* in skim yogurt fortified with iron-casein complex (0, 10, 20, and 40 mg iron/kg yogurt) over 30 d of storage. $LSD_{.05}$ for comparing days of storage for fixed iron source at a fixed level = .34. Values for the same iron level with the same superscript letter are not significantly different.

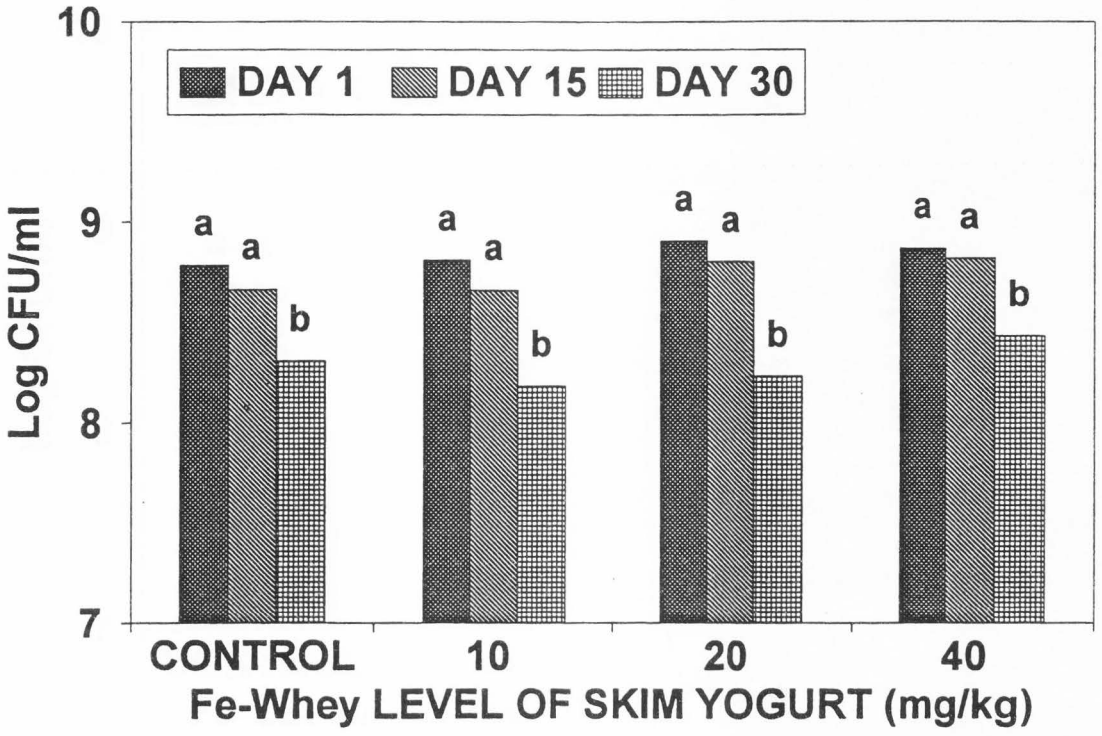

Figure 22. Mean survival of *L. delbruekii* ssp. *bulgaricus* in skim yogurt fortified with iron-whey protein complex (0, 10, 20, and 40 mg iron/kg yogurt) over 30 d of storage. $LSD_{.05}$ for comparing days of storage for fixed iron source at a fixed level = .34. Values for the same iron level with the same superscript letter are not significantly different.

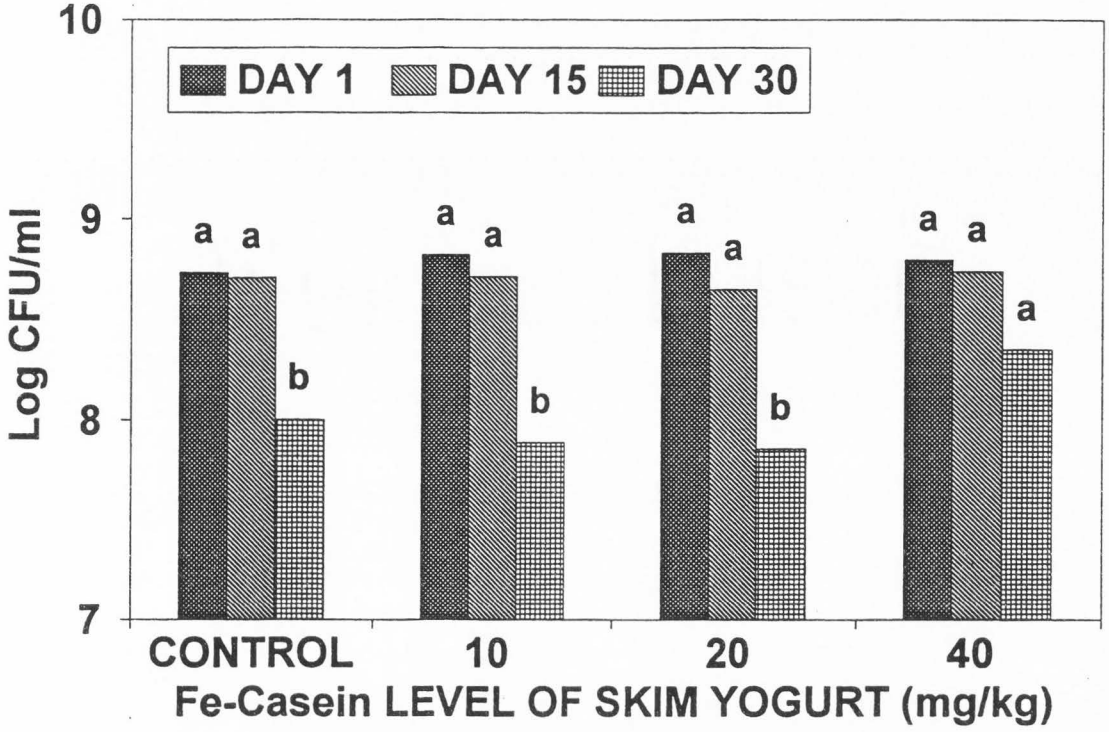

Figure 23. Mean survival of S. *thermophilus* in skim yogurt fortified with iron-casein complex (0, 10, 20, and 40 mg iron/kg yogurt) over 30 d of storage. Values for the same iron level with the same superscript letter are not significantly different ($LSD_{.05}$ = .49).

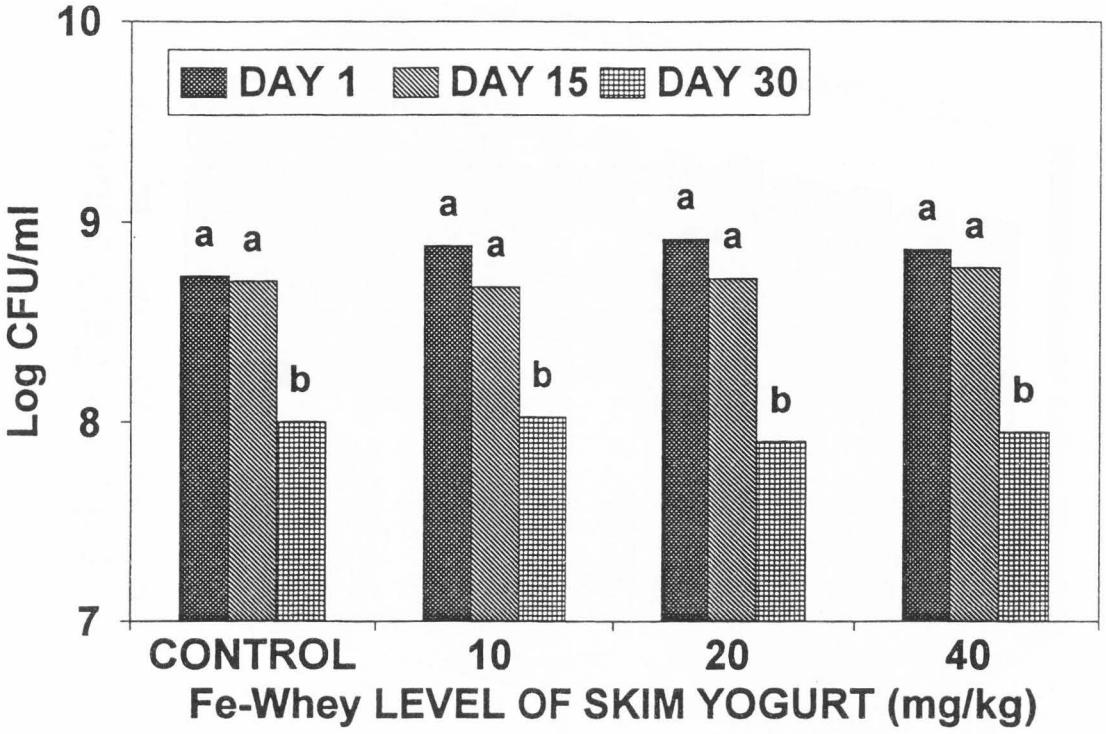

Figure 24. Mean survival of S. *thermophilus* in skim yogurt fortified with iron-whey protein complex (0, 10, 20, and 40 mg iron/kg yogurt) over 30 d of storage. $LSD_{.05}$ for comparing days of storage for fixed iron source at a fixed level = .49. Values for the same iron level with the same superscript letter are not significantly different.

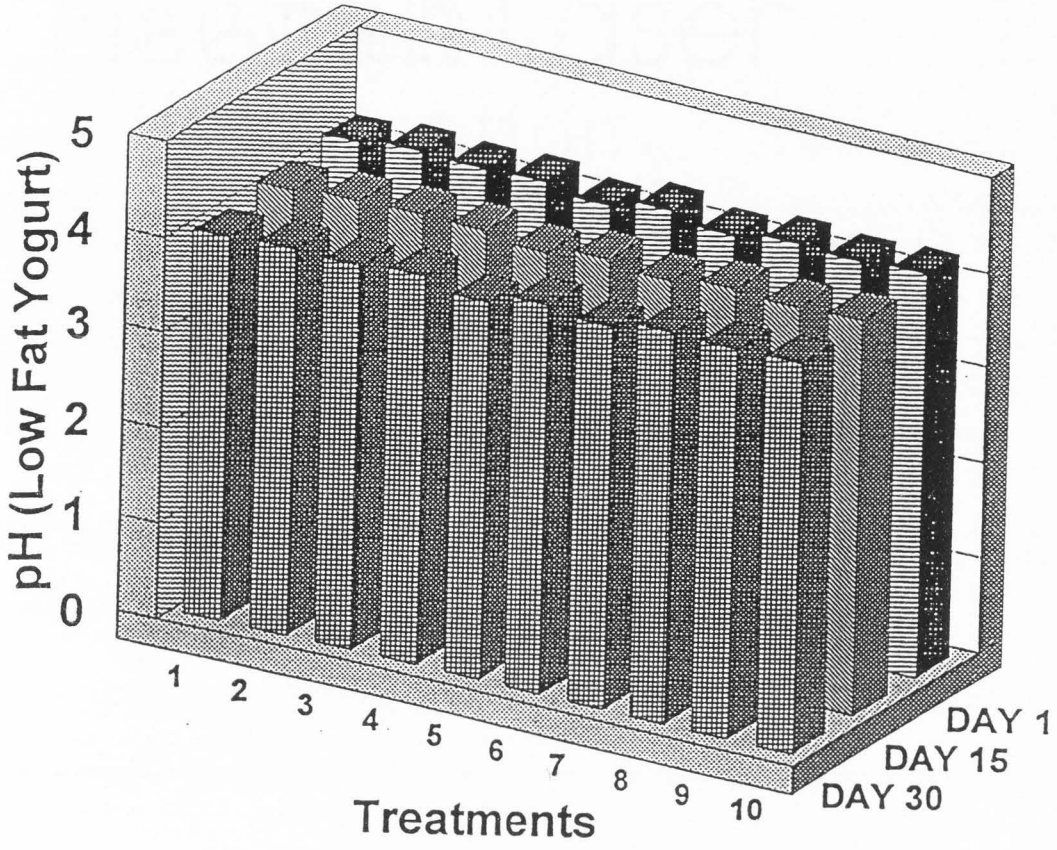

Figure 25. Comparison of acid production in regular and iron fortified low fat (2%) yogurt during 30 d of storage. Treatments; 1 = control, 2-4 = yogurt fortified with $FeCl_3$ at the rate of 10, 20, and 40 mg iron/kg yogurt, respectively, 5-7 = yogurt fortified with iron-casein complex at the rate of 10, 20, and 40 mg iron/kg yogurt, respectively, 8-10 = yogurt fortified with iron-whey protein complex at the rate of 10, 20, and 40 mg iron/kg yogurt.

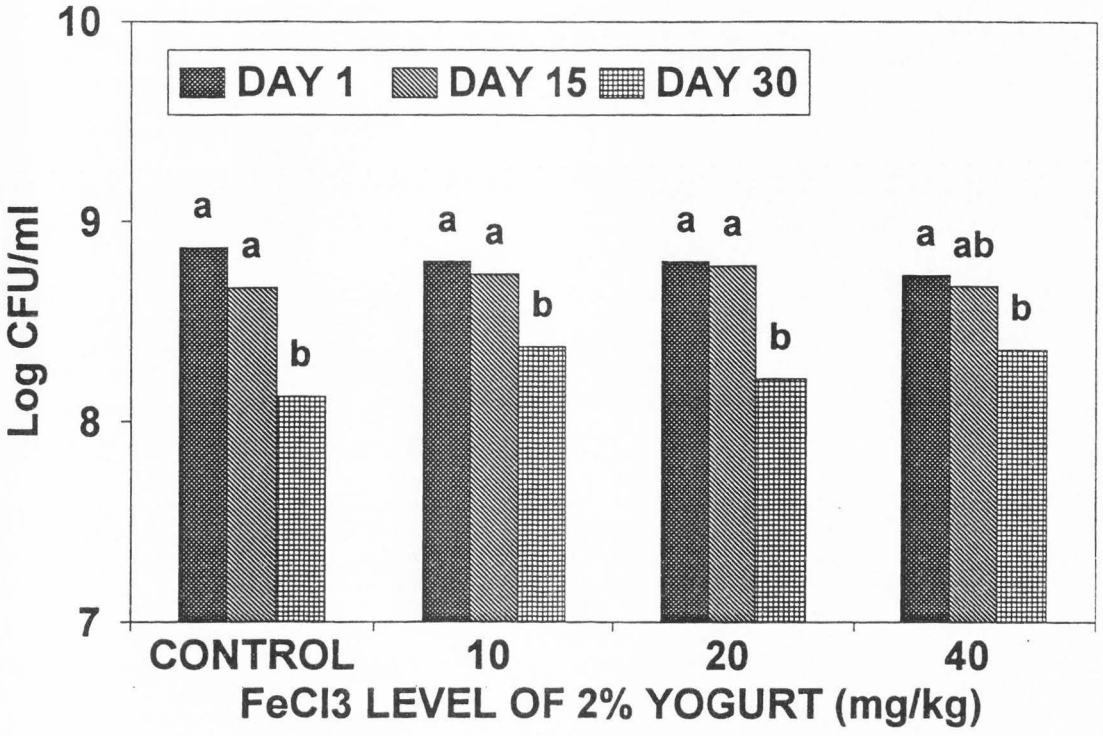

Figure 26. Mean survival of *L. delbruekii* ssp. *bulgaricus* in low fat (2%) yogurt fortified with $FeCl_3$ (0, 10, 20, and 40 mg iron/kg yogurt) over 30 d of storage. $LSD_{.05}$ for comparing days of storage for fixed iron source at a fixed level = .34. Values for the same iron level with the same superscript letter are not significantly different.

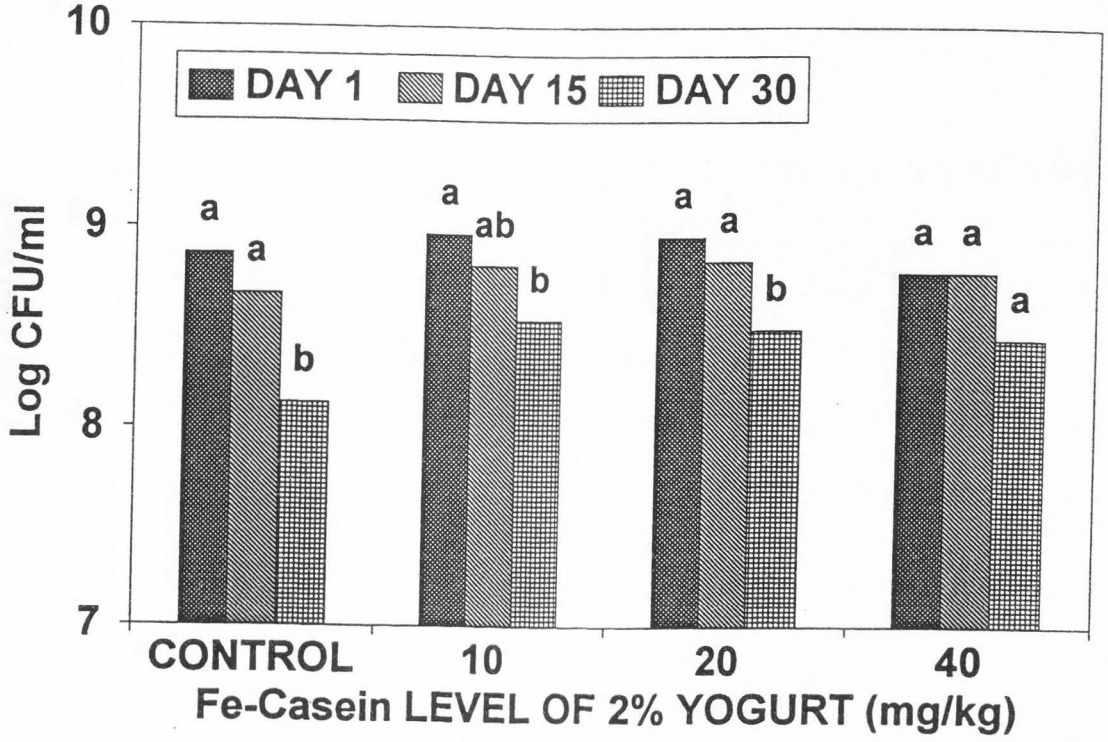

Figure 27. Mean survival of *L. delbruekii* ssp. *bulgaricus* in low fat (2%) yogurt fortified with iron-casein complex (0, 10, 20, and 40 mg iron/kg yogurt) over 30 d of storage. $LSD_{.05}$ for comparing days of storage for fixed iron source at a fixed level = .34. Values for the same iron level with the same superscript letter are not significantly different.

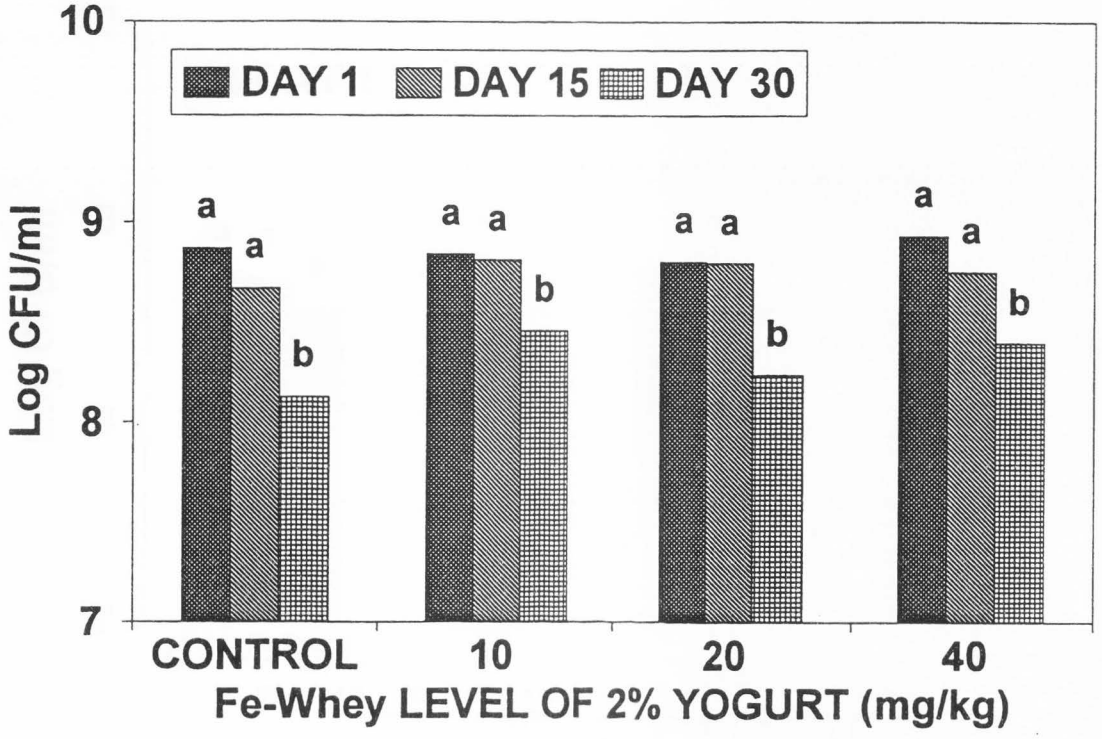

Figure 28. Mean survival of *L. delbruekii* ssp. *bulgaricus* in low fat (2%) yogurt fortified with iron-whey protein complex (0, 10, 20, and 40 mg iron/kg yogurt) over 30 d of storage. $LSD_{.05}$ for comparing days of storage for fixed iron source at a fixed level = .34. Values for the same iron level with the same superscript letter are not significantly different.

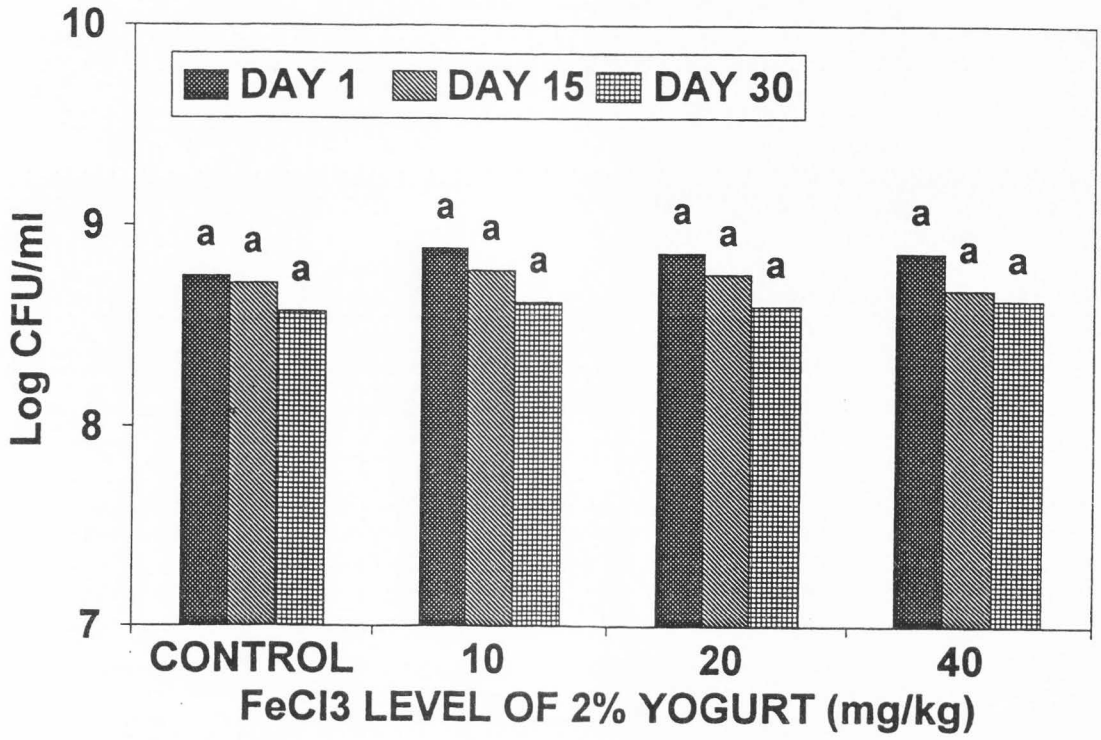

Figure 29. Mean survival of S. *thermophilus* in low fat (2%) yogurt fortified with $FeCl_3$ (0, 10, 20, and 40 mg iron/kg yogurt) over 30 d of storage. $LSD_{.05}$ for comparing days of storage for fixed iron source at a fixed level = .49. Values for the same iron level with the same superscript letter are not significantly different.

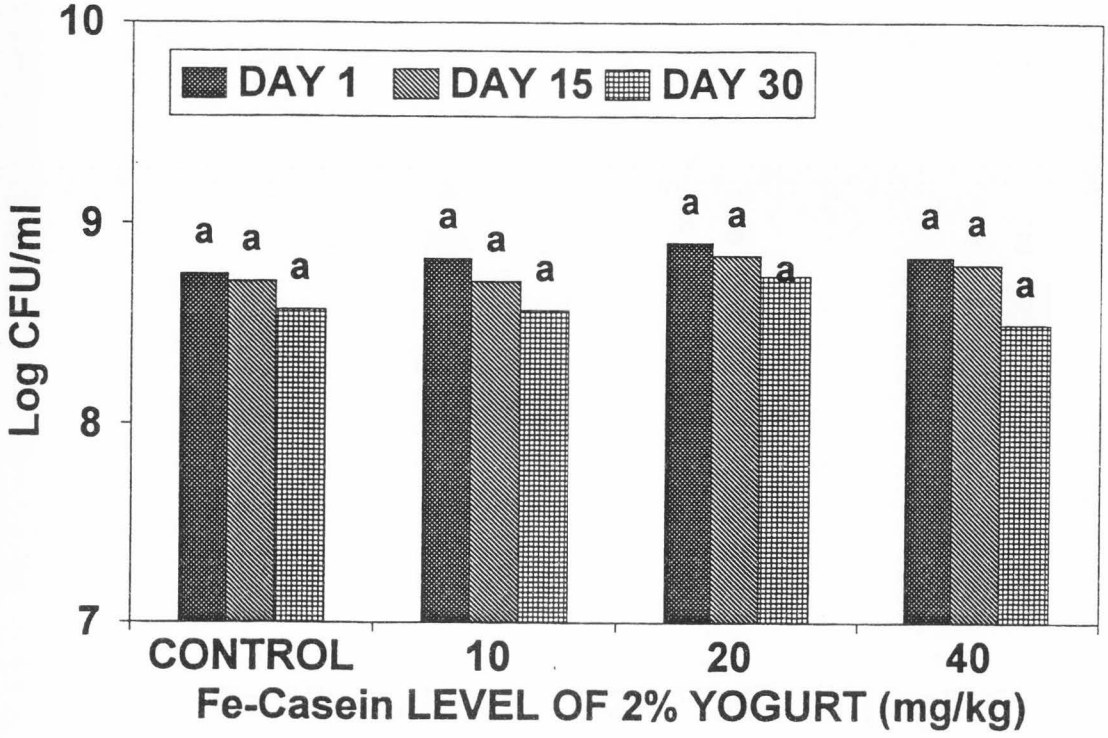

Figure 30. Mean survival of S. *thermophilus* in low fat (2%) yogurt fortified with iron-casein complex (0, 10, 20, and 40 mg iron/kg yogurt) over 30 d of storage. $LSD_{.05}$ for comparing days of storage for fixed iron source at a fixed level = .49. Values for the same iron level with the same superscript letter are not significantly different.

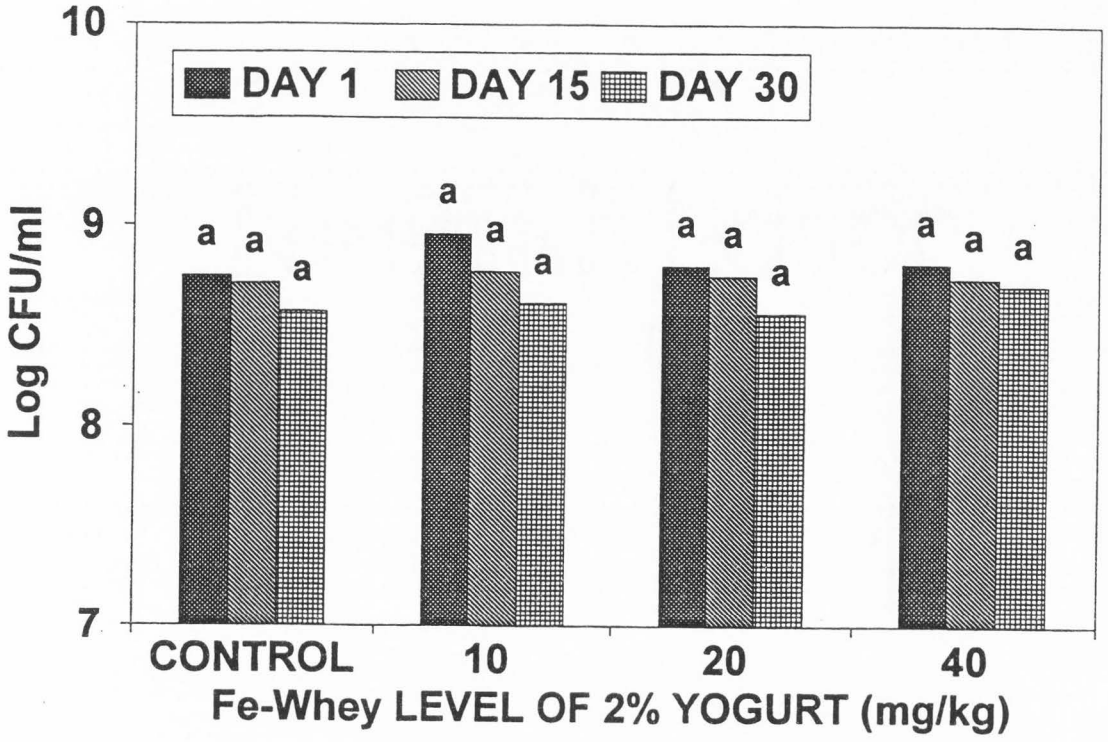

Figure 31. Mean survival of S. *thermophilus* in low fat (2%) yogurt fortified with iron-whey protein complex (0, 10, 20, and 40 mg iron/kg yogurt) over 30 d of storage. $LSD_{.05}$ for comparing days of storage for fixed iron source at a fixed level = .49. Values for the same iron level with the same superscript letter are not significantly different.

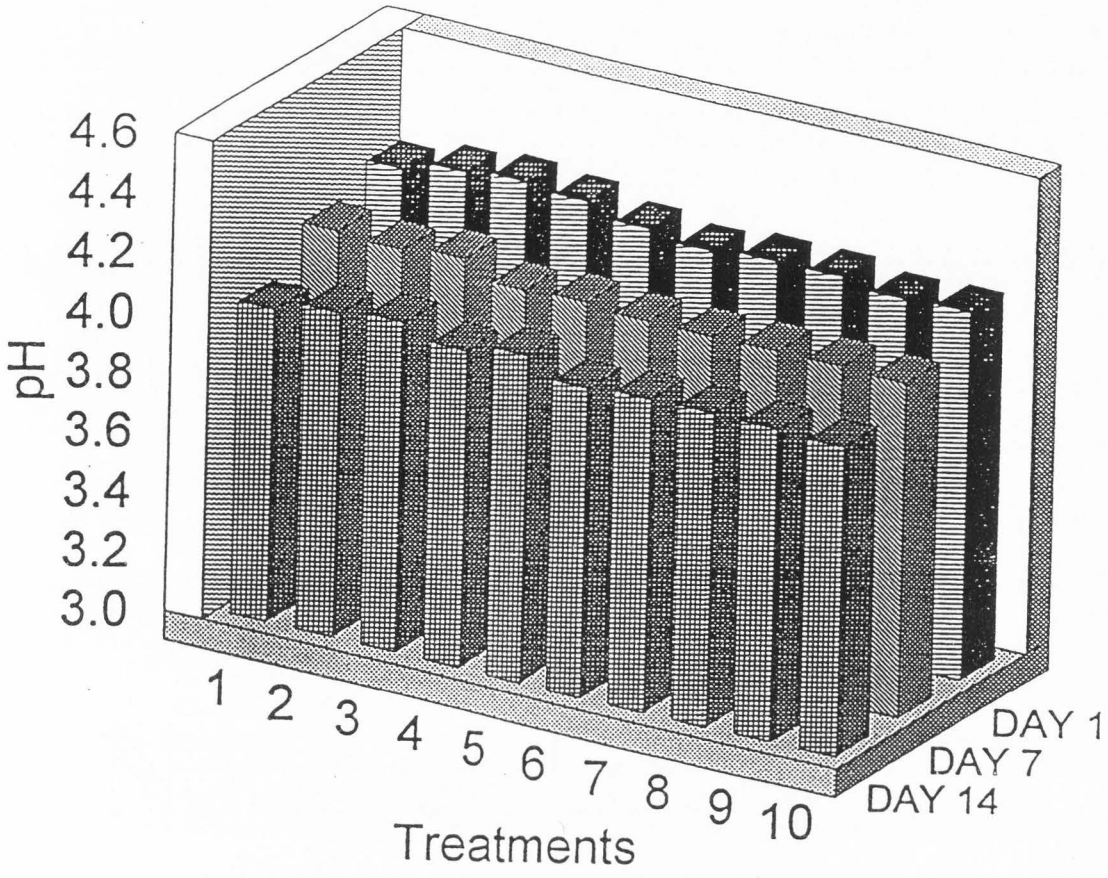

Figure 32. Comparison of acid production in regular and iron fortified skim yogurt that was inoculated with various levels of *E. coli* and *P. fluorescens* over 14 d of storage. Treatments 1-5 are yogurts fortified with 20 mg iron/kg yogurt and 6-10 are unfortified yogurts. Treatments 1 and 6 are controls, 2 and 7 are inoculated with *E. coli* at the rate of 10^3 CFU/ml of yogurt, 3 and 8 are inoculated with *E. coli* at the rate of 10^5 CFU/ml of yogurt, 4 and 9 are inoculated with *P. fluorescens* at the rate of 10^3 CFU/ml of yogurt, 5 and 10 are inoculated with *P. fluorescens* at the rate of 10^5 CFU/ml of yogurt ($LSD_{.05}$ for comparing between days means for day = .079).

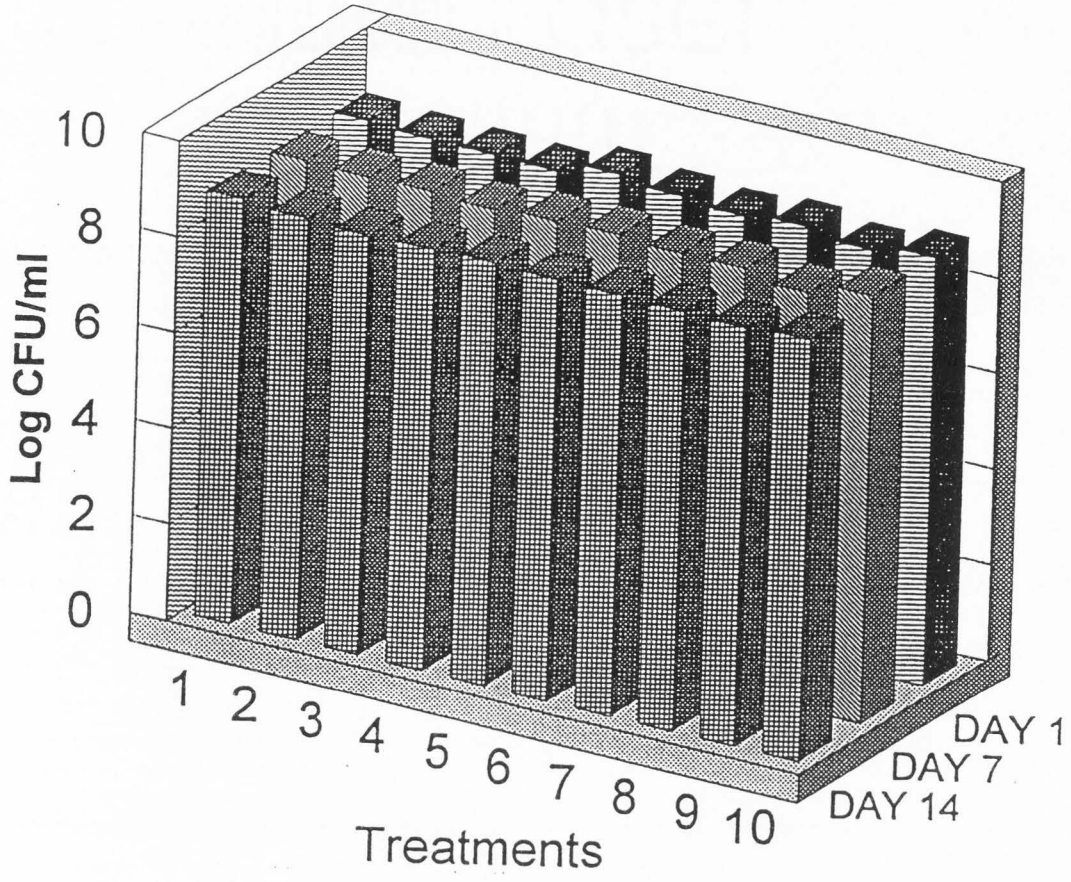

Figure 33. Mean survival of *L. delbruekii* ssp. *bulgaricus* in regular and iron fortified skim yogurt that was inoculated with various levels of *E. coli* and *P. fluorescens* over 14 d of storage. Treatments 1-5 are yogurts fortified with 20 mg iron/kg yogurt and 6-10 are unfortified yogurts. Treatments 1 and 6 are controls, 2 and 7 are inoculated with *E. coli* at the rate of 10^3 CFU/ml of yogurt, 3 and 8 are inoculated with *E. coli* at the rate of 10^5 CFU/ml of yogurt, 4 and 9 are inoculated with *P. fluorescens* at the rate of 10^3 CFU/ml of yogurt, 5 and 10 are inoculated with *P. fluorescens* at the rate of 10^5 CFU/ml of yogurt.

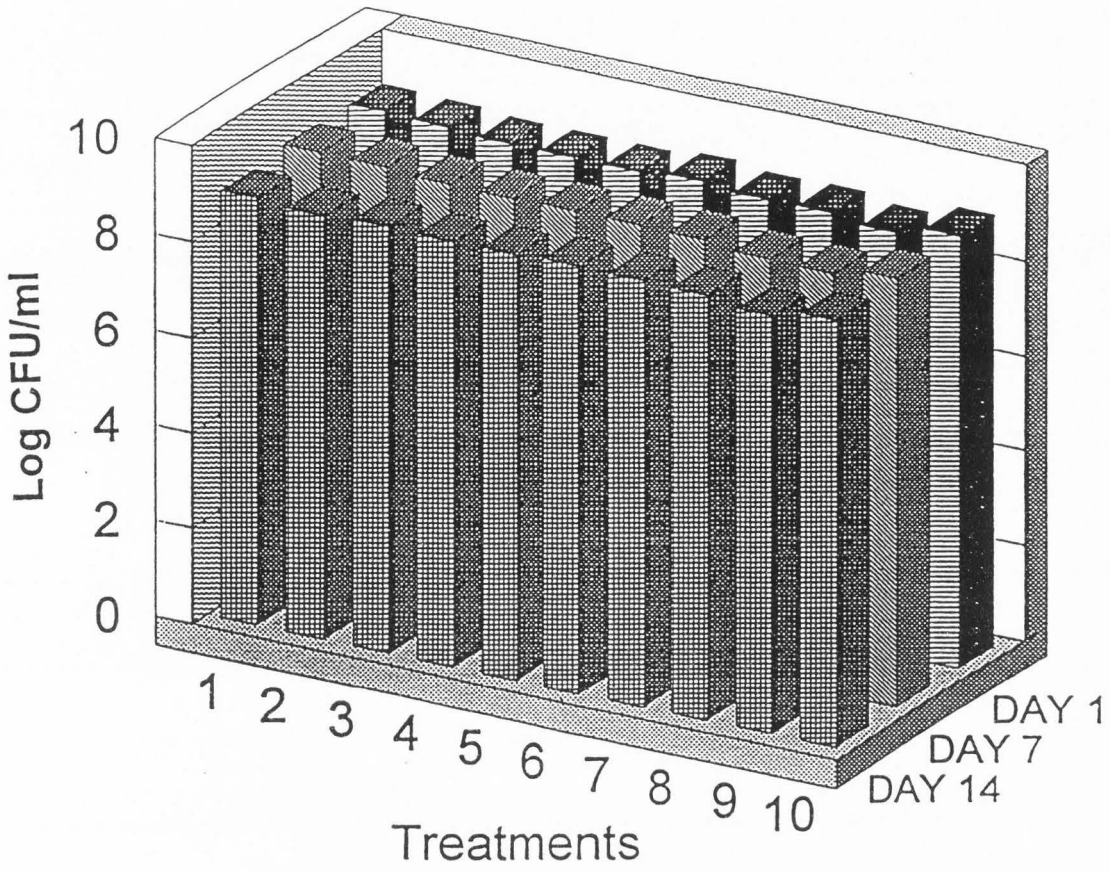

Figure 34. Mean survival of *s. thermophilus* in regular and iron fortified skim yogurt that was inoculated with various levels of *E. coli* and *P. fluorescens* over 14 d of storage. Treatments 1-5 are yogurts fortified with 20 mg iron/kg yogurt and 6-10 are unfortified yogurts. Treatments 1 and 6 are controls, 2 and 7 are inoculated with *E. coli* at the rate of 10^3 CFU/ml of yogurt, 3 and 8 are inoculated with *E. coli* at the rate of 10^5 CFU/ml of yogurt, 4 and 9 are inoculated with *P. fluorescens* at the rate of 10^3 CFU/ml of yogurt, 5 and 10 are inoculated with *P. fluorescens* at the rate of 10^5 CFU/ml of yogurt ($LSD_{.05}$ for comparing between treatments means for level = .084).

APPENDIX C

TABLE 24. Analysis of variance of trained panelists for bitter flavor in iron-fortified yogurt.

Source	df	MS	F	*P*
Judge	10	1.142	4.034	.0000
Fat (F)	1	.970	3.424	.0659
Source (S)	2	.869	3.067	.0490
Level (L)	2	.338	1.195	.3052
F x S	2	.242	.856	.4266
F x L	2	.247	.874	.4191
S x L	4	.215	.758	.5540
F x S x L	4	.732	2.585	.0388
Error (a)	170	.283		
Day (D)	2	.289	.633	.5413
Error (b)	20	.454		
F x D	2	1.197	4.082	.0177
S x D	4	.747	2.549	.0391
L x D	4	.300	1.025	.3943
F x S x D	4	.258	.878	.4772
F x L x D	4	.179	.611	.6549
S x L x D	8	.192	.654	.7318
F x S x L x D	8	.179	.611	.7686
Error (c)	340	.293		
Total	593			

$LSD_{.05}$ for comparing main effect means for source = .107.

TABLE 25. Analysis of variance of trained panelists for oxidized flavor in iron-fortified yogurt.

Source	df	MS	F	*P*
Judge	10	15.894	13.059	.0000
Fat (F)	1	1.052	.864	.3539
Source (S)	2	4.136	3.398	.0357
Level (L)	2	1.914	1.573	.2104
F x S	2	.678	.557	.5739
F x L	2	.668	.549	.5785
S x L	4	.982	.807	.5222
F x S x L	4	1.711	1.406	.2340
Error (a)	170	1.217		
Day (D)	2	9.651	1.159	.3340
Error (b)	20	8.324		
F x D	2	3.325	2.295	.1023
S x D	4	3.871	2.671	.0321
L x D	4	1.149	.793	.5303
F x S x D	4	1.792	1.237	.2948
F x L x D	4	1.039	.717	.5807
S x L x D	8	1.323	.913	.5056
F x S x L x D	8	1.537	1.061	.3901
Error (c)	340	1.449		
Total	593			

TABLE 26. Analysis of variance of trained panelists for off-flavor in iron-fortified yogurt.

Source	df	MS	F	*P*
Judge	10	8.996	8.602	.0000
Fat (F)	1	3.259	3.117	.0792
Source (S)	2	.163	.156	.8556
Level (L)	2	.188	.180	.8354
F x S	2	3.901	3.730	.0260
F x L	2	.492	.470	.6258
S x L	4	.923	.883	.4754
F x S x L	4	.913	.873	.4814
Error (a)	170	1.046		
Day (D)	2	14.269	4.189	.0302
Error (b)	20	3.406		
F x D	2	.087	.106	.8994
S x D	4	1.004	1.220	.3020
L x D	4	.128	.155	.9606
F x S x D	4	1.524	1.852	.1185
F x L x D	4	.956	1.161	.3278
S x L x D	8	.465	.565	.8064
F x S x L x D	8	.298	.362	.9399
Error (c)	340	.823		
Total	593			

TABLE 27. Analysis of variance of trained panelists for metallic flavor in iron-fortified yogurt.

Source	df	MS	F	*P*
Judge	10	17.353	12.585	.0000
Fat (F)	1	4.209	3.052	.0824
Source (S)	2	.924	.670	.5130
Level (L)	2	2.399	1.740	.1786
F x S	2	6.264	4.543	.0119
F x L	2	.012	.008	.9920
S x L	4	.725	.526	.7167
F x S x L	4	1.302	.944	.4399
Error (a)	170	1.379		
Day (D)	2	.853	.120	.8875
Error (b)	20	7.094		
F x D	2	3.557	2.991	.0515
S x D	4	3.376	2.839	.0244
L x D	4	.063	.053	.9947
F x S x D	4	.514	.432	.7855
F x L x D	4	.171	.144	.9655
S x L x D	8	1.071	.900	.5165
F x S x L x D	8	1.067	.897	.5190
Error (c)	340	1.189		
Total	593			

TABLE 28. Analysis of variance of trained panelists for acid flavor in iron-fortified yogurt.

Source	df	MS	F	*P*
Judge	10	10.176	7.521	.0000
Fat (F)	1	7.113	5.257	.0231
Source (S)	2	3.618	2.674	.0719
Level (L)	2	.577	.426	.6538
F x S	2	15.921	11.767	.0000
F x L	2	1.042	.770	.4646
S x L	4	1.072	.792	.5318
F x S x L	4	.532	.393	.8134
Error (a)	170	1.353		
Day (D)	2	446.689	45.317	.0000
Error (b)	20	9.857		
F x D	2	48.961	22.295	.0000
S x D	4	.661	.301	.8772
L x D	4	.461	.210	.9328
F x S x D	4	22.989	10.468	.0000
F x L x D	4	.269	.122	.9745
S x L x D	8	.403	.183	.9931
F x S x L x D	8	1.706	.777	.6233
Error (c)	340	2.196		
Total	593			

$LSD_{.05}$ for comparing main effect means for day = .658.

TABLE 29. Analysis of variance of open sensory evaluation for appearance scores in iron-fortified yogurt.

Source	df	MS	F	*P*
Fat (F)	1	6.480	4.10	.043
Source (S)	2	.062	.04	.961
F x S	2	.427	.27	.764
Error	444	1.581		
Total	449			

$LSD_{.05}$ for comparing main effect means for fat level = 2.01.

TABLE 30. Analysis of variance of open sensory evaluation for mouth feel scores in iron-fortified yogurt.

Source	df	MS	F	*P*
Fat (F)	1	.889	.43	.511
Source (S)	2	1.349	.66	.520
F x S	2	.616	.30	.742
Error	444	2.058		
Total	449			

TABLE 31. Analysis of variance of open sensory evaluation for flavor scores in iron-fortified yogurt.

Source	df	MS	F	*P*
Fat (F)	1	.009	.00	.960
Source (S)	2	.347	.10	.908
F x S	2	1.742	.48	.616
Error	444	3.595		
Total	449			

TABLE 32. Analysis of variance of open sensory evaluation for overall scores in iron-fortified yogurt.

Source	df	MS	F	*P*
Fat (F)	1	1.389	.50	.482
Source (S)	2	.927	.33	.719
F x S	2	.616	.22	.803
Error	444	2.804		
Total	449			

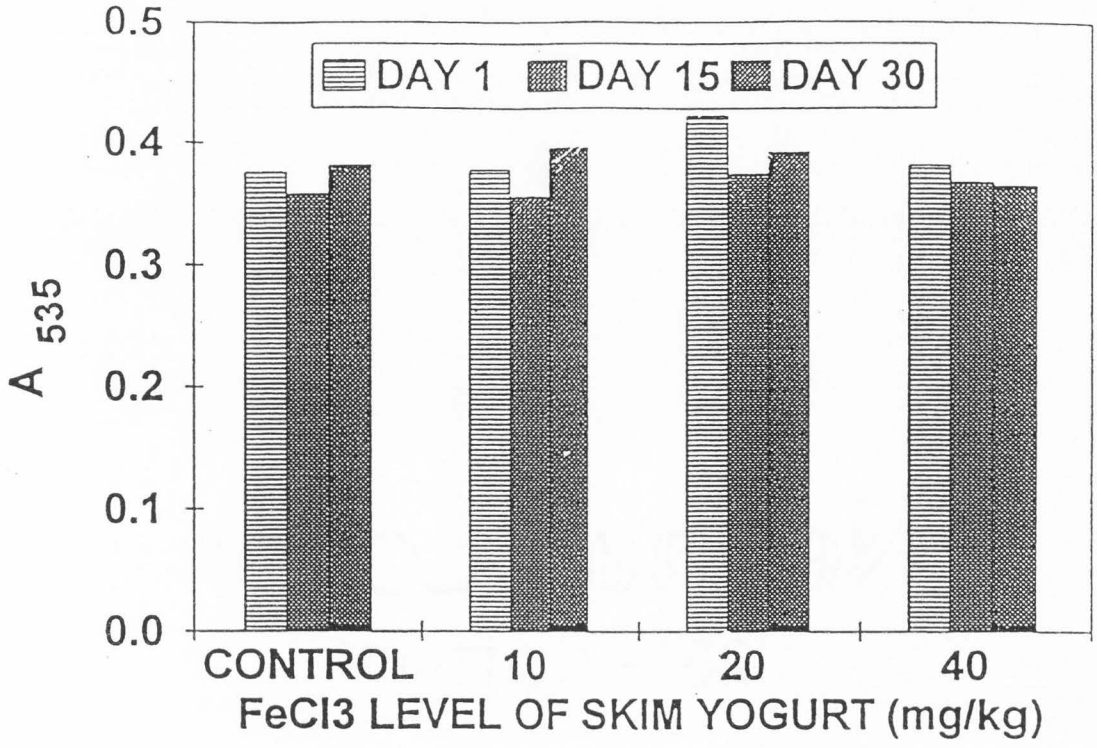

Figure 35. Comparison of chemical oxidation between unfortified yogurt and yogurts fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of $FeCl_3$ during storage.

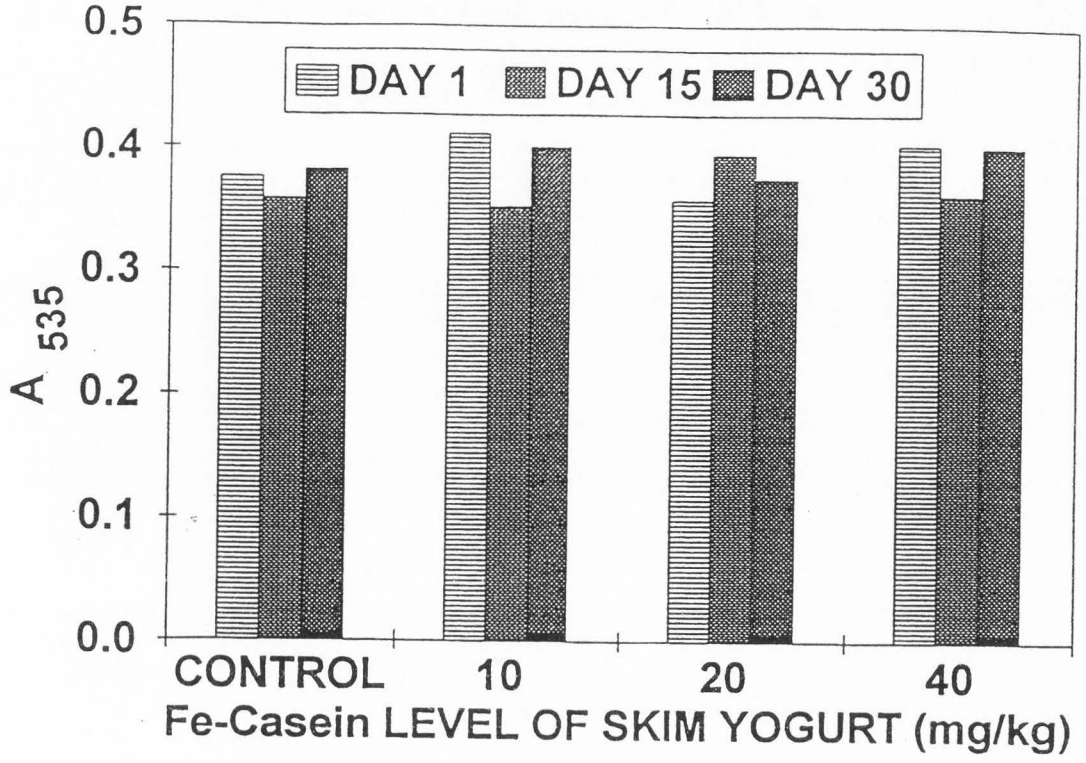

Figure 36. Comparison of chemical oxidation between unfortified yogurt and yogurts fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of iron-casein complex (Fe-CN) during storage.

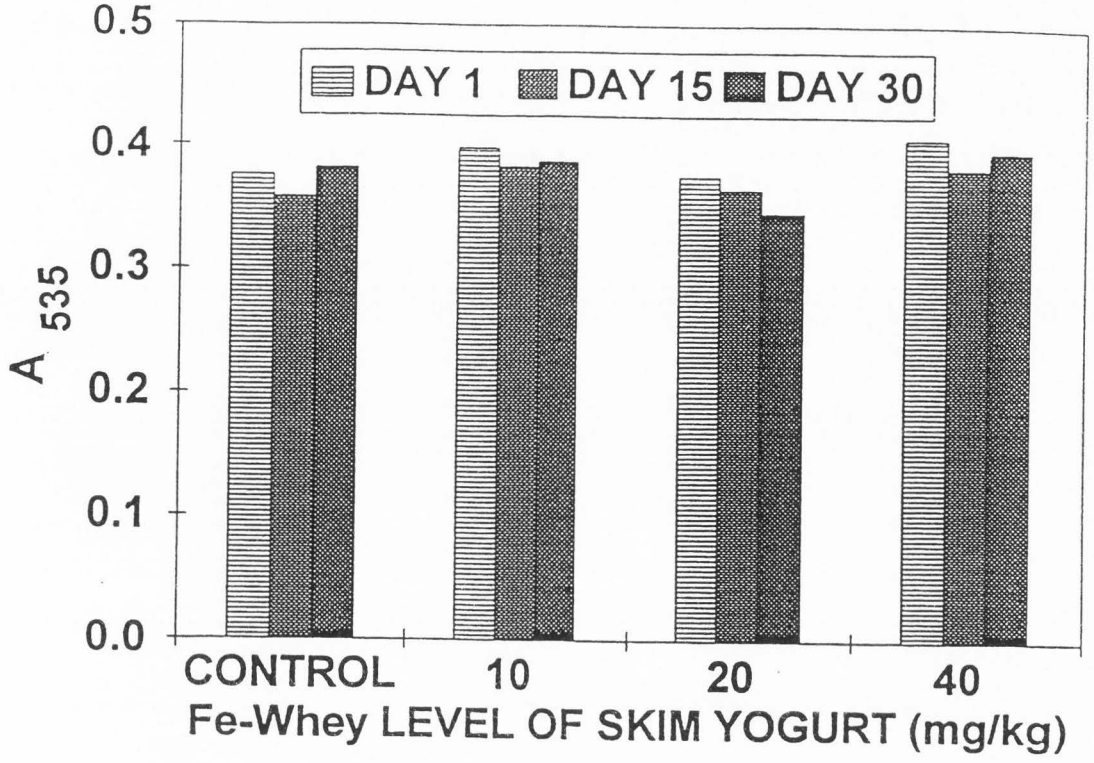

Figure 37. Comparison of chemical oxidation between unfortified yogurt and yogurts fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of iron-whey protein complex (Fe-WP) during storage.

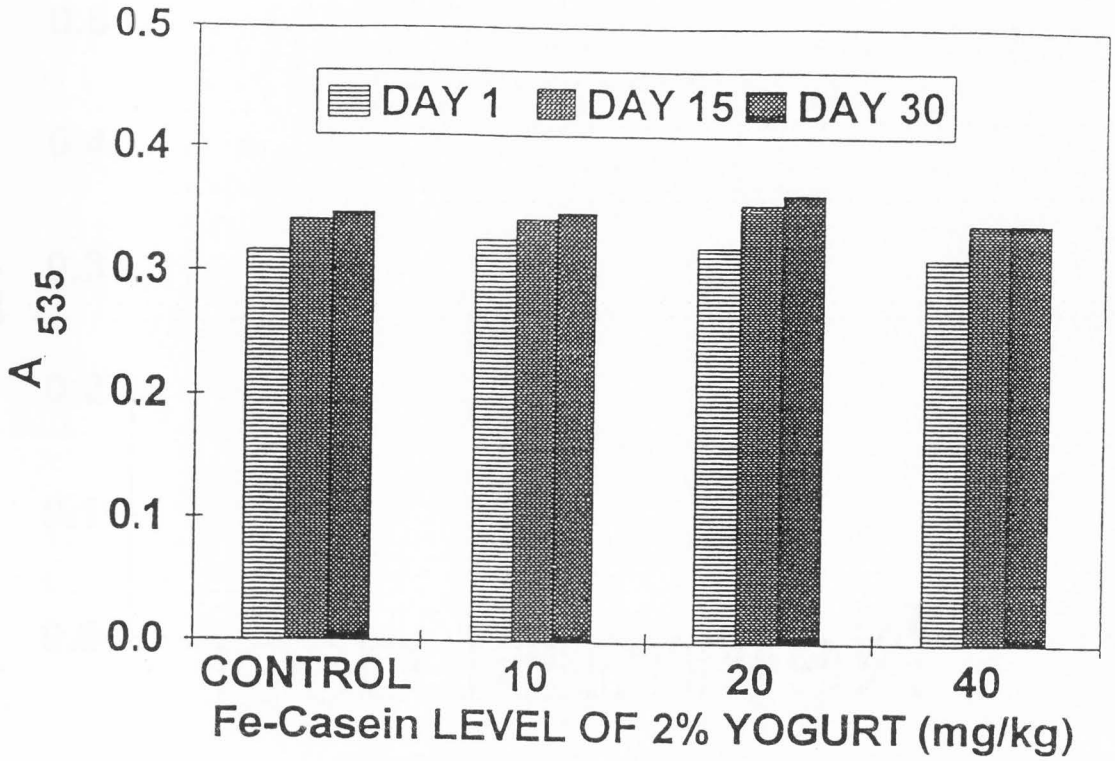

Figure 38. Comparison of chemical oxidation between unfortified yogurt and yogurts fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of iron-casein complex (Fe-CN) during storage.

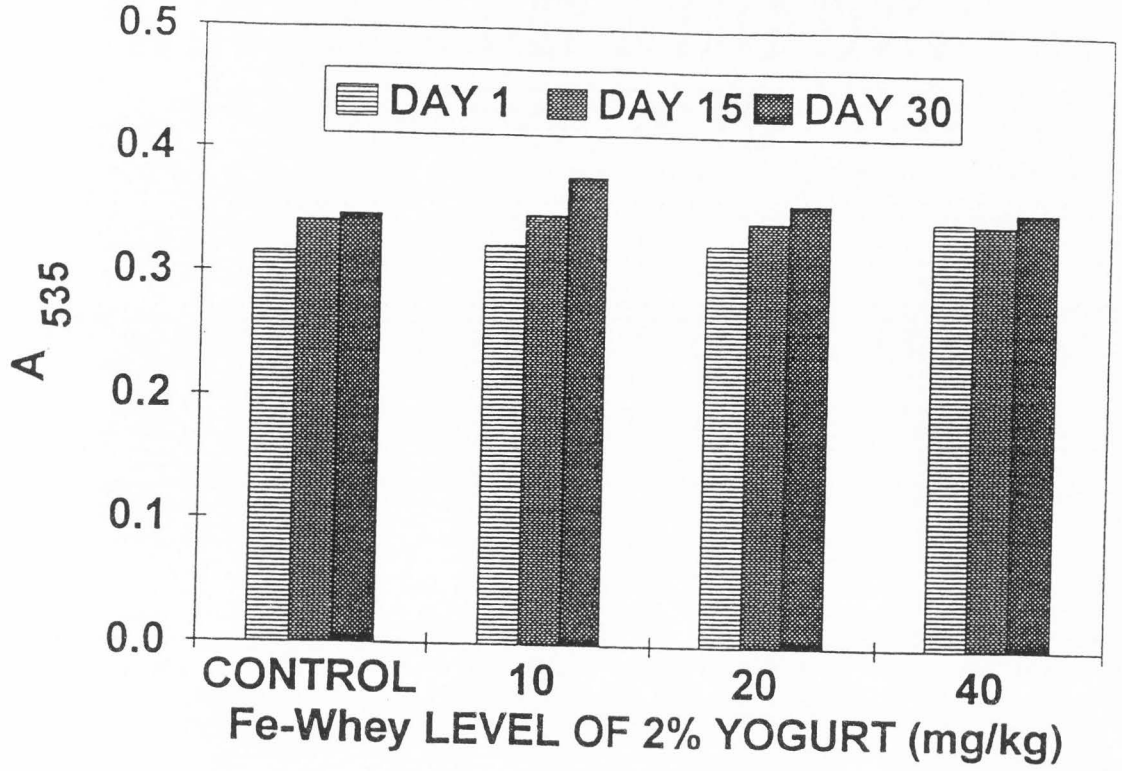

Figure 39. Comparison of chemical oxidation between unfortified yogurt and yogurts fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of iron-whey protein complex (Fe-WP) during storage.

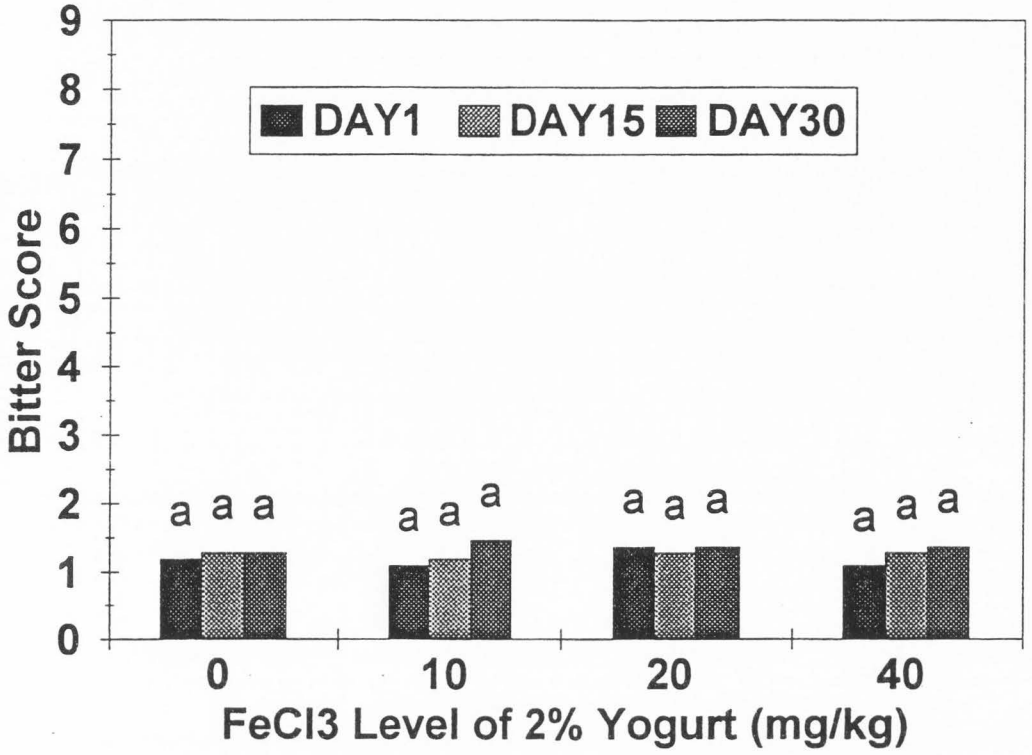

Figure 40. Bitterness scores of low fat (2%) unfortified yogurt and yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of $FeCl_3$ during storage. (Data points with the same letter are not significantly different.)

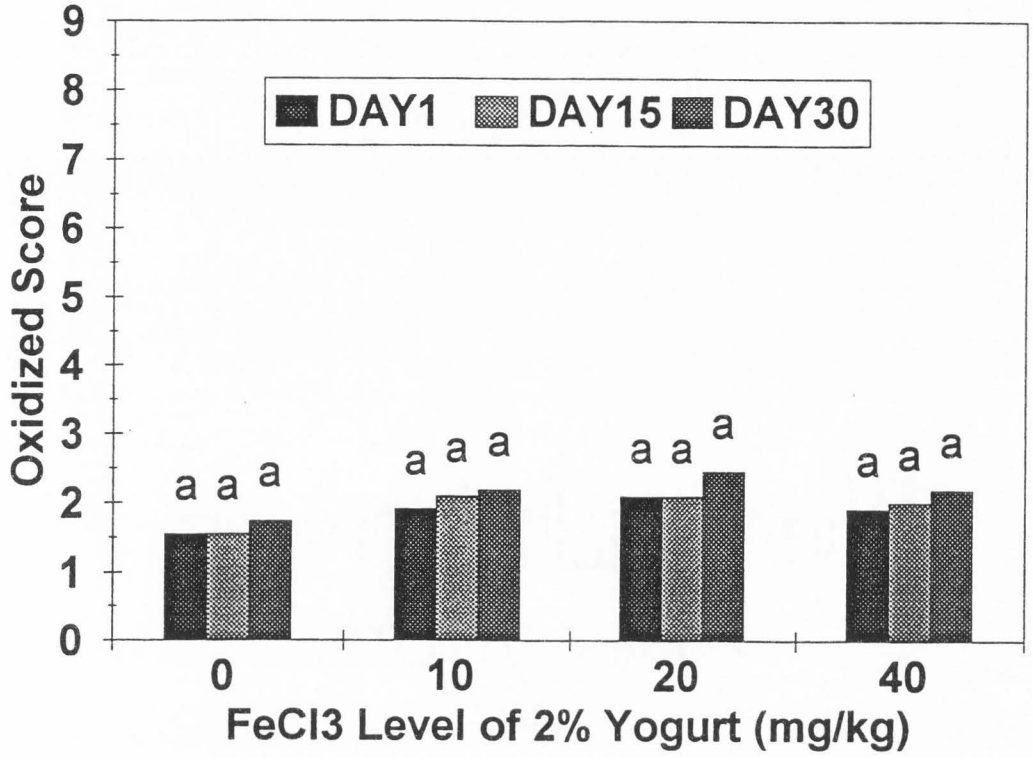

Figure 41. Oxidized flavor scores of low fat (2%) unfortified yogurt and yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of $FeCl_3$ during storage. (Data points with the same letter are not significantly different.)

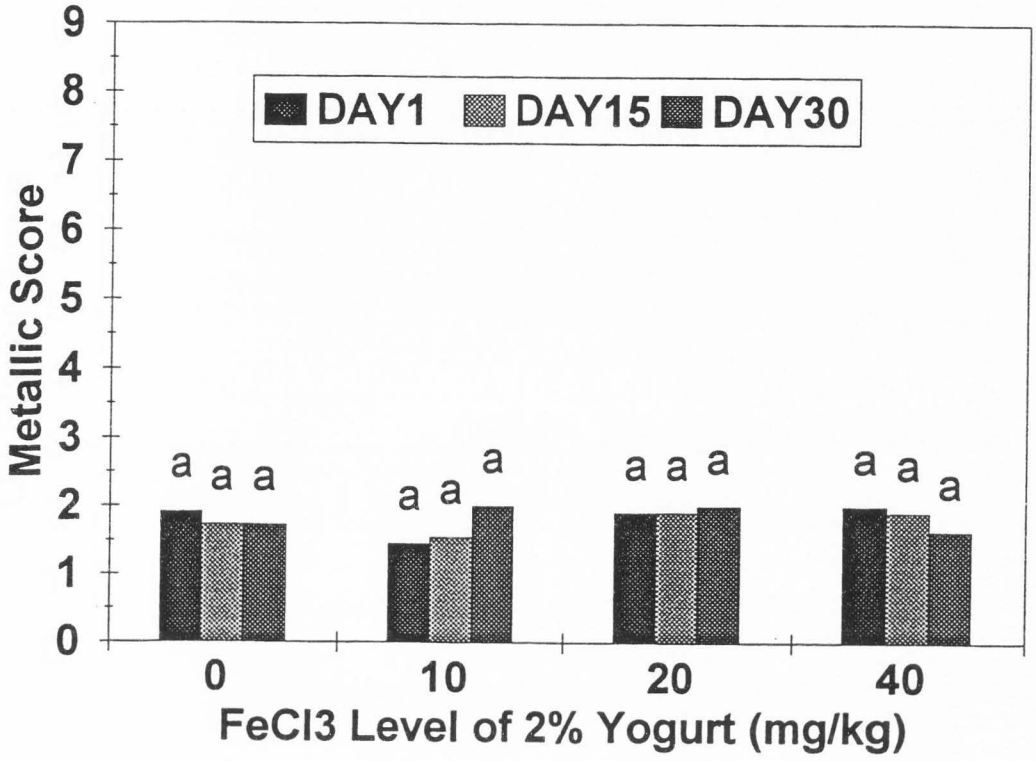

Figure 42. Metallic flavor scores of low fat (2%) unfortified yogurt and yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of $FeCl_3$ during storage. (Data points with the same letter are not significantly different.)

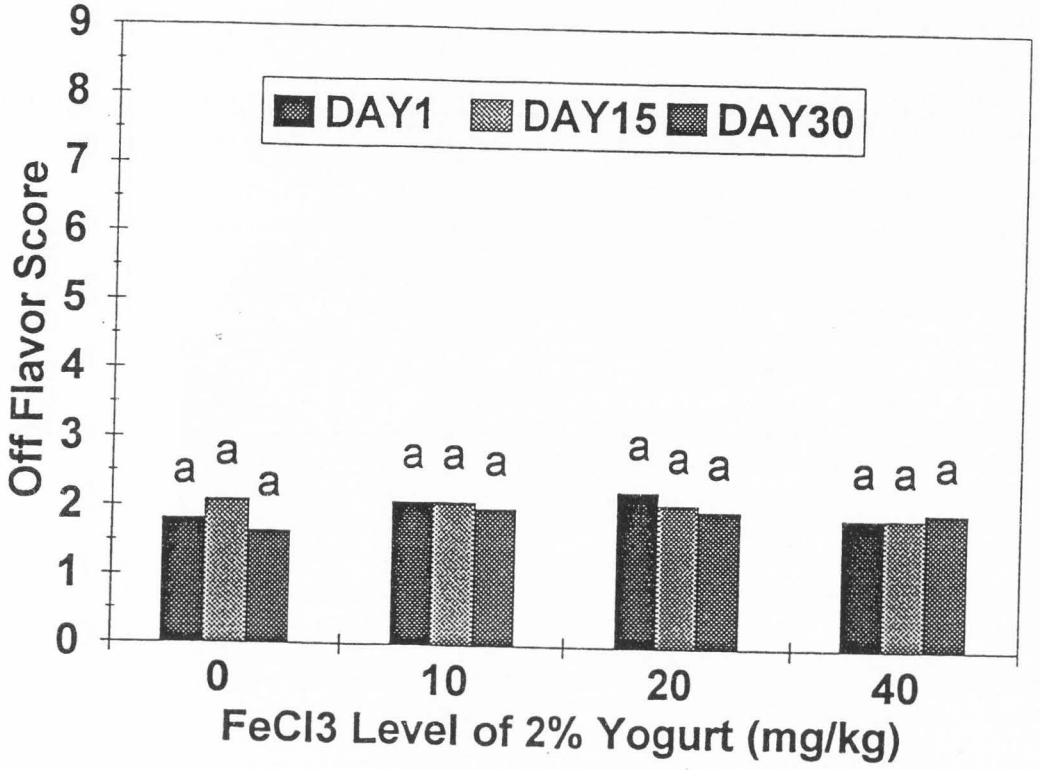

Figure 43. Off-flavor scores of low fat (2%) unfortified yogurt and yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of $FeCl_3$ during storage. (Data points with the same letter are not significantly different).

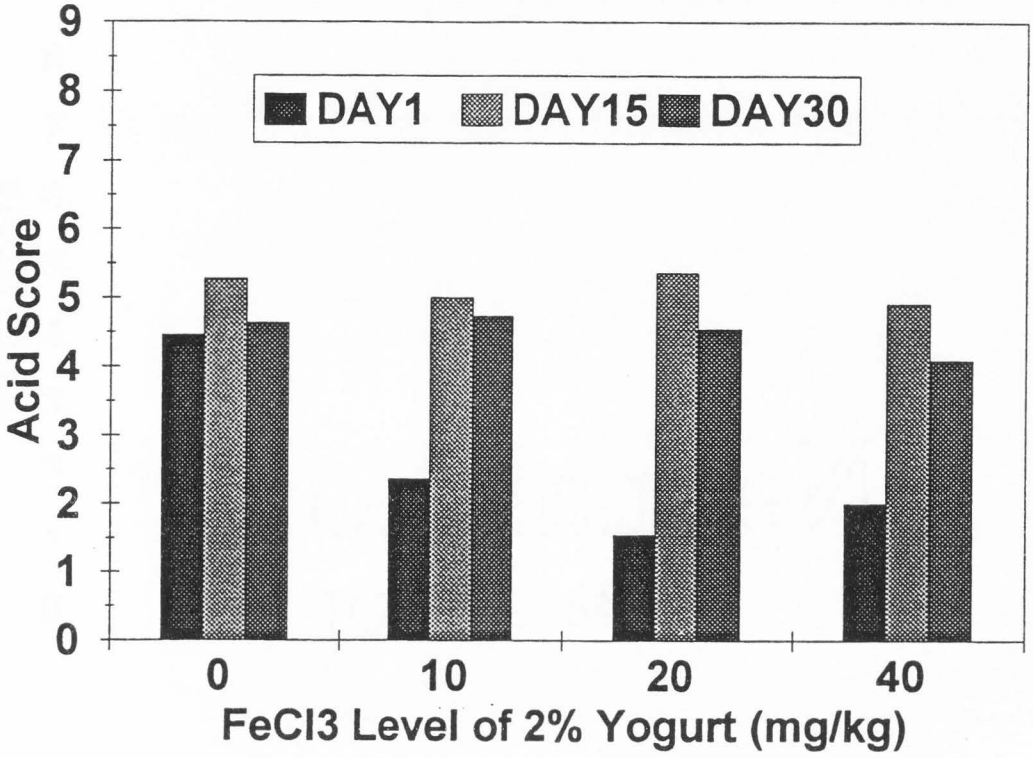

Figure 44. Acid flavor scores of low fat (2%) unfortified yogurt and yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of $FeCl_3$ during storage.

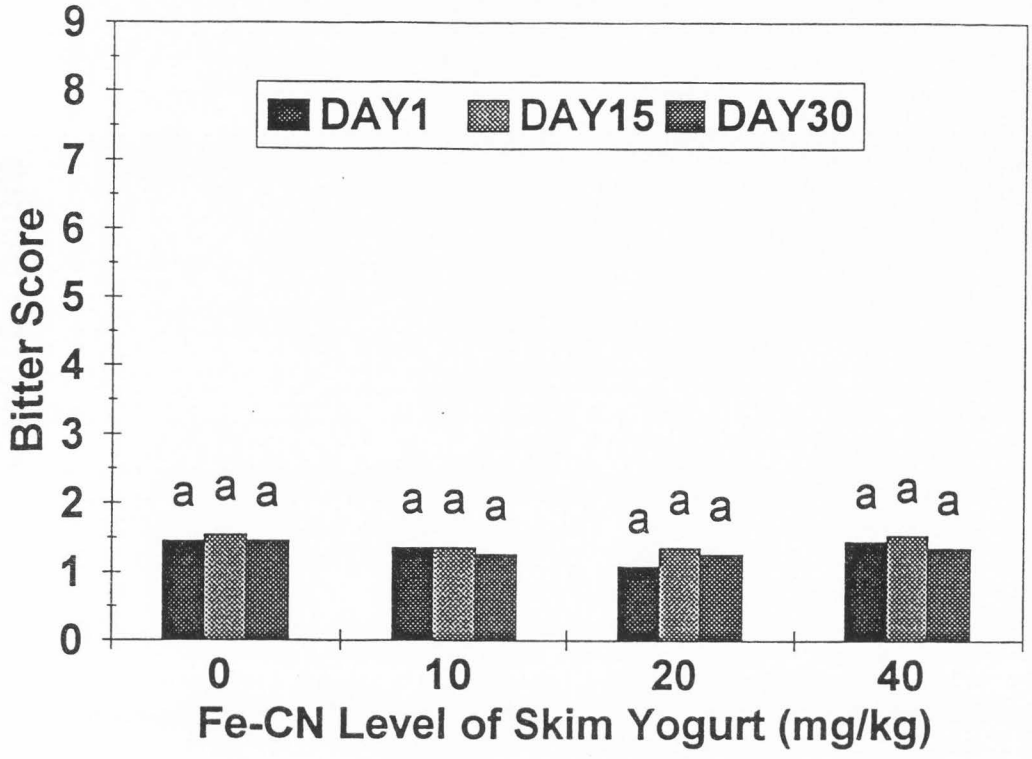

Figure 45. Bitterness scores of skim unfortified yogurt and yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of iron-casein complex (Fe-CN) during storage. (Data points with the same letter are not significantly different.)

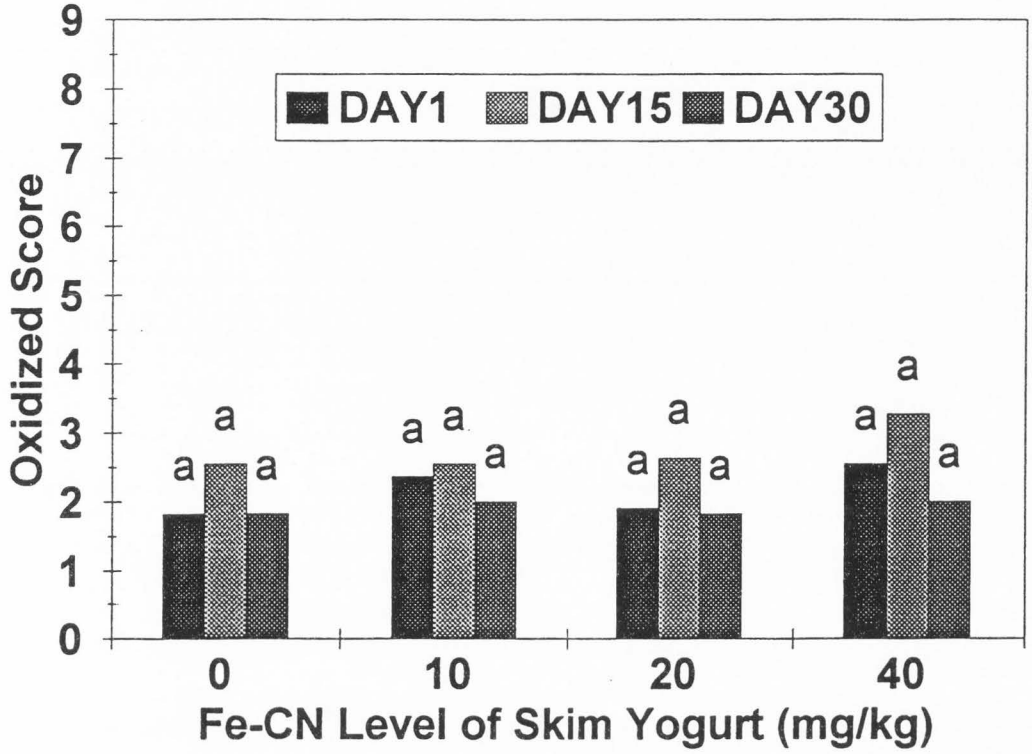

Figure 46. Oxidized flavor scores of skim unfortified yogurt and yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of iron-casein complex (Fe-CN) during storage. (Data points with the same letter are not significantly different.)

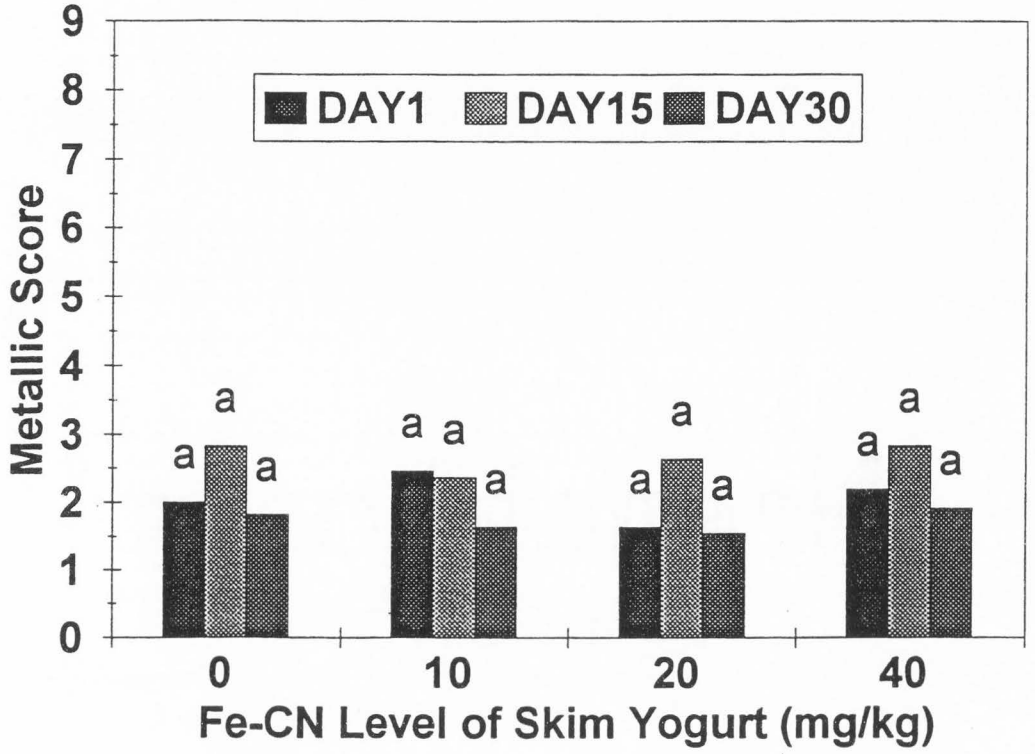

Figure 47. Metallic flavor scores of skim unfortified yogurt and yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of iron-casein complex (Fe-CN) during storage. (Data points with the same letter are not significantly different.)

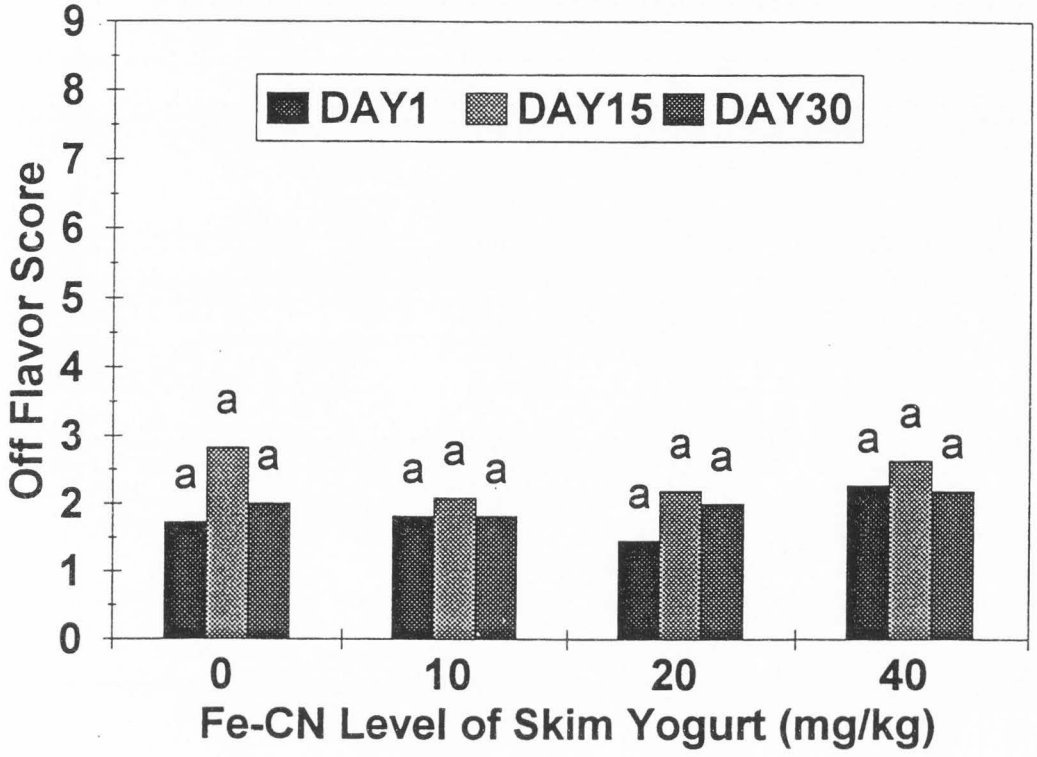

Figure 48. Off-flavor scores of skim unfortified yogurt and yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of iron-casein complex (Fe-CN) during storage. (Data points with the same letter are not significantly different.)

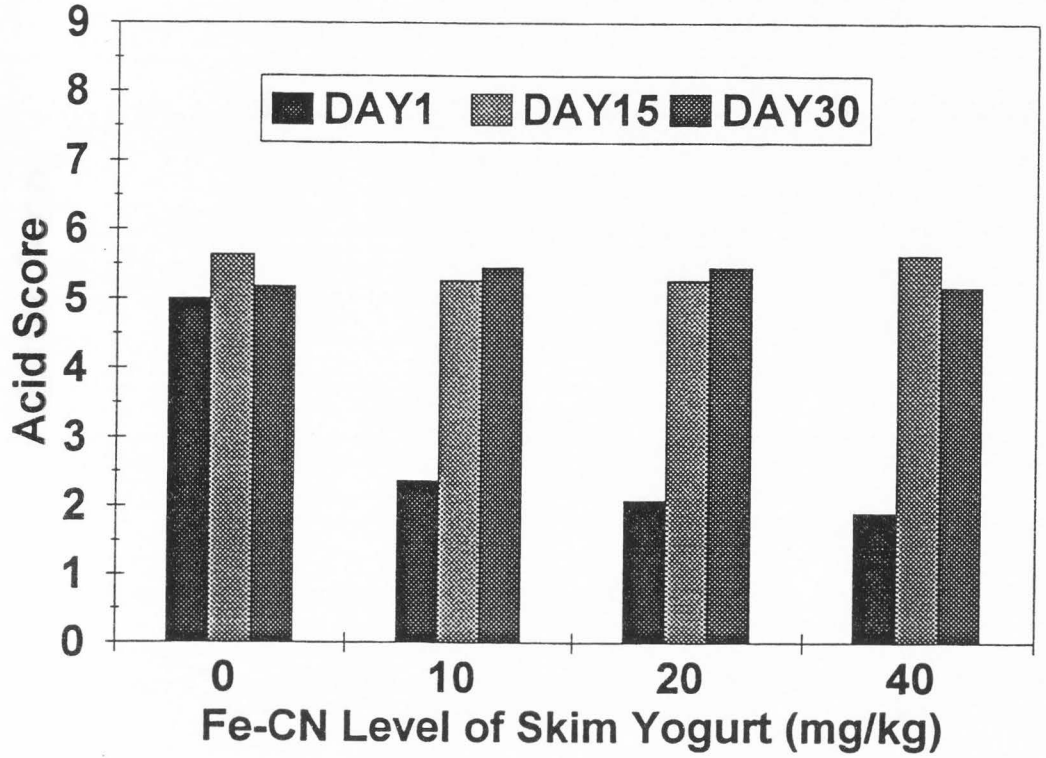

Figure 49. Acid flavor scores of skim unfortified yogurt and yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of iron-casein complex (Fe-CN) during storage.

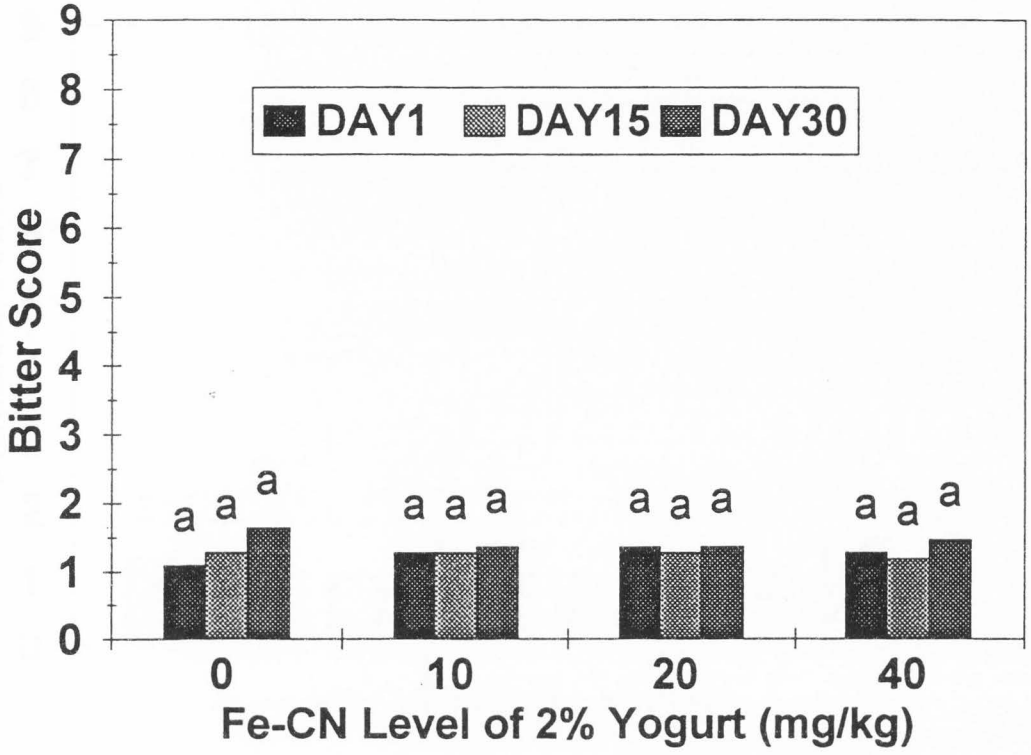

Figure 50. Bitterness scores of low fat (2%) unfortified yogurt and yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of iron-casein complex (Fe-CN) during storage. (Data points with the same letter are not significantly different.)

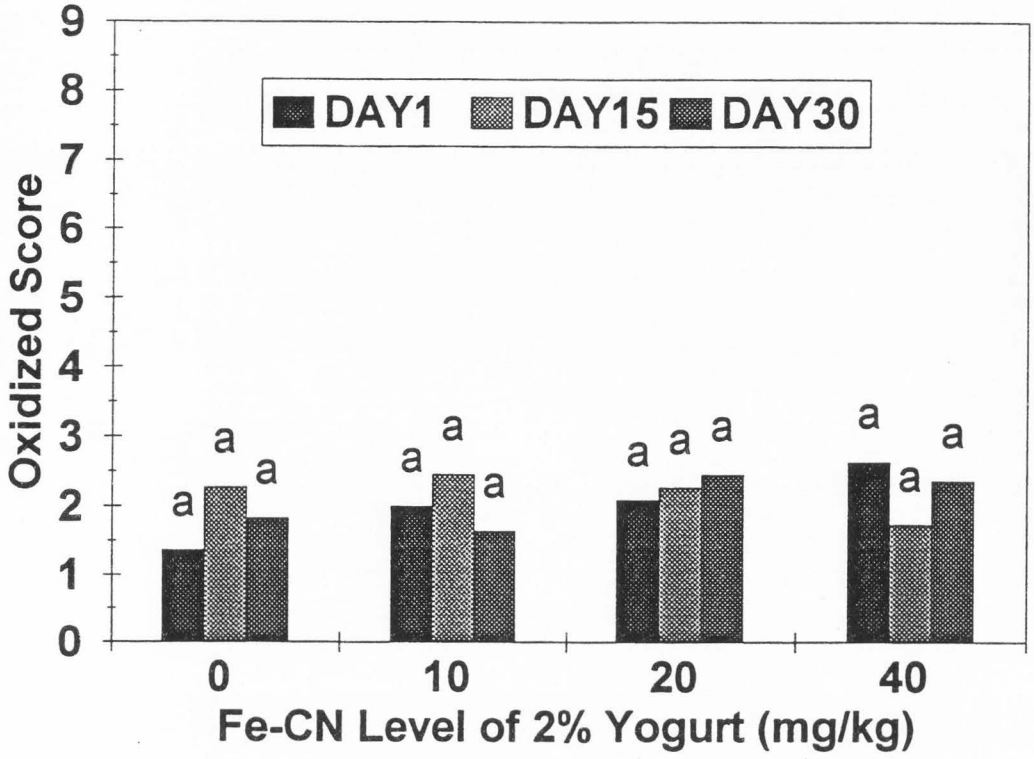

Figure 51. Oxidized flavor scores of low fat (2%) unfortified yogurt and yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of iron-casein complex (Fe-CN) during storage. (Data points with the same letter are not significantly different.)

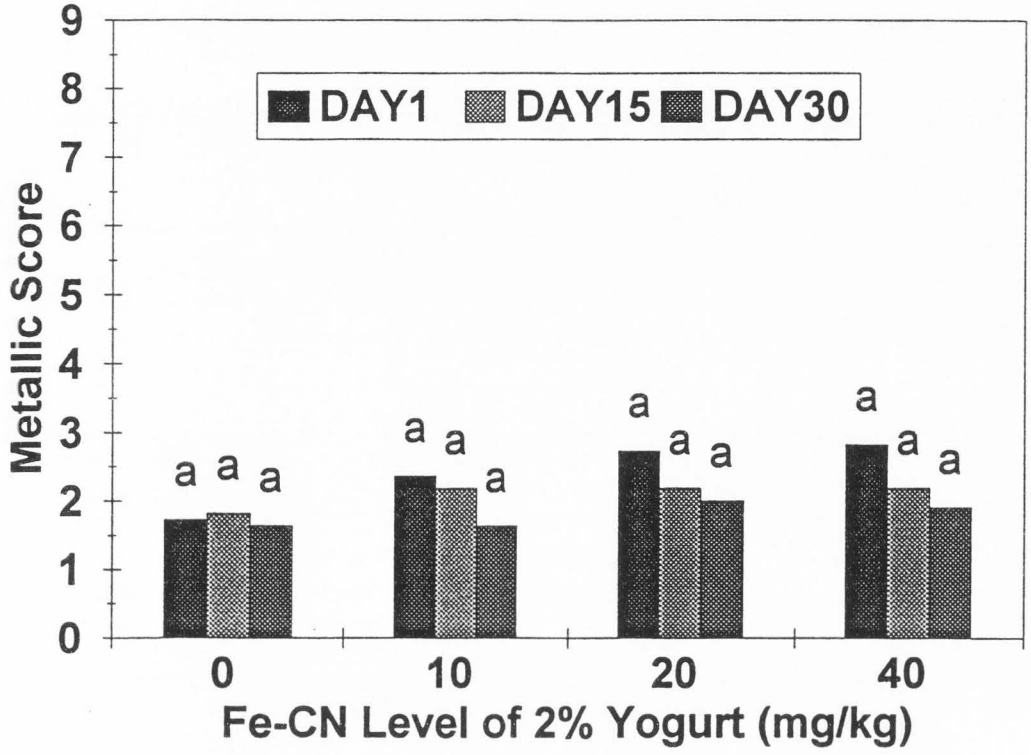

Figure 52. Metallic flavor scores of low fat (2%) unfortified yogurt and yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of iron-casein complex (Fe-CN) during storage. (Data points with the same letter are not significantly different.)

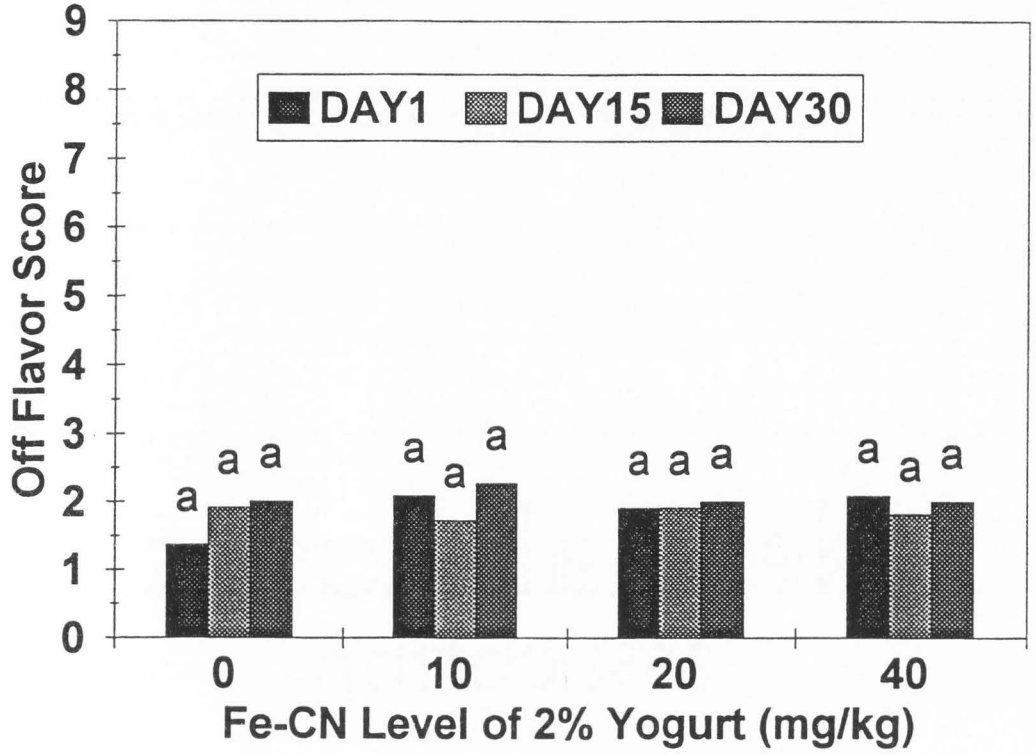

Figure 53. Off-flavor scores of low fat (2%) unfortified yogurt and yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of iron-casein complex (Fe-CN) during storage. (Data points with the same letter are not significantly different.)

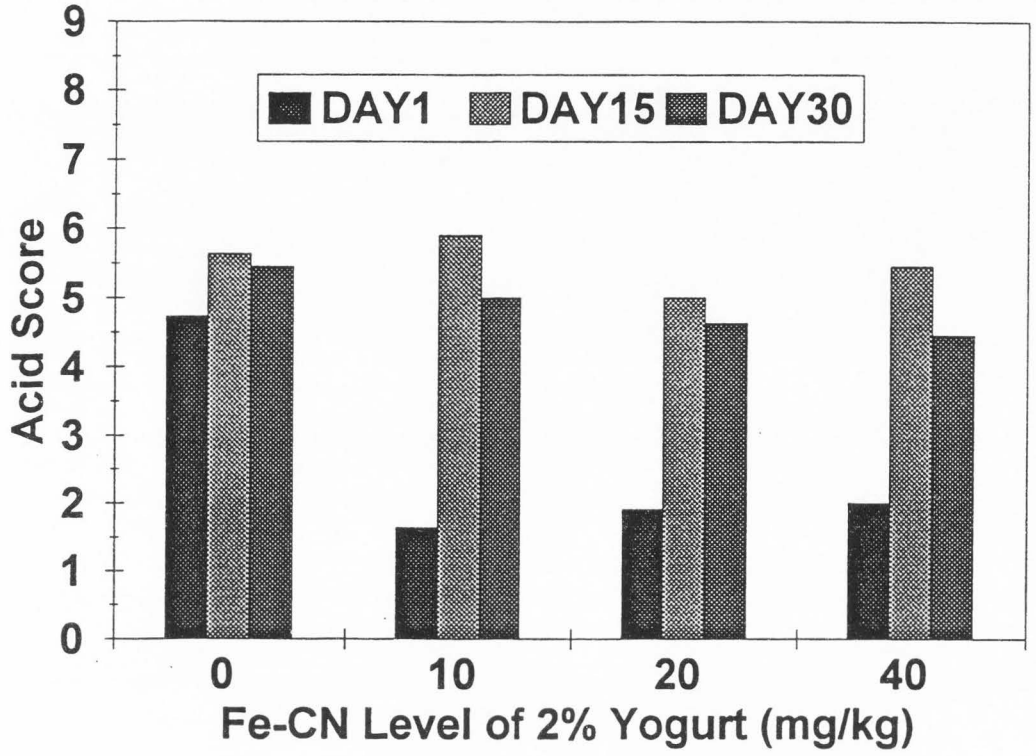

Figure 54. Acid flavor scores of low fat (2%) unfortified yogurt and yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of iron-casein complex (Fe-CN) during storage time.

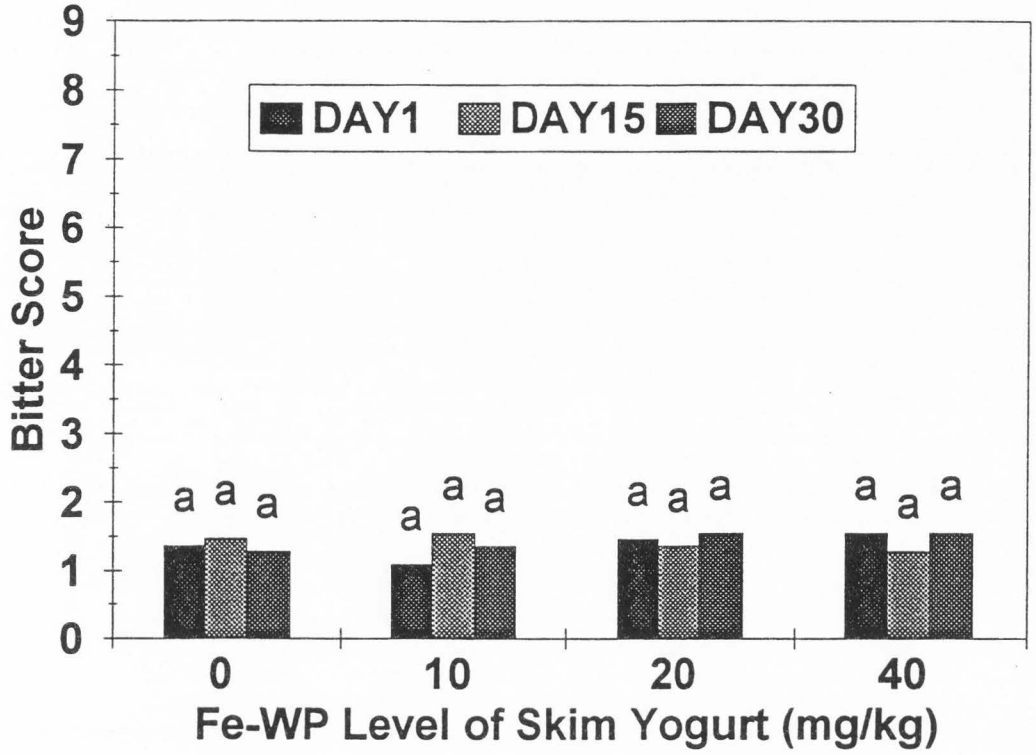

Figure 55. Bitterness scores of skim unfortified yogurt and yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of iron-whey protein complex (Fe-WP) during storage. (Data points with the same letter are not significantly different.)

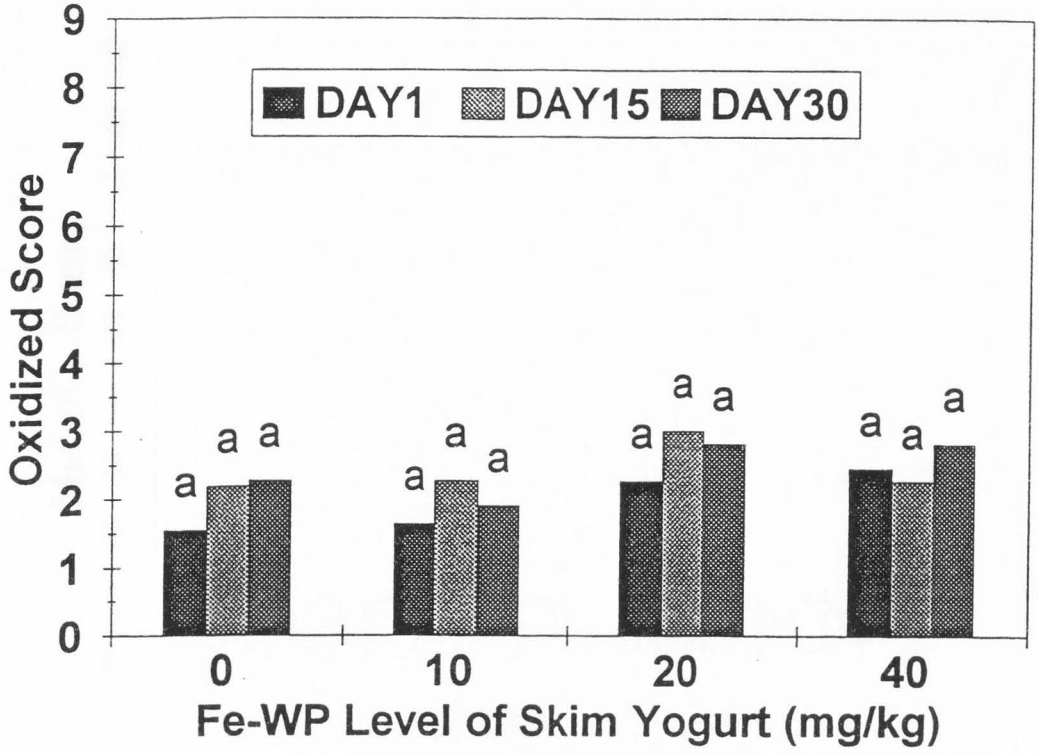

Figure 56. Oxidized flavor scores of skim unfortified yogurt and yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of iron-whey protein complex (Fe-WP) during storage. (Data points with the same letter are not significantly different.)

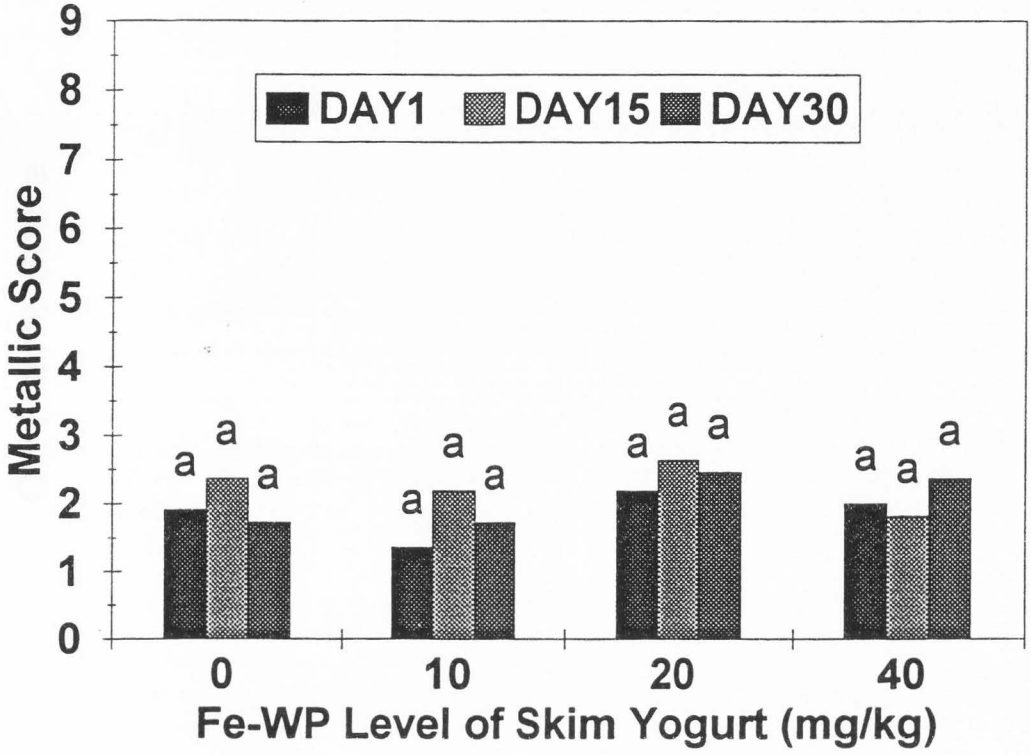

Figure 57. Metallic flavor scores of skim unfortified yogurt and yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of iron-whey protein complex (Fe-WP) during storage. (Data points with the same letter are not significantly different.)

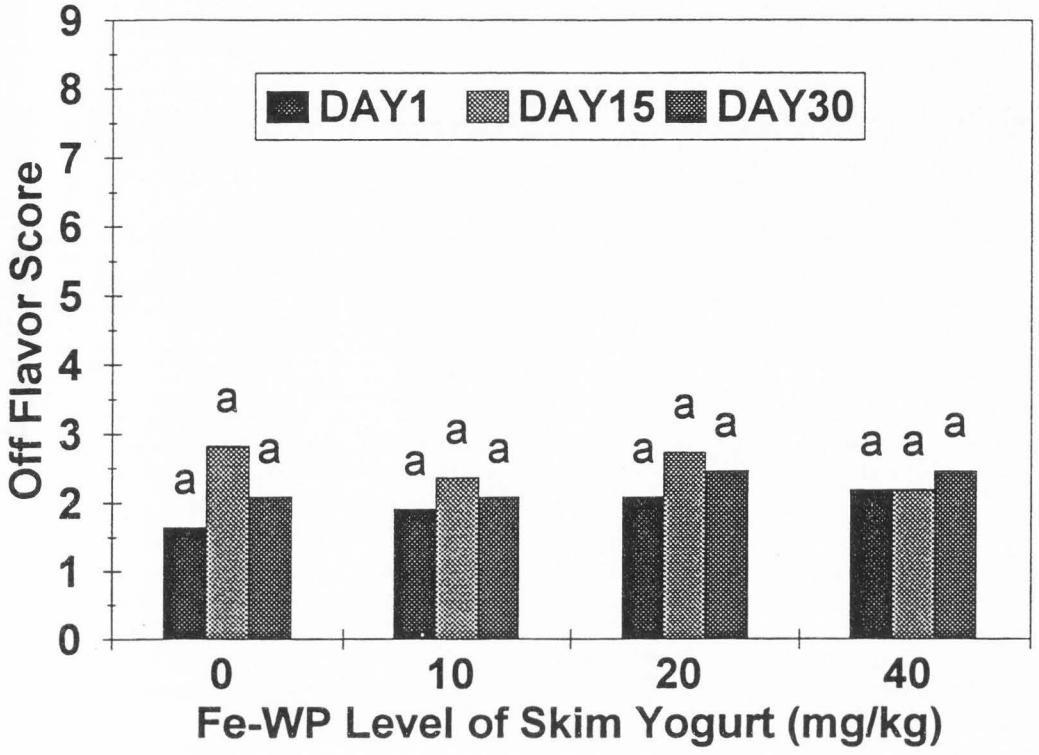

Figure 58. Off-flavor scores of skim unfortified yogurt and yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of iron-whey protein complex (Fe-WP) during storage. (Data points with the same letter are not significantly different.)

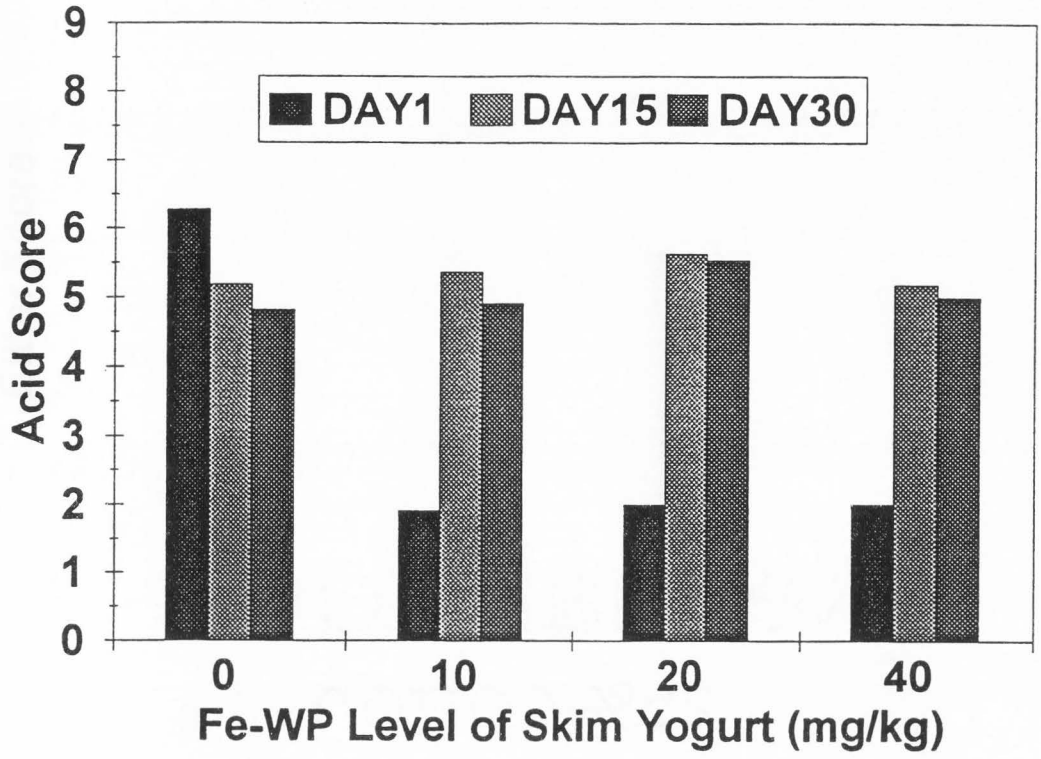

Figure 59. Acid flavor scores of skim unfortified yogurt and yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of iron-whey protein complex (Fe-WP) during storage.

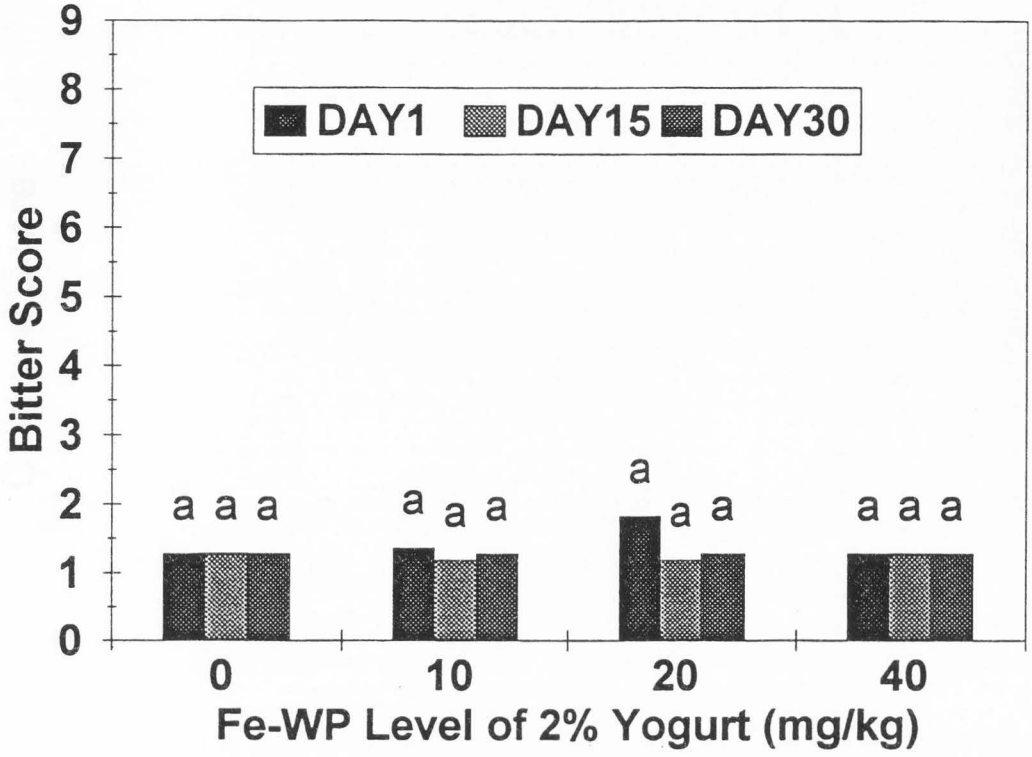

Figure 60. Bitterness scores of low fat (2%) unfortified yogurt and yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of iron-whey protein complex (Fe-WP) during storage. (Data points with the same letter are not significantly different.)

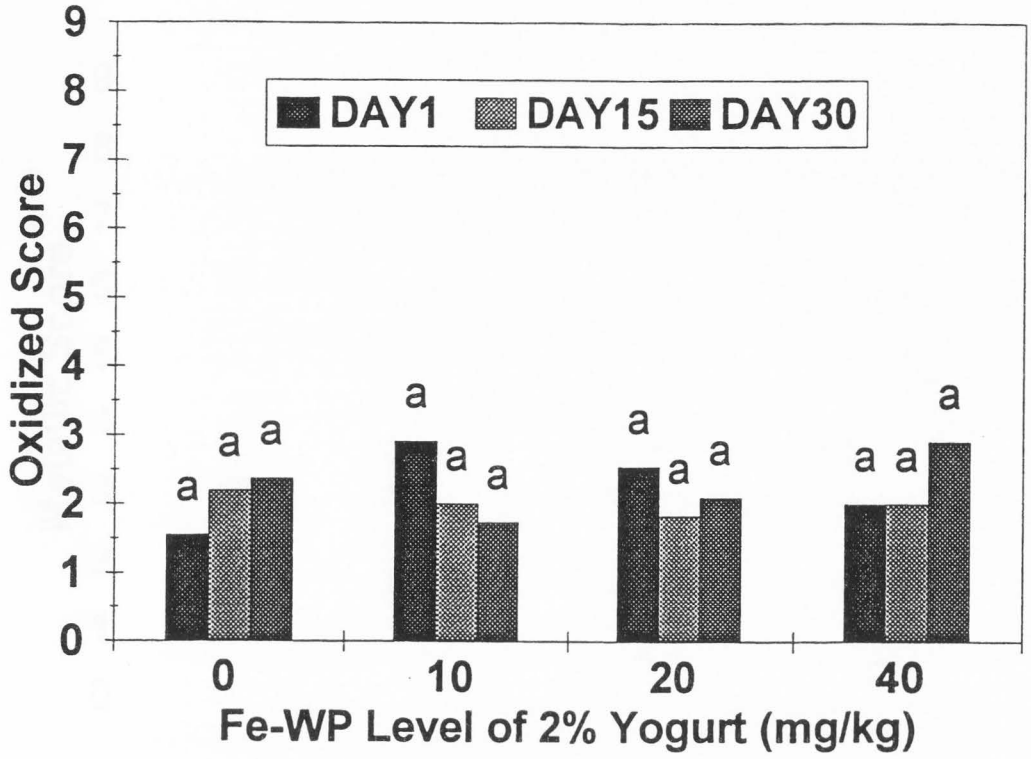

Figure 61. Oxidized flavor scores of low fat (2%) unfortified yogurt and yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of iron-whey protein complex (Fe-WP) during storage. (Data points with the same letter are not significantly different.)

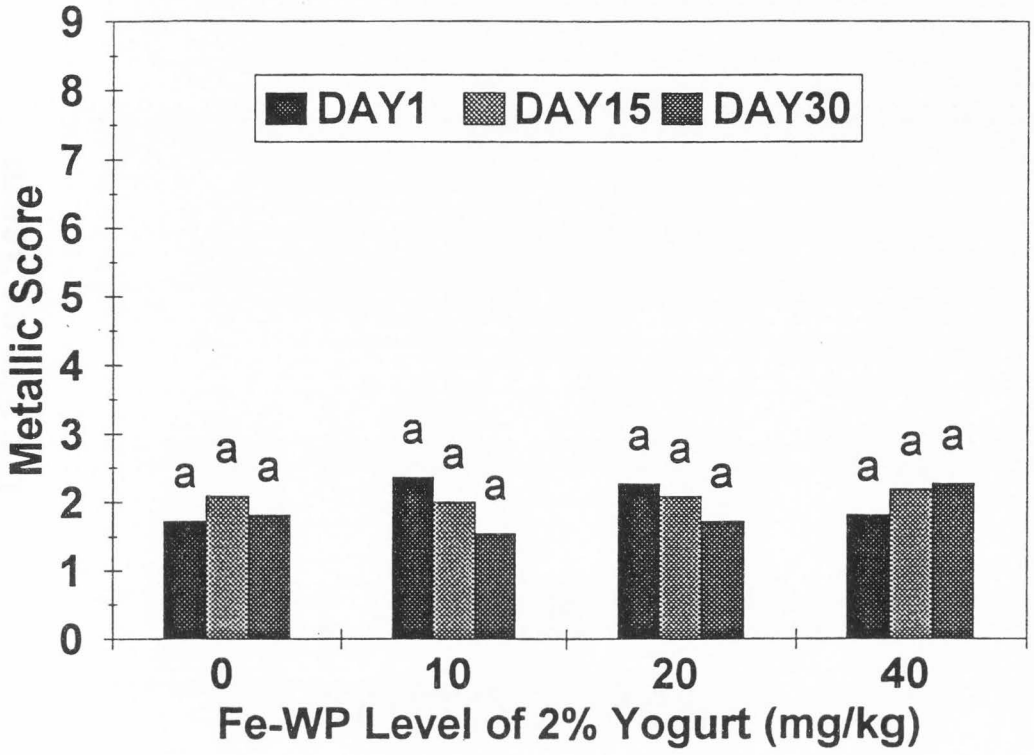

Figure 62. Metallic flavor scores of low fat (2%) unfortified yogurt and yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of iron-whey protein complex (Fe-WP) during storage. (Data points with the same letter are not significantly different.)

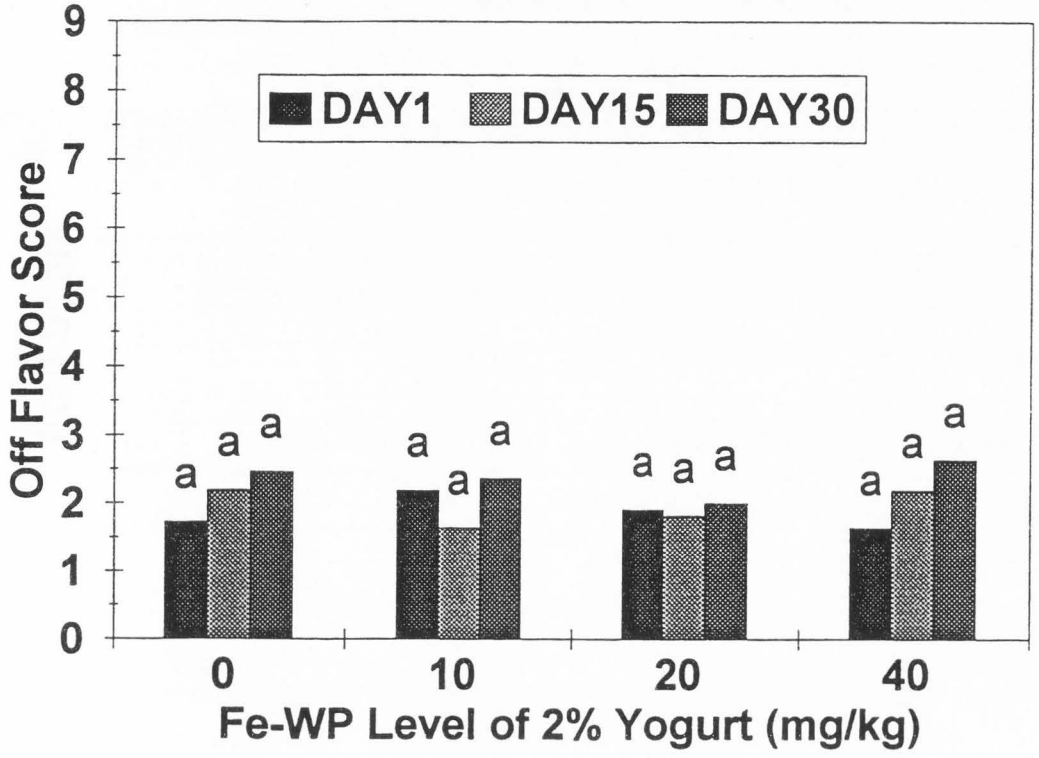

Figure 63. Off-flavor scores of low fat (2%) unfortified yogurt and yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of iron-whey protein complex (Fe-WP) during storage. (Data points with the same letter are not significantly different.)

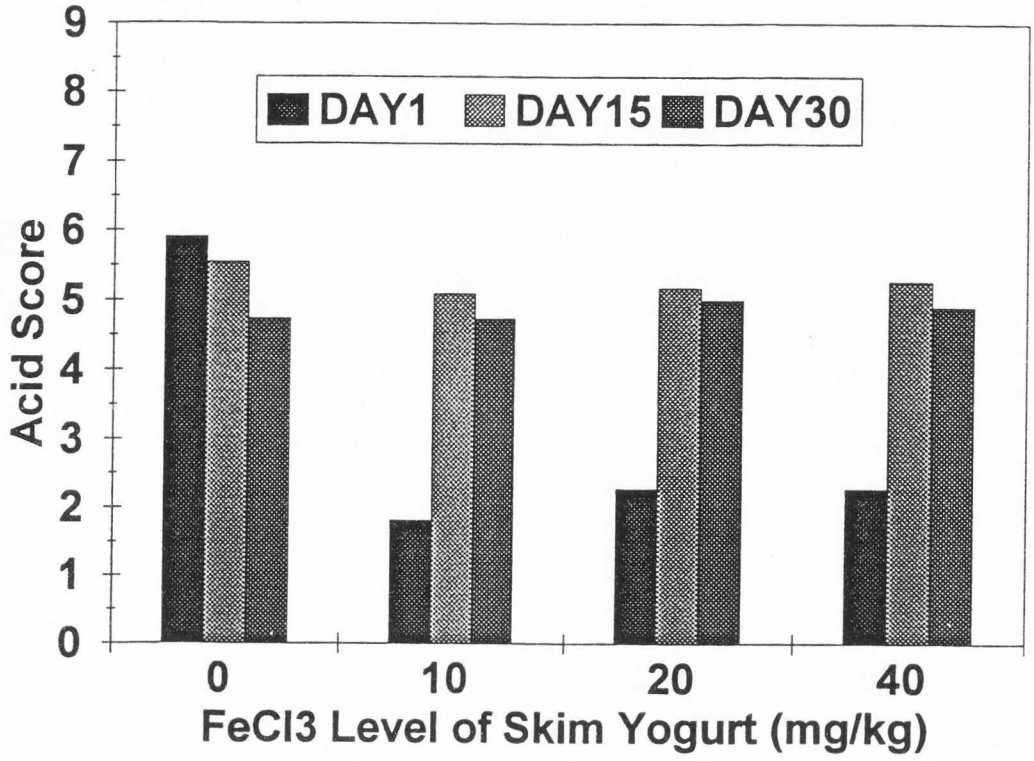

Figure 64. Acid flavor scores of skim unfortified yogurt and yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of $FeCl_3$ during storage.

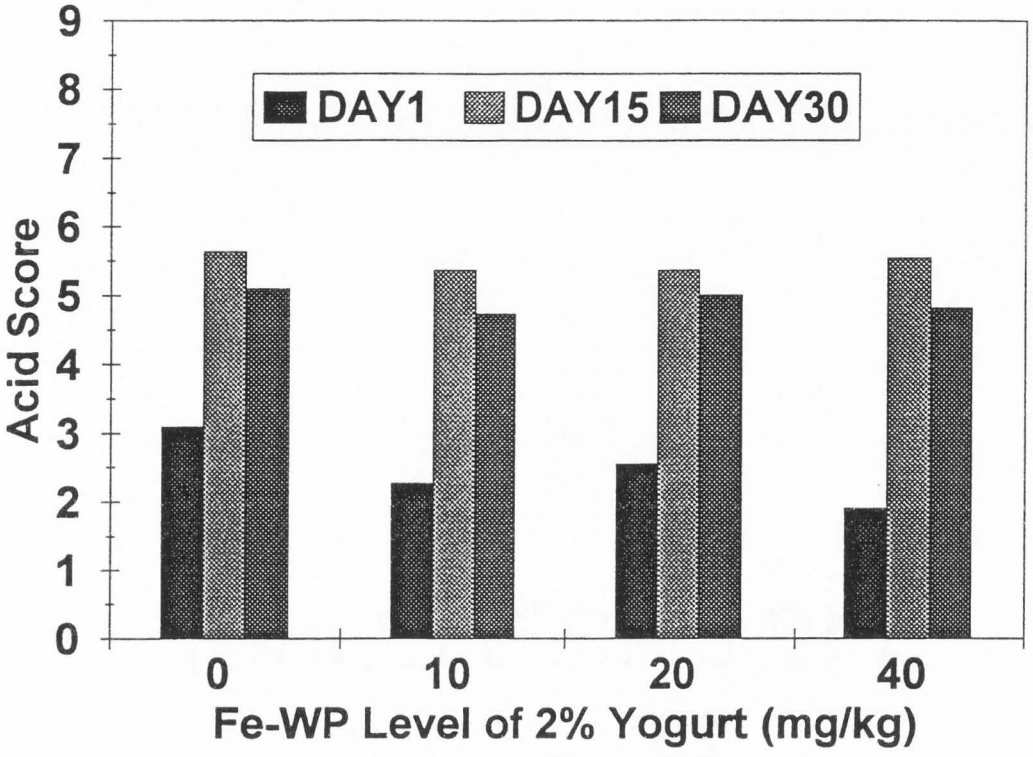

Figure 65. Acid flavor scores of skim unfortified yogurt and yogurt fortified with three levels (10, 20, and 40 mg iron/kg yogurt) of iron-whey protein complex (Fe-WP) during storage.

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Sharareh Hekmat

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Utah State University

Dissertation: Effects of iron fortification on microbiological, chemical, physical, and organoleptic properties of yogurt.

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M.S.	1991, Utah State University, Logan, Nutrition and Food Sciences, emphasis in food microbiology and food chemistry.
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Biology Academic Achievement Award, Utah State University (1985, 1986, 1987, 1988).

Department of Biology Scholarship, Utah State University 1988.

Women Center Scholarship Award, Utah State University (1987, 1988, 1990).

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Publications:

Hekmat, S., and D. J. McMahon. 1992. Survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in ice cream for use as a probiotic food. J. Dairy Sci. 75:1415.

Alleyne, M. C., D. J. McMahon, N. N. Youssef, and S. Hekmat. 1993. An apparatus for a new microcube encapsulation of fluid milk in preparation for transmission electron microscopy. Food Structure. 12:21.

Haws, W. J., S. Hekmat, M. Kalpalathika, A. W. Mahoney, and D. J. McMahon. 1994. Iron fortification of Mozzarella cheese. J. Dairy Sci. 77 (Suppl. 1):5.