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YOGURT FORTIFICATION WITH
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YOGURT FORTIFICATION WITH PREDIGESTED/GERMINATED WHOLE SOYBEAN POWDER FOR ENHANCED THERAPEUTIC BENEFITS

By

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ABSTRACT

YOGURT FORTICATION WITH PREDIGESTED/GERMINATED WHOLE SOYBEAN POWDER FOR ENHANCED THERAPEUTIC BENEFITS

By

Obianuju Nwamaka Nsofor

Growth (CFU/ml), changes in pH and titratable acidity (%TA) measurements were used to study culture activities and growth of 3 lactic acid bacteria (LAB) namely *Streptococcus salivarius* subsp. *thermophilus* (St 133*), Lactobacillus delbruekii* subsp. *bulgaricus* (Lr 78) and *Lactobacillus acidophilus* (La NCFM). These cultures were grown in blends (3:7, 1:1, 7:3) of reconstituted non-fat dry milk (NFDM) and germinated (GSP) or non-germinated (NGSP) whole soy powder obtained from three soybean varieties (Vinton 81, DF 222 and E05276-T). Cultures grown in most milk blends that contained both germinated and non-germinated whole soy powder gave low pH, high %TA and growth values. The lowest pH and highest %TA and growth were obtained when the cultures were grown in 1:1 NFDM+GSP or NGSP blend. Cultures grown in germinated whole soy powder blends produced more acid and growth ($p \leq .001$) than its non-germinated counterparts. The 1:1 blends of NFDM and GSP or NGSP were utilized to make low-fat Swiss-style strawberry yogurt. Sensory evaluation by 112 untrained panelists showed that there was no consumer preference for cow's milk yogurt over whole soy- fortified cow's milk yogurt.

The concentrations of isoflavone isomers genistein, daidzein, genistin and daidzin were determined in raw soy powder (RSP), NGSP and GSP of Vinton 81, DF 222 and

E05276-T soybean varieties using reverse-phase high-performance liquid chromatography (HPLC). Stachyose (oligosaccharide) contents of NGSP and GSP were analyzed using HPLC. The combined four isoflavone isomers and stachyose concentrations were highest in non-germinated (soaked) DF 222. All the germinated and non-germinated soy powders contained high quantities of isoflavone irrespective of soybean variety. Germinated soy powders had significantly lower amount of stachyose than non-germinated powders ($p < 0.001$). Also HPLC analysis of yogurt samples showed that total isoflavone contents increased from 1st to 6th week of cold storage. The genistein and daidzein contents of the soy-fortified yogurts remained the same throughout the 6 weeks of storage.

Shelf life studies of the yogurt samples indicated that growth and viability of all the cultures were above 10^7 CFU/g of yogurt. A concentration of at least 10^6 viable probiotic cultures is needed in fermented foods in order to exert health benefits to the consumers. Soy-fortified yogurts had the highest concentration of cell counts, which were statistically different from all dairy or soy yogurts.

To my late parents

Herbert and Mercy Ikpeze who taught me the value of education and sacrificed so much to make sure all their 10 children were educated. Thanks mom for teaching me patience and the importance of always running life's race in my assigned lane so that I will never be disqualified.

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"Learning is the fountain of youth. No matter how old you are, you musn't stop growing."

365 Tao: Daily Meditations

Deng Ming-Dao

Harper, San Francisco, 1992.

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INTRODUCTION

During the past decade, there has been an increased interest in nutrition, medical and food sciences concerning biologically active compounds or health-promoting components including peptides, which are hidden in the amino acid sequences of food proteins. These compounds represent potential functional foods or nutraceuticals for food and pharmaceutical applications. A nutraceutical is defined as a substance that is a food or part of a food and provides medical or health benefits, including the prevention and treatment of disease (Kalra, 2003). There is an increased awareness of the health benefits associated with soybean consumption and the functional foods market currently generate a lot of dollars in developed countries like the United States. It has been projected that the nutraceutical market in the United States will increase to about $20 billion by the year 2010 thus contributing to 10% of the U.S food market (Sloan, 2002).

Numerous natural foods such as milk, soymilk and their derivatives contain proteins and other compounds which could be made biologically active to form compounds like antioxidants, conjugated linoleic acid, opioid peptides, hydrolyzates, and other biologically active compounds as a result of fermentation by lactic acid bacteria or enzyme hydrolysis (Meisel and Bockelmann, 1999). Several studies have shown that there is a lower incidence of chronic diseases such as cardiovascular disease, colon, breast and prostate cancers, with population that consume soy products regularly (Erdman 2000; McCue and Shetty 2004). In these studies epidemiological association was established between soybean consumption and improved health. Some of these health benefits have been attributed to bioactive compounds in soybeans known as

isoflavones. Research has shown that these isoflavones are more prominent in fermented soy products. Germination of soybean seeds closely resembles fermentation such that enzymes inherent in the soybeans can hydrolyze the non-bioavailable compounds into bioactive compounds and further fermentation during yogurt making will increase their yield. Yogurt is traditionally made from cows' milk and is consumed in both developed and developing countries. Recently there has been an increased desire for innovations in yogurts such as including a variety of ingredients like new strains of probiotics, fibers, and other components for fortification e.g. soy protein isolate, needed for increased health benefits, hence the interest in soy-based or soy-fortified yogurt.

The main goal of this proposal is to test the hypothesis that incorporation of germinated whole soybean powder into cow milk substrate will produce increased yield of biologically active compounds in the yogurt mix to meet recommended requirements for added health claims. Five objectives are being proposed to address this hypothesis:

1. Growth and activity of lactic acid bacteria and a probiotic in reconstituted germinated whole soy powder (GSP), non-germinated whole soy powder (NGSP) and non-fat dry milk (NFDM) + GSP or NGSP.
2. Development and properties of yogurt from blends of cow's milk and whole soymilk base for consumer acceptance.
3. Production and concentration of genistein, diadzein, genistin, daidzin and oligosaccharide (stachyose) in germinated and non-germinated soy powder.
4. Effect of processing and refrigerated storage on isoflavone and stachyose contents of yogurt fortified with non-germinated and germinated (predigested) whole soy powder.

5. Shelf life studies and viability of whole soy-fortified yogurts stored at 4 °C.

Overall, I wish to establish the fact that better health benefits could be conferred to consumers by providing bioactive compounds from both soy and cow's milk in form of yogurt.

CHAPTER 1

LITERATURE REVIEW

1.1 COW'S MILK YOGURT

1.1.1 Composition and nutritional value of milk

Generally, milk and dairy products have always been considered as important components of a balanced diet because they provide a wide range of important nutrients. Proximate composition of cow's milk contains about 3.3-4.00% protein, 3.65- 4.35% fat, 82.55-88.0% moisture, 0.77-0.81% ash, and 4.0-4.5% carbohydrate calculated as the difference from 100% (Yadav and others, 2003). Among the minerals present in cow's milk, calcium and phosphorous are the most abundant (122.2 and 76.3mg/100g respectively). Milk proteins consist mostly of casein in conjunction with other minor proteins. The caseins supply the amino acids and several studies have shown the bioactive potentials of these compounds. Proteolytic enzymes can hydrolyze caseins easily therefore milk proteins can produce several bioactive peptides such as opoid peptides, immunostimulating peptides, angiotensin I converting enzyme inhibitors and antibacterial peptides (Yamamoto, 1997; Clare and others, 2003).

Milk proteins have been known to be the source of different biologically active peptides but recently several researchers have isolated bioactive peptides from plant sources such as campesterol, stimasterol and β-sitosterol (Quliez and others, 2003). The major physiological attributes of these bioactive compounds from milk include their ability to act as potential modulators of various regulatory processes in the body

(hormonal), immune modulation, antibacterial and antitumor activities (Meisel and Bockelmann, 1999).

The carbohydrate content of cow's milk is exclusively lactose sugar. The cholesterol content is about 14mg/100g and it does not contain any dietary fiber. Different processing, methods including pasteurization do not destroy a great portion of the nutrients including the vitamins (A, B, D, E, K) in the milk although it is fortified with vitamins (e.g. vitamin D) sometimes before consumption (Brenda, 2004).

1.1.2 Nutritional and health benefits of yogurt

1.1.2.1 Definition and history of yogurt

According to Codex Alimentarius of 1992, yogurt is defined as a coagulated milk product resulting from the fermentation into lactic acid from milk sugar lactose by *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (Adolfsson and others, 2004). Other lactic acid bacteria (LAB) could be combined with the traditional yogurt bacteria. The entire LAB used for yogurt making is expected to be alive and present in large amounts in the finished products. Scientific evidence has shown that cultured milk was initially made as far back as 4,500 years ago (Van de Water and others, 1999). The earliest yogurt was made by wild fermentation by the Bulgars in the 2nd century. It remained primarily a food for the South, Central and Western Asia, South Eastern Europe and Central Europe. The observation that frequent consumption of fermented milk containing *Lactobacillus bulgaricus* increased health and longevity was only made in the 20th century by Metchnikoff, who claimed that the intake of yogurt decreases the toxic

effect of the putrefactive bacteria in the colon by decreasing their growth (Metchnikoff, 1907; Wollowski and others 2001).

Cow's milk alone lack or contain insufficient amounts of amino acids (e.g. arginine, isoleucine and glutamic acid) and low molecular weight peptides, therefore cannot support the growth of probotics (Gomes and others, 1998; Hofman and Thonart, 2001; Shah, 2000). Alternatively, milk supplemented with soy protein isolate supported the growth of these probiotics and this could be as a result of enhanced lactose utilization and acetic acid production due to the presence of these amino acids in the protein isolates (Pham and Shah, 2008).

1.1.2.2 Special ingredients of yogurt

Yogurt base, which is milk, is rich in proteins, several B vitamins and essential minerals. Yogurt is a rich source of calcium and contains as much fat as the milk it is made from. Overall the nutrient composition of yogurt is based on the nutrient composition of the milk from which it is made. Several factors affect the nutritional value of the final product. These factors include changes in milk constituents, species and strains of bacteria used in fermentation, source and type of milk solids, temperature and time of fermentation (Adolfsson and others 2004). Yogurt is a good vehicle for many nutritional ingredients because of its high-refrigerated shelf life, flexibility to add any particular ingredient or nutrient before or after setting.

Yogurt can be easily fortified with a range of vitamins, antioxidants, probiotics and other beneficial nutrients. Most of the yogurt proteins come from nonfat milk, ultra filtered nonfat milk or whey protein. Other nutritional ingredients added to yogurt include

lycopene, omega-3 eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and CoQ10 (Gerdes, 2007). Most of these ingredients have well-recognized health claims. Recently, some yogurt manufacturers add probiotics, prebiotics and phytosterols to the yogurt mix (Monu and others, 2008). The prebiotics function as dietary fibers and are mostly polydextrose. Examples of such prebiotics are inulin and oligofructose (Aryana and others, 2007;Vasiljevic and others, 2007). Inulin is commonly present in plants such as artichokes, leeks and garlic and is made up of glucose and fructose chains. Oligofructose is produced from enzymatic hydrolysis of inulin or partial enzymatic hydrolysis of sucrose (transfructosylation by β-fructofuranosidase). Both inulin and oligofructose provide nutritional and functional benefits in foods. Their ascribed contributions to health benefits include the ability to selectively promote the growth of probiotics such as bifidobacteria and lactobacillus. thereby improve digestive efficiency and health (Bruno and others, 2002).

In 2005, Popa studied the influence of sweetener type on growth, activity, and viability of yogurt cultures. Her findings showed that yogurt ingredients had no inhibitory effect on the growth of the cultures used. The results also showed that viability of the lactic acid bacteria and probiotics used was retained at high percentage (85% and 90% respectively), except in the yogurt sample sweetened with sourwood honey. Vasiljevic and others (2007) improved probiotic (*Bifidobacterium animalis* ssp. *lactis*) viability and stability by adding β-glucan from oat and barley to yogurt.

An increasing popular ingredient in yogurt is phytosterols. These are sterol compounds, which occur naturally in plants but have long been used to treat hypercholesterolemia (Monu and others 2008). Phytosterols have become important

ingredients in functional foods because of perceived health potentials. Due to the ability of these compounds to block cholesterol absorption, thereby reduce coronary heart disease, it is recommended that consumption of a low-fat yogurt (0.7% fat) that contains 3g/day of plant stanols will reduce LDL cholesterol by 13.7% (Noakes and others 2005).

1.1.2.3 Biodefense properties of yogurt

The biodefensive properties of yogurt and fermented milk have been well described by several researchers (Van de Water and others, 1999:Adolfsson and others, 2004). Numerous protective proteins and peptides found in cow's milk yogurt with unique biological activities have been documented. The knowledge of dairy science and technology has afforded scientists the ability to recognize, recover and maintain these compounds as bioactive functional ingredients that could improve the quality of foods. Bioactive peptides such as casokinins or angiotensin-converting enzyme (ACE) I peptides play a role in reducing blood pressure by inhibiting ACE and blocking the conversion of angiotensin I to angiotensin II, which is a potent vasoconstrictor (Rogelj 2000). Milk peptides have also been associated with anti-carcinogenic properties. Some of these peptides, according to previous studies, may retard the development of colon tumors and tumor precursors by providing biologically active methionine and cysteine. These amino acids are involved in the cellular methylation and DNA stabilization as well as cellular synthesis of gluthathione, which plays a crucial role in the defense mechanisms that protect against cancer (Rogelj 2000). The evidence of a higher content of amino acids supports the argument that proteins from yogurt are more digestible than proteins from milk. This could be due to bacterial predigestion of the milk proteins in the

yogurt thus releasing free amino acids such as proline and glycine (Shahani and Chandan, 1979).

Previous studies have shown that yogurt and LAB contribute immensely to gastrointestinal health and thus supports Metchnicoff's theory that yogurt may indeed be beneficial to health. The benefits of yogurt have been shown in both animal and human studies. These promising health benefits include potential reduction of lactose intolerance, constipation, diarrheal diseases, colon cancer, inflammatory bowel disease, *Helicobacter pylori* infection and allergies (Adolfsson and others 2004; Van de Water and others 1999). The potential health benefits of LAB will be fully discussed later under health benefits of probiotics.

Several investigators have studied the effect of yogurt consumption on the immune system of animals. Their findings indicate that there is a great potential on enhancing the immune system by feeding yogurt to mice in terms of improving the immune signaling compounds. Tejada-Simon and others (1999) studied the effect of consuming yogurt that contain *L. acidophilus* and *Bifidobacterium* by mice and their ability to potentiate immunoglobulin A responses to cholera toxin in those mice. Their results suggested that ingestion of yogurt supplemented with *L. acidophilus* and *Bifidobacterium* increased mucosal and systemic IgA responses to cholera toxin, unlike the control yogurt, which contained only the conventional starter cultures, where less IgA was produced.

Ha and others (1999), investigated the potential of yogurt consumption to modulate the immune system by assaying mucosal and systemic cytokine gene expression in mice. Again these yogurts were supplemented with or without *L. acidophilus* and *Bifidobacterium*. They determined relative mRNA for interferon-γ, tumor necrosis factor-

α, interleukin-2, interleukin-4 and interleukin-6 from the spleen, mesenteric lymph nodes or Peyer's patches. The results showed that long time feeding of some yogurt led to either a decreased expression of some cytokine mRNAs or had no effect on the test organs, suggesting that yogurt formulation is critical since different lactic acid bacteria can differentially affect basal cytokine expression. In another related study, the spleen and Peyer's patch lymphocyte populations in mice fed probiotic-supplimented yogurts were determined (Pestka and others, 2001). Mice were fed for 14 days, after which flow cytometry was used to determine the phenotypes of immune cells obtained from the mentioned organs. Results indicated that there was no difference on the amount of CD8+ (cytotoxic T cells), B220+ (B cells), IgA+, or IgM+ cells due to yogurt ingestion, but there was a significant increase in the percentage of CD4+ (T helper) cells from the treatment groups containing *Bifdobacterium* and *L. acidophilus* and other probiotic-supplimented yogurts but not in the control groups i.e. mice that were fed conventional yogurts. These findings according to the investigators meant that short term feeding (2weeks) of conventional or probiotic-supplimented yogurts produce minor effect on lymphocyte distribution in the systemic or mucosal immune compartments.

In the United States, most of the yogurts sold are low-fat or non-fat varieties, hence lipid hydrolysis makes little contribution to yogurt products attributes. Research has shown that yogurt contains more conjugated linoleic acid (CLA), a long-chain biohydrogenated derivative of linoleic acid than just cow's milk (Rudel, 1999). CLA has been reported to have both immunostimulatory and anticarnogenic properties (Whigham and others, 2000). Due to the lower content of lactose in yogurt, the bioavailability of minerals such as calcium, magnesium and zinc may be reduced since lactose enhances

the absorption of these minerals (Bronner and Pansu 1999). Since yogurt is acidic (low pH), calcium still exists in the ionic form therefore improves intestinal calcium uptake (Bronner and Pansu 1999). Bacterial cultures used during yogurt making (fermentation process), influence the vitamin content of the final product. Some of the LAB strains do not require vitamin B for growth but instead are capable of synthesizing such vitamins. As such vitamin losses as a result of processing could be corrected by utilizing such cultures.

1.2 SOYBEAN PRODUCTS

1.2.1 Composition and nutritional value of soybean and soy products

There is an increased awareness of the health benefits associated with soybean consumption and the functional foods market currently generate a lot of dollars in developed countries like the United States (Sloan 2002). The use of soy ingredients is receiving significant attention from the food industries and consumers. Soymilk and their derivatives contain proteins and other compounds, which could be made biologically active via processing. Several studies have shown that there is lower incidence of chronic diseases such as cardiovascular disease, colon, breast, and prostate cancers, with population that consume soy products regularly (Erdman, 2000; McCue and Shetty, 2004). In these studies epidemiological association was established between soybean consumption and improved health.

1.2.1.1 Chemical composition of soybean

Among the recommended alternatives to animal proteins, soybeans remain the main choice because it contains nutritionally significant quantities of isoflavones and have protein profile similar to eggs or red meat (Beasley and others, 2003). The acceptability of soybean proteins could be attributed to its being nearly equal in biological value to casein, very affordable and having good functional properties in food systems. Soybean usage has been part of most Asian culture both as food and medicine (Messina 1995). Historical documents by the Chinese suggest that soybeans have been grown and consumed for thousands of years (11^{th} to 7^{th} century BC). Recent findings involving their bioactivity in health maintenance has led to increased usage and consumption. Soybean is a legume which is rich in phenolic compounds and is widely consumed worldwide especially in Japan, Korea, China and Indonesia and Samuel Bowen first planted soybeans in the United States in Georgia in 1760s in his plantation.

Soy foods rich in isoflavones, proteins and some oligosaccharides have been reported to be involved with many health benefits to human beings (Wiseman and others 2000). Overall, soybeans contain different nutritional components that promote health benefits and represent an inexpensive and healthy component of consumer diets. Soybean based products have always been known to be inferior in sensory characteristics due to beany and off-flavors (Wu and others, 2005). The whole soy plant including the leaves, stalk and seeds could be utilized as foods, medicine or animal feed. The protein content of soybean is 38-40% and it is presumed according to previous study to be the most important nutrient that determines the qualities of most soy products e.g. soymilk, tofu etc. (Min and others, 2005). The fat content is about 18% (85% unsaturated fatty acids

mainly linoleic and linolenic acids). Due to its high content of unsaturated fatty acids, soybean and its products are susceptible to oxidation (Penalvo and others, 2004). The carbohydrate content of soybeans is about 30% (made up of 15% each of insoluble and soluble carbohydrates). Apart from the high content of phytoeostrogens (isoflavones), soybeans are known to be a good source of anti-nutritional factors such as saponins, phospholipids, protease inhibitors, phytates and trypsin inhibitors.

There are different varieties of soybeans and they vary in protein and oil contents as well as flavor, seed coat, cotyledon and helium colors and other physical properties (Min and others, 2005). Also the growing environment apart from bean varieties can affect the sensory and physical properties of soybeans, which in turn affect the outcome of the final products made from the beans (Table 1.1).

Soybean seed is typically made up of about 90% cotyledon, 8% seed coat and 2% hypocotyl axis or germ. The cotyledon and germ are excellent sources of macronutrients. About 80-90% of isoflavone is in the germ (Min and others, 2005). Soybean is successfully cultivated in hot summer climates with optimum temperature between 20°C to 30°C (68°F to 86°F) and it takes about 80-120 days between sowing and harvesting.

1.2.1.2 Soybean protein

Soy proteins are used in a variety of foods such as salad dressings, soups, imitation meats, beverage powders, cheeses, non-dairy creamers, frozen desserts, whip topping, infant formulas, breads, breakfast cereals, pastas and pet foods. They are also used in non-food products such as adhesives, asphalts, resins, cleaning materials, cosmetics inks,

paints, paper coatings, pesticides/fungicides, plastics, polyesters and textile fibers (Anderson and wolf, 1995)

Table 1.1 Effects of soybean varieties and growing environments on soybean protein, the protein and total solid contents of soymilk (Min and others, 2005)

Soybean/	**Soy bean**	**Soymilk**	
variety	**Protein %**	**Protein %**	**Total Solids %**
Location ***(Columbus)***			
OH-1	44.3	3.41 ± 0.13^{bc}	6.66 ± 0.12^{b}
OH-2	40.9	3.37 ± 0.04^{b}	6.60 ± 0.09^{b}
OH-3	44.2	3.73 ± 0.08^{d}	6.75 ± 0.06^{b}
OH-4	42.8	3.59 ± 0.05^{cd}	6.67 ± 0.05^{b}
OH-5	38.5	3.01 ± 0.13^{a}	6.30 ± 0.14^{a}
Location ***(Lakeview)***			
OH-1	40.9	3.19 ± 0.02^{b}	$6.80 \pm 0.05d^{e}$
OH-2	38.8	2.91 ± 0.05^{a}	6.56 ± 0.07^{a}
OH-3	39.7	2.97 ± 0.10^{a}	6.68 ± 0.07^{bc}
OH-4	39.9	2.98 ± 0.12^{a}	6.70 ± 0.06^{bc}
OH-5	40.3	3.21 ± 0.06^{b}	6.74 ± 0.01^{cd}

Soybean protein belong to the globulin family of seed storage proteins called leguminins (11S fraction) and vicilins (7S fractions). These fractions are known as glycinin and beta-conglycinin in soybeans. Soybeans also contain biologically active proteins such as enzymes, trypsin inhibitors, hemaglutinins and cysteine proteases (Yagasaki and others, 1997).

The soybean protein could be made available in two main forms i.e. soy protein isolate and soy protein concentrate. Soy protein isolate contains a minimum of 90% highly refined or purified form of soy protein, while the concentrate is about 70% soy protein and it retains most of the original fibers in the soybeans. As previously mentioned, the biological value of soy protein isolate is comparable to animal proteins such as egg, casein etc. although it is lacking in the essential amino acid, methionine which is a sulfur-containg amino acid. The recent approval of soybean protein extract as dietary supplement by the FDA has further increased the demand for soy foods (U.S.FDA 1999).

1.2.1.3 Soy isoflavones

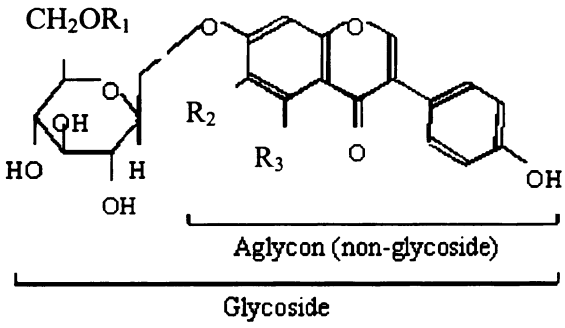
Soybean is rich in phenolic compounds called isoflavones (flavonoids). These isoflavones, which belong to the class of phytoestrogens, occur in reasonably high amount in soybeans and purified soy proteins. The lack of basic knowledge on the bioavailability and metabolism of these isoflavones has resulted into a lot of controversies regarding its health related benefits and adverse effects (Cassidy, 2005). Dietary phytoestrogens are plant derived and have weak and anti-estrogenic properties. Isoflavones are similar structurally to the human estrogens (Tsangalis and others, 2003)

and there are twelve chemical forms found in soybeans and soyfoods with varying concentration in each product (Figure 1.1). The chemical forms include 3 aglycones (daidzein, genistein and glycitein), 3 β-glycosides (daidzin, genistin and glycitin), 3 acetylglucosides (6"-O-acetyldaidzin, 6"-O-acetylgenistin and 6"-O-acetylglycitin) and 3 malonylglucosides (6"-O-malonyldaidzin, 6"-O-malonylgenistin and 6"-O-malonylglycitin (Xu and others, 2002; Preinerstorfer and Sontag, 2004).

Soybeans and non-fermented soy foods contain predominantly the glucoside conjugates of these isoflavones, which make up to 80-95% of the total isoflavone concentration (King and Bignell, 2000). The glucoside conjugates are biologically inactive large molecule and therefore are not easily absorbed directly into the blood system. The aglycone forms are rarely found in soybeans and soy foods unless the foods are fermented (Wang and Murphy, 1994). Fermentation of soybeans has been shown to increase the aglycones and enhance bioavailability of isoflavones (Fukutake and others 1996; Izumi and others 2000; Chien and others 2006). These workers indicated that the aglycones were absorbed faster and in higher amounts than their glycosides in humans.

In vitro fermentation studies have shown that when isoflavone glucosides were incubated with human fecal samples, they were converted into aglycones by the enzyme glucosidase produced by the fecal bacteria. (Hou and others 2000; Hur and others 2000; Setchell and others 2002). These same aglycones were further hydrolyzed into dihydrodaidzein, dihydrogenistein or equols by anaerobic intestinal bacteria. These studies show that biotranformation of isoflavones in the gut are mostly dependent on the type of intestinal microflora, thus suggesting that a change in the microflora could lead to a change in isoflavone bioavailability.

Figure 1.1 Structural formula of Isoflavones (Izumi et al. 2000)



	R1	R2	R3
[Glycoside]			
Daidzin	H	H	H
Glycitin	H	OCH_3	H
Genistin	H	H	OH
6"-0-malonyldaizin	$COCH_2COOH$	H	H
6"-0-malonylglycitin	$COCH_2COOH$	OCH_3	H
6"-0-malonylgenistin	$COCH_2COOH$	H	OH
6"-0-acetyldaizin	$COCH_3$	H	H
6"-0-acetylglycitin	$COCH_3$	OCH_3	H
6"-0-acetylgenistin	$COCH_3$	H	OH
[Non-glycoside]			
Daidzein	H	H	H
Glycitein	H	OCH_3	H
Genistein	H	H	OH

Certain lactobacilli and bifidobacteria are known to hydrolyze β-glucosides, although the actual bacteria responsible for the metabolism in the intestine are not yet known (Choi and others, 1999; Jeon and others, 2002). Chun and others (2007) studied the conversion of isoflavone glucosides to aglycones in soymilk by fermentation with LAB. Their results suggest that the rates of conversion from glycosides to aglycones depend on the species of LAB involved. Since *Bifidobacterium* and *Lactobacillus* are the dominant bacteria in the intestines, several workers have studied the production of the enzyme β-glucosidase by *Bifidobacterium* and its ability to transform isoflavones into bioavailable and bioactive forms (Tsangalis and others, 2002, 2004; Wei and others, 2007). Also the ability of certain commercial probiotic lactic cultures (*Lactobacillus acidophilus* L10, *B. lactis* B94 and *L. casei* L26) to equally produce β-glucosidase and biotransform these isoflavones in soymilk was studied (Donkor and Shah, 2008). Their data showed that all the bacteria produced this enzyme and also hydrolyzed β-glucoside to aglycones in fermented soymilk thereby suggesting an improved biological function of such soymilk.

Previous investigations have also shown that supplementation of soymilk with certain ingredients such as skim milk powder, lactulose, calcium etc, in conjuction with LAB enhanced the biotransformation of glycosides to aglycones (Pham and Shah 2007, 2008; Otieno and Shah 2007). The unprocessed soybeans have about 1.2-4.2mg/g of total isoflavones but this amount can vary due to seed variety, crop year and growth location (Grün and others 2001). During processing either into fermented or unfermented products, some concentrations of the isoflavones are lost and also heat, moisture and β-glucosidases could change isoflavone forms and distribution (Wang and Murphy, 1996;

Simonne and others 2000; Grün and others 2001; Murphy and others 2002). Some of these workers observed that up to 61, 44 and 53% of total isoflavones, were lost in the manufacturing of tempeh, tofu and soy isolate respectively.

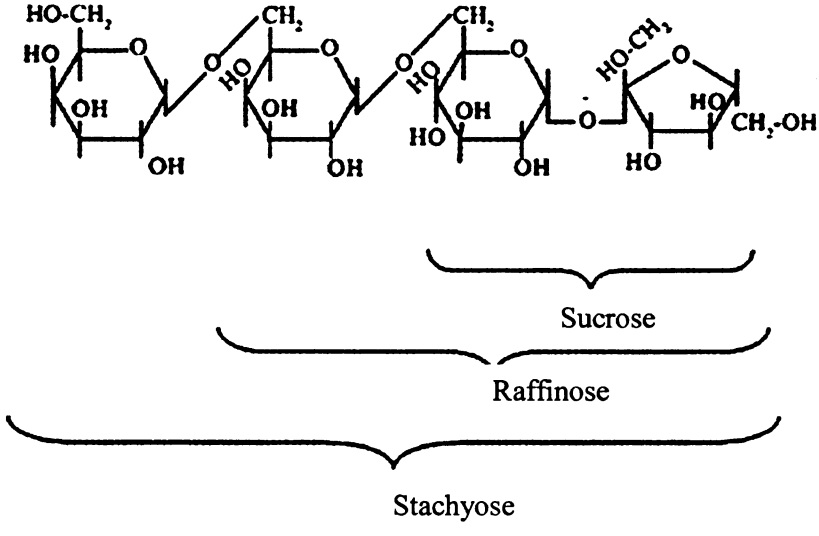
Overall studies have shown that unfermented soy products contain more isoflavones (total), than fermented soy products. Coward and others (1998) reported that individual isoflavones changed their profiles as a result of high heat but the total isoflavone contents still remained the same. According to them, baking and frying release more of malonyl conjugates, moist heat increases β-glucoside conjugate contents, while dry heat leads to an increase in the acetyl conjugates. Conversion to the aglycone form and subsequent loss in total isoflavone concentration occurred when the product was heated excessively during manufacturing. In summary, it can be concluded from these studies that isoflavone content in soy based foods is dependent on the variety of soybeans used, the storage conditions of the raw materials and products and the processing conditions used during manufacturing. More data are still needed to fully understand the chemistry and kinetics of isoflavones reaction in a model food system.

1.2.1.4 Soy oligosaccharides

Oligosaccharides belonging to the raffinose family, α-galactosides or galactooligosaccharides (GOS) are non-digestible carbohydrates that commonly occur in different foods and legumes such as soybeans (Espinosa-Martos and Ruperez, 2006). Soybean carbohydrates (oligosaccharides) are made up of about 30% sucrose, 18% stachyose, 6% raffinose and 22% of other saccharides (fructose, rhamnose, arabinose and glucose) and they are the second largest components in soybeans. The overall properties

of an oligosaccharide are dependent on its chemical structure, molecular weight and contents of contaminating mono- and disaccharides. They are water-soluble and are typically 0.3 to 0.6 times as sweet as sucrose (Espinosa-Martos and Ruperez, 2006). Humans are unable to digest these oligosaccharides because they lack the enzyme α-galactosidase needed to hydrolyze or digest these carbohydrates. The GOS in soybeans are called raffinose and stachyose. Raffinose is a trisaccharide containing galactose linked α- (1-6) to the glucose unit of sucrose while, stachyose is a tetrasaccharide containing a galactose linked α-(1-6) to the terminal galactose unit of raffinose (Figure 1.2). Intact oligosaccharides are preferentially fermented in the colon of the consumer by bifidobacteria resulting in production of gases such as carbon dioxide, hydrogen, methane etc. Other compounds produced as a result of fermentation are short chain fatty acids, which have been associated with prebiotic activity with health benefits (Espinosa-Martos and Ruperez, 2006).

Figure 1.2 The structures of oligosaccharides (Connes and others, 2004)



As a result of the associated health benefits of these non-digestible oligosaccharides (prebiotics), they are now regarded as important functional food ingredient especially in Japan where the Ministry of health and welfare has approved it as functional foods in 1991. Other uses of soybean oligosaccharides include sweeteners (oligosaccharide syrup) since it has about 60% of the sweetness of sucrose with 50% fewer calories. Hence potential applications include beverages, baked goods, confections, ice cream, desserts and health foods.

1.2.1.5 Soybean germination

Seed germination or sprouting is a biological process in which the plant becomes fully enzymatically active. This process is known to offer numerous nutritional and health advantages over non-germinated seeds such as increased nutrient bioavailability, reduction in cooking time and temperature (Khalil and others, 2006; Bau and others, 2000). The fact that soybeans are known to contain large amount of isoflavones compared to other seeds does not guarantee their presence in sufficient amounts in products made from soybeans. Germination of whole soybeans consequently leads to activation of endogenous enzymes of the soybeans resulting to hydrolysis of soy protein, isoflavones, carbohydrates and other molecules, to release bioactive compounds. According to Bau and others, (2000) soybean germination leads to substantial increase in certain biochemical and biologically active components of the beans. Some of the bioactive compounds include lecithin, phytosterols, saponins and oestrogenic compounds which increased in concentration as a result of germination. Alternatively germination leads to decreases in levels of raffinose saccharides (Kuo and others, 1988).

Soybean germination is also known to increase the activity of the enzyme α-galactosidase that is responsible for hydrolyzing significant amount of low molecular weight oligosaccharides primarily, stachyose and raffinose into more digestible carbohydrates such as sucrose and galactose. The inability to digest raffinose and stachyose generally is due to lack of α-galactosidase, which leads to a reduction in metabolizable energy and an increase in flatulence and diarrhea. These oligosaccharides decreased rapidly during germination thus, ultimately leads to reduction of flatulence-causing properties of non-germinated soybean foods and increase the potential uses of soybeans in both developed and developing countries. Studies have also shown that short period of germination can improve the odor and flavor of soy products as a result of reduced lipoxygenase activities thereby increase product acceptability in developed world (Bordingnon and others, 1995). Also germination or sprouting deactivates anti-nutritional factors such as trypsin inhibitors and phytates and transforms soy into a fully enzyme active safe health food (Zhu and others, 2005).

There is limited information on the effect of soybean germination on isoflavone contents and composition. Terrence (1991) and Zhu and others (2005) reported that the total isoflavone concentration in soybeans increased around 24 hours of germination, and then concentration decreased slowly thereafter. The total isoflavone content at varying length of hypocotyls of different varieties of soybean seed during germination is shown in Table 1.2 (Zhu and others, 2005). According to these workers the higher total isoflavones at 0.5 and 2.5mm hypocotyl length could be as a result of increased induction of the metabolic pathways of naringenin chalcone and isoliquiritigenin, which are the precursors of isoflavonoids in legumes.

Table 1.2 Total isoflavone contents in soybean during various stages of germination in mg/g ground seed, dry basis* (Zhu and others, 2005)

	Soybean variety	
Seed type	Hutcheson	Caviness
Dry	2.190^{b}	2.286^{b}
Soaked	2.235^{ab}	2.368^{b}
Germinated (hypocotyl 0.5mm)	2.491^{a}	2.700^{a}
Germinated (hypocotyl 2.5mm)	2.442^{ab}	2.78^{a}
Germinated (hypocotyl 6.5mm)	2.304^{ab}	2.486^{ab}
Nongerminated	2.035^{b}	2.174^{b}

*Values in a column with different superscript letters are significantly different ($p < 0.05$)

Overall, germination significantly increased the total genistein and its β-glucoside conjugates, daidzein and its β-glucoside conjugates but had little effect on glycitein and its β-glucoside conjugates. Apart from isoflavone concentration change during germination, other bioactive compounds such as saponins are found more in germinated soybeans (Jyothi and others, 2007). Soybean protein hydrolysates with antioxidant properties have been produced by germination (Khalil and others, 2006). In general, soybean germination offers a rare opportunity of using the whole soybean seed and this will lead to improved human health, increased soy marketability and decrease in environmental wastes.

1.2.1.6 Soy and health

In 2006, consumer awareness of health benefits of soy products was about 82% (United Soybean Board, 2006). The health promoting effect of soybean consumption was initially thought to be due to the protein content of soy alone, but recent studies have also linked these attributes to the biological activities of a specific group of phenolic compounds known as isoflavonoids (Yamakoshi and others 2000). Soy-based foods provide benefits for the consumer due to their anticarcinogenic effect, prevention of cardiovascular disease, prevention of osteoporosis and reduced allergenicity (Favaro-Trindade and others 2001; Messina and Messina 2000). The effect of lowering cholesterol by soy foods has been attributed to the isoflavone contents of the food although the beneficial effects of individually isolated isoflavones on lipid markers of cardiovascular disease have not been fully established yet (Hall and others 2006). These isoflavones, which belong to the class of phytoestrogens, occur in reasonably high amount in soybeans and purified soy proteins. The lack of basic knowledge on the bioavailability and metabolism of these isoflavones has resulted into a lot of controversies regarding its health related benefits and adverse effects (Cassidy 2005).

People with severe allergies to cow's milk including infants were treated with soy based formulas or products. In addition, soy formulas have been used to treat medical indications such as post diarrhea lactose intolerance, galactosemia and primary lactose deficiency (Badger and others, 2002). Soy has been implicated in cancer prevention in several studies and different workers have shown that early intake of soy could prevent cancer that would have developed later in life e.g. breast, colon or prostrate cancer. Due to concerns about hormone replacement therapy (HRT), many pre- and post menopausal

women are looking for natural alternatives to reduce or erase the symptoms of menopause. Clinical studies have shown the potential roles of soy foods and supplements to combat these adverse effects of menopause. The hypocholesterolemic effects of soy proteins have been documented and extensively reported, hence the FDA has recommended intake of soy protein at 25g per day for health claims in soy foods. This is as result of research by Bakhit and others (1994), where as little as 25g of soy protein were able to lower cholesterol in hypercholesterolemic individuals. Soy meal replacement study suggests that serum cholesterol, LDL-cholesterol, serum triglycerides were significantly reduced as well as the weights of the subjects when compared to casein meal replacement (Anderson and Hoie, 2005).

Isoflavones have effects, which are estrogen receptor-mediated and non-estrogen receptor-mediated (Patisaul and others 2001; An and others 2001; Setchell and Cassidy, 1999) even though they are regarded as weak estrogens. These compounds bind estrogen receptors and function as estrogen agonists, antagonists or selective estrogen receptor modulators. According to these workers, these actions or effects are dependent on tissue or cell types, isoflavone concentration and other conditions like hormonal status, age etc of the individuals. Some studies show that isoflavones play a role in inhibiting angiogenesis, cell differentiation induction, apoptosis and enhancing healthy cardiovascular function including immune function (Scallet and others, 2003). Recent studies have indicated that soy isoflavones indirectly improved cognitive functions in women (Lethaby and others, 2007). Based on the studies on the health benefits of soy proteins and isoflavones, the importance of the synergistic effects of all bioactive components of soy on protection from or prevention of diseases cannot be over looked.

1.2.1.7 Soy products

Different types of soy foods are now available throughout the world but the most common is soy beverage (e.g. soymilk) especially in the developed world. Two major types of soy food exist namely fermented and non-fermented soy foods. Traditional non-fermented soy foods include soymilk, soy sprouts, soy nuts, tofu, soy flour etc. (Golbitz, 1995). Fermented soy foods include tempeh, miso, soy sauces and natto. Increased interest in cow's milk yogurt alternative, soy isoflavones and probiotics has led to soy yogurt formulations (Farnworth and others, 2007; Lee and others, 2000; Drake and Gerard, 2003; Donkor and others 2005).

1.3 PROBIOTICS AND HEALTH

A lot of studies have indicated the potential therapeutic effects of LAB including the probiotics and yogurt. Some of these effects include immunostimulation due primarily to yogurt or LAB-induced changes in the gastrointestinal microecology (Fiander and others, 2005). Probiotic bacteria are defined as "live microorganisms which when administered in adequate amounts confer health benefits to the host" (FAO/WHO, 2002). These probiotics must meet certain requirements viz: (1) have the ability to colonize the host's intestine; (2) have the ability to survive and withstand exposure to low pH and bile acids; (3) have the ability to adhere to intestinal epithelium; (4) be nonpathogenic and nontoxic; (5) host must be able to benefit from it; (6) must be human-specific organisms (except, veterinary probiotics); (7) must be stable during storage (Isolauri and others 2001). Research over the past two decades has shown that probiotic

administration could be used to alleviate gut diseases and also prevent and treat other forms of diseases such as allergies and immune related diseases (Gill and Guarner, 2004).

Research findings from experimental animals and mostly short-term human studies have shown that probiotics such as lactobacilli and bifidobacteria have the ability to modulate a host's immunity and thus making fermented dairy products with probiotics very popular due to their health benefits (Shah 2006). Consumption of yogurt or lactic acid bacteria modulates the production of cytokines that play different roles in regulating immune functions. For example, the use of fermented foods and cultured milk products containing live microbes has been in existence a long time ago and is believed recently, to offer a possible means of controlling allergies. The observation that frequent consumption of sour milk containing *Lactobacillus bulgaricus* increased health and longevity was only made in the 20th century by Metchnikoff, who claimed that the intake of yogurt decreases the toxic effect of the putrefactive bacteria in the colon by decreasing their growth (Metchnikoff, 1907; Wollowski et al. 2001). Probiotics have shown some potential in thwarting the food-borne infections such as salmonella and *E. coli.* The antimicrobial effects of probiotics have extensively been studied and their effects on the microflora of the gut cannot be overemphasized. Some probiotics produce short chain fatty acids, which contribute to the low pH of the colon, thus inhibiting the growth of pathogenic microorganisms and favoring the growth of the less virulent microorganisms (Rolfe 2000). Several workers have shown that the health benefits of these probiotics are dose dependent.

Researches have shown that generally probiotics do not grow very well in cow's milk, therefore in yogurt samples these microorganisms do not attain high numbers

unlike the starter cultures (Champagne and others, 2005; Sodini and others, 2002). However several studies have shown that soy could be a good substrate for probiotic growth (Mital and others 1974; Nsofor and others 1992; Scalabrini and others 1998), although they indicated poor growth for traditional yogurt cultures in soy. These studies suggest that some selected probiotics could compete in the same soy-based substrate as yogurt cultures, even though limited information is available on the growth of probiotics in mixed cultures with yogurt starters in soy substrates. The reason is that most studies are based on the growth of pure cultures on soy substrates or extracts (Kamaly, 1997; Hou and others 2000; Desai and others, 2002). Farnworth and others (2007) studied the growth of *Lactobacillus* sp and bifidobacteria in a soy yogurt formulation. Their data suggest that probiotic bacteria and the bifidobacteria were using different sugars for metabolism when grown in cow's milk or soymilk. Previous studies on the growth and metabolism of selected strains of probiotic bacteria in milk also emphasized the importance of fermentation time since probiotic strains produced different amounts of metabolic products at various fermentation times (Østlie and others, 2003). Possible interactions between starter cultures and probiotics in fermentation of dairy product should be taken into account when such products are being manufactured since studies have shown that probiotics are more inhibitory to LAB than vice versa (Vinderola and others, 2002).

Recent clinical studies have shown that consumption of probiotic drink containing *L. casei*, *L. bulgaricus* and *S. thermophilus* could decrease the incidence of antibiotic associated diarrhea in adults (Hickson and others, 2007). Some workers suggest that traditional yogurt cultures should be considered as probiotics since these cultures are able

to eliminate symptoms of lactose intolerance hence improve digestion which is a health benefit (Guarner and others, 2005). Questions still exist on whether all yogurt cultures can exert these health benefits and therefore may require further research. Several factors that affect the effectiveness of probiotics include inclusion of other potentially bioactive ingredients, the use of probiotic blends, mixing probiotics and prebiotics, growth and preservation methods for the probiotic strain(s), concentration of probiotics or prebiotics used, and method of delivery of probiotics to the consumer (Sanders and others, 2005). The assertion of these workers is that the mode of delivery of probiotics to humans influences the target site of the intended product for example using enteric-coated capsules may be more protective to stomach acid although the biological activity of the cultures might be unknown. Acid, bile and heat tolerance of eight free and microencapsulated strains of probiotic bacteria were studied by Ding and Shah (2007). Their data suggest that microencapsulated probiotic bacteria survived better than free bacteria in acidic environment, bile salts, and thirty minutes of heat treatment (65° C). When the cultures were exposed up to one hour of heat treatment, both the free and microencapsulated probiotic strains had equal viability losses.

Earlier work by Sheil and others (2004) indicated that oral route for probiotic delivery might not be essential for anti-inflammatory effects and also concluded that responses of probiotics are not disease specific. These workers injected *L.salivarius* 118 subcutaneously into IL-10 KO mice in order to attenuate colitis and suppress collagen-induced arthritis. There was significant decrease in colonic inflammatory scores compared to control animals.

In summary, since human health issues are very difficult to study directly, different end points are usually utilized e.g. blood cholesterol is used is an indicator of heart disease risk. Another burning issue is that not much is known about the mechanisms by which these probiotics exert their health benefits and that leads to a lot of general assumptions that can be misleading. Some doubts still exist on the safety of microbial food supplements thus more work is needed on safety evaluation. Cases of bacterial translocation and bacteraemia and sepsis have been reported but are rare (Isolauri and others, 2004). The potential mechanisms of probiotic intervention are shown in Table 1.3.

Currently, the Food and Drug Administration (FDA) has not established a formal regulatory category for functional foods including probiotic cultures (Teitelbaum and Walker, 2002). More attention is called for to ensure the viability of the cultures, prior to ingestion. Among the most promising factor that could assure culture viability especially during the storage of probiotic foods (shelf life) is presence of prebiotics in the food substrate. A prebiotic is defined as "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" (Roberfroid, 2000). The main prebiotic with sufficient scientic data to show for its efficacy in terms of functional food is inulin. Most European countries have officially recognized chicory inulin and oligofructose as natural food ingredient, while in the United States, these compounds have a self-affirmed safe status (Roberfroid, 2000, 2002).

Table 1.3 Potential clinic targets of probiotic intervention (Isolauri and others 2004)

Effect	Potential mechanism	Potential risks
Nutritional management of acute diarrhea	Reduction in the duration of rotavirus shedding, normalization of gut permeability and microbiota	Risk related to host and strain characteristics
Nutritional management of allergic disease inflammatory bowel disease	Degradation/structural modification of enteral antigens, normalization of the properties of aberrant indigenous microbiota & of gut barrier functions, local & systemic inflammatory response, increase in the expression mucins	Strains with proinflammatory effects/adverse effects on innate immunity. Translocation/ infection
Reducing the risk of infectious disease	Increase in IgA-secreting cells against rotavirus, the expression of mucins	Risk related to host & strain characteristics
Reducing the risk of allergic/inflammatory disease	Promotion of gut barrier functions, anti-inflammatory potential, regulation of the secretion of inflammatory mediators & promotion of development of the immune system	Directing the microbiota towards other adverse outcomes/ directing the immune responder type to other adverse outcomes

Prebiotics are food sources that are chosen preferentially by the probotics and the body does not produce digestive enzymes to hydrolyze it, therefore they serve as sources of fiber or bulk (Lim and others, 2005). The mechanisms for the beneficial effects of prebiotics have been speculated to include change in the activity of exogenous carcinogens by modifying metabolic activities and detoxification. Also, prebiotics have the ability to stimulate the production of butyrate (a short-chain fatty acid), and ability to stimulate certain cytokines in order to modify immune response (Schley and Field, 2002; Lim and others, 2005). Some prebiotics are considered normal constituents of the diet therefore prudent selection of such diets would be beneficial to health (Cummings and Macfarlane, 2002).

Despite all the above health benefits of soy products, a lot of inconsistencies and controversies still abound. Epidemiological data suggest that population that consume soy regularly over a long period of time, have lower incidence of the chronic diseases earlier mentioned. The need to understand the role of each bioactive component of soy cannot be overemphasized in order to appreciate their roles in health care.

CHAPTER 2

GROWTH AND ACTIVITY OF LACTIC ACID BACTERIA AND A PROBIOTIC IN RECONSTITUTED GERMINATED WHOLE SOY POWDER (GSP), NON-GERMINATED WHOLE SOY POWDER (NGSP) AND NON-FAT DRYMILK (NFDM) + GSP OR NGSP

2.1 ABSTRACT

There is an increased awareness in the role of dietary supplements and nutraceuticals (bioactive compounds) as regulatory compounds with hormone-like functions in the human body. Some natural foods such as cow's milk, soymilk and their derivatives could be made biologically active via fermentation or enzyme treatment. Health benefits of soybeans have been attributed to bioactive compounds in the beans. Incorporation of these compounds into foods such as yogurt will enhance health benefits. The objective of this study was to evaluate the growth and activity of three lactic acid bacteria (LAB) in blends of reconstituted non-fat dry milk (NFDM) and germinated (GSP) or non-germinated (NGSP) whole soy powder obtained from three soybean varieties (Vinton 81, DF 222 and E05276-T. Growth (CFU/ml), changes in pH and titratable acidity (%TA) of *Streptococcus salivarius* subsp. *thermophilus* (St 133), *Lactobacillus delbruekii* subsp. *bulgaricus* (Lr 78) and *Lactobacillus acidophilus* (La NCFM) grown in 12% reconstituted NFDM, GSP, NGSP and blends (3:7, 1:1, 7:3) of reconstituted NFDM and soy powder were investigated. All the bacterial strains exhibited comparable growth and acid production in all milk substrates. There was a

significant interaction ($p \leq .001$) between the milk blends, soybean variety and culture type, although the blends i.e. NFDM/soy powder was the most significant factor ($p \leq 0.001$) that affected the activities and growth of LAB strains. Overall, cultures grown in most milk blends that contained whole GSP or NGSP gave the lowest pH, highest %TA and growth (CFU/ml) values. However, highest culture activity and growth were obtained when the cultures were grown in 1:1 NFDM+GSP orNGSP blend. Cultures grown in GSP blends produced more acid and growth ($p \leq .001$) than its NGSP counterparts. The significance of this study is that partial substitution of cow's milk with germinated ornon-germinated whole soy powder increased growth and activities of LAB and probiotic that might lead to increase in bioactive compounds to enhance health benefits of the consumers.

2.2 INTRODUCTION

Some of the probiotic LAB belonging to bifidobacteria and lactobacilli have been introduced into food products for human consumption so as to enhance health benefits. Consumption of living bacteria such as *Lactobacillus rhamnosus* GG, *Lactobacillus casei* Shirota, *Lactobacillus acidophilus* NCFB 1748, NCFM, LA5, or *Lactobacillus johnsonii* LA1 were shown to positively affect the health of the consumers (Fonden and others, 2000). A minimum number of viable bacteria are required to exert positive health benefits after consumption. Several studies done have shown that a daily consumption of fermented dairy products (about 100g), should contain between 10^6 CFU/g to 10^9 CFU/g of these cultures (Vanderhoof and Young, 1998; Donnet-Hughes and others, 1999). Unfortunately, this is not the case with some commercially available products (Rybka

and Fleet, 1997). The explanation to this anomaly is that probiotics grow very slowly in these commercial products because they cannot compete with the traditional starter cultures. Also, it is observed that the probiotics are unstable during storage. A 3-log cycle decrease was reported for *L. johnsonii* (LA 1) in fermented milk, after only two weeks of storage at 4° C hence the need to modify the milk base so as to support probiotic growth.

Milk is considered to be an unsuitable substrate for growing probiotics in the presence of other lactic acid bacteria because it does not contain adequate amounts of amino acids and lower molecular weight peptides (Shah, 2006). Based on the poor growth of probiotic bacteria in the presence of these starter cultures, studies are being done to improve their growth on milk bases. Nitrogenous compounds, oxygen scavengers, oligosacchrides, sugars from different sources, milk protein concentrates and casein hydrolysates have all been used in different studies to maintain growth of probiotics (Dave and Shah, 1996; Shah 2000; Shin and others, 2000; Sodini and others 2002). The results from these studies indicate that there was better survival and stability of these probiotics.

Soymilk has been reported to be an adequate medium for growth and metabolism of lactic acid bacteria (Angeles and Marth, 1971). Several amino acids such as arginine, isoleucine and glutamic acids are low in milk but are higher in soy protein isolate, therefore probiotic cultures are expected to grow abundantly in milk supplemented with soy protein isolate (Gomes and others, 1998). The results obtained by Pham and Shah (2008) show that fermentation of reconstituted skim milk supplemented with soy protein isolate enhanced lactose utilization and acetic acid production but reduced lactic acid production and viable microbial population by the probiotics utilized. The growth rates

and changes in pH of two strains of bifidobateria namely *Bifidobacterium longum* and *B. bifidum* were investigated in reconstituted skimmed milk, soy milk and modified MRS broth (Kamaly, 1997). His data showed that the bifidobacterial strains had more proteolytic activity in soymilk than in reconstituted skimmed milk but growth and pH changes were more in reconstituted skimmed milk than in soymilk. This study also indicated that enrichment of soymilk with lactose, galactose, glucose, yeast extract, proteose peptone, casitone (pancreatic digest of casein), polypeptone and phytone highly stimulated the growth and acid production by *B. bifidum*. The objective of this study therefore, is to investigate the suitability of germinated and non-germinated whole soy powder blends with NFDM as substrates for growth and acid development by yogurt starter cultures and *L. acidophilus* NCFM.

2.3 MATERIALS AND METHODS

2.3.1 Materials

2.3.1.1 Cultures

Streptococcus salivarius subsp. *thermophilus* (St-133), *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lr-78), from System Bio-Industries (Waukesha, WI) and *Lactobacillus acidophilus* (La NCFM) from Danisco (formerly Rhodia, Madison Wisconsin) were used. The first two organisms are the traditional lactic acid bacteria used in yogurt manufacturing for acid production and flavor, while the third organism was selected based on the information strongly supporting its probiotic capabilities (Sanders and Klaenhammer, 2001; Barrangou and others, 2003).

2.3.1.2 Soybean

Soybean varieties utilized in this study were Vinton 81, E05276-T and DF 222. Vinton 81 and DF 222 soybean varieties were procured from Michigan Crop Improvement Association (Lansing, MI). Vinton 81 variety was chosen because it is locally grown and documented evidence shows uniformity of genetic traits, seed purification, increased growth yield, competitive growth advantages over some other varieties such as high protein content, disease resistance and performance. A newly developed variety E05276-T from the Department of Soil and Crop Sciences, Michigan State University was also used. This is a high yielding variety with high protein content similar to Vinton 81 and low fat content. DF 222 variety is also grown locally and naturally high in protein content and functionality.

2.3.2 Germinated and non-germinated whole soy powder preparation

An optimized germination process was utilized (patent #US 7,067,163 B2). Five kilograms of each soybean variety were washed twice in tap water before soaking. The soak water was acidified with citric acid to about pH 4.8 and the beans were steeped for 14 to16 hours at 24 to 28°C. The steeped water was drained and the hydrated beans incubated (germinated) for 18 to 24 hours at 24 to 28°C. At the end of incubation period, the soybeans were de-acidified with five times volume/weight water at 45°C for one hour. The beans were mechanically wet dehulled and milled. The slurry was gelatinized at 92°C for 15 minutes and cooled to 60°C before homogenization at 3,000psi (Homogenizer-200, Cherry Burrell Corp. Chicago, IL) and 12,000psi (Rannie 12.56 VH Homogenizer, APV Americas, Willington, MA). The homogenized whole soymilk was

spray dried at an inlet temperature of 400°F and outlet temperature of 160°F and stored in the cold room prior to further studies (Figures 2.1 to 2.7).

The non-germinated whole soybean preparation was similar to the germinated process except that the soybeans were not allowed to germinate after steeping in acidified water for about 16 hours.

Figure 2.1. Schematic diagram germinated soy powder (GSP) preparation (Patent#US7, 067,163 B2)

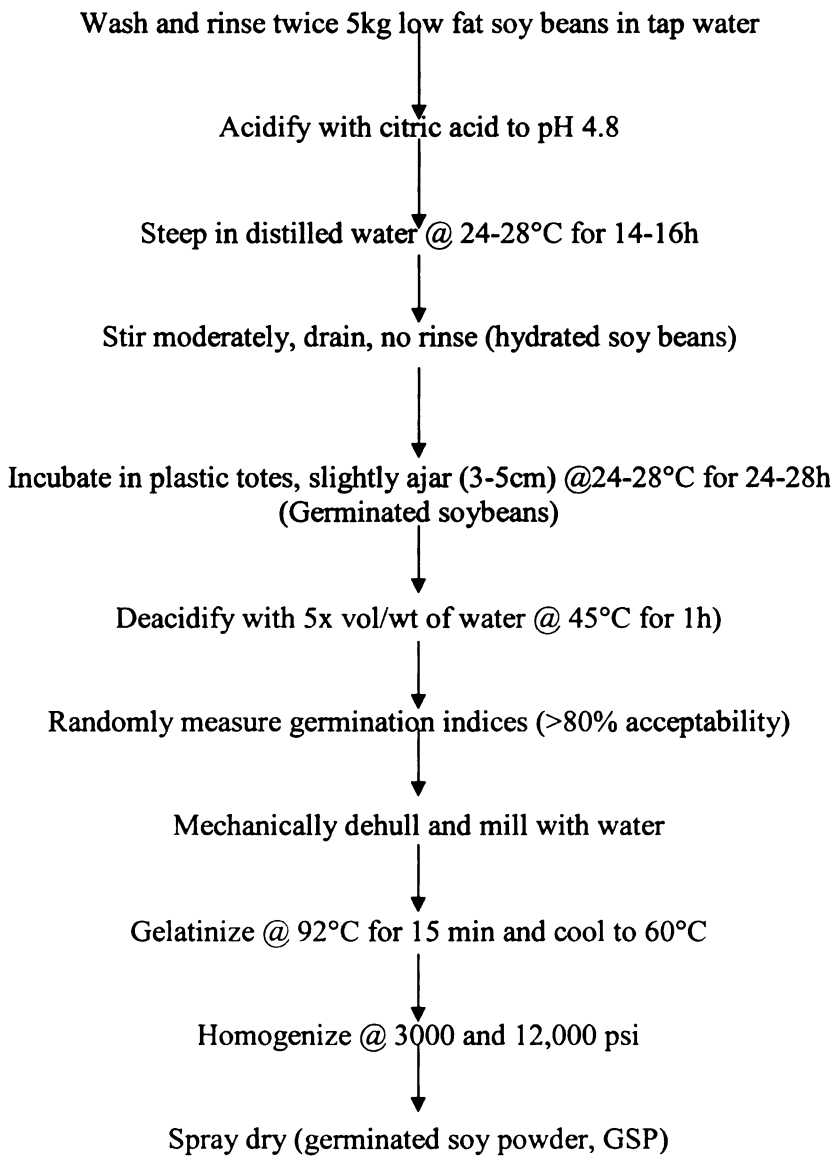


Figure 2.2 Germinated soybeans after steeping in acidified water

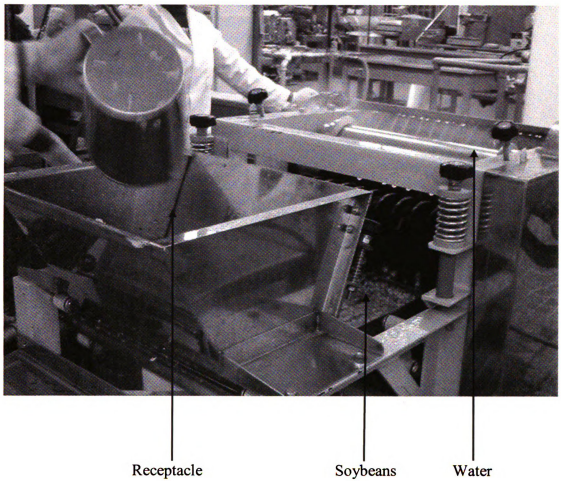


Figure 2.3 Wet dehulling process (A wet-type model BB soybean dehuller, BAR, N.A, Inc., Seymour IL

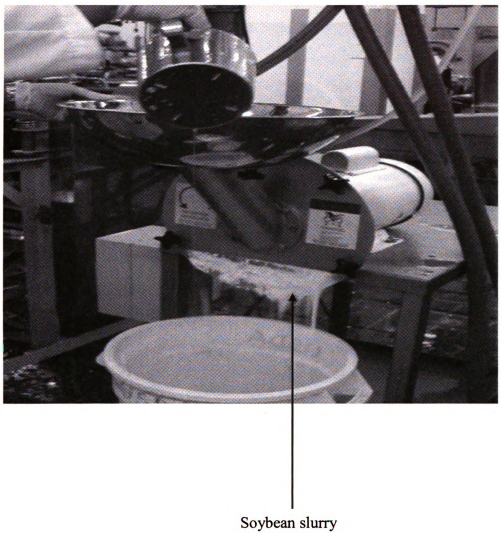


Figure 2.4 Wet milling process (Model 150 BMI Stainless Steel Mill, BAR, N.A. Inc., Seymour, IL

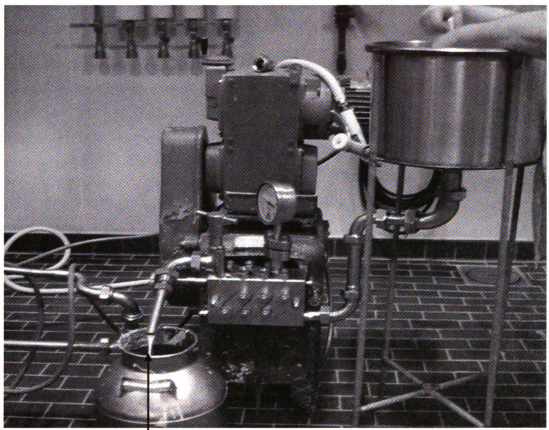


Figure 2.5 First stage homogenization process at 3,000psi (Homogenizer-200, Cherry Burrel Corp. Chicago, IL

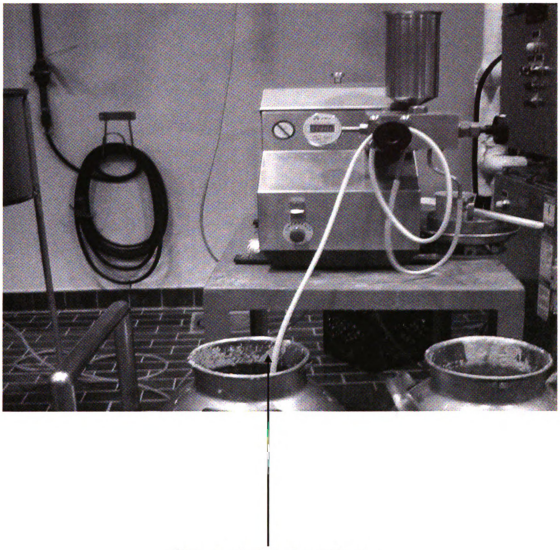


Figure 2.6 Second stage homogenization at 12,000psi (Rannie 12.56 VH Homogenizer, APV Americas, Willington, MA

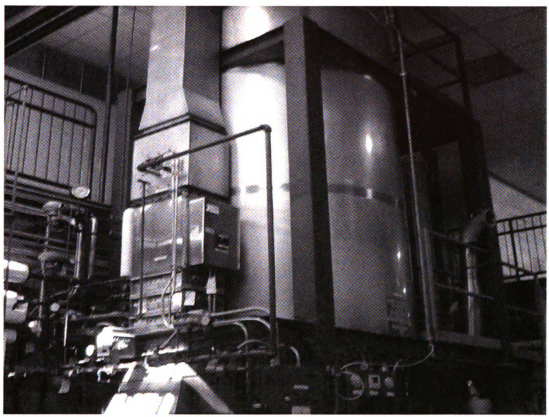


Figure 2.7 Spray dryer

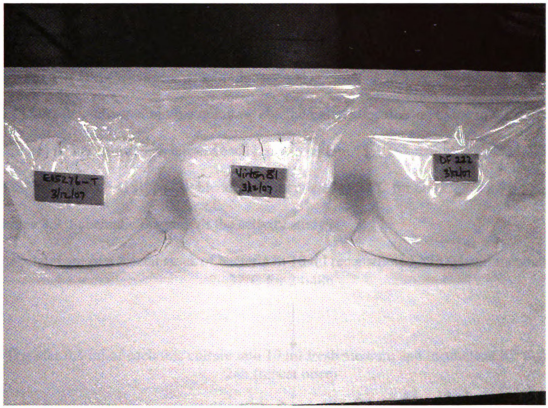
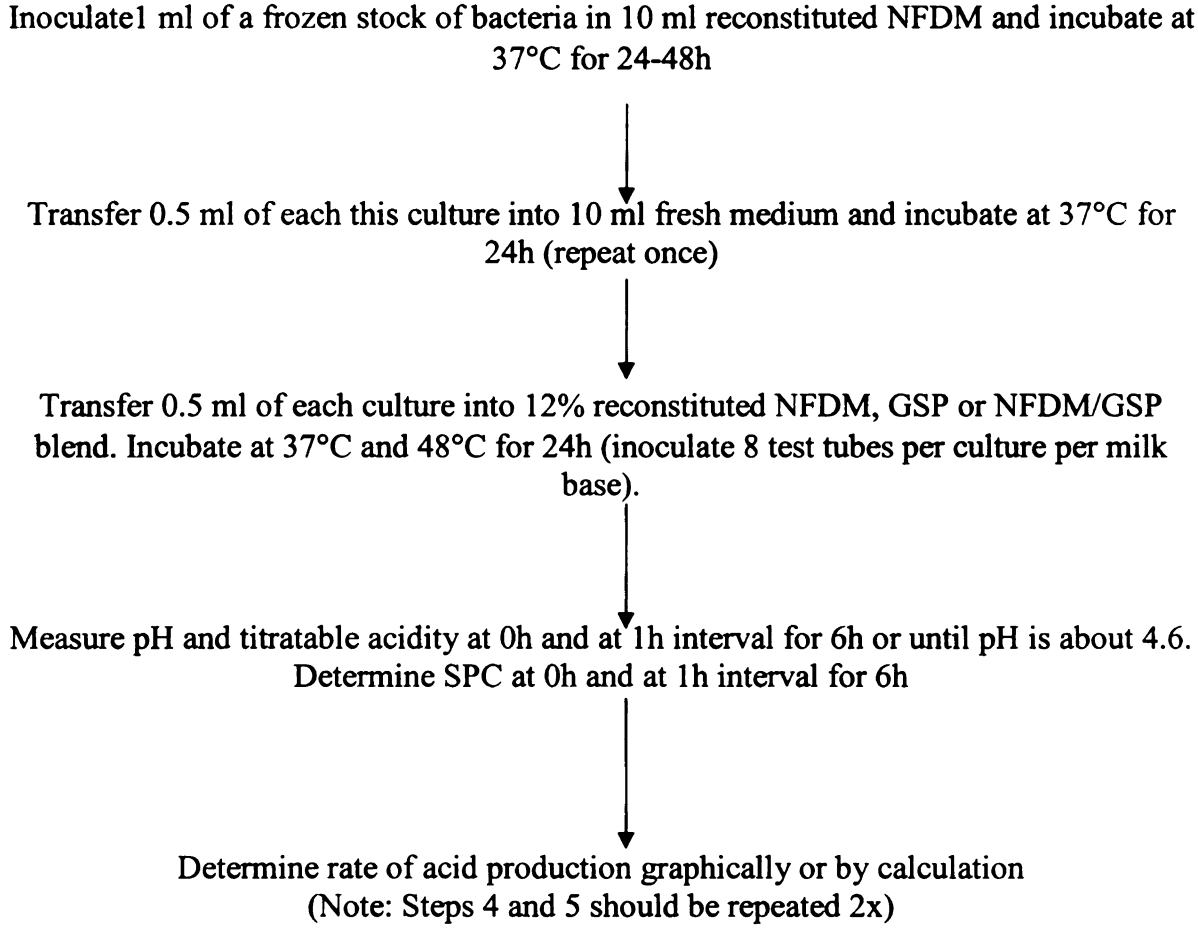


Figure 2.8 Samples of spray dried whole soy powders

2.3.3 Growth and activity evaluation

Conventional yogurt cultures, namely *Lactobacillus delbreuckii* subsp. *bulgaricus* (Lr 78) and *Streptococcus salivarius* subsp. *thermophilus* (St 133), and a probiotic, *Lactobacillus acidophilus* (La NCFM), were used. Frozen stock cultures (-80°C) of the LAB were separately inoculated into pasteurized (80 °C for 30 min.) 12% reconstituted high-heat NFDM (Michigan Milk Producers Association, Ovid MI), and incubated at 37°C for 24h. Activated mother cultures (obtained after subculturing twice in the same medium) at 5% culture levels were each inoculated into 12% reconstituted NFDM, GSP, NGSP or NFDM + GSP/NGSP blends, and incubated at 37°C for 6h (Figure 2.9).

Figure 2.9 Schematic diagram of the activity study



The following blends (ratios) were utilized:

GSP/NGSP	NFDM
1	0 (control)
0.7	0.3
0.5	0.5
0.3	0.7
0	1 (control)

The pH and titratable acidity (%TA) were measured at 0h and every hour for 6h for each sample. Change in pH for each sample was determined by subtracting the final pH value (at 6h) from the initial pH (at 0h). Simultaneously, 9ml of each sample was titrated against 0.1N sodium hydroxide using phenolphthalein as an indicator to determine %TA. Standard plate count (SPC) was done, reported as CFU/ml at 0h and up to 6h incubation. One milliliter of each sample was diluted in 99ml of sterile 0.1% w/v bacto-peptone (Difco Laboratories, Detroit, MI) to determine viable counts of the LAB strains. The cultures were plated on De Man, Rogosa, Sharpe (MRS) agar (Difco Laboratories, Detroit, MI) and incubated at 37°C for 24 to 48h. The colonies were counted using a Quebec colony counter (Fisher Scientific, Pittsburgh, PA).

2.3.4 Statistical analysis

Each of the treatments was independently replicated 2 times in a randomized block experiment. All the data were statistically analyzed using Sigma Stat 3.1 (Jandel Scientific, San Rafael, CA) and Tukey's test was used for multiple comparisons where $p < 0.05$ was considered statistically significant.

2.4 RESULTS AND DISCUSSION

There was a statistically significant interaction between the NFDM and soy powder blends, soy powder variety and culture types. Table 2.1 shows the analysis of variance (ANOVA) for the independent variables (main effects) i.e. medium blends i.e. milk, soy powder ratio (1:0; 0.7:0.3; 0.5:0.5; 0.3:0.7: 0:1), bean variety (Vinton 81, DF 222, E05276-T) and culture types (Lr 78, St 133, La NCFM), their two-way and three-way interactions on final pH (i.e. after 6h incubation).

The most significant factor according to the F-value obtained in a 3-way ANOVA that affected the activities and growth of the LAB strains is the blends (i.e. milk ratios; F-value = 41023.820) at p-value $\leq$ 0.001. This was followed by the culture variety (F-value = 4941.238) at p-value $\leq$ 0.001, and bean variety (F-value = 2364.470) at p-value $\leq$ 0.001. The most statistically significant interaction was between the blend and culture variety (F-value = 10165.008) at p-value $\leq$ 0.001.

Table 2.2 and Figures 2.1, 2.2, and 2.3 show the change in pH of *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lr 78), *Streptococcus salivarius* subsp. *thermophilus* (St 133) and *Lactobacillus acidophilus* (La NCFM) in the different medium blends after 6h incubation.

Table 2.1 Analysis of variance for the main factors (independent variables) and interactions on pH of lactic acid bacteria and *L. acidophilus* NCFM after 6h of incubation

Main factors	DF	SS	MS	F	P
[1]Medium (milk) blend	4	19.801	4.950	41023.820	<0.001
[2]Soy powder	4	1.141	0.285	2364.470	<0.001
[3]Culture	2	1.192	0.596	4941.238	<0.001
Medium blend x Soy powder	16	1.388	0.0867	718.862	<0.001
Medium blend x Culture	8	9.813	1.227	10165.008	<0.001
Soybean powder x Culture	8	0.267	0.0333	276.300	<0.001
Medium blend x Soybean powder x Culture	32	1.308	0.0409	338.822	<0.001
Residual	75	0.00905	0.000121		
Total	149	34.919	0.234		

[1]Medium blend = NFDM/soy powder ratios

[2]Soy powder = germinated and non-germinated Vinton 81, DF 222 and E05276-T

[3]Culture = *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lr 78), *Streptococcus salivarius* subsp. *thermophilus* (St 133) and *Lactobacillus acidophilus* (La NCFM)

It is evident that the cultures grown in all the media that contained soy powder and NFDM gave the lowest pH values except the control that contained only soy powder. However the highest activity and growth were obtained when they were grown in the 50:50 NFDM + GSP or NGSP blends (e.g. pH = 4.90 while pH of 0 soy: 100 NFDM = 5.97) irrespective of the cultures or bean variety. This observation was similar to the study by Pham and Shah (2008) when they fortified reconstituted skim milk with soy protein isolate. Their study showed that addition of soy protein isolate in the medium enhanced acid production and growth of probiotic cultures. This could be as a result of more bioavailable sugars and peptides in the blend for the microorganisms to metabolize.

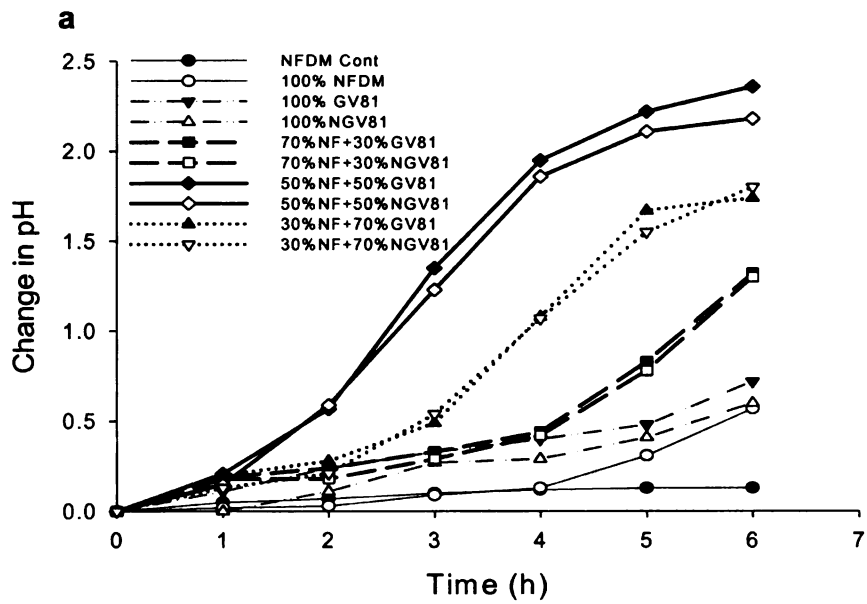
The results also showed that the cultures grown in GSP blends significantly ($p \leq 0.001$) produced more acid overall than in its NGSP counterparts (e.g. pH for germinated Vinton 81 = 5.41; pH for non-germinated Vinton 81 =5.51) (Table 2.3). Since the non-germinated soybeans were soaked for a significant amount of time before processing into powder, it was expected that the endogenous enzymes might have contributed to the release of bioavailable nutrients for the growth of the LAB. The lower pH values observed with the germinated samples is in agreement with previous study on germinated soybean and its effect on fermentation (Ariahu and others 1999). Sodini and others (2002) made similar observation in a preliminary study on the effect of enriching milk base with casein hydrolysate for culture growth. They reported that fermentation time was reduced in some strains due to this effect. Other workers also discovered that the change in pH was more in milk supplemented with nutrients such as tryptone than in non-supplemented milk (Østlie and others, 2003).

Table 2.2 Differences in pH, % titratable acidity and growth in blends of reconstituted 12% non-fat dry milk (NFDM) and germinated or non-germinated soy powder after 6h incubation.

Blends (ratios)	pH	%TA	Growth (CFU/ml) $x10^8$
0.0 Soy: 1.0 NFDM	5.97^{e*}	0.41^{e}	1.89^{d}
0.3 Soy: 0.7 NFDM	5.68^{d}	0.68^{a}	1.97^{cd}
0.5 Soy: 0.5 NFDM	4.90^{a}	0.60^{b}	2.81^{a}
0.7 Soy: 0.3 NFDM	5.29^{b}	0.58^{c}	2.21^{b}
1.0 Soy: 0.0 NFDM	5.57^{c}	0.47^{d}	2.04^{c}

* Different letters column wise denote significant difference at $p < 0.001$;$n = 2$

Figure 2.10 Change in pH of *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lr 78) in reconstituted non-fat dry milk and germinated/non-germinated (a) Vinton 81 (b) DF 222 (c) E05276-T



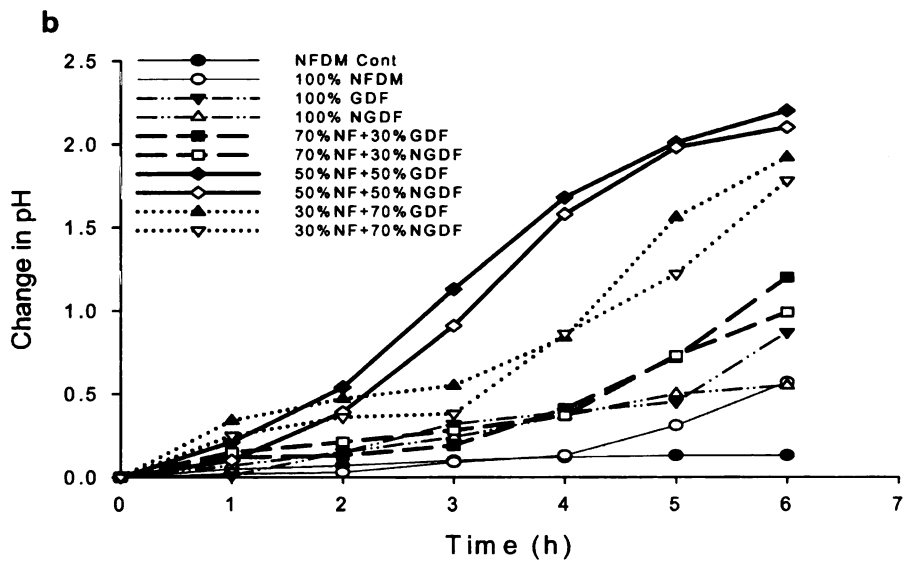


Figure 2.10 continued.

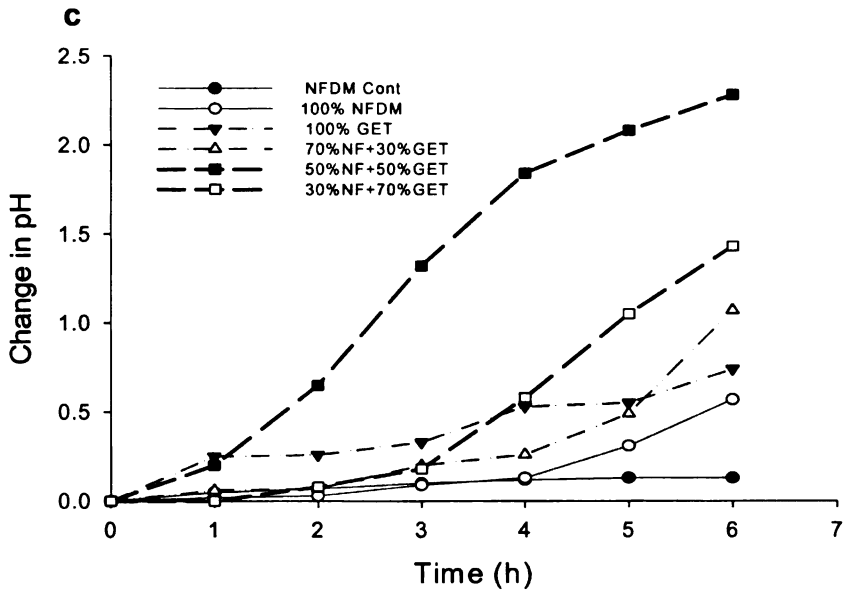
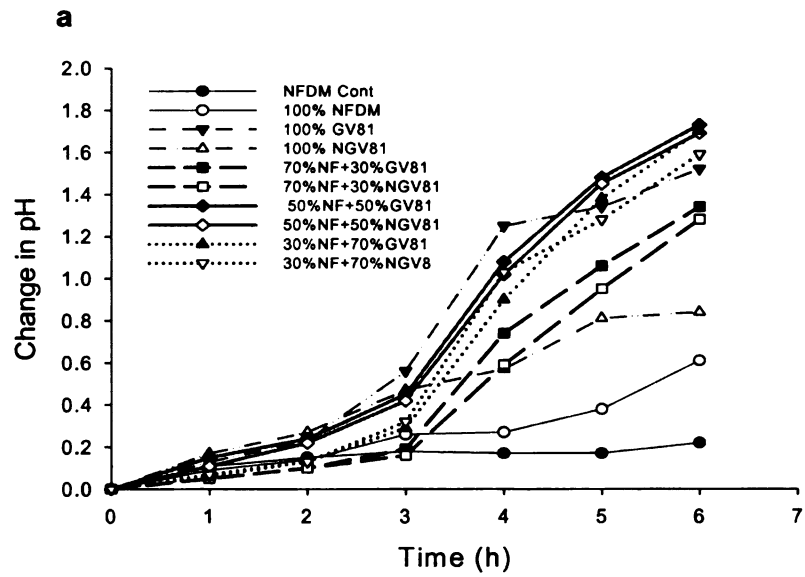


Figure 2.11 Change in pH of *Streptococcus salivarius* subsp. *thermophilus* (St 133) in reconstituted non-fat dry milk and germinated/non-germinated (a) Vinton 81 (b) DF 222 (c) E05276-T



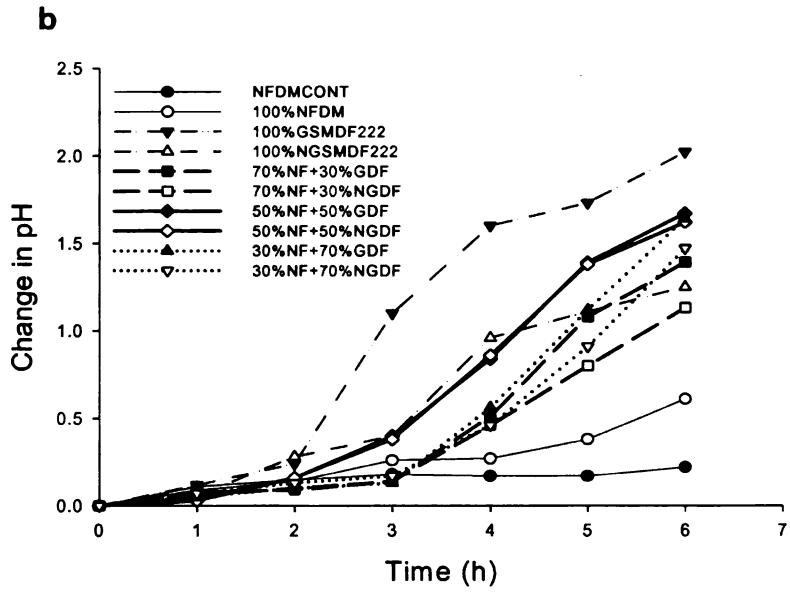


Figure 2.11 continued.

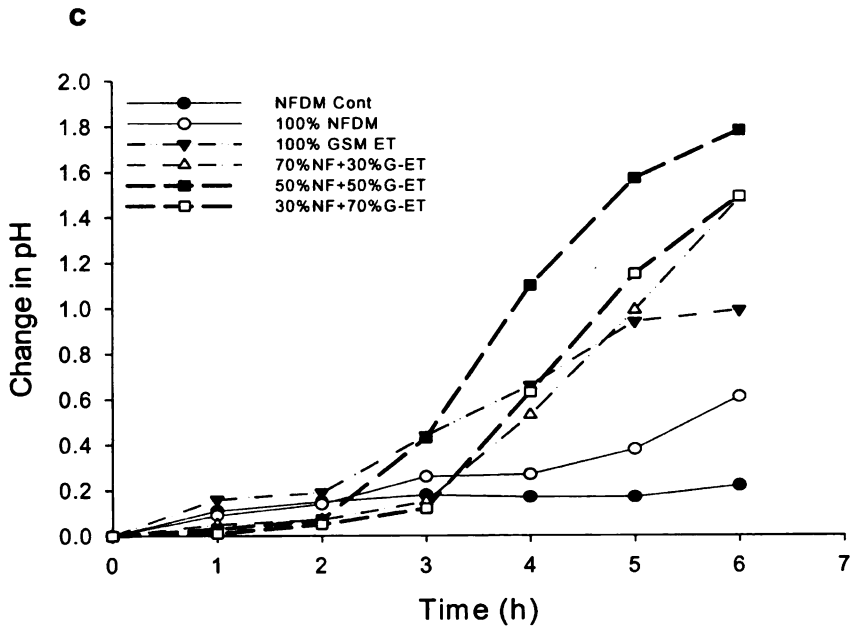
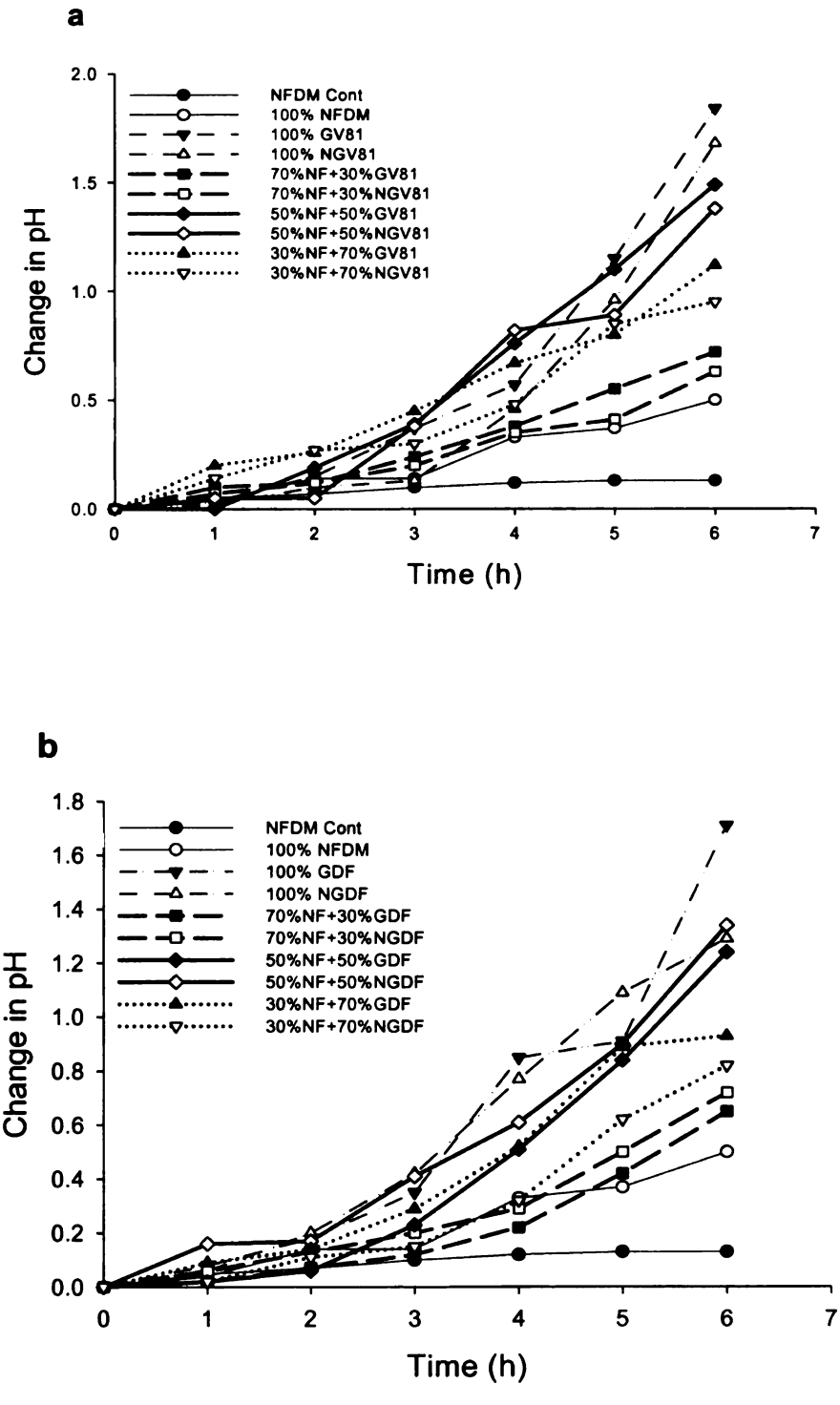


Figure 2.12 Change in pH of *Lactobacillus acidophilus* (La NCFM)) in reconstituted non-fat dry milk and germinated/non-germinated (a) Vinton 81 (b) DF 222 (c) E05276-T



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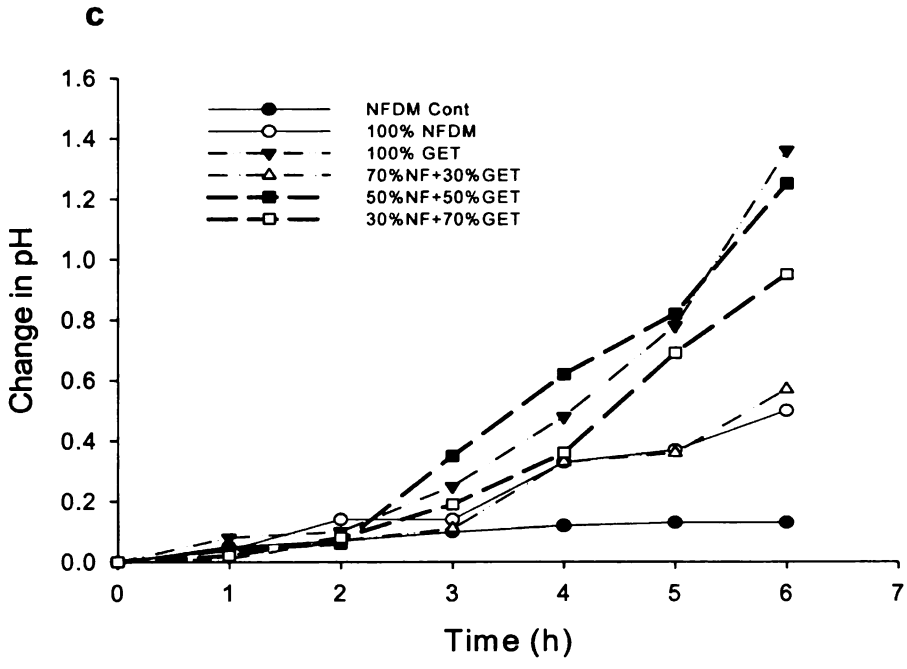


Table 2.3 Difference in pH of cultures between germinated and non-germinated soybean varieties

Soybean Variety	Germinated	Non-germinated
DF 222	5.38^{a**}	5.62^{c}
Vinton 81	5.41^{a}	5.51^{b}
E05276-T	5.52^{b}	NA*

* =Not Available

** = Different letters denote significant difference at $p \leq 0.001$.

Table 2.4 shows the analysis of variance (ANOVA) for the main effects i.e. medium blends, soy powder varieties (Vinton 81, DF 222, E05276-T) and culture types (Lr 78, St 133, La NCFM), their two-way and three-way interactions on final titratable acidity i.e. %TA after 6 hours of incubation. The F-values showed that medium blend is the most significant factor among all the independent variables (F = 8663.034) at p-value ≤ 0.001. Similarly, the most statistically significant interaction was between the medium blend and culture variety with an F-value of 7592.552 ($p \leq 0.001$). This trend is similar to the results observed with pH results. According to the data obtained, the cultures grown in medium blends gave the highest %TA (Table 2.4), however the 0.5:0.5 and 0.3:0.7 NFDM + GSP/NGSP blends produced the highest %TA (0.600 and 0.675 respectively). Meanwhile, the sample with 0 soy: 1.0 NFDM had the lowest %TA of 0.410.

Table 2.4 Analysis of variance for the main factors (independent variables) and interactions on titratable acidity (%TA) of lactic acid bacteria and *L. acidophilus* NCFM after 6h of incubation

Main factors	DF	SS	MS	F	P
[1]Medium Blend	4	1.340	0.335	8663.034	<0.001
[2]Soy powder	4	0.0802	0.0200	518.422	<0.001
[3]Culture	2	1.177	0.0886	2292.207	<0.001
Medium Blend x Soy powder	16	0.0891	0.00557	144.047	<0.001
Medium Blend x Culture	8	2.349	0.294	7592.552	<0.001
Soy powder x Culture	8	0.0218	0.00273	70.569	<0.001
Medium Blend x Soy powder x Culture	32	0.0588	0.00184	47.530	<0.001
Residual	75	0.00290	0.000039		
Total	149	4.119	0.0276		

[1]Medium blend = NFDM/soy powder ratios

[2]Soy powder = germinated and non-germinated Vinton 81, DF 222 and E05276-T

[3]Culture = *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lr 78), *Streptococcus salivarius* subsp. *thermophilus* (St 133) and *Lactobacillus acidophilus* (La NCFM)

Figures 2.13, 2.14, and 2.15 show the rate of acid production (%TA) of *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lr 78), *Streptococcus salivarius* subsp. *thermophilus* (St 133) and *Lactobacillus acidophilus* (La NCFM) in the medium blends. According to the results obtained, cultures grown in most of the blends that contained soy powder gave the highest TA values. However highest activity and growth were obtained when they were grown in the 0.5:0.5 NFDM + GSP/NGSP and 0.7:0.3 NFDM + GSP/NGSP blends (%TA = 0.6 and 0.68 respectively while pH of 0.0 soy: 1.0 NFDM = 0.41) irrespective of the culture type or soy powder variety (Table 2.2). The mean TA values among bean variety varied from 0.52 for non-germinated DF 222 to 0.58 for germinated DF 222 (Table 2.5).

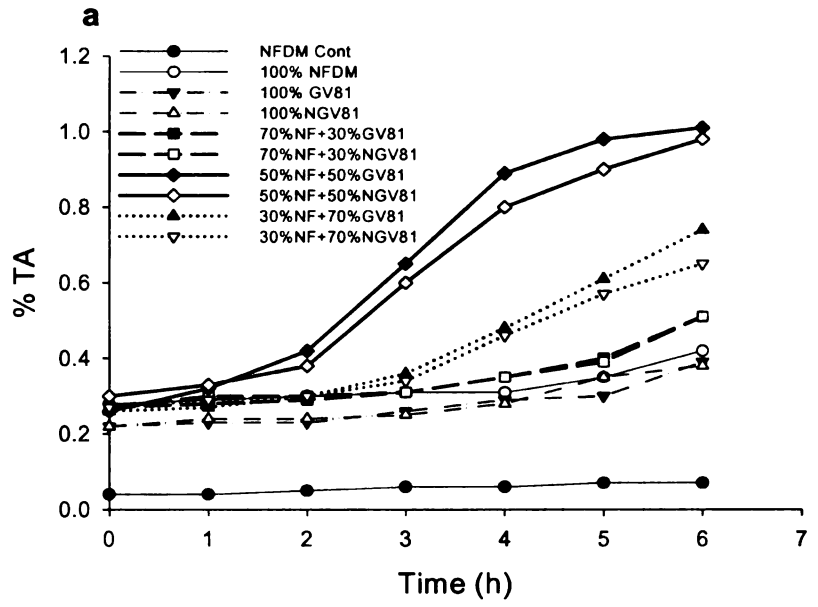
Table 2.5. Difference in percent titratable acidity (%TA) of cultures between germinated and non-germinated soybean varieties

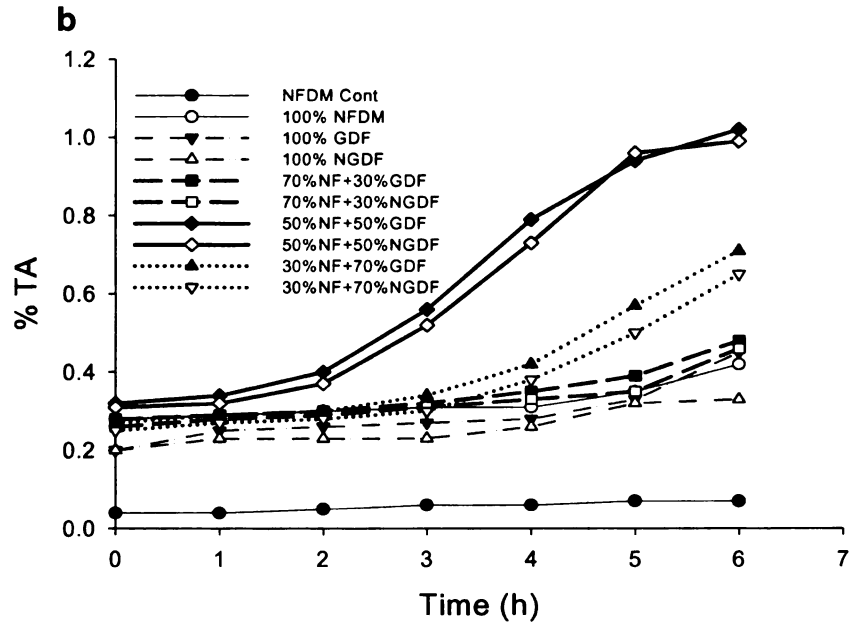
Soybean Variety	Germinated	Non-germinated
DF 222	0.58^{a**}	0.52^{d}
Vinton 81	0.56^{b}	0.53^{c}
E05276-T	0.56^{b}	NA^{*}

* =Not Available

** = Different letters denote significant difference at $p \leq 0.001$

Figure 2.13 Culture activity of *Lactobacillus delbruekii* subsp. *bulgaricus* (Lr 78) in reconstituted non-fat dry milk and germinated/non-germinated (a) Vinton 81 (b) DF 222 (c) E05276-T





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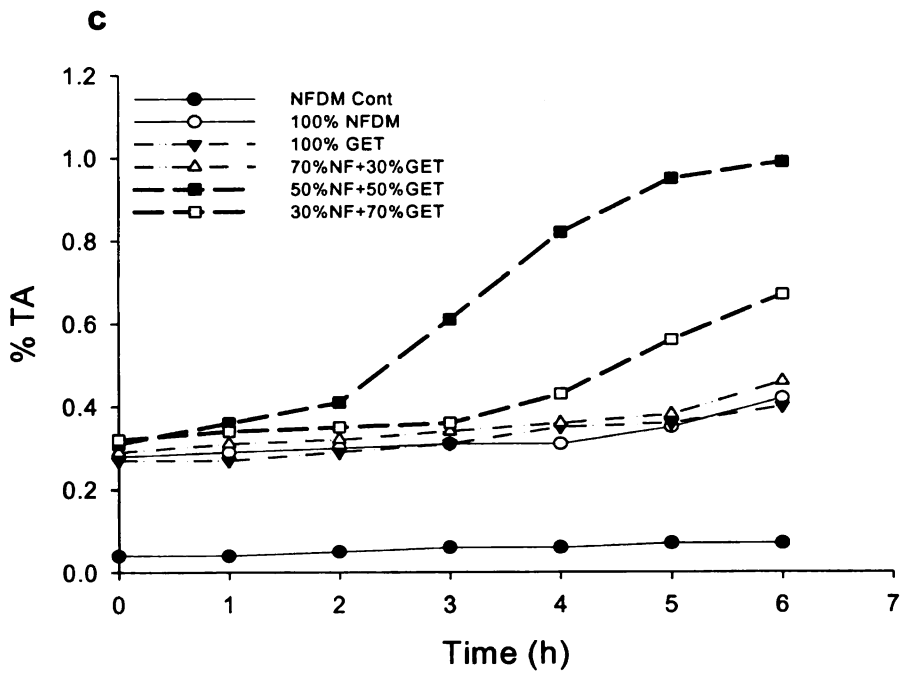
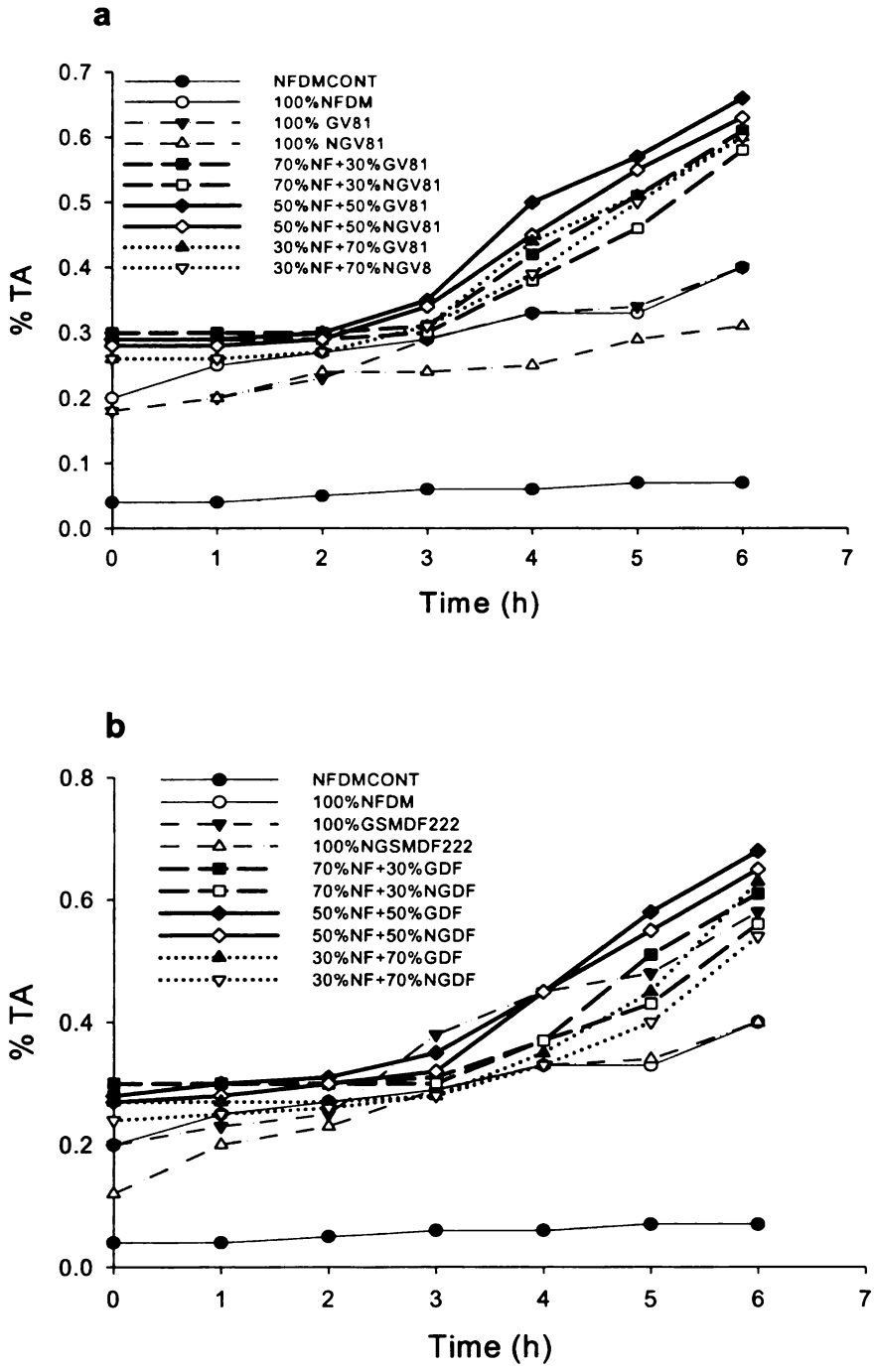


Figure 2.14 Culture activity of *Streptococcus salivarius* subsp. *thermophilus* (St 133) in reconstituted non-fat dry milk and germinated/non-germinated (a) Vinton 81 (b) DF 222 (c) E05276-T



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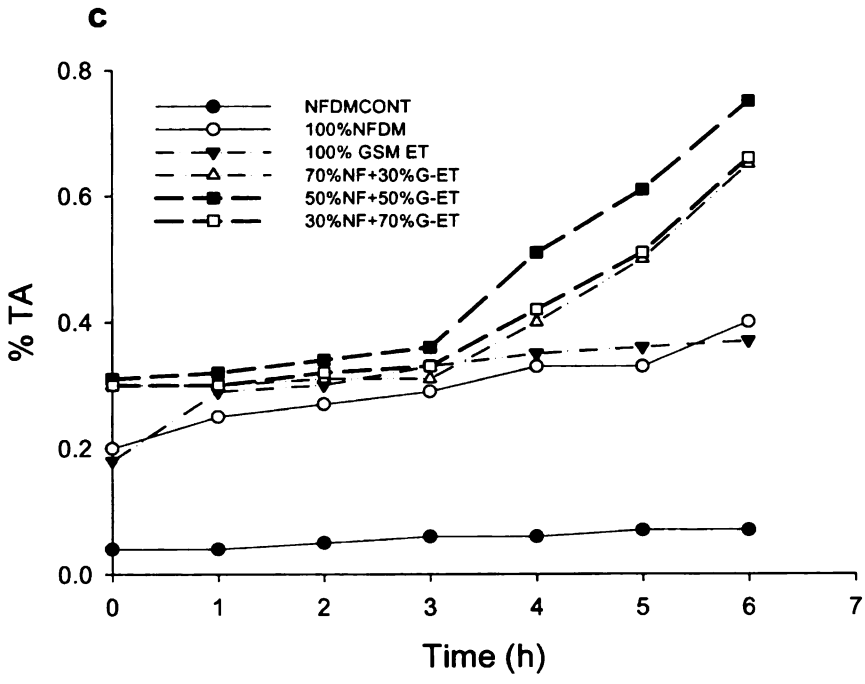
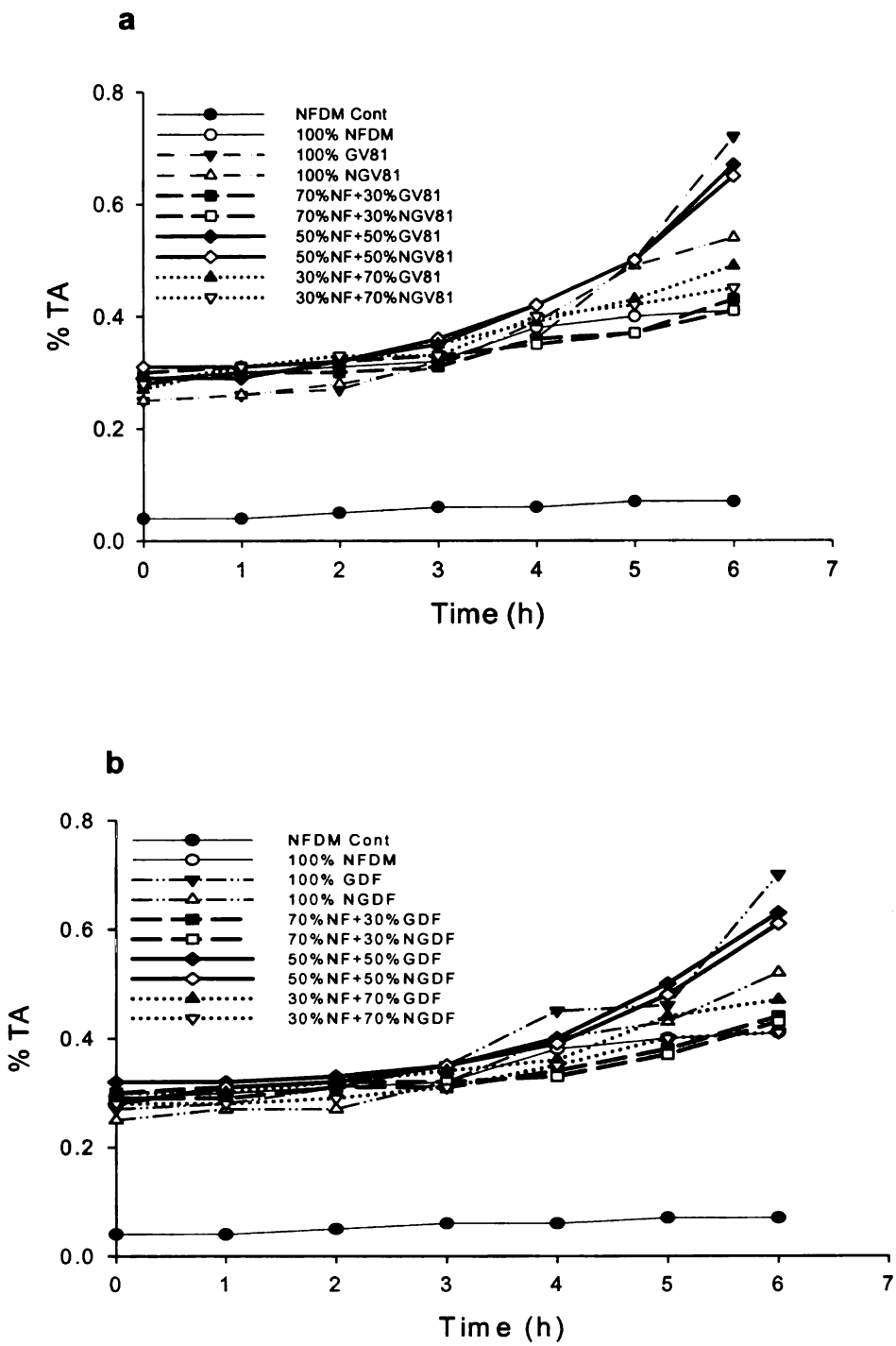


Figure 2.15 Culture activity of *Lactobacillus acidophilus* (La NCFM) in reconstituted non-fat dry milk and germinated/non-germinated (a) Vinton 81 (b) DF 222 (c) E05276-T



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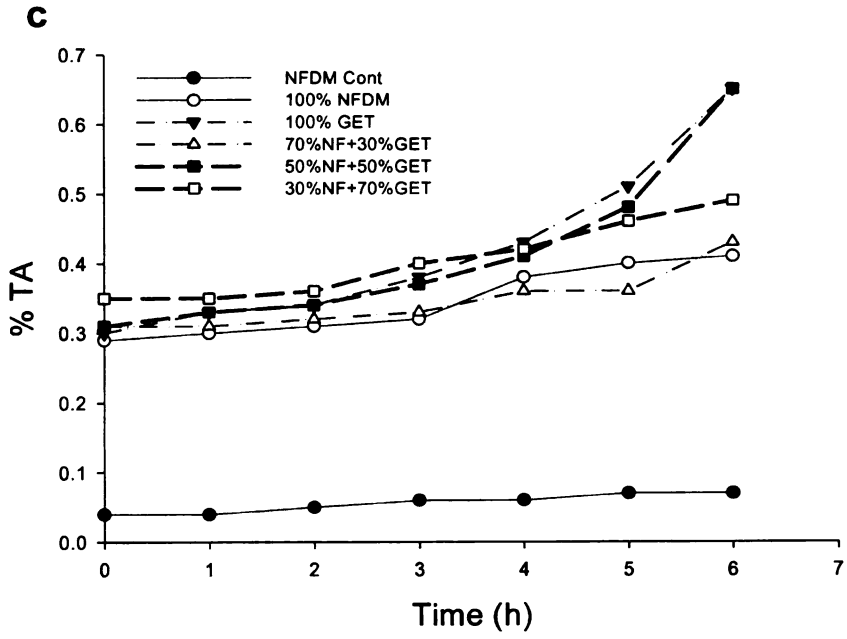


Table 2.6 shows the ANOVA for the independent variables and their various interactions on the growth of *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lr 78), *Streptococcus salivarius* subsp. *thermophilus* (St 133) and *Lactobacillus acidophilus* (La NCFM) in the medium blends. Consistent with the results of pH and titratable acidity, medium blend was the most significant factor with an F-value of 112.735 ($p< 0.001$). Unlike pH and %TA, soybean powder variety (F-value = 16.028; $p< 0.001$) had more significant effect on growth than culture type (F-value = 4.967; $p= 0.009$), although the interaction between medium blend and culture variety was most significant which is consistent with the other parameters measured. Cultures grown in fortified NFDM had highest growth (CFU/ml). The results showed that all the cultures used in this study successfully attained a desired level, achieving about 10^8 CFU/ml of each strain in each blend (Table 2.2; Figures 2.16, 2.17 and 2.18). A concentration of at least 10^6 CFU/ml viable cells in product are needed in order to exert health benefits (Ostlie and others, 2003). The 0.5:0.5 NFDM and GSP/NGSP blend overall had the highest growth (2.81 x 10^8 CFU/ml).

Table 2.6 Analysis of variance for the main factors (independent variables) and interactions on the growth (log CFU/ml) of lactic acid bacteria and *L. acidophilus* NCFM after 6h of incubation

Main factors	DF	SS	MS	F	P
[1]Medium Blend	4	1.648	0.335	112.735	<0.001
[2]Soy powder	4	0.0802	0.0200	16.0287	<0.001
[3]Culture Variety	2	1.177	0.0886	4.96	<0.009
Medium Blend x Soy powder	16	0.0891	0.00557	2.793	<0.001
Medium Blend x Culture	8	2.349	0.294	12.871	<0.001
Bean Variety x Culture Variety	8	0.0218	0.00273	7.102	<0.001
Medium Blend x Bean Variety x Culture variety	32	0.0588	0.00184	3.142	<0.001
Residual	75	0.00290	0.000039		
Total	149	4.119	0.0276		

Similar to the pH and %TA data, the germinated soybean varieties supported the growth of the cultures better than their non-germinated counterparts (Table 2.7). This result substantiates the fact that germination induced a substantial increase in the amount of bioactive compounds as well as increase in enzyme activities (Bau and others, 2000), thus providing more bioavailable nutrients for more acid and growth.

Figure 2.16 Growth of *Lactobacillus delbruekii* subsp. *bulgaricus* (Lr 78) in reconstituted non-fat dry milk and germinated/non-germinated (a) Vinton 81 (b) DF 222 (c) E05276-T

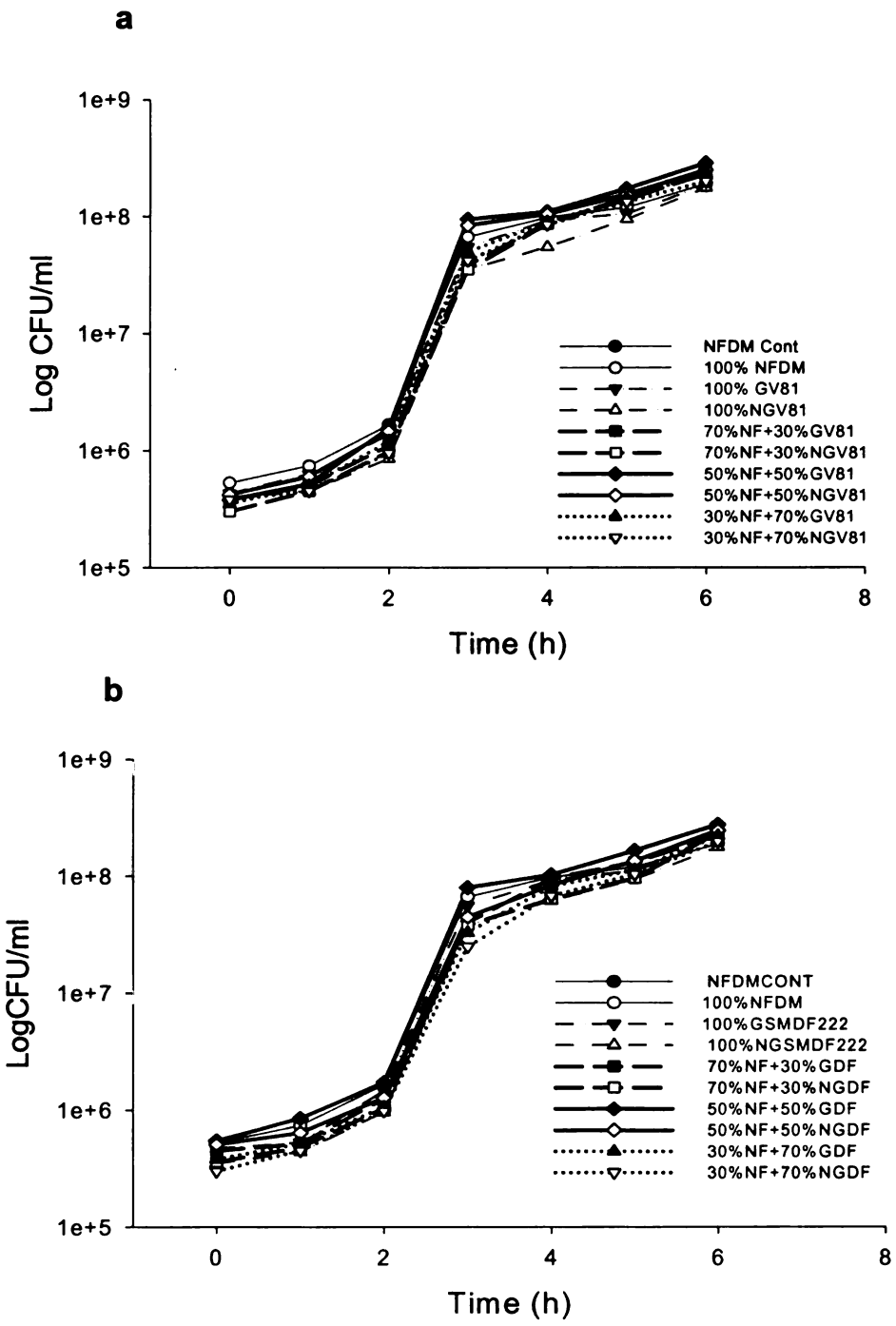


Figure 2.16 continued.

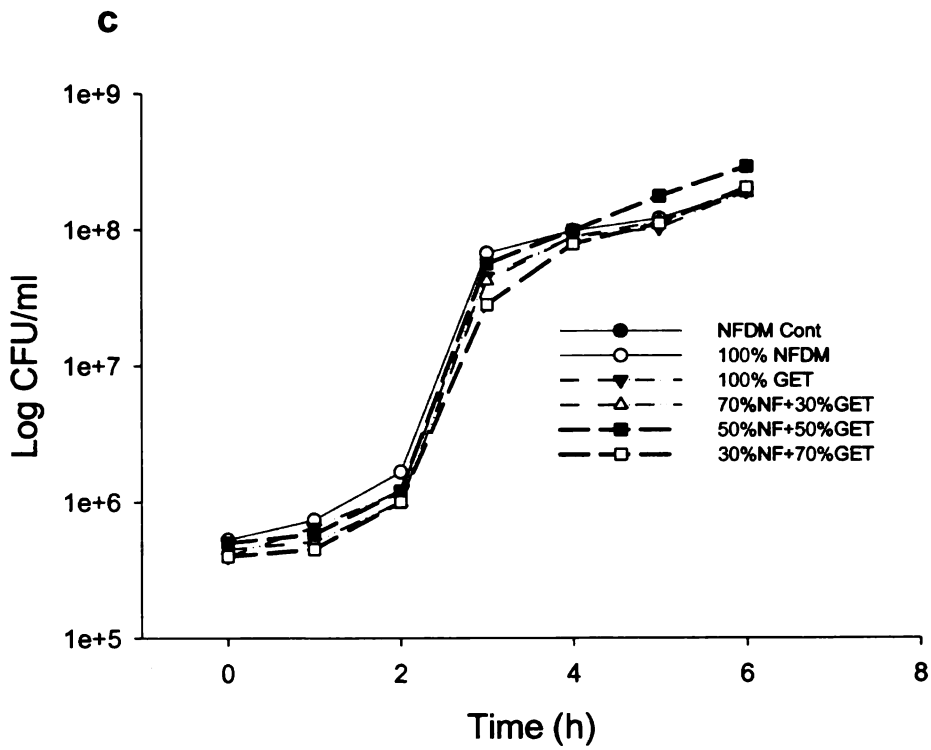
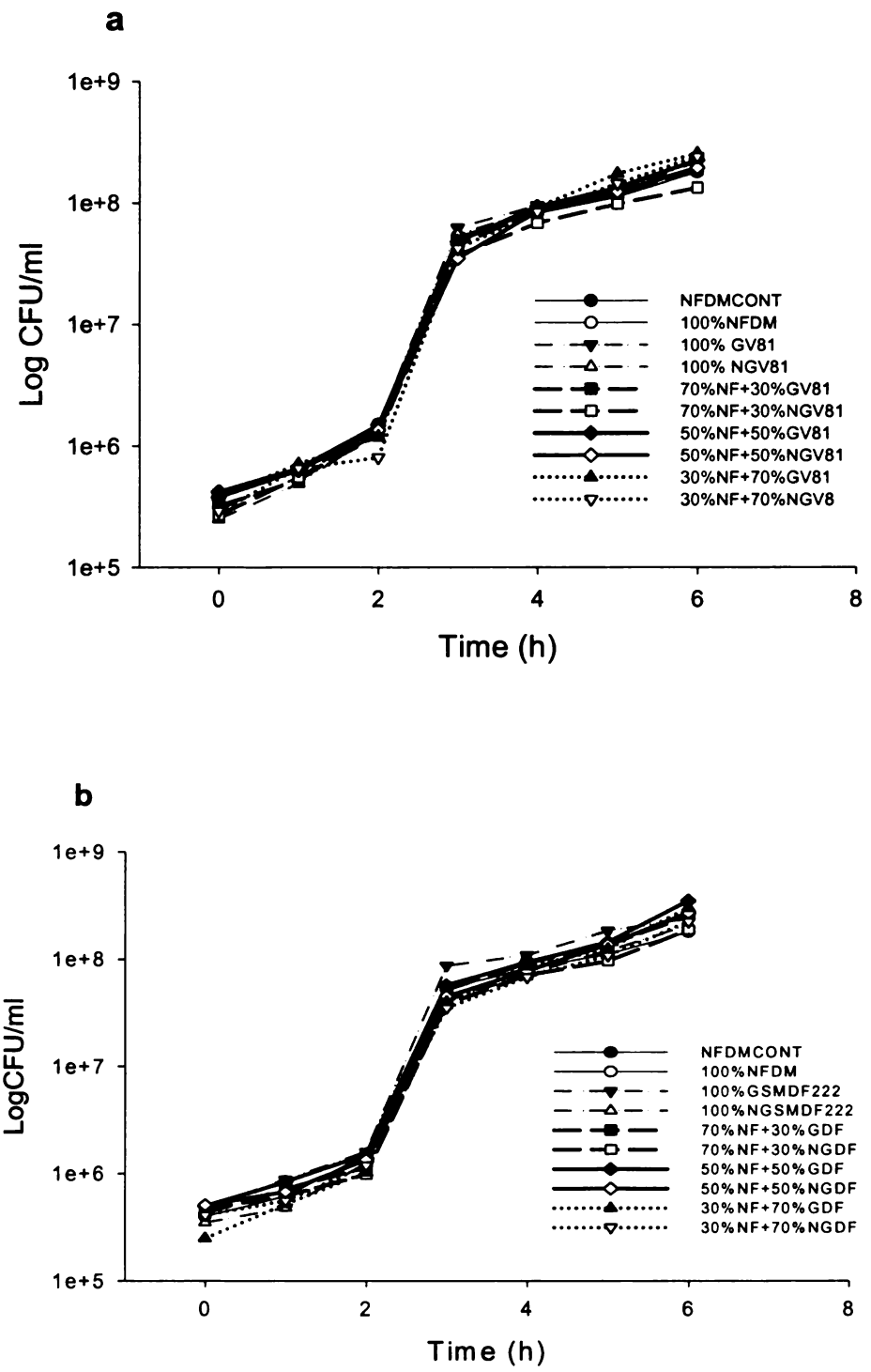


Figure 2.17 Growth of *Streptococcus salivarius* subsp. *thermophilus* (St 133) in reconstituted non-fat dry milk and germinated/non-germinated (a) Vinton 81 (b) DF 222 (c) E05276-T



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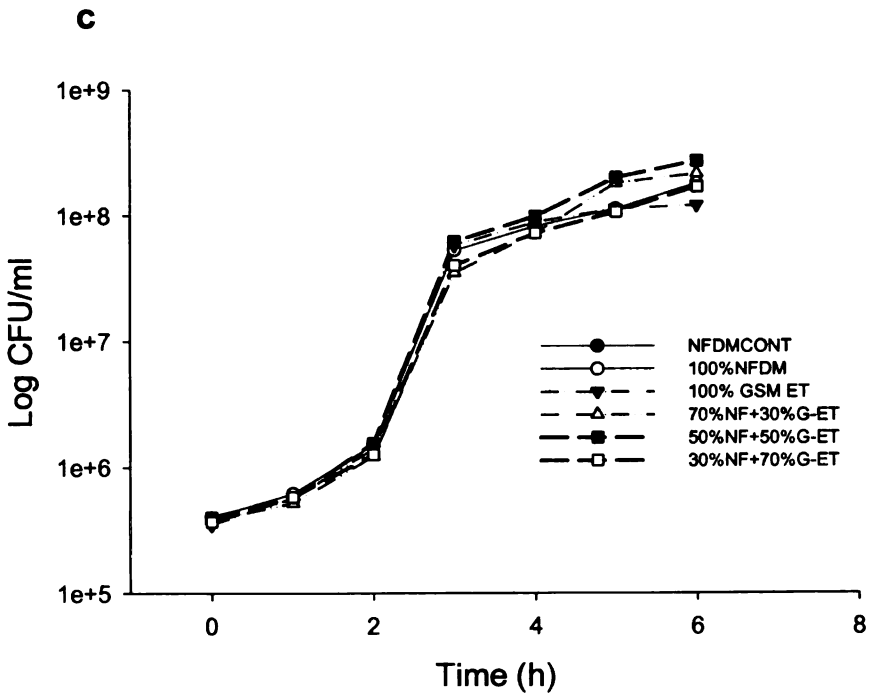


Figure 2.18 Growth of *Lactobacillus acidophilus* (La NCFM) in reconstituted non-fat dry milk and germinated/non-germinated (a) Vinton 81 (b) DF 222 (c) E05276-T

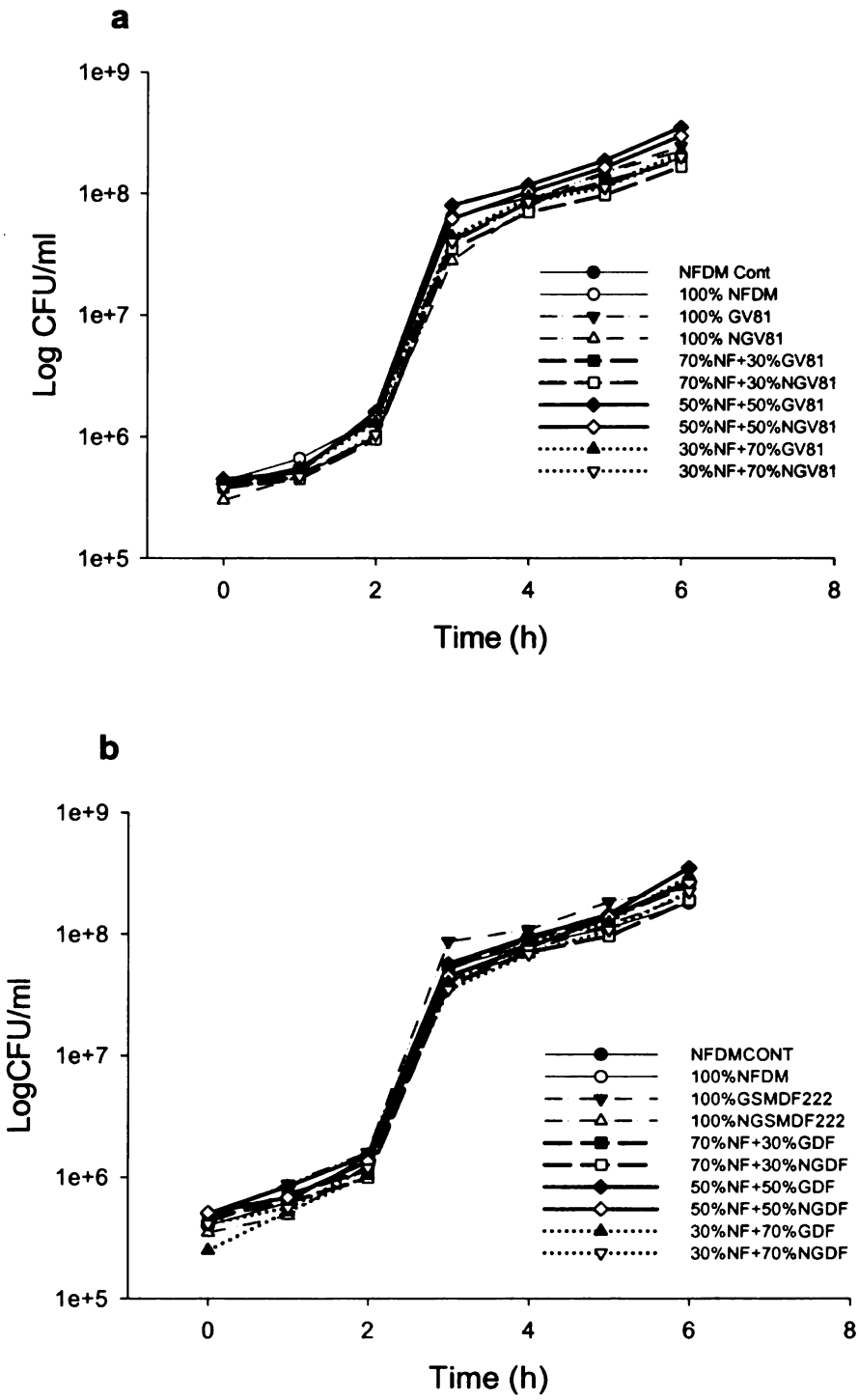


Figure 2.18 continued.

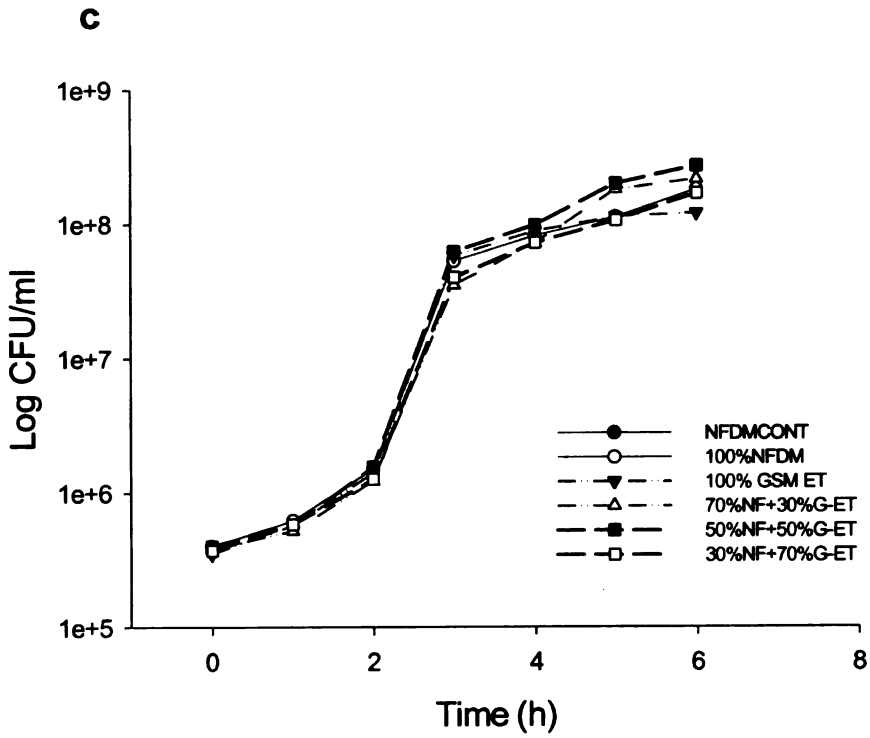


Table 2.7 Difference in growth (CFU/ml) of cultures between germinated and non-germinated soybean varieties

Bean Variety	Germinated ($x10^8$)	Non-germinated ($x10^8$)
DF 222	2.39^{a**}	2.11^{c}
Vinton 81	2.26^{b}	2.05^{d}
E05276-T	2.10^{c}	N/A*

* =Not Available

** = Different letters denote significant difference at $p \leq 0.001$

Overall these data strongly support the view of several workers that non-fortified milk does not support LAB growth as much as fortified milk (Kamaly 1997; Pham and Shah, 2008). Recent work showed that pH drop during the fermentation of soy beverages was faster by *Streptococcus thermophilus* (ATCC 4356), *Lactobacillus delbruekii* subsp. *bulgaricus* (IM 025) and certain probiotics such as *Lactobacillus johnsonii* NCC533 (LA-1), *Lactobacillus rhamnosus* ATCC 53103 (GG), bifidobacteria than in cow's milk (Farnworth and others 2007). From their research, these workers concluded that probiotic bacteria utilized different sugars to support growth when grown in either cow's milk or soy beverage. Our study indicates that addition of whole soy powder promoted cell growth and acid production. This work shows that more than 10^6 CFU/ml needed to exert health benefits to consumers was obtained when NFDM was fortified with whole soy powder.

CHAPTER 3

DEVELOPMENT AND PROPERTIES OF YOGURT FROM BLENDS OF COW'S MILK AND WHOLE SOYMILK BASE FOR CONSUMER ACCEPTANCE

3.1 ABSTRACT

Consumer awareness of soy as a healthy food has increased substantially in the past decade, therefore the growth potential has enticed food manufacturers to extend products by replacing larger quantities of dairy ingredients with soy. 50:50 blends of high heat non-fat dry milk (NFDM) and germinated (GSP) or non-germinated (NGSP) whole soy powders were utilized for yogurt manufacturing for preliminary sensory evaluation. Experienced yogurt consumers screened the fortified yogurt samples and selected samples made with soy powders from Vinton 81 and DF 222 soybean varieties as the most acceptable for pilot plant yogurt production. Six different strawberry flavored yogurts were manufactured as follows: GSP Vinton 81 + NFDM, NGSP Vinton 81 + NFDM, GSP DF 222 + NFDM, NGSP DF 222 + NFDM, NGSP Vinton 81- all soy control and NFDM- all dairy control. A total of 112 untrained panelists evaluated each sample for appearance, body texture, flavor and overall acceptance on a 9-point hedonic scale. There was no statistically significant difference between 100% NFDM and the 50:50 blended yogurts for flavor and overall acceptance ($p=0.0001$). Overall the 100%

soy yogurt had lower sensory scores than the other yogurts. Also, the pH of the 100% soy yogurt was significantly higher than the other samples (4.67) even though it was still within acceptable pH range for yogurt.

Nutrient composition analysis indicates that the protein contents of all the yogurt samples ranged from 4.80% (100% soy yogurt) to 6.82% (50% E05276-T powder + 50% non-fat dry milk). There was no statistical difference between the protein content of 100% dairy yogurt and the remaining soy-fortified yogurt samples. On the other hand 100% soy yogurt had 1.32% fat while 100% dairy yogurt had 0% fat since it was manufactured from non-fat dry milk only. The carbohydrate contents ranged from 13.62% for yogurt fortified with non-germinated Vinton 81 powder and 19.39% for yogurt fortified with germinated E05276-T powder. The ash contents varied from 2.08% for 100% soy yogurt to 4.09% for 100% dairy yogurt. Alternatively, the neutral detergent fiber varied from 0% for 100% dairy yogurt to 1.6% for 100% soy yogurt.

3.2 INTRODUCTION

Traditionally, yogurt is usually made with a base of dairy milk with high protein content. Food scientists and manufacturers view yogurt as a perfect delivery vehicle for vitamins, fibers, essential fatty acids, antioxidants, probiotics etc, thus several added health benefits have been attached to it especially to a specific population e.g. aged individuals. The nutritional value of yogurt is dependent on the nutrient content of the milk it is made from although some minerals are more bioavailable due to fermentation (Adolfsson and others, 2004). Some vitamins like B-6 and B-12 are decreased while peptides, free amino acids, free fatty acids, folic acid and choline contents are increased

in yogurts (Meydani and Ha, 2000). Addition of phytosterols to foods such as yogurt is gaining popularity as a result of its ability to reduce serum cholesterol (Hansel and others 2007; Monu and others, 2008). Lactic acid bacteria especially probiotics are used both to preserve foods as well as promote good health, but strict strain dependence and poor growth and survival under different processing conditions have resulted in limited use of cultures and probiotics addition and has increased the use of microencapsulated cultures leading to extra manufacturing cost (Dave and Shah, 1996; Bruno and others, 2002; Donkor and others, 2006; Aryana and others 2007).

The therapeutic effects of cow milk yogurt consumption on gut function have been investigated and reviewed (Van de Water and others 1999; Adolfsson and others 2004). Substantial evidence supports the beneficial effects of yogurt and probiotics although there are still some inconsistencies in reported data for the use of probiotics. These inconsistencies could be attributed to strain differences, routes of administration of cultures or improper research design. The addition of dietary fibers into foods is becoming popular. Due to FDA's approval for soy protein health claim concerning heart disease, the soy market has doubled. In addition, many studies supporting the ability of soy to improve or protect against postmenopausal symptoms, osteoporosis, hyperlipidemia, prostrate enlargement, bladder cancer, hypertension, as well as other types of cancer have been published (Bager and others 2002). According to USDA Nutrient data base for Standard Reference, 100g of soybeans provides 36.5g of protein, 277mg of calcium, 15.7g of iron, 280mg of magnesium, 704mg of phosphorous, 1797mg of potassium, 4.9mg of zinc and varying amounts of vitamins A, E, C, B1, B2, B3, B5, B6 and folic acid. Despite all the claims about soy benefits to health a lot of controversies

still exist concerning its safety to consumers such as estrogenicity and increased cancer risk as a result of high isoflavone consumption (Tsangalis and others 2002).

Culture and familiarity play important roles in the perception, description and acceptance of food products. A study done in France and Vietnam showed that only a small cultural difference was observed on perception of soy and cow milk yogurts (Tu and others 2007). Meanwhile in the same study, a significant difference was observed in the two groups in the verbal description of yogurt aroma especially with soy yogurt. Their investigation simply suggested that globally, the perception of soy yogurt characteristics is not influenced by culture but by use of familiar terminology unique to each culture to describe the same attribute e.g. aroma. Another study on culture-specific variation in the flavor profile of soy product concluded that culture-specific preferences are the determining factor in flavor profiles of soymilk from distinct geographical regions (Keast and Lau, 2006). Presently, little if any research on the fortification of dairy yogurt with soy protein or whole soy is known.

The aim of this study therefore is development and properties of yogurt from blends of cow's milk and whole soymilk base with consumer acceptance.

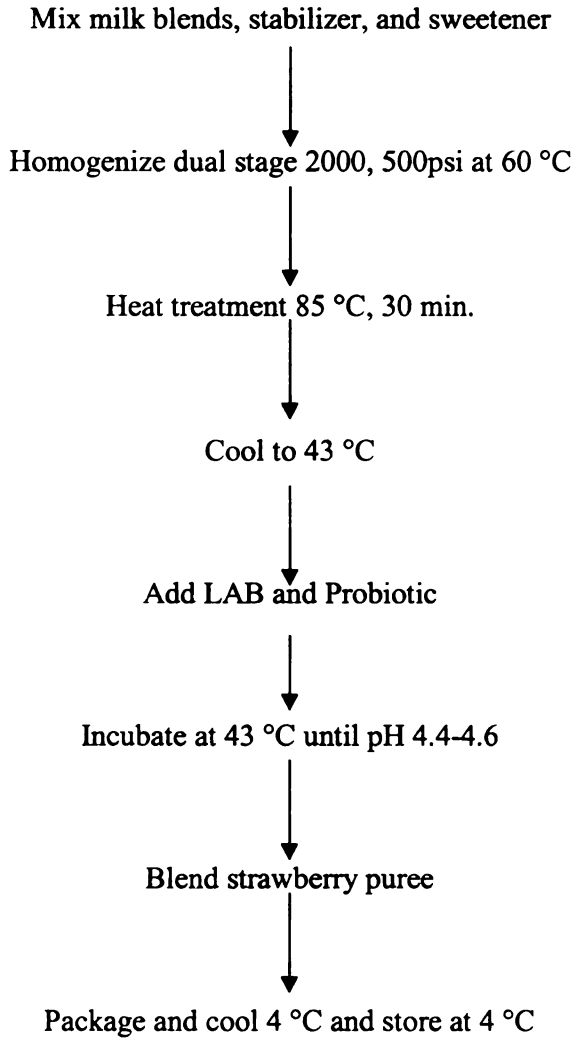
MATERIALS AND METHODS

3.2.1 Low fat yogurt formulation and manufacture

Swiss-style strawberry flavored low fat yogurt was made using 50:50 blends of all the soybean powder varieties of germinated (GSP) and non-germinated (NGSP) Vinton

81, germinated (GSP), non-germinated (NGSP) DF 222 and germinated (GSP) E05276-T and NFDM. Figure 3.1 shows the flow diagram for the yogurt manufacturing process.

Figure 3.1 Flow diagram for manufacture of cow's milk/soymilk yogurt.



Seven percent high heat NFDM (Michigan Milk Producers Association, Ovid, MI) was blended with 7% (total solids) soy powder, 0.5% stabilizer (Tate and Lyle Custom Ingredients, Sycamore, IL) and 7% sucrose (Michigan Sugar Company, Saginaw, MI). Moisture content of the soy powders was taken into consideration and adjustment was made for each powder to give 7% total solids (Table 3.1). All the yogurt milk bases were homogenized at 2000 and 500 psi (Homogenizer-200, Cherry Burrell Corp. Chicago, IL)

at 60°C and batch pasteurized at 85°C for 30 minutes. The mixed bases were cooled to 43°C and inoculated with 0.5% (w/v) commercial yogurt cultures YC-X11 (Chr. Hansen Laboratories, Milwaukee, WI), and a probiotic *Lactobacillus acidophilus* culture NCFM (Danisco US, Madison, WI). Inoculated yogurt bases were incubated at 43 °C until pH was about 4.4-4.6. At the end of incubation period 13% strawberry puree (Kraus & Co., Walled Lake, MI) was added to each yogurt batch and mixed thoroughly. All the yogurt samples were packaged into 8oz cups and stored at refrigeration temperature (4°C) for further analysis including sensory and shelf life evaluations.

Table 3.1 Low-fat whole soy-fortified yogurt formulation

Ingredients (%)	**GV 81**[2]	**NGV 81**	**GDF 222**	**NGDF 222**	**GET**
Soy powder	7.58	7.59	7.58	7.51	7.56
NFDM[1]	7.00	7.00	7.00	7.00	7.00
Sucrose	7.00	7.00	7.00	7.00	7.00
Stabilizer	0.50	0.50	0.50	0.50	0.50
Strawberry puree	13.00	13.00	13.00	13.00	13.00
Added water	64.92	64.91	64.92	64.99	64.94
Total	100.00	100.00	100.00	100.00	100.00

[1]NFDM = Nonfat dry milk

[2]GV 81 = Germinated Vinton 81 powder

NGV 81 = Non-germinated Vinton 81 powder

GDF 222 = Germinated DF 222

NGDF 222 = Non-germinated DF 222

GET = Germinated E05276-T

3.2.2 Screening of Swiss-style strawberry flavored low-fat yogurt by experienced consumers

Experienced yogurt consumers made up of Dairy and Food Processing Professors, Dairy Plant Manager and Food Technologists and some members of Dairy Products Evaluation Team screened the yogurt samples made from 50:50 blends of all the different soybean powder varieties namely GSP and NGSP Vinton 81, GSP and NGSP DF 222 and GSP E05276-T. An evaluation form was provided for each screener to simply indicate acceptable (score of 1) or unacceptable (score of 0) (Appendix 1) for the following yogurt attributes: flavor, body and texture, appearance and color, and overall acceptance. Five varieties of strawberry flavored yogurts were manufactured and randomly labeled with three-digit number as 252 (GSP Vinton 81 + NFDM), 169 (NGSP Vinton 81 + NFDM), 344 (GSP DF 222 + NFDM), 159 (NGSP DF 222 + NFDM), and 817 (GSP E05276-T). Samples containing GSP/NGSP Vinton 81 and GSP/NGSP DF 222 varieties were chosen as the most acceptable by the experienced screeners for yogurt production. These selected samples were then manufactured on a larger scale at the Michigan State University dairy pilot plant for the sensory evaluation using untrained consumer panelists.

3.2.3 Sensory evaluation of Swiss-style strawberry flavored low-fat yogurt: Untrained Panel

The untrained panelists were recruited through e-mails containing the flyers (Appendix 2) to students (graduate and undergraduate), faculty and staff of different departments but mostly from Food Science and Human Nutrition Department. Also flyers were posted around the Food Science Department buildings. Prior to the initial screening

of yogurt samples, permission was sought and approved for the use of human subjects for this study by the University Committee on Research Involving Human Subjects (UCRIHS) (Appendix 3). A total of 112 untrained panelists took part in the sensory evaluation exercise. The evaluation was conducted in booths located in the sensory laboratory in the Department of Food Science and Human Nutrition at Michigan State University (MSU). Before participating in the actual testing, the consent form (Appendix 4) was given and explained to each panelist for signing.

Six varieties of strawberry flavored yogurts were manufactured. These were randomly labeled with three-digit numbers as follows: 252 (GSP Vinton 81 + NFDM), 169 (NGSP Vinton 81 + NFDM), 344 (GSP DF 222 + NFDM), 159 (NGSP DF 222 + NFDM), 894 (NGSP Vinton 81-all soy control), and 949 (NFDM-all dairy control). The yogurt samples were scooped into 4oz plastic cups and labeled accordingly with the selected three-digit numbers. Each panelist was presented with six yogurt samples (maintained at refrigeration temperature) in a random order so as to eliminate bias across different individuals. The panelists were asked to evaluate all six samples for appearance, body texture, flavor and overall acceptance and indicate their degree of liking on a nine-point hedonic scale from 1 = dislike extremely to 9 = like extremely; 5 = neither like nor dislike (Appendix 5). Drinking water was provided for panel members to rinse their pallet in between samples in order to avoid carry over of taste from one sample to another.

3.2.4 Proximate analysis of yogurt samples

The following parameters were measured namely crude protein, fat and carbohydrate (determined by subtraction). Also measured were ash, dietary fiber and dry

matter. All the measurements were carried out in the Dept. of Animal Science, Michigan State University. Standard or slightly modified standard methods were employed. Ether extraction method was used for the crude fat analysis (AOAC 2005), neutral-detergent fiber method was utilized for dietary fiber analysis (Goering and Van Soest, 1970; Robertson and Van Soest, 1977; Cherny and others, 1989). The total nitrogen (modified Kjeldahl digestion) method was used for the protein analysis (Hach and others, 1987). Ash content was determined using the AOAC (2005) method 945.46 and carbohydrate was estimated by difference.

3.2.5 Statistical analysis

All the data were analyzed using one-way analysis of variance (ANOVA). Tukey's test was used for multiple comparisons of the means. SIM 2000 Sensory Evaluation Software, version 6.0(Sensory Computer Systems, Morristown, N.Y., U.S.A.) was used for the sensory evaluation analysis. Sigma Stat 3.1 was utilized for the analysis of nutrient composition data (replicate). ANOVA data with $p < 0.05$ were considered as statistically significant.

3.3 RESULTS AND DISCUSSION

3.4.1 Sensory evaluation of yogurt samples

Table 3.2 summarizes the results of the sensory analysis by a consumer panel. The sensory parameters measured were appearance, body texture, flavor and overall acceptance.

Table 3.2 Overall acceptability of the yogurt samples as determined by untrained consumer panel (n = 112)

Attribute	252[1]	169	949	344	159	894	p-value	Significance
Appearance	6.80^{ab}	6.80^{ab}	6.57^{cb}	6.96^{ab}	7.01^{a}	6.24^{c}	0.0001	***
Body Texture	6.34^{ab}	6.07^{ab}	6.51^{a}	6.25^{ab}	6.40^{a}	5.81^{b}	0.064	**
Flavor	5.43^{a}	5.21^{a}	5.58^{a}	5.39^{a}	5.67^{a}	4.43^{b}	0.0001	***
Overall Acceptance	5.48^{a}	5.32^{a}	5.71^{a}	5.59^{a}	5.72^{a}	4.55^{b}	0.0001	***

[a-c] Means in the same row with different small letter superscripts are significantly different.

Scale: 9 = like extremely; 5 = neither like nor dislike; 1 = dislike extremely.

[1]252 = germinated Vinton 81 soy powder + NFDM

169 = non-germinated Vinton 81 soy powder + NFDM

949 = all dairy NFDM-control

344 = germinated DF 222 soy powder + NFDM

159 = non-germinated DF 222 soy powder + NFDM

894 = all non-germinated Vinton 81-control

In this particular study, the treatment effect was associated with the addition of 50% whole soy powder to the yogurt base, while the two controls consisted of either 100% NFDM (sample 949) or 100% whole soy powder (sample 894). The mean score values for overall acceptance for all the treatment and control samples were between 4.55 and 5.72, which corresponded to "dislike slightly" and "neither like nor dislike" respectively. Overall, based on the acceptability mean score, there was no statistically significant difference between the 100% all dairy (NFDM) and the 50:50 blended yogurts

for flavor and overall acceptance ($p = 0.0001$). Overall consumer preference for NFDM whole soy-fortified yogurts was similar to 100% dairy yogurt. The 100% soy yogurt (NGSP Vinton 81) scored lower overall in all the sensory attributes measured than the other yogurts. This could be attributed to the higher fiber contents present in the whole soy powder utilized, which contributed to increased viscosity, and thus thick body (Drake and others 2000). This observation is also reflected in the scores for body texture where 100% soy yogurt (sample 894) had the lowest score (5.81), while 100% NFDM yogurt (sample 949) had the highest score (6.51) although it was not statistically different from the soy-fortified yogurts ($p = 0.064$) (Table 3.2).

In terms of yogurt appearance, the mean scores ranged from 6.24 for sample 894 (100% soy) to 7.01 for sample 159 (non-germinated DF 222 and NFDM). This simply means that the subjects "liked slightly to "moderately liked" the appearance of all the yogurt samples. The data also showed that the consumers in some cases significantly preferred the appearance of the soy-fortified yogurt to the non-fortified yogurt. Yogurt samples made from DF 222 had higher ratings than samples made from either Vinton 81 or NFDM. This could be as result of the lighter yellow (creamy) color of DF 222 soy powder as opposed to a more intense yellow color of the Vinton 81 soy powder. Previous study by Min and others (2005) indicated that soybean varieties and growing locations could significantly affect chemical and physical properties of soybean and soy foods especially the protein and total solids contents of the corresponding soy foods. The strawberry puree was responsible for the pink color of the yogurt samples.

3.4.2 Effect of whole soy powder fortification on pH of yogurts

The pH values of the yogurt samples are shown in Table 3.3. Prior to the sensory evaluation (a week after manufacturing), the pH values of the yogurt samples were measured and there was no significant difference in pH that was originally measured immediately after incubation.

Table 3.3 Mean pH of yogurt samples at the time of manufacturing and at the time of sensory evaluation

Sample	**pH** at time of manufacturing	**pH** at time of sensory evaluation
[1]252	4.54 ± 0.01^{c}	4.550 ± 0.01^{c}
169	4.60 ± 0.01^{c}	4.590 ± 0.01^{c}
344	4.40 ± 0.01^{a}	4.390 ± 0.01^{a}
159	4.480 ± 0.01^{b}	4.490 ± 0.01^{b}
894	4.65 ± 0.01^{d}	4.670 ± 0.01^{d}
949	4.41 ± 0.01^{a}	4.405 ± 0.01^{a}

[a-d] Different superscript letters denote significant difference ($p \leq 0.0001$), n = 2 for all samples.

[1]252 = germinated Vinton 81 soy powder + NFDM

169 = non-germinated Vinton 81 soy powder + NFDM

949 = all dairy NFDM-control

344 = germinated DF 222 soy powder + NFDM

159 = non-germinated DF 222 soy powder + NFDM

894 = all non-germinated Vinton 81-control

Sample 894 (100% soy yogurt) had the highest pH but overall, all yogurt samples were within the acceptable range of yogurt pH. Yogurt fortified with germinated DF 222 had the lowest pH and was not significantly different from pH of 100% dairy yogurt. This result is consistent with the result obtained in objective 1 on culture activity where germinated DF 222 had the lowest pH when the cultures were grown in it. The higher pH values obtained in sample 894 could be attributed to the fact that it has less hydrolyzed carbohydrates for the lactic acid bacteria to metabolize.

3.4.3 Nutrient composition of yogurt samples

The protein, fat, carbohydrate and ash contents of the soy-fortified yogurts are shown in Table 3.4. The results on the proximate analysis indicate that there are statistically significant differences in the mean values ($p \leq 0.001$), of each parameter measured.

3.4.3.1 Effect of soy fortification on protein content of yogurt

The data in this study show that there was no significant difference ($p< 0.05$) in protein content between the all-dairy control (sample 949) with 5.56% protein and the germinated and non-germinated Vinton 81 fortified yogurts (samples 252, 5.98%protein and 169, 5.87% protein), respectively. Also the 100% dairy yogurt was not statistically different ($p< 0.05$) from germinated DF 222 fortified yogurt (5.16%) but was different from non-germinated DF 222 fortified yogurt (5.08%) and 100% soy yogurt (sample 894, 4.80% protein). Germinated E05276-T fortified yogurt (sample 817, 6.82% protein) was

significantly higher ($p < 0.05$) than the rest. Overall 100% soy yogurt contained the lowest percentage of protein among all the yogurt samples.

Table 3.4 Compositional analysis (%) of germinated and non-germinated soy-fortified yogurts after manufacturing

Sample	Protein	Fat	Carbohydrate	Dry Matter	Ash	Neutral Detergent Fiber
[1]**252**	5.98 ± 0.03^{b}	0.98 ± 0.06^{a}	13.99 ± 0.01^{d}	23.92 ± 0.14^{e}	3.02 ± 0.11^{b}	0.92 ± 0.02^{b}
169	5.87 ± 0.11^{b}	1.13 ± 0.02^{a}	13.62 ± 0.04^{d}	23.73 ± 0.04^{f}	3.12 ± 0.13^{b}	0.74 ± 0.11^{b}
344	5.16 ± 0.00^{cd}	0.14 ± 0.06^{bc}	17.78 ± 0.01^{b}	25.63 ± 0.04^{c}	2.58 ± 0.05^{bc}	0.74 ± 0.11^{b}
159	5.08 ± 0.05^{d}	0.46 ± 0.23^{b}	17.49 ± 0.29^{b}	25.21 ± 0.01^{d}	2.20 ± 0.56^{c}	0.54 ± 0.13^{c}
817	6.82 ± 0.16^{a}	0.40 ± 0.08^{b}	19.39 ± 0.10^{a}	29.52 ± 0.04^{a}	2.92 ± 0.02^{b}	0.63 ± 0.01^{bc}
894	4.80 ± 0.18^{d}	1.32 ± 0.02^{a}	15.72 ± 0.37^{c}	23.91 ± 0.05^{e}	2.08 ± 0.16^{c}	1.60 ± 0.01^{a}
949	5.56 ± 0.11^{bc}	0.00 ± 0.00^{c}	17.75 ± 0.03^{b}	27.40 ± 0.01^{b}	4.09 ± 0.08^{a}	0.00 ± 0.00^{d}

[a-f] Mean values in a column with different letters are significantly different ($p < 0.05$); n = 2 for all samples

[1] 252 = 50% germinated Vinton 81 + 50% non-fat dry milk

169 = 50% non-germinated Vinton 81 + 50% non-fat dry milk

344 = 50% germinated DF 222 + 50% non-fat dry milk

159 = 50% non-germinated DF 222 + 50% non-fat dry milk

817 = 50% germinated E05276-T + 50% non-fat dry milk

894 = 100% non-germinated Vinton 81 (all soy yogurt- control)

949 = 100% non-fat dry milk (all dairy yogurt- control)

3.4.3.2 Effect of soy fortification on fat content of yogurt

Since only non-fat dry milk was utilized for the formulation of 100% dairy yogurt, the fat content was 0%. On the other hand, 100% soy yogurt formulated with non-germinated Vinton 81 spray dried soy powder and non-germinated/germinated Vinton 81 fortified yogurts had the highest fat contents (1.32%, 1.13%, and 0.94% respectively), which were statistically significant from the rest of the yogurt samples. Yogurts fortified with soy powder varieties of germinated E05276-T, germinated and non-germinated DF 222 had lower fat contents than yogurts fortified with Vinton 81 powders (Table 3.3). This result is expected since the yogurts were formulated from non-fat dry milk and low fat soybean powders. This data suggest that whatever fat is in the yogurt was from the soy powder utilized.

Although the fatty acid profile of these fats in the present study is not known, previous studies indicate that the fat contents of soy contain mostly unsaturated fatty acids (USDA, 1979; Eitenmiller, 1997). The yogurts made from these formulations could be regarded as non-fat (less than 0.5 g/serving milk fat) or low fat (not more than 2 g/serving milk fat) soy fortified yogurts, which could be beneficial to consumers with cardiovascular diseases. In the United States of America, yogurts are identified according to the standards of identity listed in the U S. Code of Federal Regulations (CFR), in sections 21 of the Food and Drug Administration (NYA, 2003).

3.4.3.3 Effect of soy fortification on carbohydrate content of yogurt

The carbohydrate contents were obtained from the difference between the dry matter and the protein, fat and ash contents. Germinated E05276-T fortified yogurt

sample had the highest carbohydrate content that was statistically significant from the rest. All dairy control (100% non-fat dry milk) yogurts' carbohydrate content was 17.75% and it was similar to the carbohydrate contents of sample 344 (17.78%) and sample 159 (17.49%). The carbohydrate contents of the yogurt varieties made from germinated and non-germinated Vinton 81 (samples 252 and 169) were the lowest i.e. 13.99% and 13.62% respectively.

3.4.3.4 Effect of soy fortification on the ash and neutral detergent fiber contents of yogurt

The 100% dairy yogurt, sample 949 contained the highest amount of ash (4.09%), but had 0% neutral detergent fiber. Overall, the ash contents of soy fortified and all soy yogurt samples were similar. This trend was also seen in the fiber contents although the fiber content of 100% soy yogurt was the highest (1.6%). Sensory evaluation of some these yogurt samples have suggested likely acceptance of products. With the increase in public's interest in wellness, health and functional foods, the future of soy-fortified yogurts is promising.

Dairy yogurt fortification with fibers, iron and other selected nutrients in order to enhance health benefits have been studied (Fernandez-Garcia and others 1998; Hekmat and McMahon 1997). Yogurts fortified with 2.5% soy protein were found to be similar in sensory, chemical and microbiological properties to traditional dairy yogurts (Drake and others, 2000). Our sensory evaluation showed similar results where all the blended yogurts were similar in overall acceptance with the all-dairy control yogurt. Some workers in their study showed that consumer attitudes towards acceptability of yogurts fortified with soy powder increased with increased frequency of dairy yogurt

consumption as well as increased knowledge of the health claim associated with soy products (Drake and Gerard, 2003). This observation was made during the sensory exercise for this study based on verbal comments made by the panelists.

In summary, the results obtained in this objective clearly show that dairy yogurt could be fortified up to 50% with whole soy powder. The most desirable yogurt was manufactured with soybean variety DF 222. Flavor is a major challenge faced by product developers especially when ingredients containing proteins are incorporated into food products. More work is needed to increase the acceptability scores of the blended yogurt samples for example by increasing the moisture content (decrease viscosity) and flavoring compound without compromising the nutrient composition of the yogurt base.

CHAPTER 4

PRODUCTION AND CONCENTRATION OF GENISTEIN, DAIDZEIN, GENISTIN, DAIDZIN AND STACHYOSE IN PREDIGESTED/GERMINATED AND NON-GERMINATED SOY POWDER

4.1 ABSTRACT

The concentrations of isoflavone isomers genistein, daidzein, genistin and daidzin were determined in raw, non-germinated and germinated soy powders of Vinton 81, DF 222 and E05276-T soybean varieties. Reverse-phase high-performance liquid chromatography (HPLC) with ultraviolet (UV) detector was used to determine the concentration of the isoflavones present in the soy powders. Standard curves were made for each isoflavone standard and these curves were utilized to ascertain the concentration of the isoflavones in the samples. The extracted analytes were separated on a C-18 reverse phase column, eluted with acetonitrile/HPLC grade water/Acetic acid (75/25/1, v/v/v), and detected by UV detector at 262nm wavelength. Similarly oligosaccharide, stachyose contents of the soy powders was analyzed using HPLC.

Nutrient composition analysis showed that the protein contents of all soy powders were between, 50.28% to 56.03%. All the isoflavones measured were present but genistin and daidzin were more abundant than genistein and daidzein in all the powders analyzed. Mean total isoflavones in the soybean varieties ranged from 290 μg/g (in raw E05276-T) to 726 μg/g (in non-germinated DF 222). Soaking and germination increased ($p < 0.05$) the isoflavone contents and decreased ($p < 0.05$) the stachyose contents of all soybean

varieties, thus suggesting that processing methods could influence the concentrations of these compounds. The genistein and daidzein contents of all the raw powders were similar statistically and ranged from 0.0 μg/g (genistein in E05276-T) and 5.1μg/g (genistein in Vinton 81); 2.1μg/g (daidzein in Vinton 81) and 3.7 μg/g (daidzein in DF 222). There was a significant reduction in stachyose content (53.7% for Vinton 81; 66.9% for DF 222) after germination.

4.2 INTRODUCTION

Isoflavones are phenolic compounds with similar structure to human estrogen (Tsangalis and others, 2002). Soybeans and non-fermented soy foods contain these isoflavones mostly in the form of biologically inactive conjugates consisting of 83.90% to 98.37% of the total isoflavones (King and Bignell, 2000). These isoflavones especially the aglycone isomers confer health benefits against several aging diseases, cardiovascular diseases, high cholesterol, osteoporosis, prostrate, colon and breast cancers. Isoflavones can induce biological responses that are capable of mimicking endogenous estrogens by binding to estrogen receptors (Messina and Hughs 2003). The concentration range of isoflavones is < 50μg/g to > 3500μg/g in both soybean seeds and products but the glucoside forms are higher in concentration in these products. Also, the molecular weight of the glucoside is twice that of the corresponding aglycones.

The concentrations of the aglycones namely genistein, daidzein and glycitein are low in soy foods, about 0.2-1.5mg/g (Wang and Murphy, 1994). These aglycones are known to be the most bioactive phytochemicals in soy because of their unique properties. They are easily absorbed in higher amounts than the other isomers in the gut (Izumi and

others, 2000). According to these workers, the gut microflora and the enzyme glucosidases produced by these microorganisms convert the glucosides into corresponding aglycones. The type and quantity of each isoflavone varies according to the product and processing procedures utilized (Song and others, 1998). Overall genistein is more abundant in soy foods than daidzein. Fermentation, heat and enzyme treatment could significantly change the isomeric forms of isoflavones (total of 12 isomers) (Song and others 1998). In order to claim health benefits, the suggested amount of isoflavone aglycone required is about 30 to 40mg/day (Malnig and Brown 2007), hence it is important to provide foods with a considerable amount of aglycones. Germination leads to endogenous enzyme activation resulting in hydolysis of soy proteins, isoflavones and other boactive molecules (Bau and others 2000).

Several techniques have been employed in isoflavone measurement in the past but most are time consuming and challenging. One of the methods utilized is gas chromatography with mass spectrometric detection (GC-MS), but the disadvantage of this method is that the compounds need to be derivatized before injection (Preinerstofer and Sontag, 2004). As a result, the AOAC international developed a more reliable method that could be used to measure isoflavone levels in foods using reverse phase-high performance liquid chromatography (RP-HPLC) and ultraviolet (UV) detector (Verbruggen and others 2002). RP-HPLC is very popularly used for the identification of isoflavones in soybeans and soy foods because it does not require any derivatization as aglycones or glycosides. This method uses mixtures of methanol or acetontrile and aqueous acids or buffers as mobile phase.

The extraction of isoflavones from food matrices is generally done with glacial or common organic solvents such as methanol, acetonitrile, ethanol and acetone in aqueous medium e.g. distilled water (Murphy and others 2002; Song and others, 1998). Different temperatures with or without agitation could be used during extraction in order to enhance efficiency. Isoflavone conjugates are very unstable and investigations by Coward and others (1998), revealed that elevated temperatures enhance isoflavone recovery and de-esterify the malonyl group, where as room temperature extraction slowed down the conversion of one form to another. This study also showed that isoflavone extraction at refrigeration temperature (4 °C) for 2-4 hours gave the highest amount of malonyl glucoside conjugates and lowest amount of β-glucoside forms.

Soybeans contain indigestible oligosaccharides like raffinose and stachyose, which are usually associated with flatulence and other stomach discomfort (Rackis and others 1970). Soaking and sprouting of legume seeds can improve starch digestibility and thus reduce the amount of oligosaccharides through the release of α-galactosidase (Aranda and others 2001). Stachyose and other indigestible oligosaccharides can reach the colon intact and are capable of acting like prebiotics, therefore stimulating the growth of probiotics such as bifidobacteria and lactobacilli (Crittenden and Playne 1996). These oligosaccharides also have physiological effects similar to dietary fiber. Doses of 15g/day have been demonstrated to enhance the growth of probiotics while consumption of 40 to 50g/day can cause intestinal discomfort (Nyman 2002).

To be able to estimate how much isoflvones and stachyose are available to consumers, it is important to know the concentration levels of these compounds in soy-based products in order to maximize the health benefits of soybean consumption. The aim

of this study therefore is to determine the concentration of genistein, daidzein, genistin, daidzin and stachyose in germinated, non-germinated and raw (untreated) soy powders from Vinton 81, DF 222 and E05276-T soybean varieties as potential food ingredients.

4.3 MATERIALS AND METHODS

4.3.1 Materials

4.3.1.1 Chemicals and solutions

Genistein, daidzein, geinstin, daidzin standards, glacial acetic acid (99.99+ % purity), and dimethyl sulfoxide (99.9%% purity) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Methanol (HPLC grade) was bought from J.T. Baker (Mallinckrodt Baker Inc., Phillipsburg, NJ, USA) and acetonitrile (HPLC grade), was from EM Science (EM Industries, Inc., Gibbstown, NJ, USA). HPLC water used was obtained from nanopure infinity ultrapure water system (Barnstead/Thermolyne Corporation, Dubuque, IA, USA).

Several stock solutions of genistein, daidzein, genistin and daidzin standards were prepared. Stock glucoside standards were prepared by weighing 20 mg each of genistin and daidzin reference standard into a 10 ml volumetric flask. Similarly, stock aglycones standards were prepared by weighing 5 mg each of genistein and daidzein reference standard into 10 ml volumetric flask. Eight milliliters of dimethyl sulfoxide was added to each flask and sonicated until completely dissolved and each mix was then diluted to volume with dimethyl sulfoxide and mixed. Mixed standard dilutions were created using methanol/water (80/20) and the stock standards were diluted to create a five-point curve. The following dilutions of each glucoside and aglycone stock with methanol/water were

prepared: 2/10, 1/10, 1/25, 1/50, and 1/100 (concentration varied from 5.05 to 500μg/g). All the standards were stored at refrigeration temperature and protected from light for up to 60 days.

The mobile phases consisted of two solvents. Solvents A and B were prepared mixing water/acetonitrile/acetic acid in the following ratios respectively 75:25:1 and 25:75:1. The mixtures were degassed by sonification for 5 minutes prior to use in the HPLC analysis.

4.3.1.2 Instrumentation

The HPLC system consisted of the standard for isocratic and multiple pump gradient systems containing, two HPLC pumps (Waters 1525 Binary HPLC Pump), an HPLC autosampler (Waters 717 plus Autosampler), HPLC dual wavelength (λ) absorbance detector (Waters 2487), a hydrosphere C18 column 150 x 4.6 mm, particle size 5 μm and a hydrosphere C18 guard column 4 x 20 mm, particle size 5μm (Waters Corporation, Milford, MA, USA). A Dell compatible computer installed with Waters Breeze chromatography software (Version 3.30) was used to control the HPLC system.

4.3.2 Methods

4.3.2.1 Calibration curves and calculation of standard solutions

Five hundred microliters of each dilution was added into 1 ml clear glass shell vial with polyethylene snap cap (Waters Corporation, Milford, MA, USA). Ten microliters of each standard solution (five dilutions for each standard) were injected into the chromatographic system. The compounds were separated using the gradient system on a C 18 column attached to a C18 guard column at 37 °C using a flow rate of 1.0 ml/min. A

UV detector was used to detect the compounds at a single wavelength of 262 nm and set at 0.08 AUFS (absorbance units). The injection volume was 10 µl and the following gradient was used: 0-15 min, 100% solvent A and 0% solvent B; 15-25min, 0% solvent A and 100% solvent B; 25-27min 100% solvent A and 0% solvent B, and run cycle was 40 min.

The chromatograms were recorded using the data analysis Breeze software (Waters Corporation, Milford, MA, USA). The peak areas were used to create a plot of standard peak areas versus standard concentrations (standard curve) for each isoflavone standard (e.g. Figures 4.1 and 4.2). Replicate standard curves were obtained and these curves were used to calculate the concentrations of the isoflavone isomers that were extracted from the whole soy powders. The retention time and UV absorption patterns of pure isoflavones were used to identify isoflavones in the soy powder samples.

Figure 4.1 Standard curve for pure genistein standard

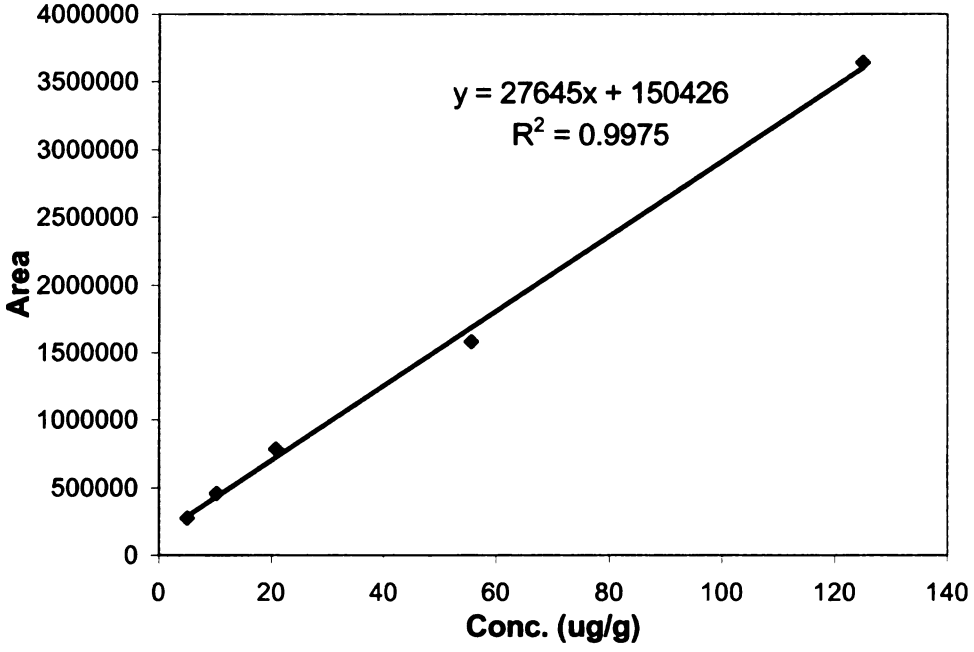
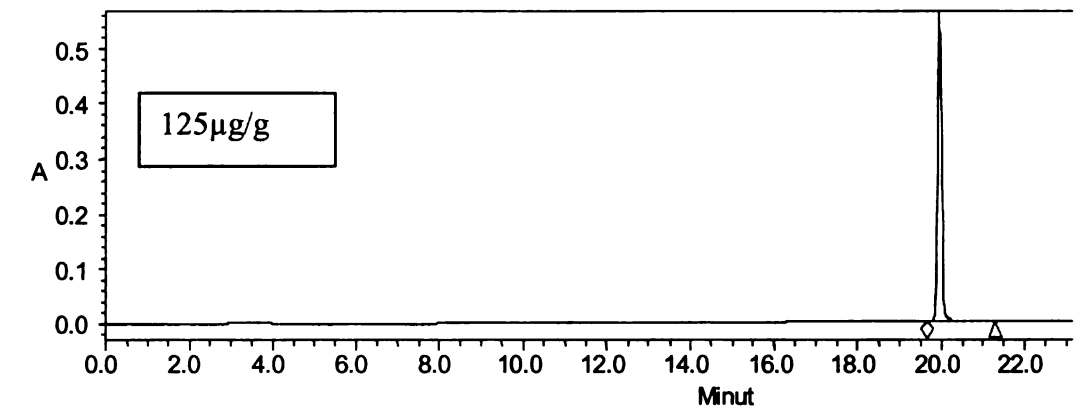
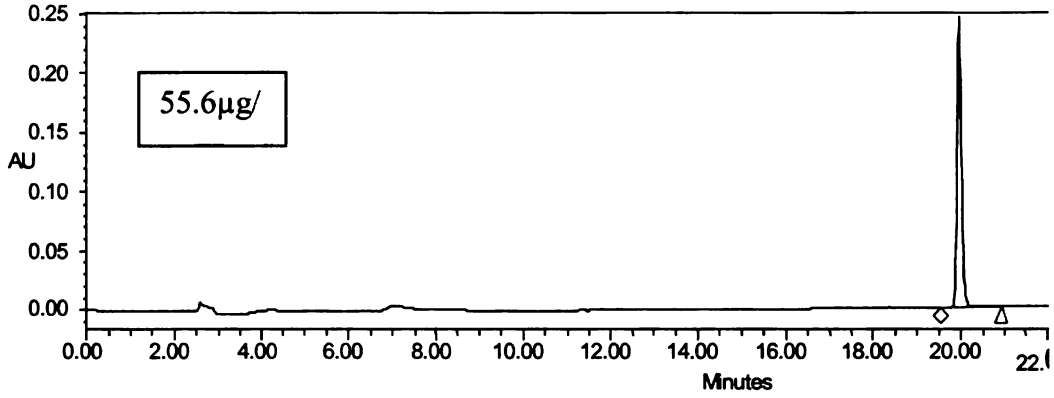


Figure 4.2 HPLC chromatogram for pure genistein standard at different concentrations (average retention time = 19.956 minutes.)





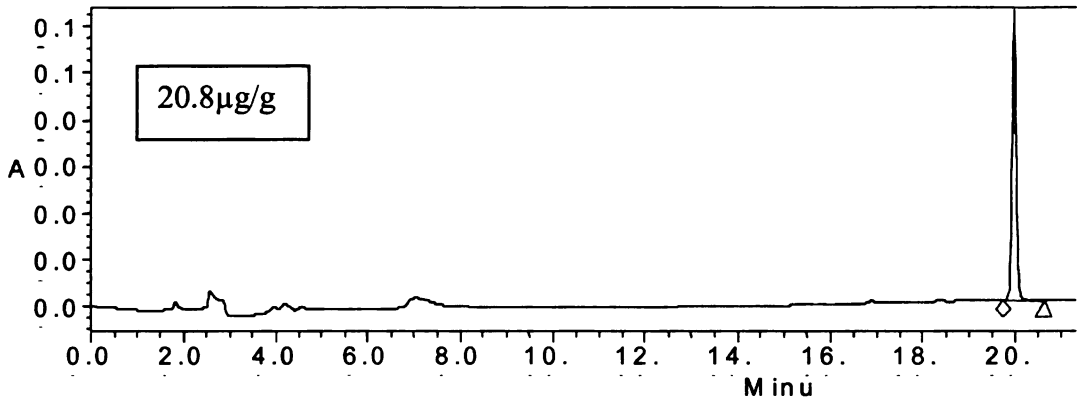
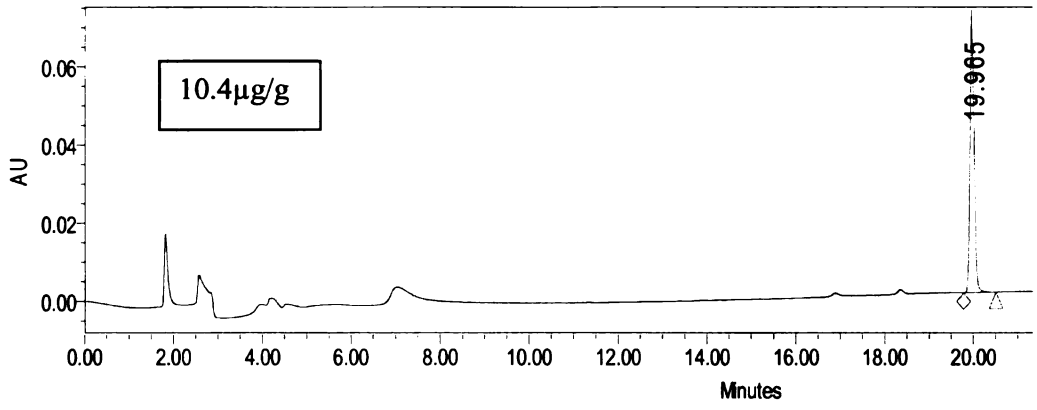
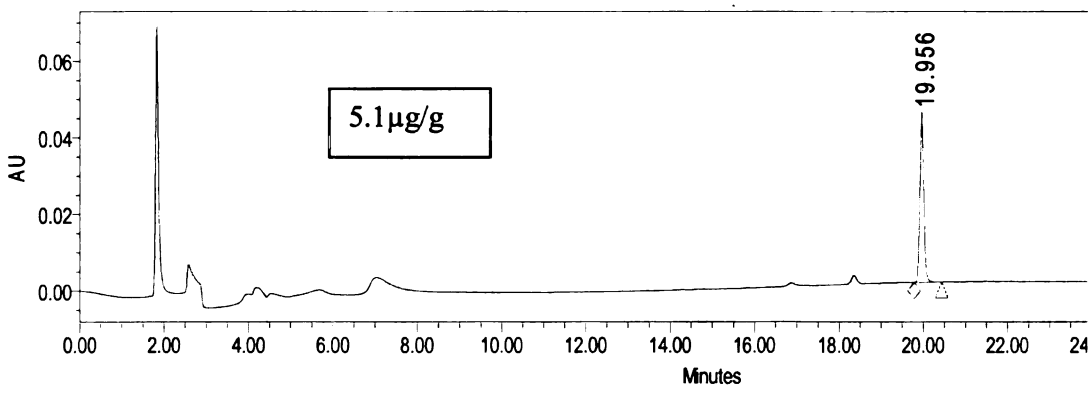


Figure 4.2 continued.





4.3.2.2 Proximate analysis of soy powders

Compositional analyses of the germinated and non-germinated soy powders were done using standard or slightly modified standard methods. These analyses were carried out in the Animal Science Department laboratories. Crude fat, protein, carbohydrate (by subtraction), ash, neutral detergent fiber and dry matter were determined. Ether extraction method was used for the crude fat analysis, neutral-detergent fiber was analyzed (Goering and Van Soest, 1970; Robertson and Van Soest, 1977; Cherney and others, 1989). The hach total nitrogen (modified kjeldahl digestion) method was used for the protein analysis (Hach and others, 1987). Ash content was determined using the AOAC (2005) method 945.46.

4.3.2.3 Isoflavone extraction

Spray-dried germinated and non-germinated soy powders and raw ground soybeans were analyzed. The soybean varieties utilized were Vinton 81, DF 222 and E05276-T. A method adopted by Bennink and Barret (2004) was used for the extraction. Two grams of each soy powder was weighed into 50 ml centrifuge tube and 35 ml of an extraction solvent made up of 50% methanol: 49% ultrapure water: 1% acetic acid was added to the powder and mixed with spatula and sonicated for 30 min. The mixture was centrifuged at 4,000 rpm for 10 min and the supernatant was poured into a centrifuge bottle (Figure 4.3). Further extractions were repeated using the following extraction solvents: 70% methanol: 29% ultrapure water: 1% acetic acid; 90% methanol: 9% ultrapure water: 1% acetic acid; 100% methanol. All the combined supernatants were kept in the centrifuge bottles overnight. After overnight storage the bottles were

centrifuged at 5,000 rpm for 15 min. The corresponding supernatants were poured into 500 ml round bottom flasks.

The filtrate for each extracted sample was dried almost completely on a rotary evaporator (Büchi; Brinkmann, Westbury, NY) at approximately 45 °C. The residue was dissolved in 15 ml 80% methanol and sonicated. The dissolved samples were filtered through 0.45 μm Supor membrane disc filters (Waters Corporation, Milford, MA, USA). All the filtered samples were stored in the freezer (-20 °C) prior to HPLC analysis.

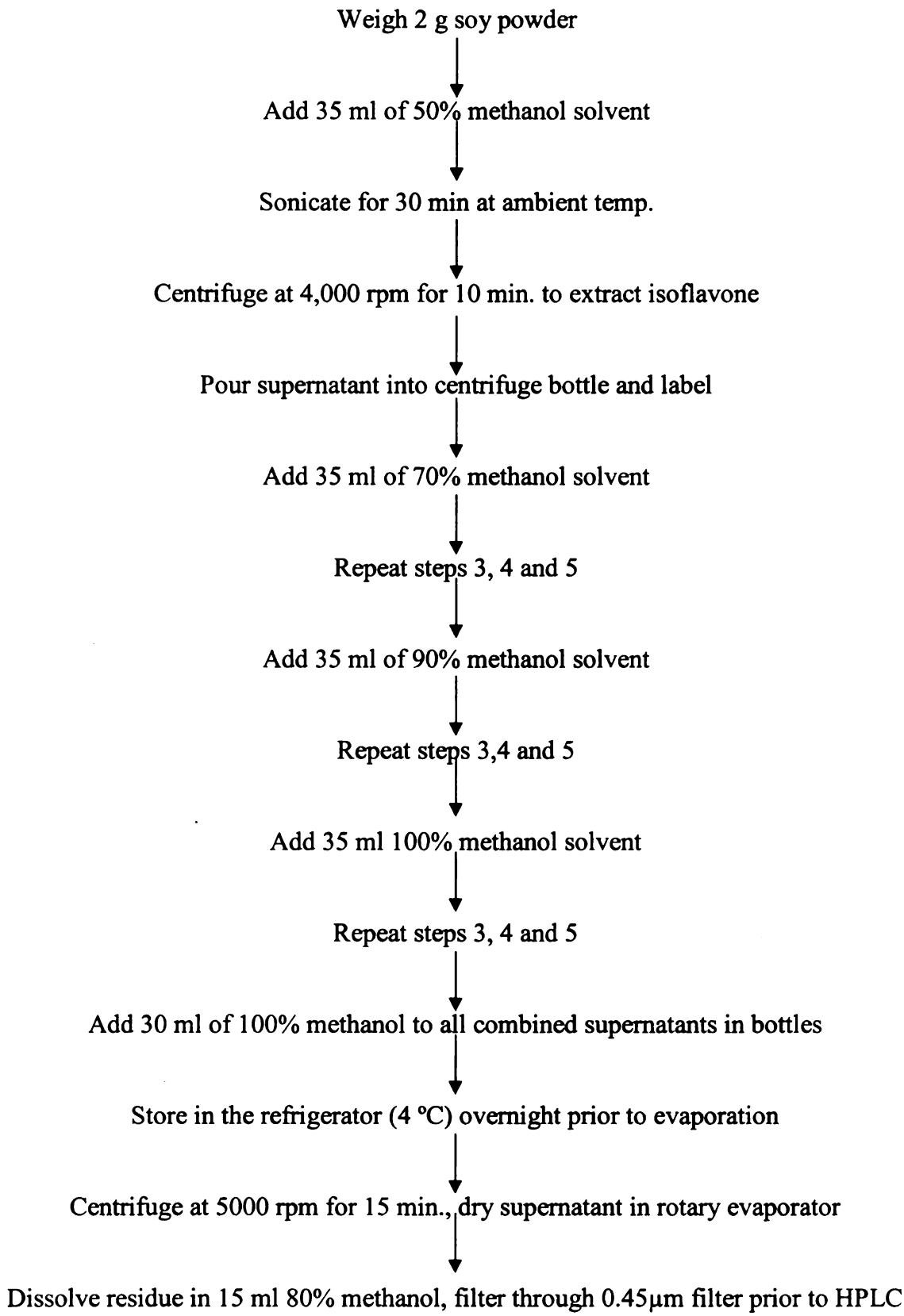
4.3.2.4 Reverse-phase high performance liquid chromatography (HPLC) of isoflavones

Waters plus C-18 SEP-PAK filter cartridges (pore size 0.22 μm) (Waters Corporation, Milford, MA, USA) were used for HPLC analysis. Each filter cartridge was conditioned with 5 ml of 80% methanol and 5 ml of HPLC grade water (nanopure water) before sample application. A portion (1 ml) of each evaporated and re-dissolved sample was micro filtered into 1 ml clear glass shell vial with polyethylene snap cap (Waters Corporation, Milford, MA, USA) and injected into HPLC system. The same running condition with the standards was also applied to the samples in the HPLC system.

4.3.2.5 Reverse-phase high performance liquid chromatography (HPLC) of stachyose

Stachyose contents of germinated and non-germinated soy powders were evaluated by ABC Research Corporation (Gainsville, FL, USA). Extraction method by Omogbai and others (2005) were utilized before HPLC analysis. It involved the use of 2-propanol for extraction. Supernatant was filtered after centrifuging before HPLC analysis.

Figure 4.3 Flow diagram for isoflavone extraction from soybean powders



4.3.3 Statistical analysis

Replicate data for nutrient composition and stachyose, and triplicate data for isoflavone analysis were used for all the analysis. Statistical analysis including linear regression, average and standard deviation (SD) was performed using the statistical function of Microsoft Excel and Sigma plot 9.0(Jandel Scientific, San Rafael, CA). Analysis of variance was done using Tukey's test for multiple comparisons of the means. Sigma Stat 3.1(Jandel Scientific, San Rafael, CA) was utilized for this analysis.

4.4 RESULTS AND DISCUSSION

4.4.1 Effect of germination on compositional analysis of soybean powder

The proximate analysis data is shown in Table 4.1. Each data represents means of two replicates. The average protein content of whole soybeans is about 40% with about 20% fat, 35% carbohydrate and 5.0% ash (Min and others 2005). Our results indicated that the protein contents of all the soybean varieties utilized were between 50.28 to 56.03% well above the average. The soybean seed varieties used for this study contained 35.5% protein and 19.8% fat (DF 222), 39.1% protein and 17.6% fat (Vinton 81) while the E05276-T variety contained 36.7% protein and 16% fat in the raw beans. According to several workers such as Khalil and others (2006), Bau and others (2000), Wang and others (2003), the nutrient contents and protein digestibility can be improved and anti-nutritional factors reduced when soybeans were soaked, germinated and dehulled.

Table 4.1 Nutrient composition (%) of germinated and non-germinated soy powders

Sample	Protein	Fat	Carbohydrate	Dry Matter	Ash	Neutral Detergent Fiber
[1]**GV 81**	56.03 ± 0.35[a]	20.85 ± 0.01[c]	18.80 ± 0.30[d]	92.41 ± 0.01[b]	4.32 ± 0.63[b]	4.54 ± 0.05[a]
NGV 81	50.76 ± 0.49[b]	18.39 ± 0.01[b]	26.06 ± 0.65[ab]	92.17 ± 0.28[b]	4.79 ± 0.16[ab]	4.39 ± 0.04[a]
GDF 222	51.00 ± 0.68[b]	22.40 ± 0.44[d]	22.20 ± 0.36[c]	92.25 ± 0.15[b]	4.40 ± 0.14[b]	4.55 ± 0.04[a]
NGDF 222	50.28 ± 0.95[b]	17.62 ± 0.05[b]	27.81± 0.88[a]	93.22 ± 0.15[a]	5.09 ± 0.02[a]	4.39 ± 0.09[a]
GET	54.97 ± 0.03[a]	15.81 ± 0.13[a]	24.20 ± 0.26[bc]	92.60 ± 0.13[ab]	5.02 ± 0.16[a]	3.84 ± 0.02[b]

[a-d] Means in the same column with small letter superscripts are significantly different ($p<0.05$); n = 2 for all samples

[1]GV 81 = Germinated Vinton 81

NGV 81 = Non-germinated Vinton 81

GDF 222 = Germinated DF 222

NGDF 222 = Non-germinated DF 222

GET = Germinated E05276-T

According to Khalil and others (2006), their study suggested that enzyme hydrolysis during and after germenation of raw soybeans resulted in a remarkable increase in the protein content. Our data clearly showed that soaking and germination greatly improved the nutrient contents especially the protein contents. Ahmad and Pathak

(2000) also reported that no significant changes were observed in protein, total sugars, reducing sugars or ash contents of germinated and non-germinated soybean flours.

Overall the germinated soybeans in this research had higher protein contents than the non-germinated counterparts although germinated Vinton 81 (56.03%) and germinated E05276-T (54.97%) were significantly higher statistically ($p < 0.05$), than the rest (Table 4.1). There was no significant difference in protein content between germinated and non-germinated DF 222 powders. Soybean proteins have played a significant role in human nutrition for a long time in both developing and developed countries. Soaking and germination process, induced increase in hydrolytic enzymes (increased water activity), which facilitated the metabolism of nitrogenous compounds from carbohydrate reserves, probably leading to increase in crude protein contents (Khalil and others 2006). On the other hand, the carbohydrate contents of the non-germinated soybeans were significantly higher than the germinated counterparts. Non-germinated Vinton 81 and DF 222 contained 26.06% and 27.81% carbohydrate respectively while their germinated varieties had 18.80% and 22.20% carbohydrate respectively. This observation could be as a result of lower α-galactosidase activity in the non-germinated soybeans hence the carbohydrate contents remained more intact than the germinated soybeans (Bau and others 2000). Such soybeans as previously discussed are expected to have flatulence-causing properties.

The total fat contents were generally low in all varieties although it is expected that the lipoxygenase activities should be lower in germinated than in non-germinated soybeans therefore improve odor and flavor of such beans. The fat content of germinated E05276-T soy powder was significantly lower than the rest of the powders. Non-

germinated Vinton 81 and DF 222 had significantly lower fat contents (18.39% and 17.62% respectively) than their germinated varieties (20.85% and 22.40% respectively). The ash contents of the non-germinated soy powders were generally higher than the germinated powders. Ash contents varied between 4.32% (for germinated Vinton 81) to 5.09% (for non-germinated DF 222). Similarly there was no significant difference between the dietary fiber contents of germinated and non-germinated Vinton 81 and DF 222 (Table 4.1). The dry matter, which is an indication of the moisture contents of the powders, varied from 92.17% (for non-germinated Vinton 81) to 93.22% (for non-germinated DF 222).

4.4.2 Effect of germination on total isoflavone content

The average total isoflavone (4 isomers, on dry matter basis) contents, namely genistin, genistein, daidzin and daidzein of raw, non-germinated (soaked) and germinated soybean varieties are shown in Table 4.2.

Table 4.2 Total isoflavone contents (on dry matter basis) in raw and spray dried germinated and non- germinated soybean powder (μg/g)

	Soybean varieties		
Seed treatment	**Vinton 81**	**DF 222**	**E05276-T**
Raw	378.74 ± 1.32^{c}	375.33 ± 19.85^{c}	290.13 ± 28.20^{b}
Non-germinated (soaked)	509.41 ± 19.41^{a}	726.16 ± 51.80^{a}	N/A*
Germinated	419.84 ± 2.55^{b}	414.77 ± 28.83^{b}	611.87 ± 21.30^{a}

[a-b] Values in a column with different letters are significantly different ($p < 0.05$)

* Not Available

The total isoflavone contents of the isomers measured in the raw beans from soybean varieties Vinton 81, DF 222 and E05276-T were 378.74, 375.33 and 290.13 μg/g, respectively. The total isoflavone contents in Vinton 81 and DF 222 varieties increased after soaking. During the course of this study, the limited availability of E05276-T variety did not allow us to process the non-germinated type hence there was no data for this treatment. Among the germinated powders, the total isoflavone content of E05276-T was the highest (611.87 μg/g). There was significant difference between the total isoflavone contents of the raw and germinated soy powders of Vinton 81 and DF 222 and E05276-T varieties.

According to Zhu and others (2005), the isoflavone contents of soybeans are affected by soybean variety, environmental changes like temperature, and amount of sunshine and moisture level. The increase of isoflavone contents within the same soybean variety could be attributed to induced metabolic pathways of the precursors of isoflavonoids commonly found in legumes or oil seeds.The formation of β-glucoside is increased as a result of de-esterification of malonyl- and acetylglycoside. As observed in Table 4.2, the increase after soaking and decrease after germination of isoflavone contents could be due to the conversion of different flavonoids to isoflavones and vice versa or conversion of an isoflavone isomer to another isomer as reported by Terrence (1991).

4.4.3 Effects of germination on Genistein and Genistin contents

Genistein and its β-glucoside conjugate, genistin contents in variously treated soybean powder varieties are shown in Table 4.3. Total genistein and genistin increased

significantly during soybean soaking and germination. The maximum amount was obtained at soaking (354.2 μg/g) for variety DF 222, and 285.2 μg/g for variety Vinton 81. Data was not obtained for the soaked version of E05276-T variety because the powder was not available but its germinated counterpart had a total content of 316.2 μg/g, which was significantly higher than the raw powder (137.6 μg/g).

Table 4.3 Total Genistein and Genistein contents in raw and spray dried germinated and non-germinated soybean powder (μg/g)

Soybean variety	Genistein	Genistin	Total
[1]**GV 81**	20.091 ± 1.38^{bc}	220.740 ± 4.44^{b}	240.831 ± 6.99^{c}
NGV 81	59.154 ± 7.77^{a}	226.055 ± 4.25^{b}	285.209 ± 20.76^{bc}
RV 81	5.069 ± 2.63^{c}	172.075 ± 1.37^{c}	177.144 ± 3.86^{d}
GDF 222	66.123 ± 8.12^{a}	177.551 ± 6.72^{c}	243.674 ± 25.21^{c}
NGDF 222	55.528 ± 9.49^{a}	298.65 ± 10.27^{a}	354.174 ± 34.19^{a}
RDF 222	3.025 ± 2.26^{c}	165.250 ± 3.10^{cd}	168.275 ± 10.35^{d}
GET	42.195 ± 12.79^{ab}	274.017 ± 6.99^{a}	316.212 ± 23.21^{ab}
RET	0.000 ± 0.00^{c}	137.608 ± 6.75^{d}	137.608 ± 11.693^{d}

[a-b] Values in a column with different letters are significantly different ($p < 0.05$); n = 3
[1]GV 81 = Germinated Vinton 81
NGV 81 = Non-germinated Vinton 81
RV 81 = Raw Vinton 81
GDF 222 = Germinated DF 222
NGDF 222 = Non-germinated DF 222
RDF 222 = Raw DF 222
GET = Germinated E05276-T
RET = Raw E05276-T

There was a highly significant difference in genistein content between the raw and soaked or germinated varieties. Raw seeds from E05276-T seem to have no detectable amount of genistein (0.00 μg/g). The differences especially in the genistein content among raw, non-germinated (soaked only), and germinated soy powders could be as a result of physiological changes that occurred during soaking and germination. Soaking and germination to a limited extent induce the hydrolysis of glucosides, which leads to an increase in genistein content (Zhu and others, 2005). The decrease in genistein content in germinated Vinton 81 when compared to non-germinated Vinton 81 could be as a result of the conversion of its genistein to other isoflavones.

4.4.4 Effects of germination on Daidzein and Daidzin contents

The effect of soybean germination on daidzein and daidzin is shown in table 4.4. All the raw soybean varieties had similar daidzein content i.e. Vinton 81 (2.13μg/g), DF 222 (3.65 μg/g) and E05276-T (2.49 μg/g). Overall, a small quantity of daidzein (aglycone) was present in the soy powders in comparison with its glucoside component i.e. daidzin. The maximum amounts of daidzein (27.95 and 27.37 μg/g) were observed after soaking and germination in DF 222, which was not significantly different ($p < 0.05$) from non-germinated (soaked only) Vinton 81 (21.52 μg/g), and germinated E05276-T (18. 99 μg/g). Germinated Vinton 81 had significantly lower daidzein content (5.65μg/g) than the non-germinated counterpart. This observation was also similar to the result obtained in the isoflavone, genistein content in germinated and non-germinated Vinton 81.

Table 4.4 Total Daidzein and Daidzin contents in raw and spray dried germinated and non-germinated soybean powder (μg/g)

Soybean variety	Daidzein	Daidzin	Total
[1]**GV 81**	5.650 ± 1.07^{b}	173.355 ± 3.53^{cd}	179.006 ± 4.08^{d}
NGV 81	21.516 ± 3.27^{a}	232.685 ± 31.82^{bc}	224.202 ± 0.81^{c}
RV 81	2.129 ± 1.07^{b}	199.468 ± 1.87^{cd}	201.597 ± 2.57^{c}
GDF 222	27.370 ± 4.44^{a}	143.733 ± 0.81^{d}	171.103 ± 5.07^{d}
NGDF 222	27.955 ± 3.61^{a}	344.044 ± 10.64^{a}	371.999 ± 10.18^{a}
RDF 222	3.651 ± 0.05^{b}	203.400 ± 5.13^{c}	207.051 ± 5.53^{c}
GET	18.994 ± 2.249^{a}	276.666 ± 2.66^{b}	295.660 ± 3.31^{b}
RET	2.494 ± 0.44^{b}	150.031 ± 9.70^{d}	152.525 ± 9.74^{d}

[a-b] Mean values in a column with different letters are significantly different ($p < 0.05$); n=3

[1] GV 81 = Germinated Vinton 81

NGV 81 = Non-germinated Vinton 81

RV 81 = Raw Vinton 81

GDF 222 = Germinated DF 222

NGDF 222 = Non-germinated DF 222

RDF 222 = Raw DF 222

GET = Germinated E05276-T

RET = Raw E05276-T

The percent increase of daidzein contents in non-germinated i.e. soaked and spray-dried soy powders from varieties Vinton 81 and DF 222 were 910.6% and 665.7% respectively compared to raw powders. Similarly, the percent increase of daidzein content in germinated powders from Vinton 81, DF 222 and E05276-T varieties were 165.4%, 649.7% and 661.6% respectively compared to raw powders. However there was a remarkable decrease in daidzein content between non-germinated and germinated soy powders for Vinton 81. Germinated DF 222 and germinated E05276-T powders had significantly higher daidzein content (27.96 and 18.99 μg/g respectively) than germinated Vinton 81 powder (5.65 μg/g), which was not significantly different ($p < 0.05$) from raw powders from all the soybean varieties. The total amount of daidzein and daidzin were significantly higher in non-germinated powders ($p < 0.05$) in DF 222 and Vinton 81. Non-germinated powder from E05276-T was not available hence no data was obtained. The maximum amount of daidzein and daidzin was observed in non-germinated DF 222 variety (372 μg/g).

The retention times of four soy isoflavone isomers measured are shown in Figures 4.4, 4.5 and 4.6. The retention times for daidzin, genistin, daidzein and genistein were approximately 13.7, 14.9, 17.6 and 19.8 minutes respectively. Overall, the β-glucosides isomers genistin and daidzin were more abundant than the aglycone isomers genistein and daidzein in all the soy powders analyzed (Figure 4.7). This could be to due the fact that the aglycone forms are easily converted during processing and storage into other isoflavone isomers that were not measured, but are equally beneficial to the health of the consumer (Coward and others, 1998). Generally, the total amount of isoflavones in each powder was high.

Figure 4.4 Representative HPLC chromatogram of isoflavones in germinated (GV 81), non-germinated (NGV 81) and raw (RV 81) Vinton 81 soybean varieties (**a**, daidzin; **b**, genistin; **c**, daidzein; **d**, genistein).

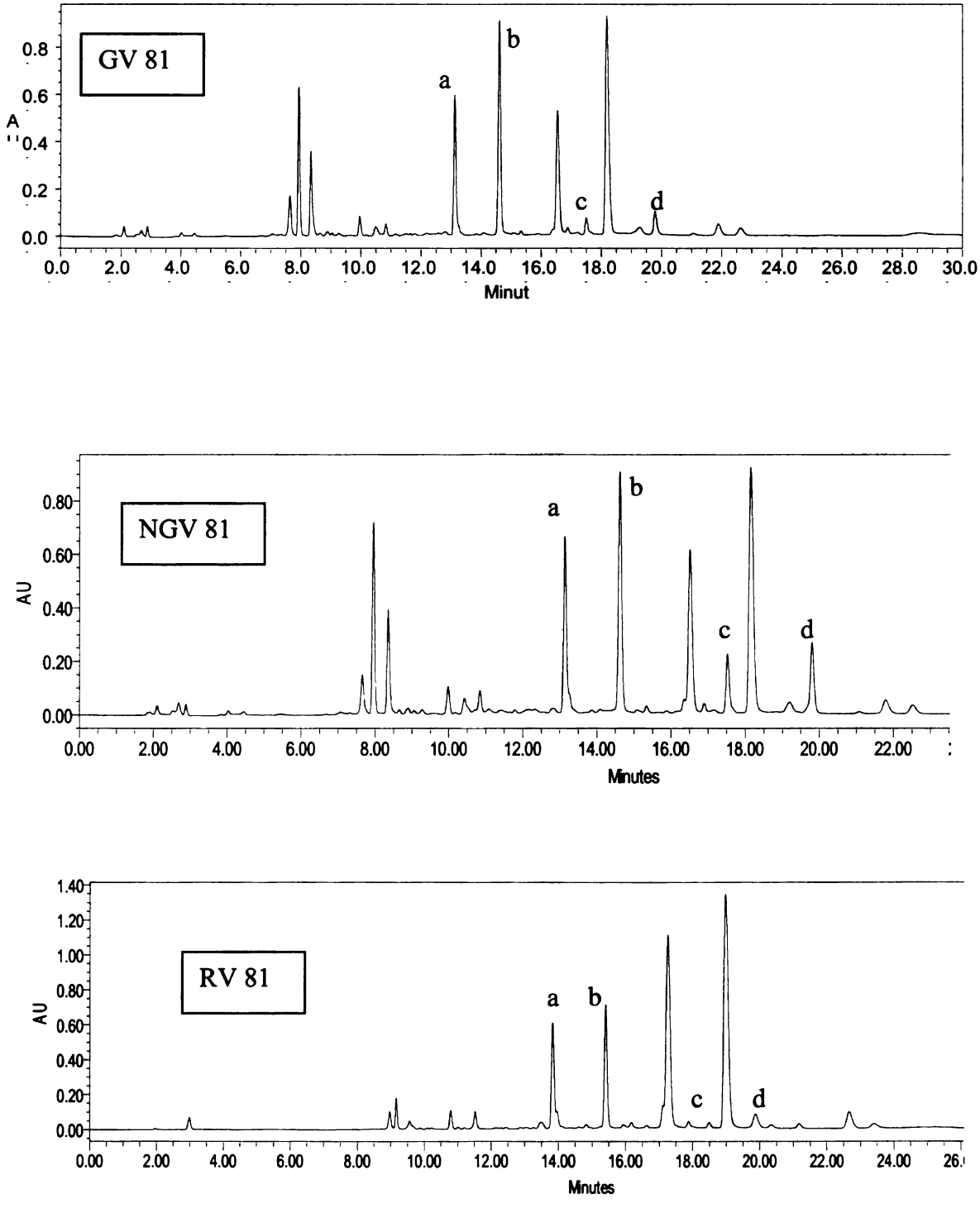


Figure 4.5 Representative HPLC chromatogram of isoflavones in germinated (GDF 222), non-germinated (NGDF 222) and raw (RDF 222) DF 222 soybean varieties (**a**, daidzin; **b**, genistin; **c**, daidzein; **d**, genistein).

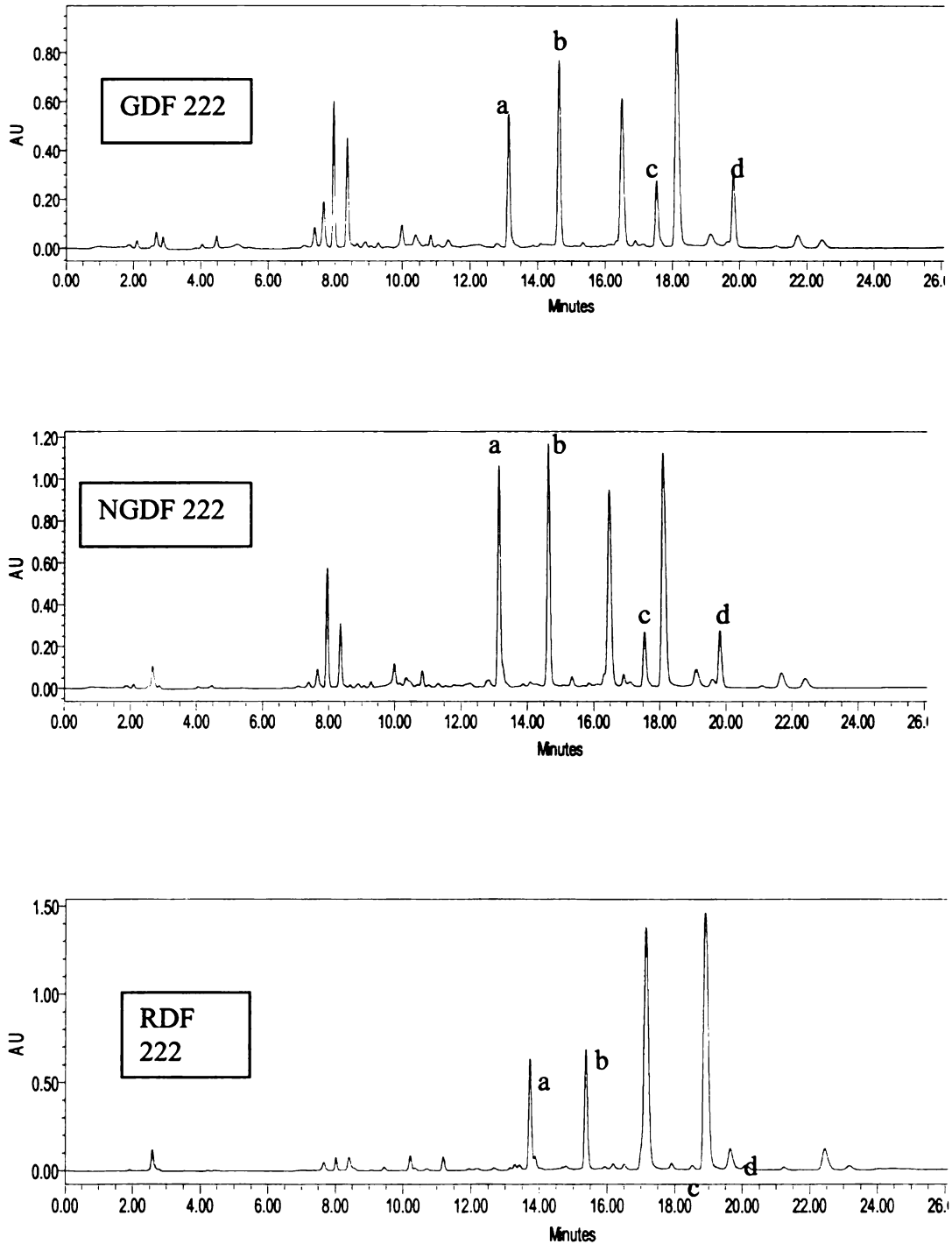


Figure 4.6 Representative HPLC chromatogram of isoflavones in germinated (GET), and raw (RET) E05276-T soybean varieties (**a**, daidzin; **b**, genistin; **c**, daidzein; **d**, genistein).

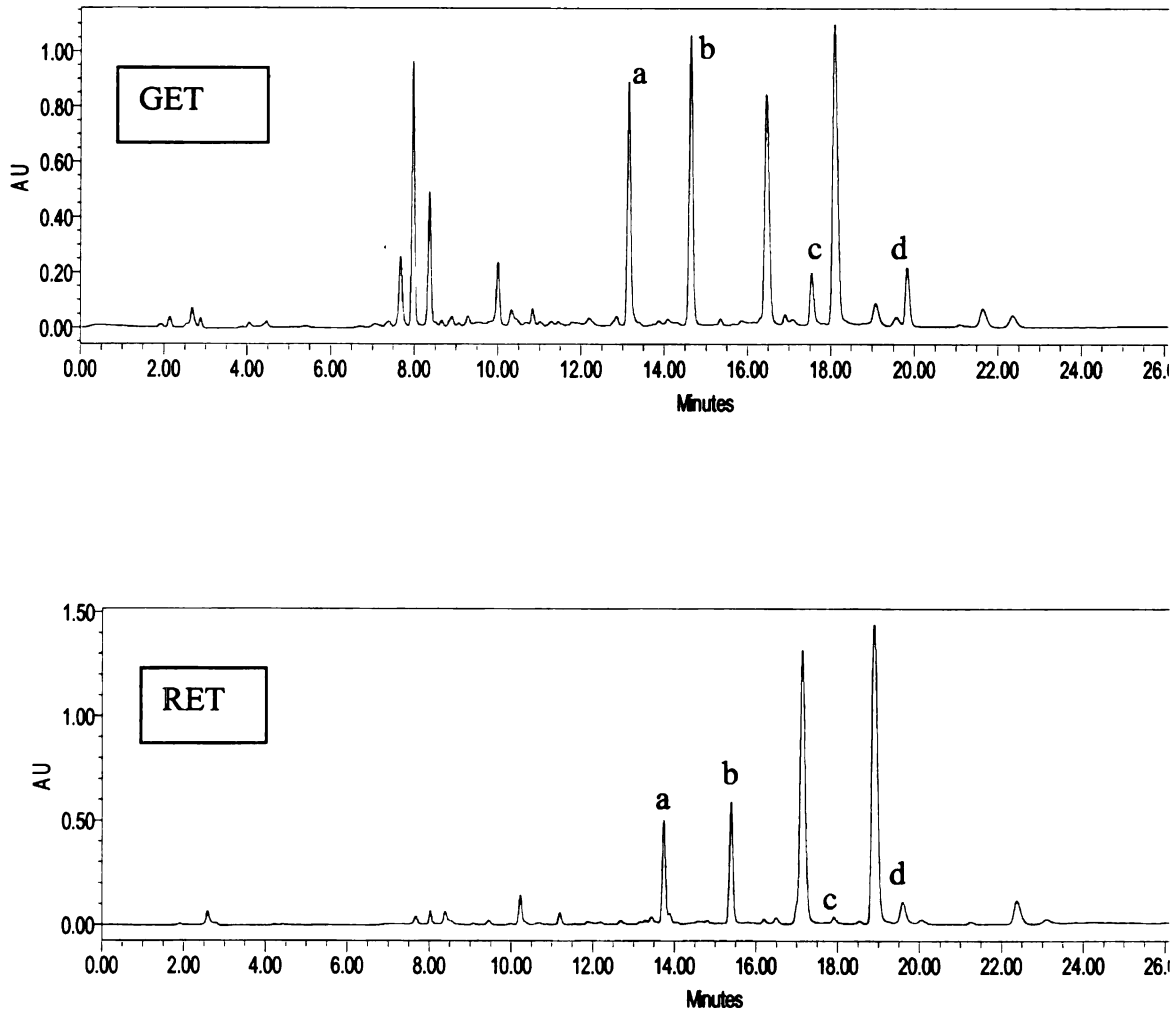
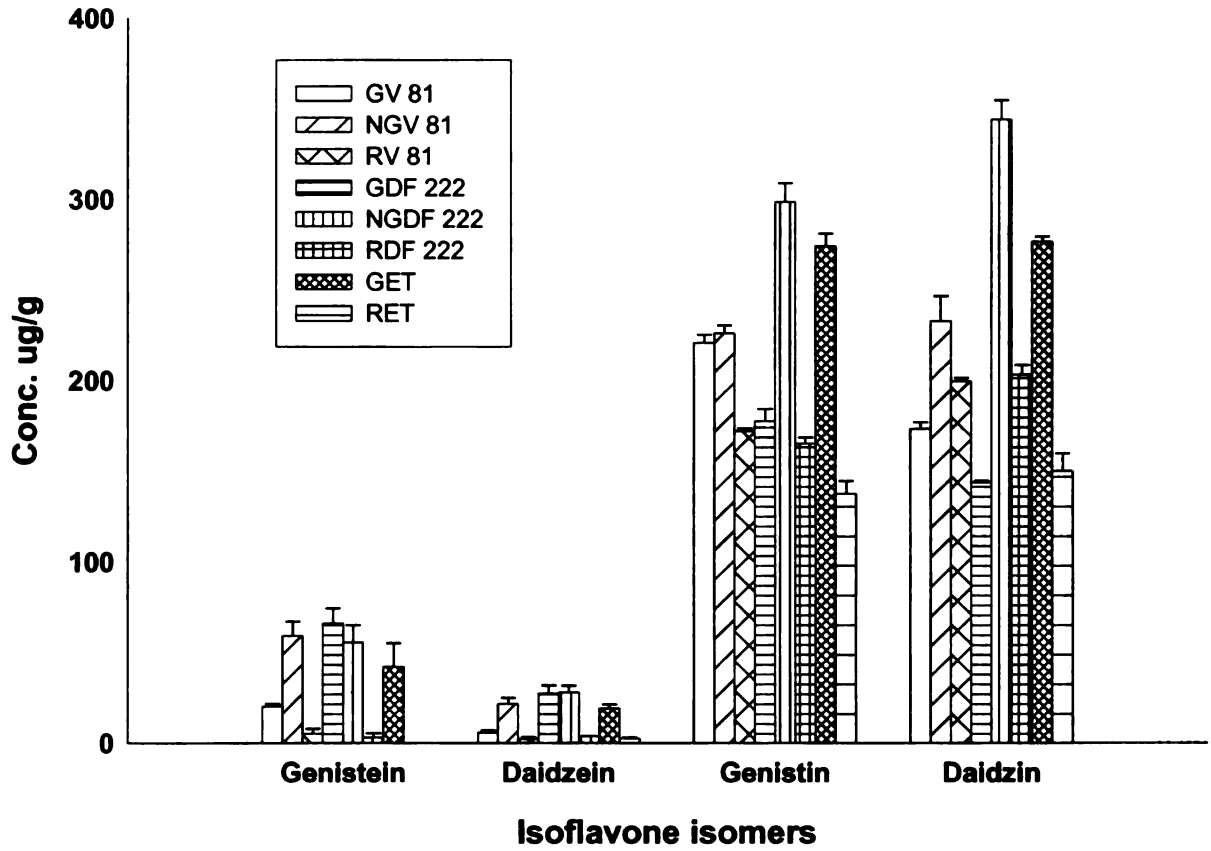


Figure 4.7 Concentrations of soy isoflavone isomers in germinated, non-germinated and raw soy powders.



GV 81 = Germinated Vinton 81

NGV 81 = Non-germinated Vinton 81

RV 81 = Raw Vinton 81

GDF 222 = Germinated DF 222

NGDF 222 = Non-germinated DF 222

RDF 222 = Raw DF 222

GET = Germinated E05276-T

RET = Raw E05276-T

4.4.5 Effects of germination on Stachyose contents

Stachyose contents of germinated and non-germinated soy powders are shown in Table 4.5. All the germinated soy powder varieties i.e. Vinton 81, DF 222 and E05276-T had significantly lower ($p < 0.05$) amounts of stachyose (19.15 mg/g, 15.45mg/g and 13.9mg/g respectively). On the other hand, non-germinated Vinton 81 and DF 222 contained 41.35 mg/g and 46.65 mg/g respectively.

Table 4.5 Stachyose contents in spray dried germinated and non-germinated soybean powder (mg/g)

	Soybean varieties		
Seed treatment	**Vinton 81**	**DF 222**	**E05276-T**
Non-germinated (soaked)	41.35 ± 0.92^{b}	46.65 ± 0.78^{a}	N/A*
Germinated	19.15 ± 0.35^{c}	15.45 ± 0.35^{cd}	13.90 ± 2.26^{d}

[a-d] Values with different superscript letters are significantly different ($p < 0.05$); n = 2

* Not Available

Mature raw soybeans contain about 1.4 to 4.1% stachyose depending on the variety. Stachyose is composed of one sucrose molecule connected to two molecules of galactose. In this study the value of stachyose contents in the germinated powders ranged from 1.39% (in E05276-T) to 1.91% (in Vinton 81). Meanwhile, the stachyose contents in non-germinated powders varied from 4.14% (in Vinton 81) to 4.67% (in DF 222), which were significantly different from the germinated powders. These data clearly showed that germination process greatly reduced the contents of stachyose by 53.7% in Vinton 81 and 66.9% in DF 222 varieties. This phenomenon could be attributed to the increase in enzyme α-galactosidase during germination (Viana and others, 2005), which

may have resulted in the conversion of the tetrasaccharide to monosaccharides and disaccharides.

The purpose of this study was to evaluate the effects of germination on isoflavone and stachyose contents and the potential importance on the inclusion of such powders in food preparations to influence optimal health benefits. In summary, soaked (non-germinated) and germinated soybeans induced substantial increase in the contents of isoflavones particularly the aglycones. Non-germinated and germinated DF 222 soybean variety gave the highest aglycone contents. Soaking caused a substantial increase in daidzin, genistin, genistein and daidzein. In most cases germination of soaked seeds decreased the aglycone content suggesting that controlled germination (e.g. germination time, temperature and sprout length) is needed to enhance the aglycone content of soybean. Also, germination reduced the stachyose contents of the powders significantly compared to non-germination process (soaking only). This suggests that flatulence factor or stomach discomfort due to stachyose would be reduced in such powders and at the same time the powders will contain necessary amounts to act as prebiotics or dietary fibers.

The above results clearly suggest that soybean seed soaking and/or germination are necessary in order to increases the more bioactive (aglycones) components of soy food products before consumption. This will lead to increased absorption in the gut of the consumer hence increase health benefits.

CHAPTER 5

EFFECT OF PROCESSING AND REFRIGERATED STORAGE ON ISOFLAVONE AND STACHYOSE CONTENTS OF YOGURT FORTIFIED WITH NON-GERMINATED AND GERMINATED/PREDIGESTED WHOLE SOY POWDER

5.1 ABSTRACT

The profiles of genistein, daidzein, genistin, daidzin and stachyose were determined in freeze-dried yogurts fortified with germinated or non-germinated spray dried whole soy powders. The isoflavones were evaluated at one week of manufacturing and also at the end of six weeks of storage at 4 °C while stachyose was evaluated at one week of manufacturing. The soybean varieties utilized for yogurt making were Vinton 81, DF 222 and E05276-T and reverse-phase high-performance (HLPC) was used for analysis. The total of four isoflavone contents increased after 6 weeks of storage compared to the 1st week. Daidzin and genistin (β-glucosides) contents contributed most to the increment during storage. The genistein and daidzein contents of the whole soy-fortified yogurts remained significantly the same throughout the shelf life period (6 weeks). The isoflavones retained in the soy and soy- fortified yogurts were significantly ($p < 0.05$) high (e.g. 94% i.e. 478.7 μg/g retained in 100% soy yogurt) at the end of 6th week storage compared to the isoflavone contents of the corresponding soy powder (509.4 μg/g for non-germinated Vinton 81 powder) that was utilized in the yogurt base.

All the yogurt samples containing germinated soy powders had lower stachyose contents than non-germinated soy-fortified yogurts (2.82 to 4.41 mg/g of stachyose). The stachyose amounts in yogurts fortified with non-germinated soy powders varied from 8.45 to 17.25 mg/g). The sample with the highest concentration of 17.25 mg/g was the 100% soy yogurt. Hence effect of soybean germination and subsequent fermentation by lactic acid bacteria during yogurt making was shown in stachyose content.

5.2 INTRODUCTION

Dairy yogurt fortification with fibers and minerals so as to provide additional health benefits has been studied (Fernandez-Garcia and McGregor, 1997; Hekmat and McMahon, 1997). Yogurt has been considered as a good vehicle to provide combined benefits of soy protein and dairy ingredients (Schmidt and others 1980). According to the Federal Register (1999), dairy yogurts that contain 5% added soy protein concentrate meet the FDA requirement for health claim (6.25g soy protein per serving), while dairy yogurts that contain only 2.5% soy protein are regarded as a "good source" claim of soy protein. Previous work showed that dairy yogurts fortified with 5.0% soy protein was found to be darker, chalky and less sweet, while yogurts fortified with 1 or 2.5% soy protein were most similar to control yogurt (Drake and others, 2000).

There has been considerable interest in soybean isoflavones aside from soy proteins, and their potential health benefits. Initially, isoflavone compositions in soy foods were thought to be dependent on whether the food was fermented or not. As such it was assumed that fermented soy foods contained the unconjugated isoflavone aglycones (daidzein, genistein and glycitein) only, while the unfermented foods contained the

β-glucoside conjugates (daidzin, genistin and glycitin) (Coward and others, 1998). Reverse-phase high performance liquid chromatography (HPLC), has shown that most soy foods contain mixtures of isoflavone isomers. Research has shown that some of these isomers are altered during food processing and extraction methods.

Major biologically active soy isoflavones associated with soy foods consumption are daidzein, genistein and glycitein as aglycones. Several authors have studied the biological activity of isoflavones and suggested many beneficial roles in the diet (Setchell, 2002; Song and others, 1998), but there are other studies indicating undesirable effects of isoflavones such as cognitive function, reproductive abilities and breast cancer risk especially when consumed in high dosages (Sirtori, 2001). Some European countries like Italy have advised consumers to maintain a daily intake of isoflavones, consumed as dietary supplement lower than 80mg/day (Morandi and others 2005). It has been reported that 30% to 50% of people in Western countries have the capacity to convert isoflavone glycosides to aglycones and then to equol in the intestinal tract (Frankenfeld and others, 2005; Setchell and others 2002).

Considerable quantities of oligosaccharides present in soy-based foods limit their biological value and acceptability due to the flatulence-causing factor. At the same time, these oligosaccharides promote the growth of probiotics in the gut, which promote gut health. A reduction in oligosaccharide level in soy-based foods will be highly desirable. Several methods used to lower oligosaccharide contents include hulling, soaking, cooking, gamma irradiation, germination and microbial or plant α-galactosidase treatments (Viana and others, 2005).

Past studies have shown that soybean soaking and/or germination increased the aglycone forms of the isoflavones. Several workers have also reported the bioconversion of isoflavone glycosides to aglycones in soymilk or fortified soymilk by probiotics such as bifidobacteria and lactobacilli (Tsangalis and others, 2002; Otieno and others, 2005; Chien and others, 2006; Shah, 2006; Pham and Shah, 2007; Pham and Shah, 2008). It is crucial to know exactly what forms and proportion of isoflavones the individuals consume, since it is proposed that aglycones are absorbed more readily than the β-glucosides. Consequently, dairy/soy-based foods would attract a broader approval if reasonable amounts of aglycones and oligosaccharides such as stachyose were present. . The objective of this study was to analyze the yogurt samples and evaluate the outcome of the bioactive compounds (genistein, daidzein, genistin and daidzin), in the samples after processing and storage at refrigerated temperature.

5.3 MATERIALS AND METHODS

5.3.1 Yogurt samples

The yogurt samples utilized for this study were freeze-dried in the department of Animal Science Laboratories, Michigan State University. Briefly, yogurts were prepared by blending non-fat dry milk with or without soy powders, stabilizer and sugar. The yogurt mixtures were homogenized and inoculated with *Streptococcus salivarius* subsp. *thermophilus*, *Lactobacillus delbreuckii* subsp. *bulgaricus*, Lactobacillus acidophilus NCFM and incubated at 42 °C until pH of 4.4-4.6. The same yogurt samples were utilized during the sensory evaluation study and randomly coded as 159 (50% non-germinated DF 222 + 50% non-fat dry milk), 344 (50% germinated DF 222 + 50% non-fat dry milk),

169 (50% non-germinated Vinton 81 + 50% non-fat dry milk), 252 (50% germinated Vinton 81 + 50% non-fat dry milk), 817 (50% germinated E05276-T + 50% non-fat dry milk), 894 (100% non-germinated Vinton 81- all soy control) and 949 (100% non-fat dry milk- all dairy control). All the samples were placed in freezable cups covered with cheesecloths and labeled appropriately. The cups were placed in a chamber of a laboratory freeze drier (Sorvall RC 6 Plus, Thermo Electron Corporation, Asheville NC, USA) to dry at –50 °C and 10 microns Hg pressure until a constant weight was reached. Total drying was estimated to be 18-26 hours for each load.

5.3.2 Instrumentation and solutions

Reverse-phase high performance liquid chromatography (HPLC) instrumentation system and conditions utilized in the analysis of isoflavone concentration in soybean powders were also utilized for this study. A gradient system consisting of two HPLC pumps (Waters 1525 Binary HPLC Pump), HPLC autosampler (Waters 717 plus Autosampler), HPLC dual wavelength absorbance detector (Waters 2487), a hydrosphere C18 column 150 x 4.6 mm, particle size 5 μm and a hydrosphere C18 guard column 4 x 20 mm, particle size 5 μm (Waters Corporation, Milford, MA, USA).

Standard curves for each isoflavone isomer i.e. genistein, daidzein, genistin and daidzin standards previously utilized for soy powder isoflavone analysis was used for this study (Figures 4.1 and 4.2). HPLC grade solutions of methanol (J.T. Baker, Mallinckrodt Baker Inc., Phillipsburg, NJ, USA), acetonitrile (EM Science, EM Industries, Inc., Gibbstown, NJ, USA) and nanopure water (Barnstead/Thermolyne Corporation, Dubuque, IA, USA) were utilized for both extraction and HPLC process. Isoflavone standards were procured from Sigma-Aldrich (St. Louis, Missouri, USA).

5.3.3 Extraction and Evaporation

Freeze dried yogurt samples were ground using porcelain mortar and a pestle prior to mixing. The extraction procedure that was used during isoflavone extraction from soy powders was utilized (Figure 4.3). Here, two grams of finely ground yogurt samples were mixed with 35ml of 50% methanol in water and mixed for 30 minutes before centrifuging at 4,000 rpm for 10 minutes. The supernatant was collected after which subsequent extractions were carried out with 70% methanol, 90% methanol and 100% methanol (Figure 4.3).

All the supernatants for each sample were combined in a centrifuge bottle and kept overnight in the refrigerator. At the end of overnight storage, the mixtures were further centrifuged at 5,000 rpm for 15 minutes and the supernatants transferred into 500 ml round bottom flasks. These samples were evaporated in the rotary evaporator (Büchi, Birnkmann, Westbury, NY) at 45 °C and each residue was dissolved in 15 ml 80% methanol and mixed thoroughly. Each mixture was filtered through 0.45 μm Supor membrane disc filters (Waters Corporation, Milford, MA, USA), and all the filtrates were stored in the freezer at –20 °C pending HPLC analysis.

5.3.4 HPLC Analysis

A linear reversed-phase HPLC gradient used consisted of solvent A, 25% acetonitrile in water with 1% acetic acid, and solvent B made up of 75% acetonitrile in water with 1% acetic acid. Same standard curves initially developed for soy powders (Figures 4.1 and 4.2) for calculation of genistein, daidzein, genistin and daidzin were utilized for the yogurt sample extracts. Prior to each run, the system was equilibrated for

40 minutes with solvent A. The flow rate was 1.0 ml/min. UV spectra (262 nm) were recorded and area responses were integrated by Breeze chromatography software (Version 3.30) (Waters corporation, Milford, MA, USA). Isoflavone contents and profiles were determined in all yogurt samples at week one and week six (end of storage at 4 °C). The eluted isoflavones were detected at 262 nm and quantitative data for genistein, daidzein, genistin and daidzin were obtained from comparison with the known standards. The stachyose contents of the yogurt samples at first week of manufacturing were evaluated using the HLPC method at ABC Research Corporation (Gainsville, FL, USA).

5.3.5 Statistical Analysis

Statistical analysis was conducted using the statistical function of Microsoft excel and Sigma plot 9.0 (Jandel Scientific, San Rafael, CA). One-way analysis of variance using Sigma Stat 3.1 (Jandel Scientific, San Rafael, CA) was conducted, and differences between the sample means were analyzed by Tukey's test for multiple comparisons of means at $p < 0.05$. Triplicate samples were used for isoflavone evaluation while replicate samples were used for the stachyose evaluation.

5.4 RESULTS AND DISCUSSION

5.4.1 Effects of soy fortification on total isoflavone content of yogurt

In this study four isoflavone isomers evaluated in the yogurt samples were geinstein, daidzein, genistin and daidzin. The HPLC results for the isomeric isoflavones are shown in Figures 5.1, 5.2 5.3 and 5.4. The respective concentrations of total isoflavones in the yogurt samples at first week and sixth week varied over wide ranges depending on the

sample. Mean total isoflavone concentration varied between 0.00-377.15 μg/g for 1st week, and 0-478.66 μg/g, for 6th week of storage (Table 5.1 and Figures 5.1 and 5.2). The 100% all dairy yogurt (sample 949) did not contain any measurable isoflavones (0.00 μg/g) at 1st and 6th week, while 100% all soy yogurt (sample 894) had the highest concentration at 1st and 6th week (377.15 μg/g and 478.66 μg/g respectively.

Figure 5.1 Representative HPLC chromatogram of isoflavones in soy-fortified yogurts at 1st week (**a**, daidzin; **b**, genistin; **c**, daidzein; **d**, genistein). Reverse-phase

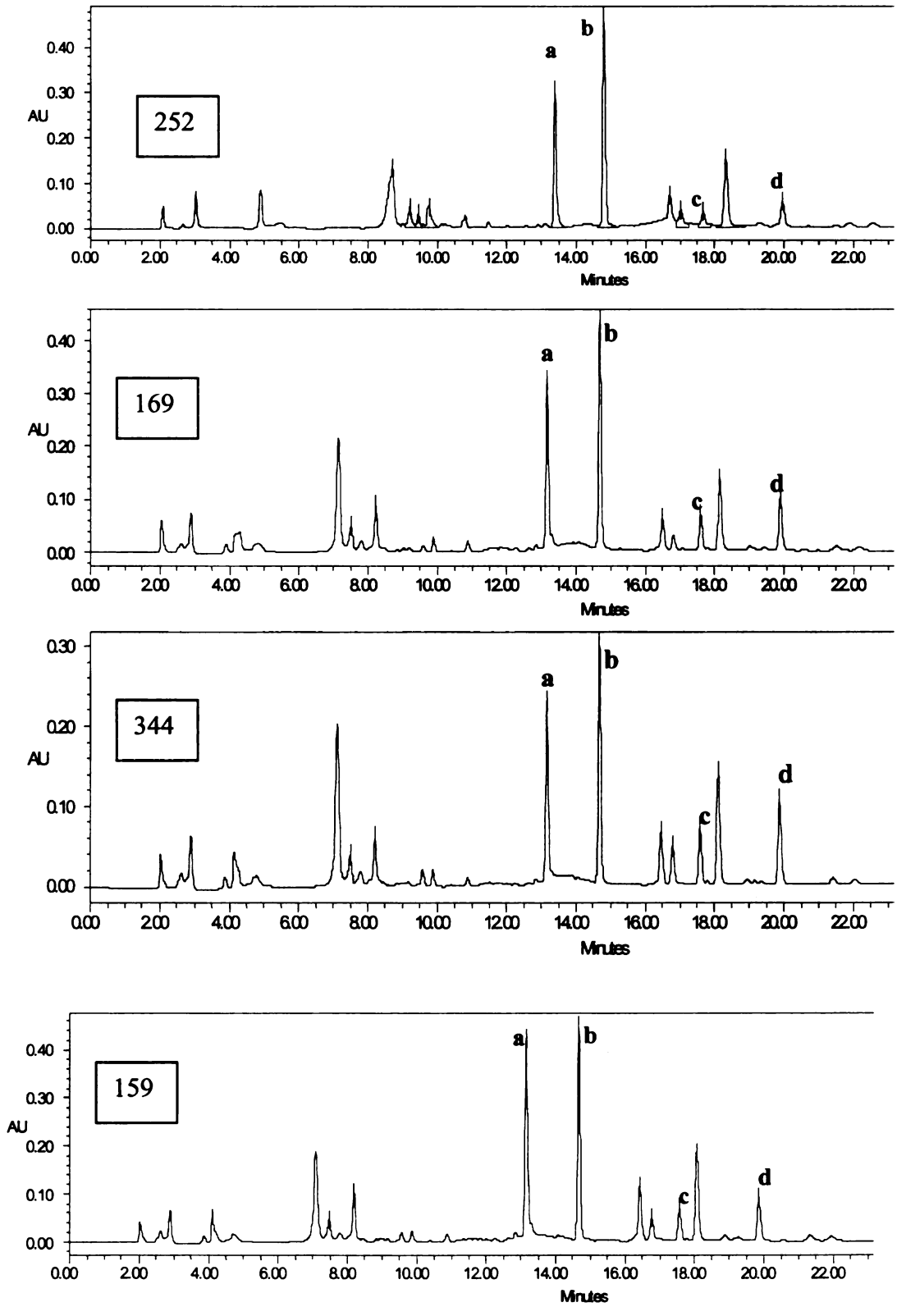


Figure 5.2 Representative HPLC chromatogram of isoflavones in soy-fortified yogurts at 1st week(**a**, daidzin; **b**, genistin; **c**, daidzein; **d**, genistein).

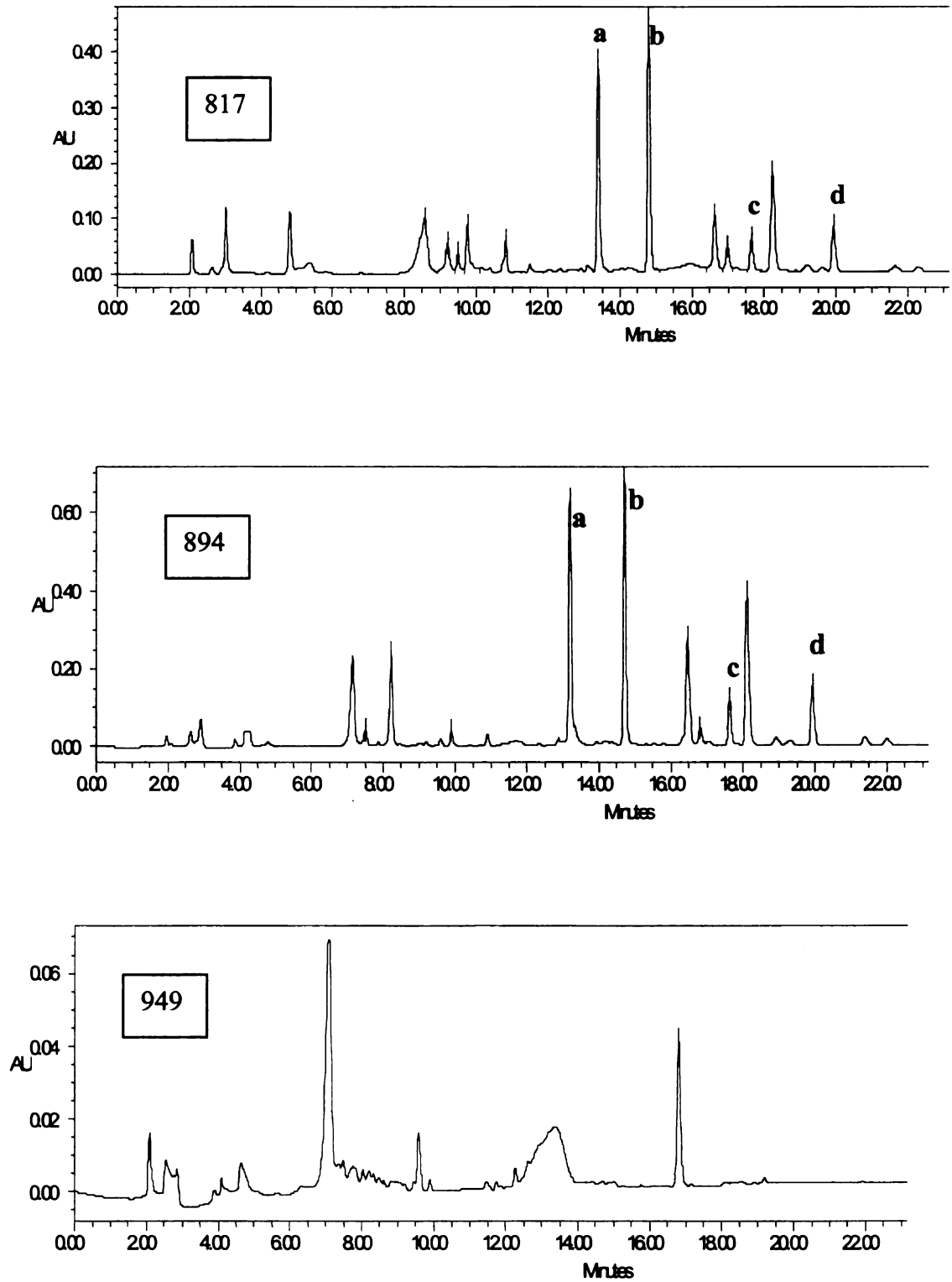


Figure 5.3 Representative HPLC chromatogram of isoflavones in soy-fortified yogurts at 6th week (**a**, daidzin; **b**, genistin; **c**, daidzein; **d**, genistein).

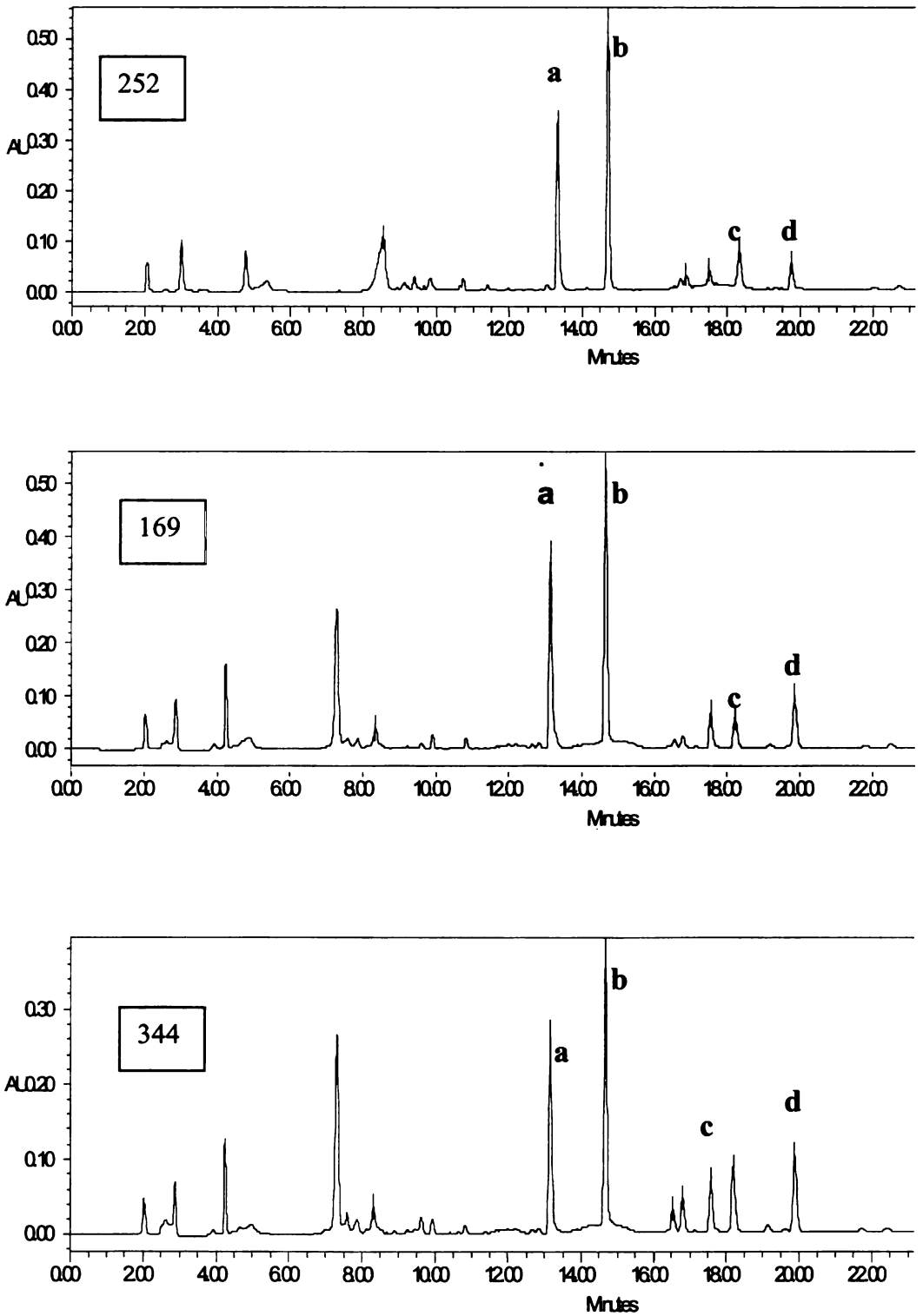


Figure 5.4 Representative HPLC chromatogram of isoflavones in soy-fortified yogurts at 6th week (**a**, daidzin; **b**, genistin; **c**, daidzein; **d**, genistein)

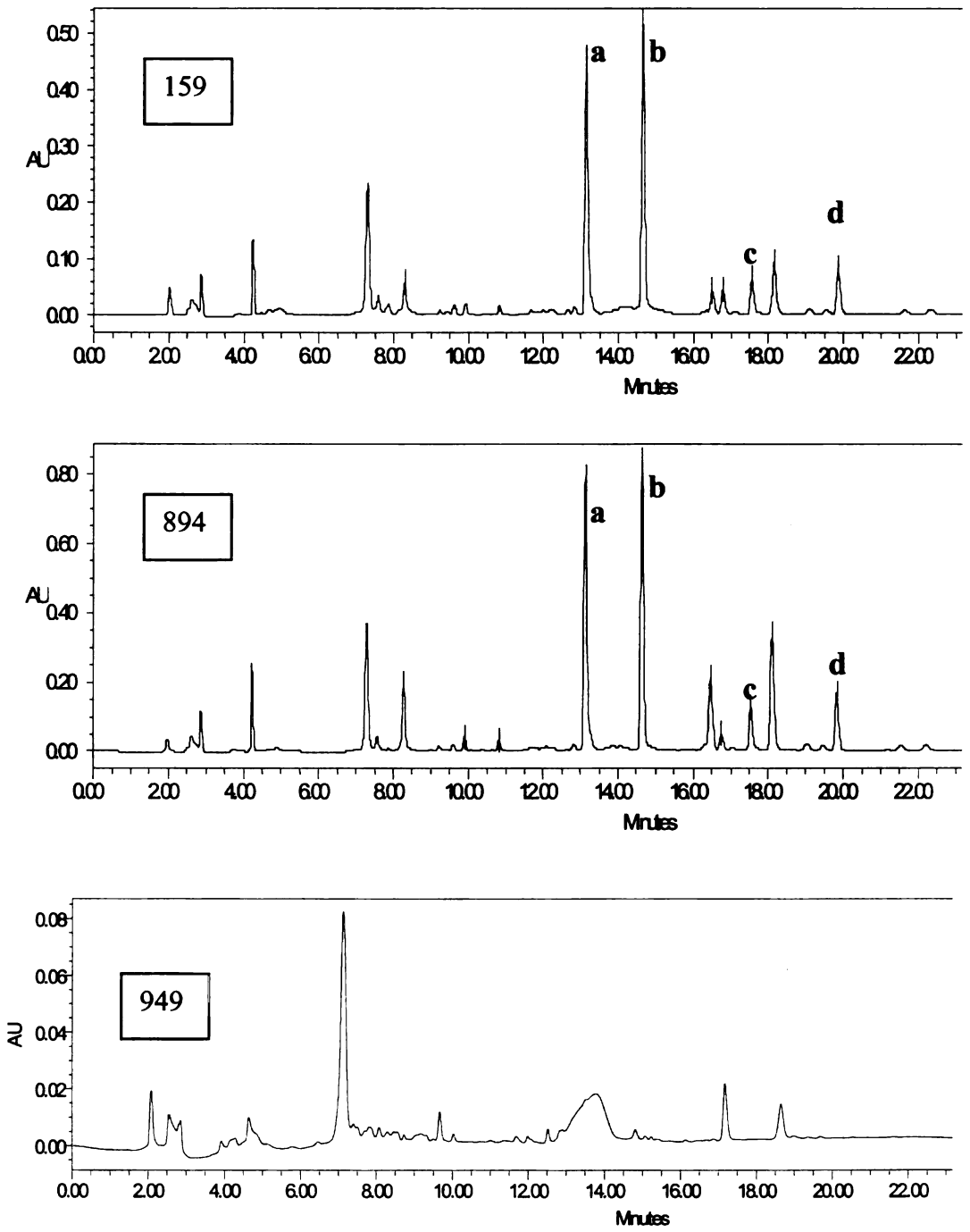


Table 5.1 Total isoflavone concentrations (on dry matter basis) in yogurts fortified with germinated or non- germinated soybean powders (μg/g) at 1st and 6th week of storage (4°C)

Yogurt samples	1st Week	6th Week	Percent increase
[1]252	131.20 ± 13.36^{cdB}	161.30 ± 8.43^{cA}	18.7
169	149.18 ± 18.31^{cB}	230.91 ± 7.58^{bA}	35.4
344	89.68 ± 5.62^{dB}	128.50 ± 15.27^{cA}	30.2
159	197.95 ± 26.33^{bB}	259.82 ± 10.26^{bA}	23.8
817	201.95 ± 24.84^{b}	N/A[2]	N/A
894	377.147 ± 6.81^{aB}	478.66 ± 27.01^{aA}	21.2
949	0.00 ± 0.00^{eA}	0.00 ± 0.00^{dA}	0.0

[a-e]Mean values in a column with different superscript small letters are significantly different ($p < 0.05$); n = 3.

[A-B] Mean values in a row with different superscript capital letters are significantly different ($p < 0.05$); n = 3

[1]252 = 50% germinated Vinton 81 + 50% non-fat dry milk

169 = 50% non-germinated Vinton 81 + 50% non-fat dry milk

344 = 50% germinated DF 222 + 50% non-fat dry milk

159 = 50% non-germinated DF 222 + 50% non-fat dry milk

817 = 50% germinated E05276-T + 50% non-fat dry milk

894 = 100% non-germinated Vinton 81 (all soy yogurt- control)

949 = 100% non-fat dry milk (all dairy yogurt- control)

[2]N/A = Not Available

An explanation to the different concentration of isoflavones between the samples could be due to the use of different soybean varieties and differently treated soybean powders utilized in the yogurt manufacturing. The respective concentration of isoflavones (on dry matter basis) in the yogurts fortified with non-germinated soy powders were149.18 ± 18.31μg/g (50:50 NGV 81:NFDM), and 197.95 ± 26.33μg/g (50:50 NGDF 222:NFDM). On the other hand, the concentration of isoflavones in the yogurt samples fortified with germinated soy powders at 1 week of manufacturing were 131.2 ± 13.36 μg/g (50:50 GV 81:NFDM), 89.68 ± 5.62 μg/g (50:50 GDF 222:NFDM) and 201.95 ± 24.84 μg/g (50:50 GET:NFDM). Aside from the control yogurts, the samples fortified with germinated E05276-T and non-germinated DF 222 soy powders had higher ($p< 0.05$) isoflavone contents than the rest. The results obtained in this study are similar to the data presented by Morandi and others (2005), in their study on the isoflavone content of Italian soy food products, which included soy yogurts.

At the end of 6 weeks of storage, the total isoflavone (4 isomers) concentrations in all the yogurt samples apart from sample 949 i.e. 100% dairy yogurt, increased significantly (Table 5.2, Figures 5.3 and 5.4). The highest percent increase occurred in sample 50:50 NGV 81:NFDM (35.4%), followed by 50:50 GDF 222:NFDM (30.2%), 50:50 NGDF 222:NFDM (23.8%), 100% soy yogurt (21.2%), 50:50 GV 81:NFDM (18.7%) and 100% dairy yogurt (0.0%).

Loss of isoflavones was determined by Wang and Murphy (1996), during processing of soybeans into tempeh, tofu, and soy protein isolate. They observed that 61, 44, and 53% of total isoflavones were lost during manufacturing of these products respectively. In our study, we observed a decrease of 26% of total four isoflavones during

processing of soy powder into yogurt in the 1st week and 6% in 6th week of yogurt manufacturing using 100% non-germinated Vinton 81 soy powder (Table 5.2).

Table 5.2 Conversion and retention of isoflavones (4 isomers) during processing of soy powders into yogurts (%)

Yogurt sample	**1st Week (%) Converted**	**1st Week (%) Retained**	**6th Week (%) Converted**	**6th Week (%) Retained**
[1]**252**	37.5	62.5	23.2	76.8
169	41.4	58.6	9.3	90.7
344	56.8	43.2	38.0	62.0
159	45.5	54.5	28.4	71.6
817	34.0	66.0	N/A[2]	N/A
894	26.0	74.0	6.0	94.0
949	00.0	00.0	00.0	00.0

. [1]252 = 50% germinated Vinton 81 + 50% non-fat dry milk

169 = 50% non-germinated Vinton 81 + 50% non-fat dry milk

344 = 50% germinated DF 222 + 50% non-fat dry milk

159 = 50% non-germinated DF 222 + 50% non-fat dry milk

817 = 50% germinated E05276-T + 50% non-fat dry milk

894 = 100% non-germinated Vinton 81 (all soy yogurt- control)

949 = 100% non-fat dry milk (all dairy yogurt- control)

[2]N/A = Not Available

The percentages of isoflavones retained in the yogurt samples fortified with soy powders were calculated based on the fact that 50% of soy powder was incorporated into the yogurt base unlike the high percentage retained when 100% soy powder was utilized for yogurt manufacturing (Table 5.2). This data clearly showed that yogurt manufacturing process did not adversely affect the isoflavone contents of the final product significantly. According to Coward and others (1998), certain processing conditions such as heating including baking and frying did not necessarily alter the total isoflavone contents of soy products but instead changed the profiles of individual isoflavones.

5.4.2 Effects of soy fortification on Genistein and Genistin content of yogurt

Recent studies have shown that genistein contributed most to the concentration of aglycones in four soybean extracts fermented with lactic acid bacteria and bifidobacteria (Pyo and others, 2005). The genistein and genistin contents of various soy-fortified yogurts at one week and six week of manufacturing are shown in Tables 5.3 and 5.4. Previous study showed that 90% of the genistein series was found to be in the conjugated forms with the β- glucoside (genistin) being higher than the malonyl- or acetylglycoside forms (Grün and others, 2001). This suggests that only about 10% exists as genistein, which is similar to our data. Genistein contents of all the yogurt samples were little affected by storage at 4 °C after 6 weeks because no significant increase or decrease was observed as seen in Table 5.3. The small decreases observed can most likely be attributed to molecular conversions.

Table 5.3 Genistein concentrations in yogurts fortified with germinated or non-germinated soybean powders (μg/g) at 1st and 6th week of storage (4°C)

Yogurt sample	1st Week	6th Week	% Increase (↑) or decreases (↓)
[1]252	11.626 ± 2.17^{dA}	9.604 ± 1.14^{dA}	17.4 ↓
169	20.437 ± 0.67^{bcA}	21.336 ± 0.83^{bA}	5.1 ↑
344	22.252 ± 1.13^{bA}	20.432 ± 0.80^{bA}	8.2 ↓
159	17.963 ± 1.36^{cA}	16.627 ± 0.65^{cA}	7.4 ↓
817	18.743 ± 1.37^{c}	N/A[2]	N/A
894	$34\ 675 \pm 0.91^{aA}$	34.460 ± 0.59^{aA}	0.6 ↓
949	0.00 ± 0.00^{eA}	0.00 ± 0.00^{eA}	0.0

[a-e]Mean values in a column with different superscript small letters are significantly different ($p < 0.05$); n = 3.

[A-B] Mean values in a row with different superscript capital letters are significantly different ($p < 0.05$); n = 3

[1]252 = 50% germinated Vinton 81 + 50% non-fat dry milk

169 = 50% non-germinated Vinton 81 + 50% non-fat dry milk

344 = 50% germinated DF 222 + 50% non-fat dry milk

159 = 50% non-germinated DF 222 + 50% non-fat dry milk

817 = 50% germinated E05276-T + 50% non-fat dry milk

894 = 100% non-germinated Vinton 81 (all soy yogurt- control)

949 = 100% non-fat dry milk (all dairy yogurt- control)

[2]N/A = Not Available

Table 5.4 Genistin concentrations in yogurts fortified with germinated or non-germinated soybean powders (μg/g) at 1st and 6th week of storage (4°C)

Yogurt sample	1st Week	6th Week	% Increase (↑) or decreases (↓)
[1]**252**	75.552 ± 7.75^{bB}	95.132 ± 3.98^{bcA}	20.6 ↑
169	71.662 ± 6.51^{bB}	123.816 ± 3.16^{bA}	42.1 ↑
344	38.895 ± 4.56^{cB}	72.440 ± 12.47^{cA}	46.3 ↑
159	79.434 ± 9.84^{bB}	120.581 ± 3.64^{bA}	34.1 ↑
817	91.527 ± 18.68^{b}	N/A[2]	N/A
894	147.971 ± 5.79^{aB}	207.239 ± 21.85^{aA}	28.6 ↑
949	0.00 ± 0.00^{dA}	0.00 ± 0.00^{eA}	0.0

[a-e] Mean values in a column with different superscript small letters are significantly different ($p < 0.05$); n = 3.

[A-B] Mean values in a row with different superscript capital letters are significantly different ($p < 0.05$); n = 3

[1]252 = 50% germinated Vinton 81 + 50% non-fat dry milk

169 = 50% non-germinated Vinton 81 + 50% non-fat dry milk

344 = 50% germinated DF 222 + 50% non-fat dry milk

159 = 50% non-germinated DF 222 + 50% non-fat dry milk

817 = 50% germinated E05276-T + 50% non-fat dry milk

894 = 100% non-germinated Vinton 81 (all soy yogurt- control)

949 = 100% non-fat dry milk (all dairy yogurt- control)

[2]N/A = Not Available

Genistin contents were significantly affected ($p < 0.05$) at the end of 6 weeks of storage. Just like total isoflavone contents, the genistin contents at 6 weeks were significantly higher ($p < 0.05$) in all the yogurt samples that contained soy powders. The increase in genistin concentration during storage can be attributed to formation of this β-glucoside by the de-esterification of the malonyl- and acetylglycosides (Grün and others, 2001; Coward and others, 1998; Coward and others, 1993). A previous study by Simonne and others (2000), suggests that the increase could also be due to further hydrolysis of the malonyl- and acetylglycosides leading to conversion into β-glucoside forms.

As expected, 100% soy yogurt had the highest concentration of genistin. Of the soy-fortified yogurts, 50:50 NGDF 222:NFDM and 50:50 NGV 81:NFDM had the most genistin contents (Table 5.4). It is worthy to note that these yogurts were made with non-germinated (soaked) soybean powders. Again sample 949 made from 100%non-fat dry milk did not contain any genistein or genistin. Some strains of lactic acid bacteria have been discovered to have β-glucosidase activities whereby they could bioconvert the glucoside isoflavones into their respective aglycones (Chun and others, 2007). In relation to the metabolism of these isoflavones in humans, researches have shown that the aglycones are more bioactive because they are easily absorbed through the gut wall than the other isoflavone isomers. Thus the need to know the chemical forms in which these isoflavones exist in foods is very important.

5.4.3 Effects of soy fortification on Daidzein and Daidzin contents of yogurt

The different concentrations of daidzein and daidzin in soy-fortified yogurts are summarized in Tables 5.5 and 5.6. Table 5.5 shows that there was no significant difference between daidzein contents in 1st week and 6th week storage at 4 °C.

Table 5.5 Daidzein concentrations in yogurts fortified with germinated or non-germinated soybean powders (μg/g) at 1st and 6th week of storage (4°C)

Yogurt sample	1st Week	6th Week	% Increase (↑) or decreases (↓)
[1]252	2.751 ± 1.69^{bA}	2.324 ± 0.88^{cA}	15.5 ↓
169	4.039 ± 0.32^{bA}	4.766 ± 0.15^{bA}	15.3 ↑
344	4.832 ± 0.40^{bA}	4.572 ± 0.75^{bA}	5.4 ↓
159	4.071 ± 0.20^{bA}	4.252 ± 0.39^{bA}	4.3 ↑
817	3.496 ± 0.39^{b}	N/A[2]	N/A
894	12.968 ± 1.00^{aA}	13.005 ± 0.77^{aA}	0.3 ↑
949	0.00 ± 0.00^{dA}	0.00 ± 0.00^{eA}	0.0

[a-e] Mean values in a column with different superscript small letters are significantly different ($p < 0.05$); $n = 3$.

[A-B] Mean values in a row with different superscript capital letters are significantly different ($p < 0.05$); $n = 3$

[1]252 = 50% germinated Vinton 81 + 50% non-fat dry milk

169 = 50% non-germinated Vinton 81 + 50% non-fat dry milk

344 = 50% germinated DF 222 + 50% non-fat dry milk

159 = 50% non-germinated DF 222 + 50% non-fat dry milk

817 = 50% germinated E05276-T + 50% non-fat dry milk

894 = 100% non-germinated Vinton 81 (all soy yogurt- control)

949 = 100% non-fat dry milk (all dairy yogurt- control)

[2]N/A = Not Available

The observation on daidzein contents made in the above table is similar to that made in Table 5.3, that showed no significant difference in genistein contents of freshly manufactured soy-fortified yogurts and six week old soy-fortified yogurts. The highest daidzein content was found in 100% soy yogurt (sample 894). There were no statistically significant differences between all the soy-fortified yogurts despite the differences in soybean variety and treatment. The overall low amounts of daidzein across the samples when compared to genistein, are consistent with the findings of other workers on the daidzein and genistein contents in soy foods (Preinerstorfer and Sontag, 2004; Morandi and others, 2005). Daidzein is a very unstable isoflavone, thus its low concentrations in soy foods could be attributed to this instability as a result of bioconversion to other forms of isoflavones (Coward and others, 1993; Coward and others, 1998; Mahungu and others, 1999; Grün and others 2001).

As a result of the health benefits attached to the consumption of isoflavones, the chemical forms in which they appear in foods is considered important since it can dramatically influence the biological activity, the bioavailability, and therefore the physiological effects of these dietary constituents. After ingestion, soybean β-glucosides are hydrolyzed by intestinal glucosidases, which release the aglycones, daidzein, genistein and glycitein (Chun and others, 2007). These could be absorbed directly or further metabolized to many specific metabolites, including equol and p-ethylphenol (3,4). Genistin and daidzin contents again were more abundant than genistein and daidzein contents (Figure 5.4). Ingestion of a diet rich in isoflavone-aglycones may be more effective in increasing health benefits associated with soybean consumption.

Table 5.6 Daidzin concentrations in yogurts fortified with germinated or non-germinated soybean powders (μg/g) at 1st and 6th week of storage (4°C)

Yogurt sample	1st Week	6th Week	% Increase (↑) or decreases (↓)
[1]252	41.267 ± 5.00^{cdB}	54.237 ± 4.07^{dA}	23.9 ↑
169	53.037 ± 11.79^{cB}	80.991 ± 5.80^{cA}	34.5 ↑
344	23.700 ± 8.09^{dB}	31.059 ± 5.27^{dA}	23.7 ↑
159	96.484 ± 25.73^{bB}	118.355 ± 11.25^{bA}	18.5 ↑
817	87.830 ± 19.62^{bc}	N/A[2]	N/A
894	181.527 ± 7.31^{aB}	232.817 ± 17.12^{aA}	22.0 ↑
949	0.00 ± 0.00^{dA}	0.00 ± 0.00^{eA}	0.0

[a-e] Mean values in a column with different superscript small letters are significantly different ($p < 0.05$); n = 3

[A-B] Mean values in a row with different superscript capital letters are significantly different ($p < 0.05$); n = 3

[1]252 = 50% germinated Vinton 81 + 50% non-fat dry milk

169 = 50% non-germinated Vinton 81 + 50% non-fat dry milk

344 = 50% germinated DF 222 + 50% non-fat dry milk

159 = 50% non-germinated DF 222 + 50% non-fat dry milk

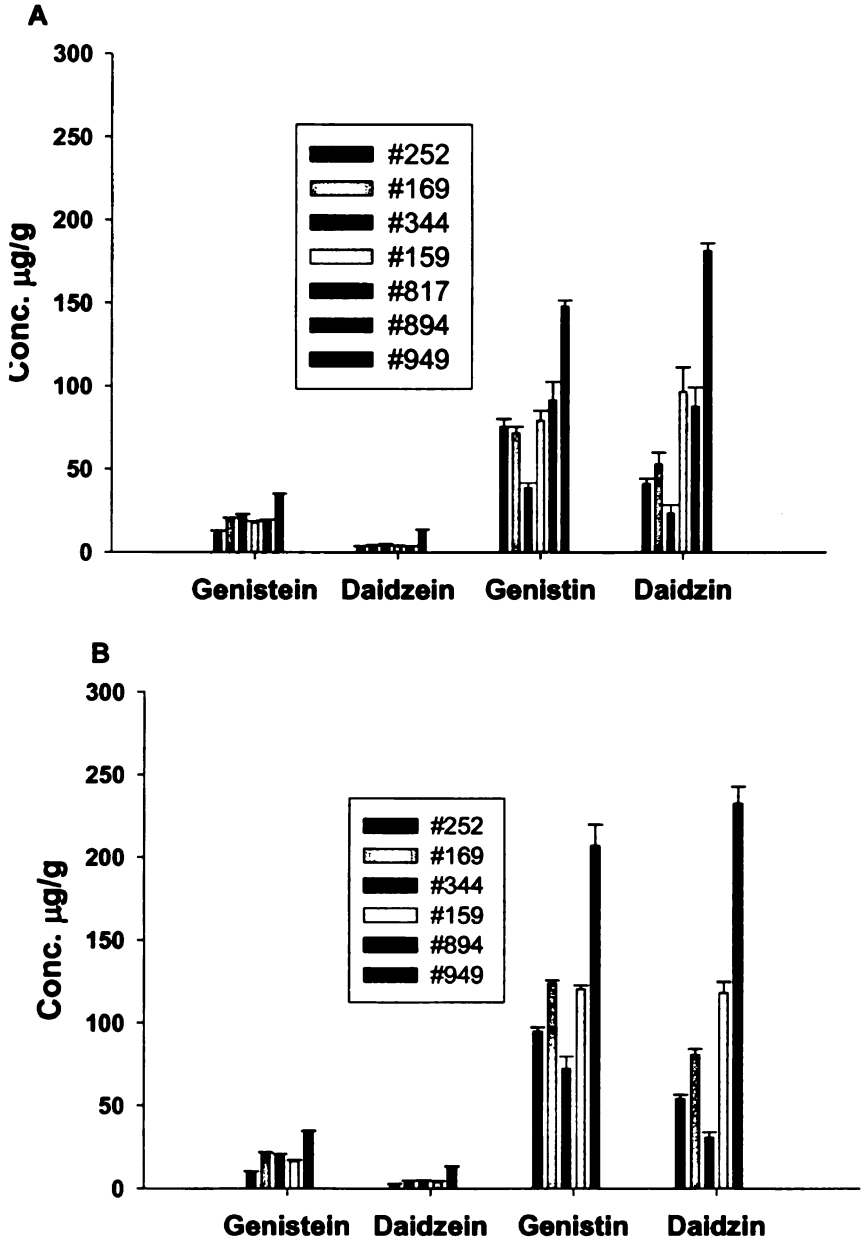
817 = 50% germinated E05276-T + 50% non-fat dry milk

894 = 100% non-germinated Vinton 81 (all soy yogurt- control)

949 = 100% non-fat dry milk (all dairy yogurt- control)

[2]N/A = Not Available

Figure 5.5 Isoflavone concentrations in 1 week (A) and 6 week (B) old yogurt samples



252 = 50% germinated Vinton 81 + 50% non-fat dry milk

169 = 50% non-germinated Vinton 81 + 50% non-fat dry milk

344 = 50% germinated DF 222 + 50% non-fat dry milk

159 = 50% non-germinated DF 222 + 50% non-fat dry milk

817 = 50% germinated E05276-T + 50% non-fat dry milk

894 = 100% non-germinated Vinton 81 (all soy yogurt- control)

949 = 100% non-fat dry milk (all dairy yogurt- control)

5.4.4 Effects of soy fortification on Stachyose content of yogurt

The stachyose contents of all the soy samples after one week of storage are summarized in Table 5.7.

Table 5.7 Stachyose contents of yogurts fortified with germinated or non-germinated whole soy powders after 1-week storage at 4 °C.

Treatment (Yogurt samples	Stachyose (mg/g)
[1]252	4.41 ± 0.19^{d}
169	10.04 ± 0.23^{b}
344	3.40 ± 0.11^{e}
159	8.45 ± 0.07^{c}
817	2.82 ± 0.13^{f}
894	17.25 ± 0.07^{a}
949	1.33 ± 0.01^{g}

[a-g] Mean values in a column with different superscript small letters are significantly different ($p < 0.05$); $n = 2$

[1]252 = 50% germinated Vinton 81 + 50% non-fat dry milk

169 = 50% non-germinated Vinton 81 + 50% non-fat dry milk

344 = 50% germinated DF 222 + 50% non-fat dry milk

159 = 50% non-germinated DF 222 + 50% non-fat dry milk

817 = 50% germinated E05276-T + 50% non-fat dry milk

894 = 100% non-germinated Vinton 81 (all soy yogurt- control)

949 = 100% non-fat dry milk (all dairy yogurt- control)

Table 5.7 shows that stachyose content in each yogurt was significantly different ($p < 0.05$) from each other. Overall yogurts made with either 50% or 100% non-germinated soy powders had higher stachyose contents irrespective of the soybean variety. Sample 894 i.e. all soy yogurt made with non-germinated Vinton 81 contained the highest amount of stachyose (17.25 mg/g). This was followed by sample 169 (50% non-germinated Vinton 81) with 10.04 mg/g of stachyose and sample 159 (50% non-germinated DF 222) with 8.45 mg/g of stachyose. Sample 949 (all dairy yogurt) had negligible amount of the compound, which was attributed to interference from sucrose since non-fat dry milk does not contain stachyose. Overall all the yogurt samples prepared with germinated soy powders had lower quantities of stachyose. This observation confirms earlier findings that oligosaccharide contents are reduced during germination due to increase in α-galactosidase activity (Viana and others, 2005) and further reduced during fermentation by lactic acid bacteria.

Table 5.8 shows the percent reduction in stachyose in yogurt samples from the base soy powders. The stachyose content of all the soy-fortified yogurts made with 50% of soy powder were highly reduced and ranged from 51.5 to 63.8% reduction in the yogurts. Also yogurt made with 100% non-germinated soy powder (i.e. sample 894) had 58.3% reduction in stachyose content. Chen and others (2004) reported that amino acids and organic acids concentrations could be increased by the increase in the activity and viability of probiotics due to the presence of prebiotics in milk to be used for yogurt manufacturing. A diet high in non-digestible carbohydrate causes increased intestinal fermentation and could result in more extensive biotransformation of phytoestrogens, leading to increased formation of equol, a mammalian isoflavone metabolite. Equol is

said to have estrogenic potency of magnitude higher than that of its plant precursor, daidzein (Joannou and others, 1995).

Table 5.8 Percent reduction of stachyose in yogurts manufactured with germinated and non-germinated soy powders

Soy powder	Yogurt sample	% Stachyose reduced
[1]**GV 81** (9.58 mg/g)	[2]**252** (4.41 mg/g)	53.9
NGV 81 (20.68 mg/g)	**169** (10.04 mg/g)	51.5
NGV 81 (41.35 mg/g)	**894** (17.25 mg/g)	58.3
GDF 222 (7.73 mg/g)	**344** (3.40 mg/g)	56.0
NGDF 222 (23.33 mg/g)	**159** (8.45 mg/g)	63.8
GET (6.95 mg/g)	**817** (2.82 mg/g)	59.4

[1]GV 81 = Germinated Vinton 81

NGV 81 = Non-germinated Vinton 81

GDF 222 = Germinated DF 222

NGDF 222 = Non-germinated DF 222

GET = Germinated E05276-T

[2]252 = 50% germinated Vinton 81 + 50% non-fat dry milk

169 = 50% non-germinated Vinton 81 + 50% non-fat dry milk

344 = 50% germinated DF 222 + 50% non-fat dry milk

159 = 50% non-germinated DF 222 + 50% non-fat dry milk

817 = 50% germinated E05276-T + 50% non-fat dry milk

894 = 100% non-germinated Vinton 81 (all soy yogurt- control)

As reported by several workers, consumption of oligosaccharides could lead to improved immune and nutritional status as well as reduced risk of Crohn's disease, colon cancer, irritable bowel syndrome etc. (Gibson and Angus, 1996; Sako and others, 1999). In summary, the data obtained in this objective indicate that each of these soy-fortified cow milk based yogurts contain the following amount of isoflavone (4 isomers) per serving (about 200 ml). On a dry matter basis, sample 252 (germinated Vinton 81 + NFDM) had 26.2 mg/serving; 169 (non-germinated Vinton 81 + NFDM) had 29.8 mg/serving; 344 (germinated DF 222 + NFDM) had 17.9 mg/serving; 159 non-germinated DF 222 + NFDM) had 39.6 mg/serving; 817 (germinated E05276-T + NFDM) has 40.4 mg/serving; 894 (non-germinated Vinton 81 only) had 75 mg/serving. Suggested amount of total isoflavones (12 isomers) per serving is 40-50 mg. Also suggested amount of dietary fiber per serving is between 3-5 grams per serving. Our study showed that the stachyose content in the yogurt samples varied from 0.6 g/serving for germinated E05276-T + NFDM to 3.45 g/serving for 100% soy yogurt.

CHAPTER 6

SHELF LIFE STUDIES AND VIABILITY OF WHOLE SOY-FORTIFIED YOGURTS STORED AT 4 °C

6.1 ABSTRACT

Fermented dairy foods produced with probiotic bacteria have attracted a lot of research interest because of their potential health benefits. The growth and viability of lactic acid bacteria and probiotic used in making whole soy-fortified yogurts were monitored at 7-day interval during 6 weeks of storage at refrigeration temperature (4 °C). MRS, MRS-sorbitol and M17 agar were utilized to enumerate *Lactobacillus delbreuckii* subsp. *bulgaricus*, *Lactobacillus acidophilus* NCFM and *Streptococcus thermophilus* respectively. The pH of each yogurt was also measured on a weekly basis during storage. The growth and viability of all the cultures were above 10^7 CFU/g of yogurt. The lowest concentrations of all the cultures were obtained in 100% whole soy yogurt. Alternatively, the highest cell concentrations occurred in whole soy-fortified yogurts especially in the sample made with germinated DF 222 soy powder.

Overall the pH values of each yogurt sample remained constant up to the 5th week of cold storage. The pH values of the sample with 100% non-germinated soy powder were significantly higher than the rest of the samples, ranging from 4.67 for 1st week to 4.82 for 6th week. In general, there was no statistically significant difference in pH between germinated DF 222 soy-fortified yogurt and 100% dairy yogurt sample during storage. There was a significant difference in pH values between 1st and 2nd week of

storage and between 3rd and 4th weeks of storage among the samples. The pH values of whole soy-fortified including the control yogurts were between 4.5 and 4.8 after 6 weeks of storage at 4 °C.

6.2 INTRODUCTION

There has been a world increase in the consumption of fermented dairy product containing probiotics. Probiotics are defined as "live microorganisms that when administered in adequate amounts confer a health benefit on the host (FAO, 2002). These probiotics were initially isolated from human sources and their health benefits have been a focus of intense research internationally (O'Sullivan and others 1992; Østlie and others 2003). Several research studies have shown the health benefits of some the well-characterized lactic acid bacteria (Salminen and others, 1996; Saarela and others 2000). It has been suggested that in order for a microorganism to exert positive health benefits, the microbial concentration must be at least 10^6 CFU/g in the product throughout the shelf life period of the product (Vinderola and others 2000, 2002; Fanworth, 2005).

Traditionally, yogurt is manufactured by the addition of two starter cultures, *Lactobacillus delbruekii* subsp. *bulgaricus* and *Streptococcus thermophilus* to milk (Tammime and Marshall 1997). Subsequent fermentation of milk as a result of the starter culture metabolism leads to the taste and bioactive metabolites, which contribute to the health promoting properties of yogurt (Rachid and others 2002; Donkor and others 2005). Dairy yogurt is widely consumed in both the developed and developing countries, but recently there is a demand to alternative to cow's milk due to problems with allergenicity, desire for vegetarian alternatives, insufficient production of dairy milk, and new products.

Hence a keen interest in soymilk-based yogurts has developed although mostly probiotic cow's milk based yogurts are currently being marketed (Lee and others 1990; Nsofor and others 1992,1996).

Probiotics have been implicated in the treatment of different types of diseases such as diarrhea (Sarker and others, 2005; Szymansky and others, 2006), Crohn's disease (Bousvaros and others, 2005), and urogenital infections (Reid and others, 2003). Yogurt is considered a good vehicle for introducing probiotics to consumers. It is now popular to use probiotics such as *Lactobacillus* sp and *Bifidobacterium* as adjunct starter cultures (Dave and Shah, 1998; Gardiner and others, 2002; Shah 2004). Several studies have shown that after probiotic ingestion, biological barriers such as stomach acid and bile prevent the survival of these probiotics in the intestinal tract (Lankaputhra and Shah, 1995) therefore their ability to confer health benefits is impeded. Also milk has been shown to be a poor medium for the growth and survival of probiotics because milk does not contain enough amino acids and lower molecular weight peptides to sustain such growth (Shah, 2006).

In order to maintain the presence of these probiotics in large numbers in the gut, many workers have supplemented the milk base with various compounds such as soy protein isolate (Pham and Shah, 2008), soy protein concentrate (Drake and others, 2000; Drake and Gerard, 2003), fibers (Fernandez-Garcia and others, 1998; Aryana and others, 2007). Prebiotics are non-digestible carbohydrates that are not easily hydrolyzed or digested in the upper part of the gastrointestinal tract but can be metabolized by some probiotics. Inulin and oligosaccharides are known to enhance the activities and growth of probiotics such as *Bifidobacterium* spp (Shin and others 2000) and *Lactobacillus*

acidophilus (Aryana and others, 2007). In 2004, Chen and others discovered that the concentrations of organic acids and amino acids can be increased in yogurts containing added prebiotics that could in turn increase the activities of the probiotics. Research evidence shows that incorporation of prebiotics to yogurt containg *Lactobacillus acidophilus* would probably lead to a healthier yogurt. The objective of this study was to evaluate the shelf life of yogurts fortified with germinated and non-germinated whole soy powders from various soybean varieties and fermented with traditional yogurt cultures and a probiotic culture, *Lactobacillus acidophilus* (La NCFM). The whole soy powders utilized contain oligosaccharides e.g. stachyose that could serve as a prebiotic, thereby eliminating supplementation of the yogurt base with extraneous fibers.

6.3 MATERIALS AND METHODS

The viability of *Lactobacillus delbruekii* subsp. *bulgaricus*, *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus acidophilus* (NCFM) used in making whole soy-fortified yogurts were monitored at 7-day interval during 42 days of storage. The yogurt samples were stored in the refrigerator (4 °C) throughout the study and these samples belonged to the same batch of yogurts utilized for the sensory evaluations and chemical analysis.

6.3.1 Media Preparation

Diluents of peptone (0.1%) and water were prepared by dissolving one gram of bacto-peptone medium (Difco Laboratories, Detroit, MI) in one liter of distilled water and sterilized by autoclaving at 121 °C for 15 minutes. In order to enumerate *S. salivarius*

subsp. *thermophilus*, M17 agar was prepared. M17 agar was prepared according to manufacturer's instructions by adding 37.25 g of M17 broth powder (Difco Laboratories, Detroit, MI), 1.5% bacto agar (Difco Laboratories, Detroit, MI) to 1 L of distilled water and also sterilized at 121 °C for 15 min., before, added 50 ml 10% sterile lactose solution, pouring into sterile agar plates. De Man, Rogosa, Sharpe (MRS) agar was used to enumerate *Lactobacillus delbruekii* subsp. *bulgaricus*. This agar was prepared by adding 70 g of MRS agar (Difco Laboratories, Detroit, MI) to 1 L of distilled water, sterilized at 121 °C for 15 min., before pouring into agar plates. Modified MRS agar was utilized to enumerate *Lactobacillus acidophilus* NCFM. This agar was prepared by adding 10% filter sterile sorbitol to sterilized dextrose free MRS agar (1% final sorbitol concentration in medium) before pouring on agar plate (Dave and Shah, 1996).

6.3.2 Enumeration of Lactic Acid Bacteria

The following yogurt samples were utilized for shelf life studies; 50% non-germinated DF 222 + 50% non-fat dry milk, 50% germinated DF 222 + 50% non-fat dry milk, 50% non-germinated + 50% non-fat dry milk, 50% germinated Vinton 81 + 50% non-fat dry milk, 100% non-germinated Vinton 81- all soy control and 100% non-fat dry milk- all dairy control. Starting at 0 week, one gram of sample was taken from each yogurt and diluted with 99 ml of sterile 0.1% (w/v) peptone and suitable serial dilutions were plated on each of the selective medium prepared (M17, MRS, MRS-sorbitol) in triplicates. Serially diluted tubes of 10^{-5} were utilized for plating on agar plates.

The diluted yogurt samples plated on M17 agar for the enumeration of *S. salivarius* subsp. *thermophilus* were incubated aerobically at 37 °C for 24h. Also samples

plated on MRS and MRS-sorbitol agars were incubated anaerobically using Gas Packs (BBL Microbiology Systems, Cockeysville, MD) at 37 °C for 24h. Plates containing 25-250 colonies were enumerated and recorded as colony-forming units per gram (CFU/g) of culture. The colonies were counted using 920A colony counter (American Bantex Corp., Burlingame, CA). The pH of each yogurt sample was also measured in replicate at 7-day interval for 42 days (shelf life period).

6.3.3 Statistical analysis

Bacterial enumeration was done in triplicate while pH monitoring was carried out using replicate samples. Fixed effects for the viability and pH of the cultures included two factors i.e. yogurt sample and storage time (weeks). Two-way analysis of variance was done to evaluate these effects and their interactions. Sigma Stat 3.1 and Sigma plots 9.0 (Jandel Scientific, San Rafael, CA) were used for this analysis and Tukey's test was used for comparisons of means. Comparisons were considered significantly different if $p < 0.05$.

6.4 RESULTS AND DISCUSSION

6.4.1 Viability of microorganisms in yogurts during cold storage

The effects of whole soy powder supplementation for milk-based yogurt on the viability of lactic cultures throughout 42 days of refrigerated storage were studied. An important parameter in evaluating viable organisms during shelf studies is the ability to differentiate each bacteria used.

6.4.1.1 Viability of Lactobacillus delbreuckii subsp.*bulgaricus during cold storage*

Table 6.1 shows the analysis of variance (ANOVA) for the independent variables i.e. yogurt samples containing *Lactobacillus delbreuckii* subsp.*bulgaricus* and weeks (0, 1, 2, 3, 4, 5, and 6) and their interactions on MRS agar.

Table 6.1 Analysis of variance for the effects of yogurt varieties and storage time (weeks) on the viability of *Lactobacillus delbreuckii* subsp.*bulgaricus* (CFU/g)

Main Effects	**DF**	**SS**	**MS**	**F**	**P**
Yogurt samples	5	2.291×10^{16}	4.582×10^{15}	28.158	<0.001
Weeks	5	9.948×10^{15}	1.990×10^{15}	12.228	<0.001
Yogurt x Weeks	25	1.14×10^{16}	4.569×10^{14}	2.808	<0.001
Residual	72	1.172×10^{16}	1.627×10^{14}		
Total	107	5.599×10^{16}	5.233×10^{14}		

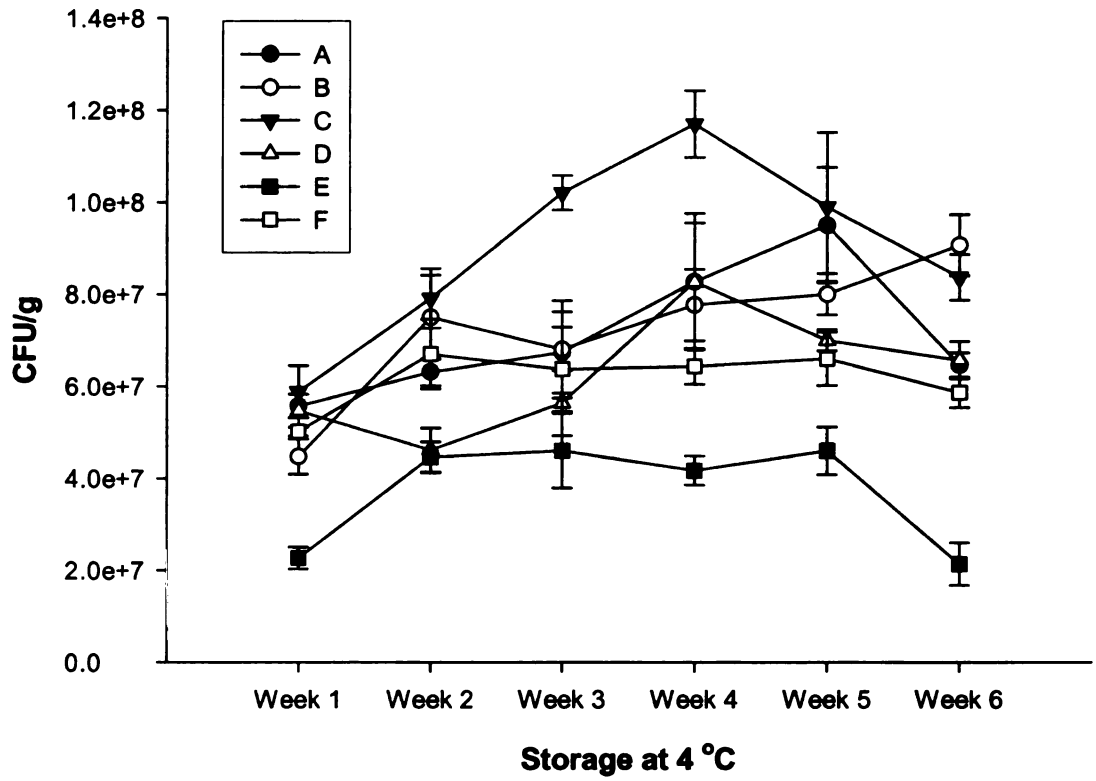
The above table suggests that the difference in the mean values among the different varieties of yogurt samples was higher and more significant with an F-value of 28.158 ($p < 0.001$), than the effects of differences in weeks (F-value =12.228, $p < 0.001$). The two-way analysis of variance also suggests that the effect of different varieties of yogurt samples depends on storage time, hence there was a statistically significant interaction between yogurt samples and weeks ($p < 0.001$). Based on this observation, one-way analysis of variance was then used to compare the means (CFU/g) of yogurt samples per week (Figure 6.1). On the 1st week of storage, there was no significant difference between 100% dairy yogurt and all the 50% soy-fortified yogurts. All these

samples were significantly different from 100% soy yogurt, which had the lowest count of *L. delbrueckii* subsp.*bulgaricus*.

The viable cell counts were between 2.3 x 10^7 CFU/g and 5.9 x 10^7 CFU/g initially. *L. delbrueckii* subsp.*bulgaricus* attained or maintained viable cell numbers after 6 weeks of cold storage (2.1 x 10^7 CFU/g to 9.1 x 10^7 CFU/g). All the yogurt samples consistently maintained high cell counts up until week 5. By week 6 few of the cell counts decreased but the cell numbers were still high enough for the recommended 10^6 CFU/g or greater for health claims (Ouwehand and Salminen, 1998). Growth of *L. delbrueckii* subsp.*bulgaricus* was slower in 100% soy yogurt than in 100% dairy yogurt but later increased by the 2nd to 5th weeks of storage. The improved growth during this period could be as result of pH drop of the soy yogurt during cold storage, which favors the growth of lactobacilli generally (Farnworth and others, 2007). Overall, the soy powder fortified samples maintained viability better than the control yogurt samples and germinated DF 222 fortified yogurt consistently maintained the highest CFU value throughout storage.

Differences in the viability of *L. delbrueckii* subsp.*bulgaricus* within each sample during 6 weeks of cold storage are shown in Table 6.2. The data revealed that there was no significant difference in viable cell counts during storage time in yogurt samples prepared with 50% non-germinated DF 222 and 100% non-fat dry milk. The remaining yogurt samples showed slight significant differences in CFU per week (increase or decrease) although the cell numbers still remained high. The 100% soy yogurt (control sample) contained the least number of viable cells especially in the 1st and 6th weeks of storage (2.3 x 10^7 and 2.1 x 10^7 CFU/g respectively).

Figure 6.1 Viability counts of *Lactobacillus delbreuckii* subsp.*bulgaricus* (CFU/g) from germinated and non-germinated whole soy-fortified yogurt samples during 6 week storage.



A (252) = 50% germinated Vinton 81 + 50% non-fat dry milk

B (169)= 50% non-germinated Vinton 81 + 50% non-fat dry milk

C (344) = 50% germinated DF 222 + 50% non-fat dry milk

D (159) = 50% non-germinated DF 222 + 50% non-fat dry milk

E (894) = 100% non-germinated Vinton 81 (all soy yogurt- control)

F (949) = 100% non-fat dry milk (all dairy yogurt- control)

Table 6.2 Viability of *Lactobacillus delbreuckii* subsp.*bulgaricus* (CFU/g) during six weeks of storage at 4 °C

Weeks of storage	*252	169	344	159	894	949
1	5.57×10^{7b}	4.47×10^{7b}	5.88×10^{7c}	5.47×10^{7a}	2.27×10^{7bc}	5.01×10^{7a}
2	6.31×10^{7b}	7.49×10^{7ab}	7.90×10^{7b}	4.61×10^{7a}	4.45×10^{7ab}	6.69×10^{7a}
3	6.73×10^{7ab}	6.80×10^{7ab}	1.02×10^{8ab}	5.63×10^{7a}	4.60×10^{7a}	6.37×10^{7a}
4	8.27×10^{7ab}	7.77×10^{7a}	1.17×10^{8a}	8.26×10^{7a}	4.17×10^{7ab}	6.43×10^{7a}
5	9.50×10^{7a}	8.00×10^{7a}	9.90×10^{7ab}	7.00×10^{7a}	4.60×10^{7a}	6.60×10^{7a}
6	6.47×10^{7b}	9.07×10^{7a}	8.37×10^{7bc}	6.57×10^{7a}	2.13×10^{7c}	5.87×10^{7a}

[a-c] Mean values in a column with different superscript small letters are significantly different ($p < 0.05$); $n = 3$.

*252 = 50% germinated Vinton 81 + 50% non-fat dry milk

169 = 50% non-germinated Vinton 81 + 50% non-fat dry milk

344 = 50% germinated DF 222 + 50% non-fat dry milk

159 = 50% non-germinated DF 222 + 50% non-fat dry milk

894 = 100% non-germinated Vinton 81 (all soy yogurt- control)

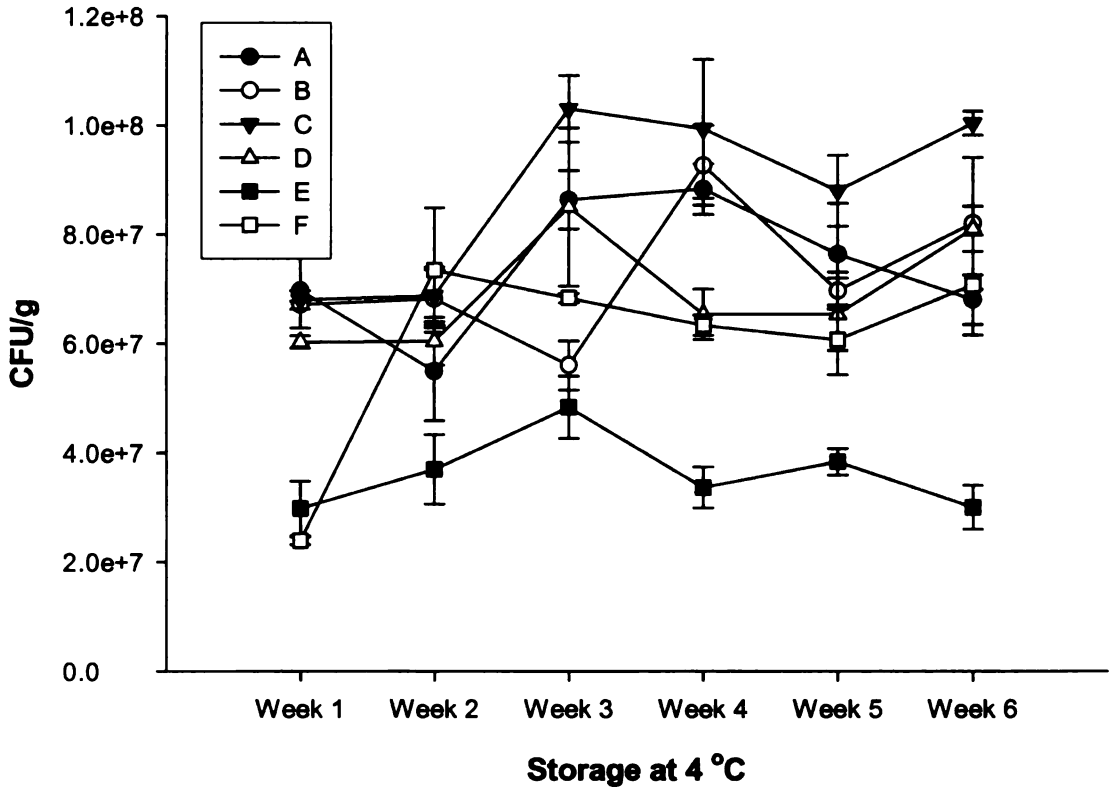
949 = 100% non-fat dry milk (all dairy yogurt- control)

6.4.1.2 Viability of Streptococcus thermophilus during cold storage

Streptococcus thermophilus cultures were enumerated on M17 agar and incubated aerobically at 37 °C for 24-48h. The differences in the mean values among the treatment groups i.e. yogurt samples and weeks of storage were greater than would be expected by chance; there is a statistically significant difference ($p = 0.002$). All the soy-fortified yogurt samples contained the highest number of *S. thermophilus* and were statistically significant from the two control samples i.e. all dairy yogurt and all soy yogurt in the first week. This could be attributed to the synergistic effect produced by the two yogurt bases i.e. milk powder and soy powder. During increased storage to six weeks, the cultures increased both in growth and viability in all the yogurts but consistently the two control samples remained the lowest. Overall, the sample containing 100% soy powder, had the lowest cell counts throughout the shelf life study ranging from 2.98×10^7 in the 1st week, then increased to 4.83×10^7 in the 3rd week and decreased to 3.00×10^7 in the last week in the refrigerator.

In general, *S. thermophilus* showed little or no significant change ($p < 0.05$) during storage in cell counts from week 1 to week 6 in all the samples (Figure 6.2 and Table 6.3). In most cases, the cell concentrations of the yogurt cultures in the soy-fortified yogurts were significantly ($p < 0.05$) higher compared with that assessed in the control dairy and soy yogurts. These results agreed with similar results obtained by Donkor and others (2005).

Figure 6.2 Viability counts of *Streptococcus thermophlilus* (CFU/g) from germinated and non-germinated whole soy-fortified yogurt samples during 6 week storage



A (252) = 50% germinated Vinton 81 + 50% non-fat dry milk

B (169) = 50% non-germinated Vinton 81 + 50% non-fat dry milk

C (344) = 50% germinated DF 222 + 50% non-fat dry milk

D (159) = 50% non-germinated DF 222 + 50% non-fat dry milk

E (894) = 100% non-germinated Vinton 81 (all soy yogurt- control)

F (949) = 100% non-fat dry milk (all dairy yogurt- control)

Table 6.3 Viability of *Streptococcus thermophilus* (CFU/g) during six weeks of storage at 4 °C

Weeks of storage	*252	169	344	159	894	949
1	$6.97x10^{7ab}$	$6.71x10^{7ab}$	$6.80x10^{7c}$	$6.03\ x10^{7a}$	$2.98x10^{7a}$	$2.39x10^{7b}$
2	$5.49\ x10^{7b}$	$6.82x10^{7ab}$	$6.87x10^{7bc}$	$6.04\ x10^{7a}$	$3.69x10^{7a}$	$7.35x10^{7a}$
3	$8.63x10^{7ab}$	$5.60x10^{7b}$	$1.03x10^{8a}$	$8.50\ x10^{7a}$	$4.83\ x10^{7a}$	$6.83\ x10^{7a}$
4	$8.83x10^{7a}$	$9.27x10^{7a}$	$9.93x10^{7ab}$	$6.53x10^{7a}$	$3.37x10^{7a}$	$6.33\ x10^{7a}$
5	$7.63x10^{7ab}$	$6.97x10^{7ab}$	$8.80x10^{7bc}$	$6.53\ x10^{7a}$	$3.83\ x10^{7a}$	$6.07\ x10^{7a}$
6	$6.80x10^{7ab}$	$8.20x10^{7ab}$	$1.00x10^{8ab}$	$8.10\ x10^{7a}$	$3.00\ x10^{7a}$	$7.07\ x10^{7a}$

[a-c] Mean values in a column with different superscript small letters are significantly different ($p < 0.05$); $n = 3$.

*252 = 50% germinated Vinton 81 + 50% non-fat dry milk

169 = 50% non-germinated Vinton 81 + 50% non-fat dry milk

344 = 50% germinated DF 222 + 50% non-fat dry milk

159 = 50% non-germinated DF 222 + 50% non-fat dry milk

894 = 100% non-germinated Vinton 81 (all soy yogurt- control)

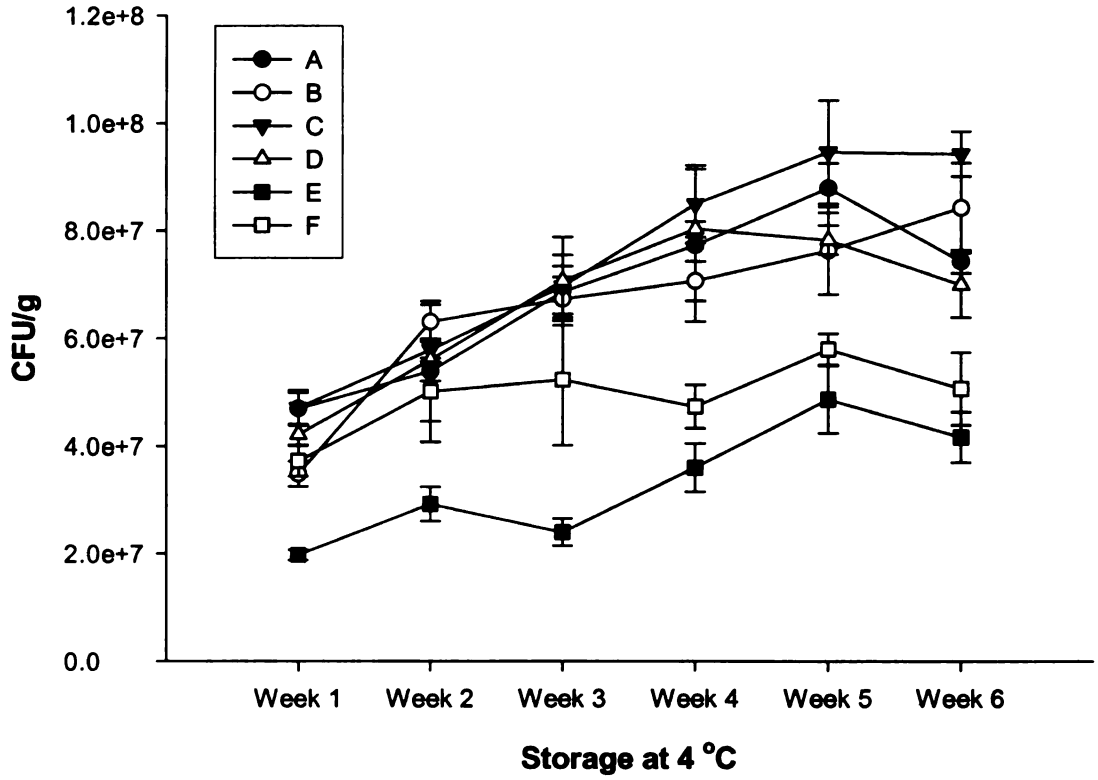
949 = 100% non-fat dry milk (all dairy yogurt- control)

6.4.1.3 Viability of Lactobacillus acidophilus during cold storage

The probiotic strain *Lactobacillus acidophilus* (La NCFM) grew well in soy-fortified yogurts reaching the health claim levels above 10^6 CFU/g. Figure 6.3 and Table 6.4 show the viable microbial number (CFU/g) during cold storage at 4 °C. As indicated, 100% soy yogurt had the lowest cell count although the counts increased steadily from 1.97 x 10^7 to 4.17 x 10^7 CFU/g during 6 weeks of storage. This observation is similar to the previous results above obtained with *Streptococcus thermophilus* and *Lactobacillus delbreuckii* subsp.*bulgaricus* cultures in the same yogurt sample (made with 100% soy). This then confirms the findings in the culture activity studies where the least activities and growth were obtained with 12% reconstituted non-germinated soy powder. Similarly, growth and viable counts of *L. acidophilus* (La NCFM) in 100% dairy yogurt also were slightly low ranging from 3.72 x 10^7 to 5.07 x 10^7 CFU/g in 6 weeks during storage.

The yogurt fortified with DF 222 germinated soy powder had the highest growth and viability throughout the 6 weeks of shelf life studies, followed by the other soy-fortified yogurts (Figure 6.3). This data indicate that there were more available nutrients in the yogurt samples to sustain the increased growth and viability of the probiotic despite the presence of the other cultures. Previous studies have indicated poor growth and viability of probiotic cultures when grown together with traditional lactic acid bacteria used for fermented foods (Vinderola and others, 2002; Sodini and others, 2002). Our data suggest that all the bacteria utilized in the yogurt making were able to survive in the presence of one another.

Figure 6.3 Growth and viability counts of *Lactobacillus acidophilus* NCFM (CFU/g) from germinated and non-germinated whole soy-fortified yogurt samples during 6-week storage



A (252) = 50% germinated Vinton 81 + 50% non-fat dry milk

B (169) = 50% non-germinated Vinton 81 + 50% non-fat dry milk

C (344) = 50% germinated DF 222 + 50% non-fat dry milk

D (159) = 50% non-germinated DF 222 + 50% non-fat dry milk

E (894) = 100% non-germinated Vinton 81 (all soy yogurt- control)

F (949) = 100% non-fat dry milk (all dairy yogurt- control

Table 6.4 shows the difference in growth and viability of La NCFM within each yogurt sample stored in the refrigerator for 6 weeks. The CFU/g of all-dairy yogurt sample (F) was statistically the same throughout the shelf life period of 6 weeks, while the CFU/g of soy-fortified yogurts generally increased after 1 week of storage. The probiotic culture grew well in the soy-fortified yogurts during storage. There was a significant increase ($p < 0.05$) in cell counts between the 1st week and 6th week of storage in yogurts containing soy powders (Table 6.4) irrespective of soybean treatment or soybean variety.

Table 6.4 Viability of *Lactobacillus acidophilus* NCFM (CFU/g) during six weeks of storage at 4 °C

Weeks of storage	*252	169	344	159	894	949
1	$4.69x10^{7b}$	$3.48 x10^{7b}$	$4.71 x10^{7c}$	$4.21 x10^{7c}$	$1.97x10^{7c}$	$3.72x10^{7a}$
2	$5.39x10^{7ab}$	$6.31x10^{7a}$	$5.79x10^{7bc}$	$5.60x10^{7bc}$	$2.92x10^{7bc}$	$5.01x10^{7a}$
3	$6.87x10^{7ab}$	$6.73x10^{7a}$	$6.97x10^{7b}$	$7.07x10^{7ab}$	$2.40x10^{7bc}$	$5.23x10^{7a}$
4	$7.73x10^{7ab}$	$7.06x10^{7a}$	$8.50x10^{7ab}$	$8.03 x10^{7a}$	$3.60x10^{7abc}$	$4.73x10^{7a}$
5	$8.80 x10^{7a}$	$7.63x10^{7a}$	$9.47x10^{7a}$	$7.83x10^{7ab}$	$4.87 x10^{7a}$	$5.80x10^{7a}$
6	$7.43x10^{7ab}$	$8.43 x10^{7a}$	$9.43x10^{7a}$	$7.00x10^{7ab}$	$4.17 x10^{7ab}$	$5.07x10^{7a}$

[a-c] Mean values in a column with different superscript small letters are significantly different ($p < 0.05$); $n = 3$.

*252 = 50% germinated Vinton 81 + 50% non-fat dry milk

169 = 50% non-germinated Vinton 81 + 50% non-fat dry milk

344 = 50% germinated DF 222 + 50% non-fat dry milk

159 = 50% non-germinated DF 222 + 50% non-fat dry milk

894 = 100% non-germinated Vinton 81 (all soy yogurt- control)

949 = 100% non-fat dry milk (all dairy yogurt- control)

6.4.2 pH changes of yogurt samples during cold storage

The pH changes of all the yogurt samples appeared to be dependent on the type of yogurt and storage period (Table 6.5) .The F-value of 772.436 at $p < 0.001$ indicate that the difference in the mean values among the different levels of yogurt samples is statistically significant.

Table 6.5 Analysis of variance for the effects of yogurt varieties and storage time (weeks) on pH

Main Effects	DF	SS	MS	F	P
Yogurt samples	5	0.826	0.165	772.432	<0.001
Weeks	5	0.205	0.0411	192.135	<0.001
Yogurt x Weeks	25	0.175	0.00700	32.741	<0.001
Residual	36	0.00770	0.000214		
Total	71	1.214	0.0171		

The above table also indicates that the difference in the mean values among the different levels of weeks is statistically significant with an F-value of 192.135 ($p < 0.001$). There is statistically significant interaction between the different types of yogurt and storage time (F-value = 32.741; $p < 0.001$). The pH values of the sample with 100% non-germinated soy powder were significantly higher than the rest of the samples, ranging from 4.67 for 1st week to 4.82 for 6th week (Table 6.6). This observation is consistent with our previous study on the activity of lactic acid bacteria. Overall, there was no statistically significant difference in pH between germinated DF 222 soy-fortified yogurt and 100% dairy yogurt

during storage. Also there was significant difference in pH values between 1st and 2nd week of storage and between 3rd and 4th weeks of storage (Table 6.6).

By the end of storage period, the pH of all products was higher than that recorded at the termination of yogurt incubation. These increases in pH could be as a result of proteolytic activities of the cultures including the probiotic during prolonged cold storage, which resulted to higher levels of liberated amino groups (Nielsen and others, 2001; Donkor and others, 2005). These data were in contrast to the data obtained by Lamoureux and others (2002) and Popa (2005), whose data showed that there were slight decrease in yogurt pH during the 42 days of storage at 4 °C, which probably led to lower cell counts (CFU/g). The pH recommended for the survival of some probiotics e.g. bifidobacteria in yogurts is 4.6 (Shah, 1996). The pH values of our whole soy-fortified including the control yogurts were between 4.5 and 4.8 after 42 days of storage at 4 °C.

Table 6.6 pH of soy-fortified yogurt samples during prolonged cold storage at 4 °C

Weeks of storage	*252	169	344	159	894	949
1	4.56^{bC}	4.59^{aC}	4.39^{aA}	4.49^{aB}	4.67^{aD}	4.41^{aA}
2	4.56^{aC}	4.60^{aC}	4.34^{aA}	4.49^{aB}	4.69^{aD}	4.37^{aA}
3	4.56^{aB}	4.62^{aC}	4.46^{cA}	4.64^{bC}	4.75^{bD}	4.47^{bA}
4	4.64^{aB}	4.67^{aBC}	4.43^{bcA}	4.62^{bC}	4.84^{cD}	4.46^{bA}
5	4.56^{aC}	4.63^{aD}	4.37^{abA}	4.60^{bCD}	4.76^{bE}	4.44^{abB}
6	4.67^{bB}	4.79^{bC}	4.68^{dB}	4.49^{aA}	4.82^{cC}	4.55^{cA}

$^{a\text{-}c;\ A\text{-}E}$ Mean values with different superscript are significantly different ($p < 0.05$); Comparisons are made within the same column $^{(a\text{-}c)}$ and within the same row $^{(A\text{-}E)}$; n = 2.

252 = 50% germinated Vinton 81 + 50% non-fat dry milk

169 = 50% non-germinated Vinton 81 + 50% non-fat dry milk

344 = 50% germinated DF 222 + 50% non-fat dry milk

159 = 50% non-germinated DF 222 + 50% non-fat dry milk

894 = 100% non-germinated Vinton 81 (all soy yogurt- control)

949 = 100% non-fat dry milk (all dairy yogurt- control)

The survival and viable numbers of probiotics in fermented dairy products are different due to differences in strains and manufacturers (Champagne and others 2005). In the present investigation, the survival of the probiotic i.e. *L. acidophilus* NCFM was excellent during the 6-week cold storage. To effectively make some health claims on the consumption of probiotics in fermented milk or soymilk, it has been suggested that the cell numbers be a minimum of 6.0 log CFU/ml (Gomes and others 1998). All the bacterial strains used in our study successfully attained a desired level of at least 7.0 log CFU/g of each strain in the yogurt samples. High proteolytic activity during storage being suggested in this study could be due to the presence of hydrolysable substrate in the form of milk and soymilk proteins.

Summarily, this investigation showed that whole soy-foritfied yogurt could be a good medium for the delivery of probiotics. The data presented in this investigation showed that germinated DF 222 soy-fortified yogurt was the best sample for the growth and viability of the microorganisms used in this study. In general, all the cultures utilized in yogurt manufacturing showed viability during prolonged cold storage at 4 °C.

CONCLUSIONS

1. There was a significant interaction ($p \leq .001$) between non-fat dry milk/soy powder blends, soybean variety and culture type, but NFDM/soy powder blend was the most significant factor ($p \leq .001$) that affected the activities and growth of lactic acid bacteria.

2. Cultures grown in most milk blends that contained whole soy powder gave the lowest pH, highest %TA and growth (CFU/ml) values.

3. Cultures grown in germinated whole soy powder blends produced more acid and growth ($p \leq .001$) than its non-germinated counterparts suggesting that germinated soybeans contained more bioavailable nutrients and other growth factors. Best results were obtained when the cultures were grown in 50% non-fat dry milk + 50% germinated soy powder blend.

4. All the cultures used in this study successfully attained a desired level, achieving at least 10^7 CFU/ml of each strain in each blend.

5. Overall data strongly support the view of several workers that non-fortified milk does not enhance lactic acid bacteria and probiotics growth as much as fortified milk.

6. Fortification of milk bases with whole soymilk or powder for fermented products will enhance bioactive compounds and the viability of the cultures, hence increases possible health benefits to consumers.

7. Sensory evaluation of soy-fortified yogurts showed no statistically significant difference between all-dairy yogurt and the 1:1 soy/NFDM blended yogurts for flavor and overall acceptance ($p=0.0001$). This suggests that there is no consumer

preference for cow's milk yogurt over whole soy-fortified cow's milk yogurt at this fortification level.

8. One hundred percent soy yogurt had the lowest sensory scores than the other yogurts in appearance and body texture although they were not significantly different. This could be attributed to the higher fiber content of the whole soy powder utilized, which increased viscosity, and thus thickness.

9. Sensory data indicated that consumer acceptable soy-fortified yogurt could be made using whole soymilk or powder blended with cow's milk.

10. Nutrient composition analysis indicated that the protein contents of the entire soy-fortified yogurt were comparable to the dairy yogurt. The fat contents were low in all yogurt samples while all soy containing yogurts had dietary fibers of up to 1.6% in 100% soy yogurt.

11. All the four isoflavone isomers measured in the soy powders were present but genistin and daidzin, the β-glucosides were more abundant than genistein and daidzein, the aglycones.

12. Soaking and germination increased the isoflavone contents and decreased the stachyose contents of all soybean varieties, thus suggesting that processing methods could influence the concentrations of these compounds.

13. The total isoflavone concentration was highest in non-germinated (soaked) DF 222 soy powder, but all the soy powders contain substantial amount of isoflavones measured.

14. All the germinated soy powders irrespective of the variety had lower amounts of stachyose. The lowest stachyose content was found in germinated E05276-T soy powder (13.90 mg/g) while the highest amount was found in non-germinated DF 222 soy powder (46.65 mg/g).

15. Nutrient composition analysis showed that the protein contents of all soy powders were between, 50.28% to 56.03% well above the average protein contents of soybeans (40%).

16. Chemical analysis of yogurt samples showed that total isoflavone contents increased at 6 weeks of storage (4 °C) compared to the 1st week. Daidzin and genistin (β-glucosides) contents contributed most to the increment during storage.

17. The genistein and daidzein contents of the whole soy-fortified yogurts remained significantly the same throughout the shelf life period (6 weeks at 4 °C). The percentage of total isoflavones retained in the yogurt samples was high.

18. All the yogurt samples containing germinated soy powders had lower stachyose contents than non-germinated soy-fortified yogurts (2.82 to 4.41 mg/g of stachyose).

19. The pH values of yogurt made with 100% non-germinated soy powder were significantly higher ($p < 0.05$) than the rest of the samples during storage.

20. The growth and viability of all the cultures were above 10^7 CFU/g of yogurt. A concentration of at least 10^6 CFU/ml viable cultures in products is needed in order to exert health benefits to consumers. Soy-fortified yogurts had the highest viable cell contents.

APPENDICES

APPENDIX 1

Questionnaire for experienced yogurt screeners

Product: Cow's milk/soymilk blended Swiss style strawberry yogurt

You will be provided with 5 yogurt samples. Please carefully evaluate each sample in the order it is presented and indicate the 3 most acceptable samples to you.

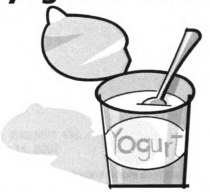
Acceptable = 1 **Unacceptable = 0**

Flavor	817 ___	626 ___	978 ___	149 ___	481 ___
Body and Texture	817 ___	626 ___	978 ___	149 ___	481 ___
Appearance and Color	817 ___	626 ___	978 ___	149 ___	481 ___
Overall Acceptance	817 ___	626 ___	978 ___	149 ___	481 ___

APPENDIX 2

Advertisement

Do you like yogurt? Come taste...



LOW-FAT YOGURT

Dairy and Soy samples

Date: Thursday January 31st, 2008

Time: 11:00 am – 4:00 pm
Sensory Lab- Room 102 Trout (Food Science) Building

Take 15-20 minutes to try some new products and earn a MSU ICE-CREAM coupon for helping out.

APPENDIX 3

MICHIGAN STATE
UNIVERSITY

Initial IRB Application Determination *Exempt*

September 17, 2007

To: Zeynep USTUNOL
2105 S. Anthony Hall
MSU

Re: **IRB# X07-857** Category: EXEMPT 1-6
Approval Date: September 14, 2007

Title: Yogurt fortification with predigested/germinated whole soybean powder for enhanced therapeutic benefits

The Institutional Review Board has completed their review of your project. I am pleased to advise you that your project **has been deemed as exempt** in accordance with federal regulations.

The IRB has found that your research project meets the criteria for exempt status and the criteria for the protection of human subjects in exempt research. **Under our exempt policy the Principal Investigator assumes the responsibilities for the protection of human subjects** in this project as outlined in the assurance letter and exempt educational material. The IRB office has received your signed assurance for exempt research. A copy of this signed agreement is appended for your information and records.

Renewals: Exempt protocols do not need to be renewed. If the project is completed, please submit an ***Application for Permanent Closure.***

Revisions: Exempt protocols do not require revisions. However, if changes are made to a protocol that may no longer meet the exempt criteria, a new initial application will be required.

Problems: If issues should arise during the conduct of the research, such as unanticipated problems, adverse events, or any problem that may increase the risk to the human subjects and change the category of review, notify the IRB office promptly. Any complaints from participants regarding the risk and benefits of the project must be reported to the IRB.

Follow-up: If your exempt project is not completed and closed after three years, the IRB office will contact you regarding the status of the project and to verify that no changes have occurred that may affect exempt status.

Please use the IRB number listed above on any forms submitted which relate to this project, or on any correspondence with the IRB office.

Good luck in your research. If we can be of further assistance, please contact us at 517-355-2180 or via email at IRB@msu.edu. Thank you for your cooperation.

Sincerely,

Peter Vasilenko, Ph.D.
BIRB Chair

APPENDIX 4

Consent form for panelists

SENSORY EVALUATION OF LOW-FAT YOGURT FORTIFIED WITH GERMINATED WHOLE SOY POWDER
UNIVERSITY COMMITTEE ON RESEARCH INVOLVING HUMAN SUBJECTS

TITLE OF THE RESEARCH
Yogurt fortification with predigested/germinated whole soybean powder for enhanced therapeutic benefits.

INVITATION TO PARTICIPATE
You are invited to participate in this study, which compares the properties of yogurt made from cow's milk to those made from blends of cow's milk and germinated soymilk.

PURPOSE OF THE STUDY
This study is being conducted in order to develop an acceptable yogurt made from blends of cow's milk and germinated soymilk.

BASIS FOR SUBJECT SELECTION
Subjects are selected based on their ability to detect differences in sensory attributes of yogurt made from cow' milk and from soymilk. Individuals with cold, sinus conditions or allergies to a specific ingredient will not be asked to participate. The general adult population is used for testing. Participants must be at least 18 years old.

POTENTIAL RISKS
The yogurt samples to be evaluated contain the following ingredients: milk, non-fat dry milk, soybean powder, stabilizer, sweeteners (sucrose), strawberry puree, starter culture and red food color. All of these ingredients are USDA and /or FDA approved for use in foods intended for human consumption and are being used at USDA/FDA approved levels. Each product has being produced in a safe and wholesome manner according to USDA and/or FDA regulations. These products samples pose no adverse health risk upon ingestion, provided the subject has not been identified as being susceptible to an allergic reaction to the previously listed product ingredients. If you believe there is a potential of an allergic reaction upon ingesting the test products, or you believe that participating will violate religious or cultural beliefs, notify the on-site sensory evaluation coordinator and/or principal investigator immediately. You will be released from participating in the study.

POTENTIAL BENEFITS
There are no direct benefits gained from participation in this study. However, your participation provides valuable data for the development of products providing compounds with increased health benefits. Information obtained from this study will be published in appropriate scientific journals to expand our current knowledge in enhancing the health values of yogurt.

EXPLANATION OF PROCEDURES
You will be asked to sit at a booth and taste a number of numerically coded yogurt samples. You will be provided with water for rinsing your mouth between samples. The tasting exercise will take a maximum of 25 minutes of your time, depending upon your speed of tasting. You will use sensory evaluation ballot forms to record responses concerning specific product attributes. Tasting will occur in the Sensory Evaluation/Human Studies Laboratory located in Room 102 of the G. Malcolm Trout (Food Science) Building.

ASSURANCE OF CONFIDENTIALITY
Any information obtained in connection with this study that could be identified with you will be kept confidential by ensuring that all consent forms are securely stored and your privacy will be protected to the

maximum extent allowable by law. All data analyzed will be reported in an aggregate format that will not permit associating subjects with specific responses or findings.

WITHDRAWAL FROM THIS STUDY
Participation in this study is voluntary. Your decision to refuse participation or discontinue participation during this study will not affect your present or future relationship with the principal investigator or Michigan State University.

COMPENSATION FOR PARTICIPATION
After you have completed your sensory testing session and turned in your sensory ballot, you will be offered a choice of treats (i.e., candy or ice cream coupon) for your time and effort.

OFFER TO ANSWER QUESTIONS
If you have any questions, please do not hesitate to contact the on-site sensory evaluation leader and /or the principal investigator. You are voluntarily making a decision to participate in this study today. Your signature certifies that you have decided to participate after having read the information provided above and that you had an adequate opportunity to discuss this study with the principal investigator and have had all your questions answered to your satisfaction. You will be given a copy of this consent form for to keep upon request.

SIGNATURE OF SUBJECT DATE

In my judgment the subject is voluntarily and knowingly giving informed consent and possesses the legal capacity to give informed consent to participate in this research study.

SIGNATURE OF INVESTIGATOR DATE

Dr Zeynep Ustunol
2105 S. Anthony Hall
Food Science & Human Nutrition
Michigan State University, E. Lansing, MI 48824
Tel: 517-355-7713 ext 184, E-mail: ustunol@anr.msu.edu

APPENDIX 5

Questionnaire

Product: Cow's milk/soymilk blended strawberry flavored low-fat yogurt

You will be provided with 6 yogurt samples followed by questions. Please evaluate each sample in the order it is presented according to the scale provided.

1. **Appearance/color**

How do you like appearance and color of the sample

252	169	949	344	159	894
___	___	___	___	___	___

9- like extremely

8- like very much

7- like moderately

6- like slightly

5- neither like/nor dislike

4- dislike slightly

3- dislike moderately

2- dislike very much

1- dislike extremely

2. **Body and Texture**

Take a scoop of the sample. Look at the sample and then taste it.

252	169	949	344	159	894
___	___	___	___	___	___

9- like extremely

8- like very much

7- like moderately

6- like slightly

5- neither like/nor dislike

4- dislike slightly

3- dislike moderately

2- dislike very much

1- dislike extremely

3. Flavor

How do you like the overall flavor of the sample

252	169	949	344	159	894
___	___	___	___	___	___

9- like extremely

8- like very much

7- like moderately

6- like slightly

5- neither like/nor dislike

4- dislike slightly

3- dislike moderately

2- dislike very much

1- dislike extremely

4. Overall Acceptance

How well do you LIKE the sample overall?

252	169	949	344	159	894
___	___	___	___	___	___

9- like extremely

8- like very much

7- like moderately

6- like slightly

5- neither like/nor dislike

4- dislike slightly

3- dislike moderately

2- dislike very much

1- dislike extremely

Instruction:

You have completed the test. Thank you for your time. Don't forget to ask for your **ICE CREAM COUPON** before leaving!

REFERENCES

REFERENCES

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