SURVIVAL OF FREEZE DRIED PROBIOTICS IN SOY PROTEIN POWDER

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SURVIVAL OF FREEZE-DRIED PROBIOITCS IN SOY PROTEIN POWDER

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ABSTRACT

A series of well diffusion assays determined that *Bifidobacterium longum* B6 (B6) and *Lactobacillus paracasei* ATCC 25598 (LP) best inhibited pathogenic bacteria. These bacteria were freeze-dried in the presence of sucrose, trehalose and soymilk to determine the best conditions for survival. Cultures were anaerobically grown overnight and centrifuged. Cell pellets were resuspended in 10 mL of 4% sucrose + 18% soymilk (SS), 4% trehalose + 18% soymilk (TS) or 4% sucrose + 4% trehalose + 18% soymilk (STS). Suspensions were freeze dried and stored at room temperature in the dark. Statistical analysis found that at 24 days, treatments SS and STS were shown to be significantly better than TS ($P \leq 0.05$).

Bacteria were then freeze-dried with 4% sucrose + 4% trehalose + 18% soymilk (STS). Freeze dried bacteria were added to isolated soy protein powder. Samples were vacuum packaged and stored in the dark at room temperature or 4^{o}C. The product was prepared for sampling on days 0, 15, 30, 45, 60, 75, and 90.

Statistical analysis of percent reduction of bacterial counts ($P \leq 0.05$) showed significant differences for bacteria, bacteria and treatment, and time. Actual counts and log reduction suggest that bacteria freeze-dried with the cryoprotectant solution STS and stored at 4^{o}C best support bacterial growth in soy protein isolate powder.

CHAPTER 1

INTRODUCTION

Probiotics are beneficial bacteria that are consumed in order to get some sort of nutritional benefit. When the organisms are present in sufficient numbers, their signature benefits can be seen in the host (Schrezenmeir and de Vrese, 2001). Health benefits from these organisms range from improved digestion to inhibition of harmful bacteria.

These organisms can be added to foods to create a functional food that has the desired health benefits characteristic of the probiotic. One such food base is soy protein. Soy protein itself carries benefits that reduce the risk of heart disease (FDA, 1999) as well as the reduction of cancerous tumors.

In order to add probiotics to a food, these organisms must be preserved so that they can survive in sufficient numbers and therefore exert their benefits. One such method of preservation is freeze drying, which removes water at low temperatures. The addition of cryoprotectants to bacteria helps to counteract the harmful effects (Leslie et al., 1995).

The objectives of this study include:

1. To determine which probiotic bacteria best inhibits pathogenic bacteria.
2. To determine which probiotic to include in a soy protein isolate powder, based on its inhibitory activity.
3. To determine which cryoprotectant solution best protects a probiotic during freeze drying and storage.
4. To fortify soy protein isolate powder with freeze dried probiotics.

5. To determine the viability of freeze-dried probiotic bacteria in soy protein isolate powder under different storage conditions.

CHAPTER 2

LITERATURE REVIEW

2.1. Functional Foods

2.1.1. Definition

A functional food can be defined as a food that has "physiological or psychological effects beyond the traditional nutritional effect (Clydesdale, 1997). Additionally, Bellisle et al. (1998) described this type of product as one that "contained a component that affects one or a limited number of function(s) in the body in a targeted way so as to have positive effects."

2.1.2. Market Value

The U.S. functional beverage market was predicted to reach $10 billion in 2004, which was an increase from $7 billion in 1999 (Mintel International Group, 2004). Sloan (2002) reported that the U.S. market for functional foods in 2002 was $18.25 billion, while it was $15.4 billion in Europe, and $11.8 billion in Japan in the same year. Stanton et al. (2001) noted a trend concerning new functional food products, including sports-related products, fortified foods and drinks. The Business Communications Company (BCC) research (2003) projected that functional beverages and teas will grow to almost $11.5 billion by 2007, an increase from $8.7 billion in 2002. In the United Kingdom, the functional foods market was worth an estimated $1.1 billion by the end of 2005, showing a growth of 143% since 2000. Probiotic yogurts and yogurt drinks rose in value from $97 million in 2001 to $275 million in 2005 (Functional Foods–UK, 2004).

Certain foods have been proven to naturally have health benefits. Examples of these include whole oat products, psyllium, soy protein, foods containing plant stanol or sterol esters, sugarless chewing gum and candy, fatty fish, cranberry juice, garlic, green tea, tomatoes, animal products with conjugated lineoleic acid, probiotics, and prebiotics (Meister, 2002). Some examples of functional food are shown in Table 2.1.

Table 2.1. Examples of functional foods.

Functional Food	Bioactive component	Potential health benefit	Recommended amount	Strength of evidence
Whole oat products	Beta-glucan	Lower cholesterol levels	3 g/day	Very strong
Psyllium	Soluble fiber	Lower cholesterol levels	1 g/ day	Very strong
Whole soy foods and foods made with soy protein	Soy protein	Lower cholesterol levels	25 g/day	Very strong
Special fortified margarines or salad dressing	Plant stanol or sterol esters	Lower cholesterol levels	3.4 g/day for stanols; 1.3 g/day for sterols; must be consumed with meals	Very strong
Sugarless chewing gums and candies	Sugar alcohols	Does not promote tooth decay	NA	Very strong
Fatty fish	Omega-3 fatty acids	Reduced risk of heart disease	Twice per week	Strong
Cranberry juice	Pro-anthocyanidins	Reduced urinary tract infections		Moderate
Garlic	Organolsulfur compounds	Lower cholesterol levels		Moderate

Green tea	Catechins	Reduced risk of some types of cancer		Weak to moderate
Tomatoes and tomato products	Lycopene	Reduced risk of some types of cancer, especially prostate cancer		Weak to moderate
Dark green leafy vegetable	Lutein	Reduced risk of age related macular degeneration		Weak
Meats and dairy products	Conjugated linoleic acid	Reduced risk of breast cancer; increased muscle mass; other possible effects		Weak
Cruciferous vegetables	Isothiocyanate, indoles	Reduced risk of some types of cancer		Weak
Fermented dairy	Probiotics	Support gastrointestinal tract health; boost immunity		Weak

Adapted from Meister, 2002.

2.2. Soy Foods

Along with the increasing demand for and consumption of functional foods, the demand for soy foods also appears to be increasing. From 2001 to 2004, food manufacturers in the U.S. introduced over 1600 new foods containing soy as an ingredient (Soyfoods: The U.S. Market, 2004). BCC research (2004) predicts that the overall U.S. market for soy-containing foods is projected to reach $202 billion in 2008, an increase from $188 billion in 2003. The market for staple soy-containing foods will

reach $197 billion in 2008. Some examples of types of soy protein products are listed below.

2.2.1. Protein Quality of Soybean Products

The quality of soy protein products depends on the amino acid composition, presence of antinutritional factors, digestibility, overall composition of the diet and nutrient requirements of the species in question (Wolf and Cowan, 1975).

2.2.2. Whole Soybeans

A soybean is made up of 8% hull, 90% cotyledon, and 2% hypocotyl and plumule (Wolf and Cowan, 1975). The oil and protein portion of the bean is about 60% and one third consists of carbohydrates, which include polysaccharides, stachyose, raffinose and sucrose (Kawamura, 1967). A whole soybean must be processed to remove the oil and to convert the defatted portion into feeds and food products (Wolf and Cowan, 1975). This is usually done with solvents that extract the desired segment from the bean.

2.2.3. Soy Protein Concentrate

This type of soy protein is made by removing a portion of the carbohydrates from defatted and dehulled soybeans (Solae®, 2006). It contains at least 65% protein (Endres, 2001) and retains most of the bean's dietary fiber. It also contains polysaccharides, that absorb water, and can be manipulated during processing to influence how tightly the water can bind to the protein in the final food product (Endres, 2001). Three processes are used to obtain soy protein concentrate which include acid leaching, extraction with aqueous alcohol and denaturation with moist heat (Endres, 2001). Soy protein

concentrates have improved flavor characteristics when compared to soy flours. Functionally, they are used to enhance fat emulsions, water and fat absorption, viscosity control, and texture control (Endres, 2001).

2.2.4. Textured Soy Protein

Textured soy protein is made by running defatted soy flour through an extruder and is popular as a meat extender. Textured soy flours and concentrates are used in meat systems based on their ability to absorb water and fat which contributes to a meat-like texture (Endres, 2001).

2.2.5. Structured Isolates

Soy protein isolate is solubilized in an alkaline medium and passed through a spinneret to form fibers (Endres, 2001). Fibers are bound together in bundles and treated with flavor ingredients so as to resemble animal products (Endres, 2001). Fibrous soy protein isolate is used to improve the texture of mechanically deboned poultry and in other meat systems to boost texture and mouthfeel (Endres, 2001).

2.2.6. Soy Flour

Soy flour is made from roasted soybeans ground into a fine powder. Removal of oil can happen before or after grinding (Endres, 2001). The flour contains about 50% protein and carbohydrate components of the soybean sugars, oligosaccharides and fiber (Solae®, 2006). There are four kinds of soy flour: full-fat, high enzyme, defatted, and lecithinated and defatted (Endres, 2001). Full fat flours are used as ingredients for replacement of non-fat dry milk and whole milk solids (Endres, 2001). High enzyme

flours include high enzyme flours which are used in bread doughs to strengthen gluten proteins (Dubois and Hoover, 1981). Defatted flours are prepared by hexane extraction of flakes (Wolf and Cowan, 1975). Other applications for dispersible defatted soy flour include bakery and cereal products. Milk replacers and pet foods, as well as fermentation media also use soy flour to enhance texture and nutritional properties. Lecithinated and defatted flours often replace eggs in bakery applications.

2.2.7. Soy Protein Hydrolyzates

Hydrolyzates result from the partial hydrolysis by enzymes, specifically pepsin (Endres, 2001). This type of protein aids in foaming capacity and stability (Endres, 2001) and is used mainly in confections, toppings and icings, and as whipping and foaming agents (Kinsella et al., 1985).

2.2.8. Soy Beverage

A soymilk is defined as an aqueous extract of whole soybeans which may contain 5 to 7 mg isoflavones per 100 g (King and Bignell, 2000). Soymilks in the U.S. are sold as regular soymilk or as fermented soymilk that may contain live probiotic bacteria. There are also soy yogurt products which contain beneficial nutrients, such as phyonutrients, isoflavones, saponins, vitamin E, folate, and omega-3 fatty aids (Meister, 2002).

2.2.9. Soy Protein Isolate

Soy protein isolate is prepared through water extraction and minimum heat on soy flakes (Solae®, 2006). This is prepared from dehulled and defatted soybeans by

removing the non-protein portion and ultimately contains >90% protein (Endres, 2001). In this process, most if not all of the beany flavor is lost. Isolates have functional properties, including emulsion and emulsion stability, fat and water binding and adhesion (Endres, 2001). The soy forms a gel which holds moisture, fat and solids and results in a texture similar to meat proteins (Endres, 2001).

2.2.10. Use of Soy Protein Isolate

The Archer Daniels Midland company suggests the use of soy protein isolate in dry beverage mixes, meal replacement drinks (dry and liquid), instant cereals, instant soup mixes, puddings, bakery mixes, and other dry blend mixes." Soy protein isolate may also be used in meat analog systems and nutritional supplements.

Soy protein is commercially popular in the form of beverage mixes or pre-mixed "shakes." Revival® offers a variety of soy shakes intended as nutritional supplements. Genisoy® also lists a number of soy powders and shakes. These are designed to provide the benefits and nutrition of soy protein as well as other nutritional compounds. The powders provide supplements demanded by target consumers, such as body builders and vegetarians.

Choi et al. (2006) combined soy protein isolate and poly-ε-caprolactone to create an adhesive used for food packaging. Soy protein isolate and ferulic acid were combined to create an edible film (Ou et al., 2005). Soy protein isolate was also studied and shown to be an effective lubricant for prosthetic joints (Ahlroos and Saikko, 1997).

2.2.11. Roles of Soy Products in Heath and Diseases

Soy products are sought out and consumed based on their health and nutritional benefits. The FDA recommended consumption of 25 g of soy protein a day to help reduce serum cholesterol levels and the risk of heart disease (2000). They are low in saturated fat and have the essential amino acids needed for human nutrition (FDA, 2002). Soy may help to reduce low-density lipoprotein (LDL) cholesterol levels, which in turn, reduces the risk of heart disease (FDA, 2000) and contains isoflavones that reduce the risk of certain cancers (USB, 2001).

Soy isoflavones decreased the fat content but increased the lean portion of pig carcasses (Payne et al., 2001). This may be due to a decreased insulin:glucose ratio cause by the isoflavones, a phenomenon that has been shown in rats (Banz et al., 1997). Soy protein with isoflavones helped to preserve the bone mineral density of the spine in older women (Newton et al., 2006). In male osteoporosis studies, soy isoflavones enhanced bone formation and suppress bone resorption (Khalil et al., 2005; Kalu et al., 1998; Ishimi et al., 2002; Wu et al., 2003).

Chemically induced rat mammary tumors were prevented by soy protein isolate (SPI) (Constantinou et al., 2002). These authors suggested that diets that include soy protein isolate might help to increase the action of the phase II detoxification enzymes, QR and GST. It was shown in rats that dietary soy protects against mammary tumorigenesis induced by a direct-acting carcinogen and alters signaling pathways (Simmen et al., 2005). Decreased progesterone levels in rats fed soy protein isolate may explain the decreased tumor incidence because progesterone acts as an anti-apoptotic (Ismail et al., 2003; Lange et al., 1999; Medina et al., 2001). The decreased tumor incidence and increased tumor latency in rats fed soy protein isolate may be caused by

distinct changes in gene expression levels in normal mammary tissues, decreased serum P levels, and increased apoptotic activity of sera towards cancer cells. Soy protein isolate may improve the pathway responsible for apoptosis and other signaling pathways (Simmen et al., 2005).

In rabbits fed soy protein products, there was lower cholesterolemia and less severe atherosclerosis versus animals fed casein (Gibney and Kritchevsky, 1983). Soy protein also reduced the progression of focal lesions on the common carotid artery of rabbits fed a high cholesterol diet (Castiglioni et al., 2003). The focal atheromatous plaque of the animals fed soy contained more macrophages, which is called soft plaque, and is more treatable with medication (Castoglioni et al., 2003). The soy was also shown to lengthen the lag phase of LDL oxidation induced by cupric oxide which "indicates dramatic resistance to oxidation, potentially leading to vascular protection" (Castoglioni et al, 2003). When compared to fish oil and its ability to reduce the severity of retinoid-induced hypertriglyceridemia, soy protein isolate was as effective as fish oil (Radcliffe and Czajka-Narins, 2004). Soy protein isolate may reduce triglyceride levels by decreasing the rate of triglyceride synthesis (Demonty et al., 2002; Iritani et al., 1988; Pfeuffer and Barth, 1986). Isoflavones may contribute to the lowering of triglyceride levels (Demonty et al., 2002). Ovarian hormone deficiency-induced hypercholesterolemia was shown to be reversed by soy protein isolate and a synthetic isoflavone (Arjmandi et al., 1997). Soy isoflavones play a role in lipid metabolism and may help to direct triglycerides away from adipose tissue to other tissues for catabolism (Arjmandi et al., 1997).

2.3. Probiotics

2.3.1. Definition

Probiotics can be added to soy and other products to create functional foods. A probiotic is described as a "substance secreted by one microorganism which stimulates the growth of another" (Lilly and Stillwell, 1965). In 1974, Parker defined probiotics as "organisms and substances which contribute to intestinal microbial balance." The most commonly used definition was introduced by Fuller (1989) which states that a probiotic is "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance." The most recent definition explains a probiotic as "a preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alters the microflora (by implantation or colonization) in a compartment of the host and by that, exert beneficial health effects in this host (Schrezenmeir and de Vrese, 2001).

2.3.2. Health Benefits

Probiotic organisms have a variety of human health benefits. One such benefit is the effect on cholesterol content. Hypercholesterolemic rats were given viable *Lactobacillus acidophilus* SBT 2062, which in turn, reduced liver cholesterol concentrations (Oda and Hashiba, 1994). Other *Lactobacillus* species were given to rats with the same condition and total cholesterol levels were reduced (Fukishima and Nakano, 1995.)

Probiotics also have an anti-tumor effect. In a study by Goldin and Gorbach (1984), *L. acidophilus* NCFM and N2 administered to humans reduced the fecal

enzymes, β-glucoronidase, nitroreductase and azoreductase, which are indicators of cancer.

Ahn and Mustapha (2004) showed that in well diffusion experiments, pathogens were inhibited by probiotics. These bacteria also competitively exclude new bacteria that try to colonize in their sites (Cummings, 2002). This was demonstrated by Barrow et al. (1980) in an experiment that showed *Lactobacillus fermentum salivarius* strain 14 with or without *Streptococcus salivarius* strain 32 reduced *Escherichia coli* numbers in the stomach and duodenum of pigs.

2.4. Commercial Probiotic Products

In the U.S., probiotics are sold as food and dietary supplements (Lee et al., 1999). In these products, the lactobacilli and bifidobacteria are the most popular probiotics (Lee et al., 1999). Lifeway SoyTreat™ is an example of a non-dairy soy beverage containing 10 live and active probiotic kefir cultures. Table 2 shows some commercial probiotic products sold in the U.S.

Table 2.4. Commercial probiotic products.

Product	Producer	Probiotic	Functional claim	Product category
Ervian Acidophilus yogurt™	Ervian Dairy	*Lactobacillus acidophilus*	Promoting health	Fermented milk
Crunch n	General Mills,	*Lactobacillus*	Promoting	Fermented milk

yogurt™	Inc.	*acidophilus with Streptococcus thermophilis* and *Lactobacillus bulgaricus*	Health	
Classic flavor	Groupe Danone	*Lactobacillus acidophilus*	Promoting health	Fermented milk
Lactinex	Hyson, Westcott and Dunning	*Lactobacillus acidophilus* with *Lactobacillus bulgaricus*	Promoting health	Powder
LGG	ConAgra Foods, Inc.	*Lactobacillus rhamnosus GG*	Promoting health	Capsules

Adapted from Lee et al., 1999.

2.5. Probiotic Bacteria

2.5.1. Lactobacillus

Lactobacillus sp. is a regular, non-sporeforming gram-positive rod with a size of 0.5-1.2 x 1.0-10.0 μm (Holt et al., 1994). Species such as *L. acidophilus*, *Lactobacillus helveticus* and *Lactobacillus johnsonii* produce compounds such as lactic acid, while *Lactobacillus casei, Lactobacillus plantarum, Lactobacillus rhamnosus,* and *Lactobacillus fermentum* produce lactic acid, acetic acid, ethanol, and formic acid (Lee et. al., 1999).

Some lactobacilli have the ability to colonize the surface of gastrointestinal epithelia (Arici M. et al., 2004; Valeur N. et al., 2003). The strain *Lactobacillus* F19 was

consumed within a yogurt product and successfully survived the human gastrointestinal (GI) tract in 100% of study subjects (Mättö et al., 2006). *L. rhamnosus* strains E800 and Lc705 had good survival ability in the GI tract when administered in a whey-based fruit juice matrix which was shown by high numbers in fecal samples (Suomalainen et al., 2006). Fermented milk containing *L. helveticus* and *Saccharomyces cerevisiae* was given to rats under exercise stress (Aoi et al., 2006). The milk helped to reduce the increased serum creatine kinase level, myeloperoxidase activity and the level of thiobarbituric-acid-reactive substances in the gastrocnemius muscle. Other activities of the milk were also observed in the increase of mRNA and levels of protective proteins, such as antioxidants and chaperone proteins (Aoi et al., 2006).

2.5.2. Bifidobacterium

Bifidobacterium sp. is an irregular, non-sporeforming gram-positive rod that can be short, curved and club-shaped. Its size is 0.5-1.3 x 1.5-8 μm (Holt et al., 1994). *Bifidobacterium infantis*, *Bifidobacterium bifidum*, *Bifidobacterium breve* and *Bifidobacterium longum* produce lactic, acetic and formic acids (Lee et al., 1999). *B. longum* showed anticarcinogenic effects in the colon of rats (Challa et. al., 1997; Singh et al., 1997). This species of bifidobacteria is present in the human intestine and provides infection resistance to the host (Garro, et al 2004). *Bifidobacterium animalis* subsp. *lactis* Bb-12, when consumed in a yogurt product, was shown to survive the conditions of the human gastrointestinal tract in 79% of study subjects (Mättö et al., 2006). *L. acidophilus* CCRC 14079, *Streptococcus thermophilus* CCRC 14085, *B. infantis* CCRC 14633 and *B. longum* B6 were used to ferment soymilk (Wang et al., 2006). Antioxidant activities,

such as the inhibition of ascorbate autoxidation, and the reducing activity and scavenging effect of superoxide anion radicals were higher than those of unfermented milk (Wang et al., 2006). The strains *Bifidobacterium lactis* Bb-12 or *B. longum* Bb-46 were added to buffalo milk yogurt and soy yogurt and resulted in lower levels of plasma total cholesterol and very low-density lipoprotein (VLDL) + low-density lipoprotein (LDL) cholesterol than the rats fed no probiotics (Abd El-Gawad et al., 2005). *B. longum* Bb-46 was more effective in lowering plasma and liver cholesterol levels than yogurt or soy yogurt containing Bb-12, and yogurts containing both probiotics resulted in more excretion of bile acids in the feces (Abd El-Gawad et al, 2005). Probiotic fresh cheese, which supports survival at 60 days after manufacture and protects against acidity, was shown to be a good medium for *B. bifidum*, *L. acidophilus* and *L. paracasei* allowing these cultures to exhibit positive immune effects in the gut (Medici et al., 2004).

2.5.3. Enterococcus

Enterococcus sp. is a gram-positive, non-sporeforming, facultative anaerobic coccus, with cells being spherical to ovoid (Holt et al., 1994). These bacteria can be found in sizes of 0.6-2.0 x 0.6-2.5 μm (Holt et al., 1994). The enterococcal species, *Enterococcus faecalis* and *Enterococcus faecium*, are considered to be probiotics (Holzapfel et al., 1998). In a study to observe the effects of *E. faecium* SF 68 compared to a placebo for treatment of acute diarrhea, the bacteria reduced the duration of the diarrhea by 18% (Marteau et al., 2001; Wunderlich et al., 1989). *E. faecium* L3 was shown to have an antagonistic activity against streptococci groups A, B, C, D and G (Yermolenko et al., 2005). This may be due to the production of enterocin A and B

which is controlled by the genes *entA* and *entB* (Yermolenko et al., 2005). Four *E. faecium* strains were selected from raw cows' milk and used to manufacture fermented milk (Muguerza et al., 2006). Doses from the whey portion were given to spontaneously hypertensive rats which resulted in significant decreases in systolic and diastolic blood pressures (Muguerza et al., 2006).

2.5.4. Others

Leuconostoc mesenteroides and *Sporolactobacillus inulins* are lactic acid bacteria that have health benefits (Holzapfel et al., 1998). Some non lactic acid bacteria such as *Bacillus cereus* var. toyoi, *E. coli* nissle, *Propionibacterium freudenreichii*, *Saccharomyces cerevisiae*, and *Saccharomyces boulardii* also exhibit probiotic qualities (Holzapfel et al., 1998). The *Saccharomyces* spp. have been used in pharmaceutical applications while *B. cereus* var. toyoi and *P. freudenreichii* have been used in pharmaceuticals as well as in animals (Holzapfel et al., 2001). *S. boulardii* was used in a premature infant formula which contributed to a fecal flora that was comparable to that of a breast fed infant (Costalos et al., 2003).

2.6. Preservation and Storage of Probiotic Cultures

Probiotics that are to be in included into a food product must be able to retain their characteristic properties that make them beneficial to consume. They must also remain in sufficient numbers so that health benefits may occur. Proper preservation and storage of said organisms help to ensure that both of these objectives are met. A variety

of methods have been used to preserve and store probiotic organisms for use in food products.

2.6.1. Encapsulation

Encapsulation is a process which uses a coating to surround tiny particles. These encapsulated particles result in capsules with useful properties. Encapsulated products include pharmacueticals and supplements, NOVOMEGA^{TM}, an encapsulated omega-3 fatty acid, flavors, and enzymes. Encapsulated probiotics have been added to yogurt in order to test their sensory effects versus that of unencapsulated bacteria (Kailasapathy, 2006). While free and encapsulated probiotic bacteria do not substantially influence the sensory characteristics of yogurts, microencapsulation helps the survival of probiotic bacteria in yogurts during storage (Kailasapathy, 2006). This process can also protect bacteria in unfavorable environments. *L. acidophilus* ATCC 4356 was encapsulated by compression coating using sodium alginate which resulted in protection from an acidic environment, similar to the human gastrointestinal tract (Chan and Zhang, 2005). *L. acidophilus* CSCC 2400 was encapsulated in calcium alginate in varying capsule sizes and bacterial loads during encapsulation (Chandramouli et al., 2004). Large capsules with higher bacterial loads showed an increase in survivors while in gastric conditions.

2.6.2. Spray Drying

Spray drying is a technique that uses a melt or polymer solution in which a particle is dissolved or suspended. Liquid products are best suited for spray drying (Heldman and Singh, 1981). Spray drying includes a rapid drying cycle, a holding drying cycle, and a final product that is package ready once it leaves the dryer (Heldman and

Singh, 1981). Flavors are often spray dried for use in products. Other spray dried products include eggs, malt extracts, animal digests for pet foods, and instant coffee. Probiotics have been spray dried as well. Skim milk, used as a carrier for *L. rhamnosus* GG during freeze-drying showed that bacterial survival was favorable at 60% but storage stability was decreased (Ananta et al., 2005).

2.6.3. Freezing

Freezing is the process by which a liquid is converted into a solid form. It also delays food decay by turning water to ice, which no longer allows microorganisms to grow, and halts chemical reactions (Heldman and Singh, 1981). Bacteria are often frozen in the presence of glycerol. Or in the case of a study by Cleland et al. (2004), glycine betadine was shown to protect a variety of prokaryotic organisms in various freezing situtations.

2.6.4. Freeze-drying

Freeze-drying involves the sublimation of water from the frozen state to the gas state (Heldman and Singh, 1981). Moisture removal or dehydration can occur without exposing the product to excessively high temperatures as well as preserving the structure of the product (Heldman and Singh, 1981). The purpose of this process is that it reduces the water content of the food so that microorganisms and enzymes are inhibited. Freeze-dried products include Astronaut Ice Cream®, designed by NASA for the Apollo missions, and instant coffee. The United States also uses freeze-drying to manufacture a shelf stable product, Meal, Ready to Eat® (MRE) for the armed forces.

2.7. Cryoprotectants

A cryoprotectant is a substance that is added to bacteria in order to increase survival and viability by preventing freeze damage to biological tissues. A number of cryoprotectants have been used in the preservation of lactic acid bacteria. Sinha et al. (1974) added skim milk fortified with ascorbic acid, thiourea, and ammonium chloride to bacteria prior to freeze-drying. The strains, *Lactococcus lactis* subsp. *cremoris* H61, *Streptococcus thermophilus* 510, and *Lactobacillus bulgaricus* B-1 exhibited maximum survival with cryoprotective solutions containing skim milk, skim milk + glutamate, skim milk + malate, and skim milk + arginine (Morichi, 1974). Zayed and Roos (2004) added sucrose and trehalose to skim milk when freeze-drying *Lactobacillus salivarius* to maximize survival during storage. The sugars, sucrose and trehalose, have been shown to protect membranes and proteins in intact *E. coli* and *Bacillus* species (Leslie et al., 1995). Gelatin was added to a milk sucrose medium and while it did not show an improvement in the survival of the four lactic acid bacteria, it helped improve the storage stability of freeze-dried *L. casei* ssp. *rhamnosus* RO11 and *B. longum* RO232 (Champagne et al., 1996).

CHAPTER 3

MATERIALS AND METHODS

The methods are discussed in two sections. The first section deals with the selection of probiotic bacteria based on their inhibitory activities against pathogenic bacteria by the use of a well diffusion assay. The second section deals with the freeze-drying of the selected probiotics, in the presence of sucrose and trehalose. Additionally, the latter section evaluates the freeze-dried probiotics after being added to a soy protein isolate powder and stored.

3.1 Pathogenic Bacteria

Pathogenic bacteria are those that can cause disease in a human host. Reduction and prevention of these organisms is a concern for the food industry. In this research, selected foodborne pathogenic bacteria were studied to see which could be inhibited by various probiotic bacteria. Twenty-six species of pathogenic bacteria were tested (Table 3.1). Cultures were obtained from the microbial collection of the Food Science Program, University of Missouri-Columbia.

3.1.1. Pathogenic Bacterial Growth Conditions

Stock cultures of the pathogenic bacteria were maintained in Tryptic Soy Broth (TSB), except for *Listeria* species which were stored in Brain Heart Infusion (BHI) broth. Species, including *Salmonella, Shigella*, *Escherichia*, *Yersinia*, *Bacillus and Staphylococcus,* were pipetted in the amount of 200 μL into 10 mL of TSB broth and incubated in a shaking incubator at 35^{o}C for 18 to 22 h. *Listeria* species were added in

the amount of 500 μL per 10 mL of BHI broth and incubated at 35°C for 18 to 22 h in a shaking incubator. Initial growth of the bacteria was followed by two transfers identical to the inoculation volume and growth conditions. All cultures were stored at 4°C.

Table 3.1. Pathogenic bacteria tested.

Pathogenic strain	
Salmonella Typhimurium 14028	*Shigella flexneri* 12022
Salmonella Binza	*Shigella sonnei* 9890
Salmonella Arizona	*Escherichia coli* O157:H7 G5310
Shigella dysentery 29028	*Escherichia coli* O157:H7 3055-93
Salmonella Pullorum	*Escherichia coli* O157:H7 C7927
Salmonella Seftenberg	*Escherichia coli* O157:H7 505 B
Salmonella Tennessee	*Escherichia coli* O157:H7 H2439
Salmonella cholerae	*Escherichia coli* O157:H7 3178-95
Salmonella Berta 18	*Salmonella enteritidis* 13076
Salmonella Rubislaw 21	*Staphylococcus aureus* 12600
Yersinia enterocolitica 35664	*Listeria monocytogenes* 7644
Bacillus cereus	*Listeria monocytogenes* Scott A
Clostridium perfringens 12195	*Clostridium perfringens* UNL

3.2. Probiotic Bacteria

The probiotic bacteria tested for inhibitory activity against the pathogens described above were *Bifidobacterium longum* B6, *Bifidobacterium infantis* 25696, *Lactobacillus rhamnosus* GG, *Lactobacillus paracasei* 25598, and *Lactobacillus bulgaricus* 12.

3.2.1. Probiotic Bacterial Growth Conditions

Stock cultures of the probiotics were maintained in de Man-Rogosa-Sharpe (MRS) (Difco Labs., BD. Diagnostics, Sparks, MI) broth at 4^{o}C. Five hundred microliters of each culture were added to 10 mL MRS broth which was then overlaid with a centimeter of sterile mineral oil. Incubation was at 37^{o}C in an anaerobic incubator for 18 to 22 h. Initial growth of the bacteria was followed by two transfers identical to the inoculation volume and growth conditions.

3.3. Bacterial Enumeration

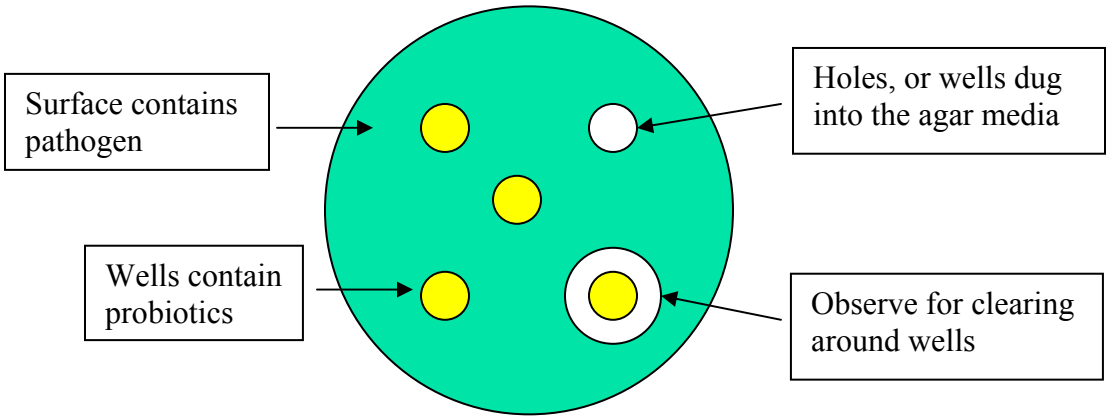
After the growth period had elapsed for the pathogenic cultures, serial dilutions were conducted. Species, except *Listeria*, were plated in duplicate using Tryptic Soy Agar (TSA), while *Listeria* species were plated in duplicate in Brain Heart Infusion (BHI) agar. These plates were incubated at 37^{o}C for 18 to 22 h. Bacterial dilutions of probiotic bacteria were pour-plated in duplicate in MRS agar and incubated anaerobically, in an anaerobic incubator, at 37^{o}C for 18 to 22 h. Average colony forming units (cfu/ml) were calculated for each strain. The optical density (OD) of each bacterial culture was also determined using a dilution factor of 1/10. These were prepared by pipetting 100 μL of probiotic broth culture into 900 μL of MRS broth, while 1000 μL of MRS was used as a control. To the check the OD of the pathogens, 100 uL of broth culture was pipetted into 900 μL of TSB or BHI for *Listeria*. As a control, 1000 μL of TSB or BHI was used. The OD was measured at a wavelength of 600 nm under visible light. (Appendix 1)

3.4. Well Diffusion Assay

3.4.1. Effect of Probiotic Culture

One hundred microliters of each pathogenic bacterium were spread onto TSA or BHI agar, respectively, using a sterile bent glass rod. The pathogenic bacterial culture was allowed 10 minutes to diffuse into the agar media. Next, five wells were dug out of the agar using a number 3 sterile brass corer, with a 5 mm diameter.

Fig 3.1. Well diffusion assay method



To the central well, 100 µL of MRS broth was added as a control. The four outer wells contained 100 µL of two types of probiotic cultures, in duplicate. These organisms were given 10 minutes to allow to diffuse into the media. Plates were incubated at 37^{o}C for 18 to 22 h. The plates were observed for clearing of the pathogenic lawn around the probiotic wells and measurements in cm were taken.

3.4.2. Effect of Probiotic Cell-Free Extracts

The cell-free extracts of *B. longum* B6, *L. paracasei* 25598, *and L. bulgaricus* 12 were tested for inhibitory properties against the pathogens as well. These three strains were chosen based on their performance as the top three probiotics to inhibit the pathogens in the previous well diffusion assay described above.

Two hundred microliters of the pathogens were transferred to TSB broth and incubated at 37^{o}C for 18 to 22 h. The bacterial culture (100 μL) was spread-plated on TSA so as to create a lawn and allowed 10 min to diffuse.

Five hundred microliters of each of these probiotic strains were transferred to 10 mL MRS broth. Cultures were incubated anaerobically for 18 to 22 h at 37^{o}C. The probiotics were then centrifuged at 6000 rpm for 10 min, each resulting supernatant was collected and the bacterial cells were discarded. Using a solution of 2.5 M NaOH, the pH of the supernatant was adjusted to 6.0. The supernatant was then filtered using a syringe and .20 μm nylon filter. One hundred microliters of the supernatant was pipetted into wells dug into TSA agar and allowed 10 min to diffuse into the media.

Plates containing both pathogens and probiotics were incubated at 37^{o}C for 18 to 22 h, zones of inhibition were observed and measurements recorded in cm.

3.5. Probiotic Strains Used for Freeze-Drying

Two probiotic strains, *L. paracasei* (LP) ATCC 25598 and *B. longum* B6 (B6) were chosen for freeze-drying based on results of the well diffusion and cell free extract studies above which showed that the aforementioned strains possessed the greatest

inhibition against pathogenic bacteria. Both strains were grown in MRS broth in an anaerobic incubator at 37°C overnight in an inoculated concentration of 1 mL strain: 10 mL media. MRS agar was used for enumerating *Lactobacillus* and *Bifidobacterium* species.

3.6. Addition of Cryoprotectants

The probiotic strains, B6 and LP, (90 ml) were added to 900 mL soymilk (8th Continent® Premium soymilk, 8th Continent LLC, Minneapolis, MN) and mixed using a magnetic stir bar. The suspension (330 mL) was then poured into sterile 500 mL centrifuge tubes and incubated anaerobically overnight at 37°C. The cells were centrifuged at 8000 rpm for 120 min at 4°C. The resulting supernatant was discarded while 330 mL cryoprotectant solution was added to each bottle containing the cell pellet. The cryoprotective solutions tested were 18% soymilk (none), 18% soymilk + 4% sucrose (SS), 18% soymilk + 4% trehalose (TS), 18% soymilk + 4% sucrose + 4% trehalose (STS). The mixtures were then divided into smaller centrifuge tubes and frozen at –17°C for 24 h.

3.7. Freeze-Drying

Once frozen, the tubes were transferred into freeze dryer cylinders and samples dried using a pilot scale freeze dryer (LabConCo Corp., Kansas City, MO) for approximately 48 h.

3.8. Shelf Life of Freeze-Dried Probiotics

The freeze dried probiotic cultures treated with SS, TS, and STS were stored in the dark at room temperature and sampled on days 0, 11 and 24. On the respective sampling date, 1 g of probiotic was used for serial dilutions and pour-plated in MRS agar. Plates were anaerobically incubated at 37^{o}C for 48 h and numbers were reported as cfu/g. Three replications were statistically analyzed using the SAS program.

3.8. Addition of Probiotic Powder to Isolated Soy Protein

After freeze-drying, the contents of each tube were combined in a stomacher bag and gently mixed by hand. The powder was added to isolated soy protein (ISP) (Pro-Fam® 825, Archer Daniels Midland, Decatur, IL) in a ratio of 4:10 g for B6 and LP fortification. For the combined B6 and LP powder, 2 g of each probiotic was added to 10 g of ISP. This amount of probiotic powder: ISP ratio was determined previously. Varying concentrations showed that 4 g of freeze-dried probiotic to 10 g ISP contained the highest numbers of viable probiotic cells (as shown in Table 3.2).

The amount of sugar to include in the powder was also considered but upon comparison to the commercial product, it was decided not to include any sugar since the commercial product appeared to have no added sweetener.

Table 3.2. Amount (cfu/g) of freeze dried probiotics (B6 and LP), treated with STS, in varying concentrations, in 10 g isolated soy protein powder.

Probiotic concentration (B6 STS)	Count (cfu/g)	Probiotic concentration (LP STS)	Count (cfu/g)
1 g	3.5×10^7	1 g	4.1×10^8
2 g	1.5×10^8	2 g	4.6×10^8
4 g[a]	***3.9 x 10^8***	***4 g***	***1.1 x 10^9***
5 g	1.9×10^8	5 g	1.2×10^9
7 g	2.5×10^8	7 g	1.1×10^9
8 g	3.5×10^8	8 g	9.8×10^8
10 g	3.1×10^8	10 g	1.3×10^9

[a]: concentration contains the highest numbers of viable probiotic cells

Samples were measured into small stomacher bags, placed into vacuum bags (Micro Layered® Vacuum Pouch, Doug Care Equipment, Springville, CA), and vacuum packaged. Bags were covered in aluminum foil and stored at room temperature (RT) or 4°C. The product was prepared for sampling at days 0, 15, 30, 45, 60, 75 and 90. A control sample of just the freeze-dried probiotic (H0) was also prepared. As a second control, isolated soy protein was prepared in 1 g samples. Vacuum packaging and storage conditions were the same as the fortified soy protein.

3.9. Sampling and Counting

On the respective sampling date, 1 g of probiotic/ISP was used for serial dilutions and pour-plated on MRS agar. Viable counts were determined for H0, D0, D15, D30, D45, D60, D75, and D90. Plates were anaerobically incubated at 37^{o}C for 48 h and numbers were reported as cfu/g.

3.10. Statistical Analysis

For the shelf life study of just the probiotic cultures, three replications of the experiment were tested by analysis of variance using the GLM procedures as well as Fisher's Differences of Least Squared Means of SAS (SAS 8.2, 2001). Data was analyzed as a randomized complete block design in which the treatments were arranged as a 2 x 3 x 4 factorial (2 bacterial levels, 3 treatments, 4 times). Data was log transformed.

Analysis of the probiotics added to the isolated soy protein powder involved two replications. Analysis of variance was analyzed by the mixed procedure and Fisher's Differences of Least Squared Means of SAS. Data was analyzed as a randomize complete block design in which the treatments were arranged as a 3 x 2 x 2 x 7 factorial (3 bacterial levels, 2 treatments, 2 temperatures, 7 times).

CHAPTER 4

RESULTS

4.1 Bacterial Enumeration

The optical density (OD) at a wavelength of 600 nm of the probiotics and pathogens after 18-22 h of growth was determined in accordance with the cfu/ml of each culture to enumerate the amount of bacteria of each. A standard curve correlating the OD values during growth with a plate count of these organisms, allowed for the estimation of culture numbers by OD determination. OD values ware subsequently used to estimate the number (cfu/ml) of probiotics or pathogens used in the well diffusion assay.

The numbers of the probiotics when used in the well diffusion assay ranged from 1.5×10^9 to 1.5×10^{10} cfu/ml (Table 4.1). Counts of the pathogens used to create the lawns in the well diffusion assay plates ranged from 1.7×10^7 to 5.3×10^9 cfu/ml. The individual OD readings and counts are listed in Appendix 1.

Table 4.1 Optical density and counts of probiotics after 18-22 h.

Probiotic	OD_{600}	Count (cfu/ml)
Control (MRS broth)	0.15	-
B. longum B6	2.92	4.5×10^9
L. rhamnosus GG	3.45	5.2×10^9
L. bulgaricus 12	3.21	1.5×10^9
L. paracasei ATCC 25598	2.39	1.6×10^9
B. infantis ATCC 25696	1.75	1.5×10^{10}

4.2 Well Diffusion Assay

4.2.1 Effect of Probiotics

The results of the well diffusion tests showed that *Lactobacillus paracasei* (LP), *Lactobacillus bulgaricus* 12 (Lb12) and *Bifidobacterium longum* B6 (B6) inhibited the pathogenic bacteria better than the other probiotics. On average, LP showed a zone of inhibition of 0.92 cm, Lb12 inhibited the pathogens at 0.99 cm and B6 at 0.95 cm. The probiotics Bi and LGG averaged zones of 0.76 cm and 0.78 cm, respectively.

The probiotics performed differently against each pathogen. On average for the total combined probiotic inhibition, *Salmonella* was inhibited at 0.87 cm, *Shigella* was inhibited at 1.02 cm, *E. coli* at 0.84 cm, *Yersinia* at 1.06 cm, *Staphylococcus* at 0.84 cm and *Listeria* at 0.76 cm.

Data for the well diffusion assays of two of the *Listeria* species and both *Clostridium* species were difficult to obtain. The pathogenic lawn that was required to grow was thin and patchy and hard to read. If inhibition was present, it was difficult to see where and how much occurred.

The inhibition of *B. cereus* was not measurable. However, the following qualitative results were observed. For all six probiotics, the pathogen that grew in a circle around the well was seen in a thicker growth around the well that thinned out as it approached the well.

Table 4.2. Average zones of inhibition for probiotic cultures against pathogenic cultures.

	Probiotic				
	LP	Lb12	Bi	BLB6	LGG
Pathogen	Average zone of inhibition(cm)				
Salmonella Typhimurium 14028	.6	.75	.85	.6	.6
S. Enteritidis 13076	1.1	.95	1.1	.75	.55
S. Berta	1.0	.75	1.0	.7	1.0
S. Rubislaw	1.3	1.35	.85	.5	1.35
S. Binza	0	1.15	.6	.65	0
S. Arizonae	.7	.6	.95	.85	.65
S. Pullorum	1.15	1.25	.6	1.9	1.1
S. Seftenberg	.9	1.15	.75	1.3	1.1
S. Tennessee 214	0	1	.4	.95	1.2
S. Cholerae	1.25	1.15	0	1.35	1.05
Shigella dysenteriae 29028	.9	1.45	.6	.7	.65
S. flexneri 12022	1.55	1.05	ND	1.45	1.1
S. sonnei 9890	.8	1.15	.8	1.05	.9
Staphylococcus aureus 12600	.95	.95	.9	.8	.6
Escherichia coli O157:H7 3055-93	.6	.8	ND	1.1	.75
E. coli O157:H7 G5310	1.8	1.1	ND	1.1	.45
E. coli O157:H7 C7927	.6	.8	ND	.6	1.3
E. coli O157:H7 505 B	.85	1.0	ND	.8	1.05

E. coli O157:H7 H2439	.75	.45	ND	.7	0
E. coli O157:H7 3178-95	1.1	1.1	.75	.95	.45
Bacillus cereus	ND	ND	ND	ND	ND
Yersinia entercolitica 35669	.95	.9	1.3	1.15	1.0
Listeria monocytogenes Scott A	ND	ND	ND	ND	.35
L. monocytogenes 7644	1.45	ND	ND	.9	ND
Clostridium perfringens UNL	ND	ND	ND	ND	ND
C. perfringens 12915	ND	ND	ND	ND	ND

4.2.2. Effect of Cell-Free Extracts

While the cell free extracts did not exhibit much inhibition against the pathogens, there was some inhibition. The three probiotics, LP, Lb12 and B6 performed similarly as above. The averages of each are as follows: LP 0.26 cm, Lb12 0.20 cm, and B6 0.35 cm. Based on these results and those above, LP and B6 were selected to be freeze-dried.

Table 4.3. Average zones of inhibition for cell-free extracts against pathogenic cultures.

	Cell-free extract		
	LP	LB12	B6
Pathogen	Average zone of inhibition(cm)		
Salmonella Typhimurium 14028	0	.5	0

S. Enteritidis 13076	1.0	0	.75
S. Berta	0	0	1.3
S. Rubislaw	.85	0	1.25
S. Binza	0	0	0
S. Arizonae	1.1	.75	0
S. Pullorum	0	.4	1.9
S. Seftenberg	ND	1.15	0
S. Tennessee 214	.45	0	0
S. Cholerae	0	0	.9
Shigella dysenteriae 29028	0	0	0
S. flexneri 12022	0	.95	.45
S. sonnei 9890	0	0	0
Staphylococcus aureus 12600	.65	0	0
Escherichia coli O157:H7 3055-93	ND	0	.4
E. coli O157:H7 G5310	0	1.35	.5
E. coli O157:H7 C7927	1.5	0	0
E. coli O157:H7 505 B	1.55	0	1.6
E. coli O157:H7 H2439	ND	0	0
E. coli O157:H7 3178-95	0	0	0
Bacillus cereus	0	0	0
Yersinia entercolitica 35669	0	0	0
Listeria monocytogenes Scott A	0	0	0
L. monocytogenes 7644	0	0	0
Clostridium perfringens UNL	0	0	0
C. perfringens 12915	0	0	0

4.3 Determination of an Optimal Cryoprotectant

The probiotic cultures, LP and B6, which were chosen based on their inhibitory qualities against pathogens, were freeze-dried. Cryoprotectants were added to a bacterial suspension prior to freeze-drying to counteract the harmful effects that dehydration and low temperature have on the cell. A cryoprotectant will protect the organism so that it will survive freeze-drying and retain its beneficial and desired characteristics. Before freeze-drying, a cryoprotectant solution of soymilk and different sugars was applied. Bacterial numbers (cfu/ml) were determined before freeze-drying (0 h) and on days 0, 11, and 24 during storage at room temperature for 24 days.

A significant difference in log cfu/ml of the freeze-dried cultures was found between the Sucrose-Soymilk (SS) and Trehalose-Soymilk (ST), and Trehalose-Soymilk (ST) and Sucrose-Trehalose-Soymilk (STS) treatments, for both probiotic strains tested ($P \leq 0.05$) (Figures 4.1 and 4.2). Treatments SS and STS resulted in higher counts of both strains than ST. There was a significant decrease in cell numbers between before freeze-drying and immediately after freeze-drying on day 0, as well as between days 0 and 11, but no significant decrease between days 11 and 24 were observed for either probiotic in all treatments tested. Between days 0 and 11, B6 and LP were reduced more in treatment SS (1.62 and 2.05 log cfu/ml, respectively) when compared to ST (1.42 and 1.57 log cfu/ml, respectively) and STS (0.71 and 1.46, respectively). However, at the end of 24 days, ST resulted in significantly greater reduction of B6 and LP (2.34 and 2.11 log cfu/ml, respectively).

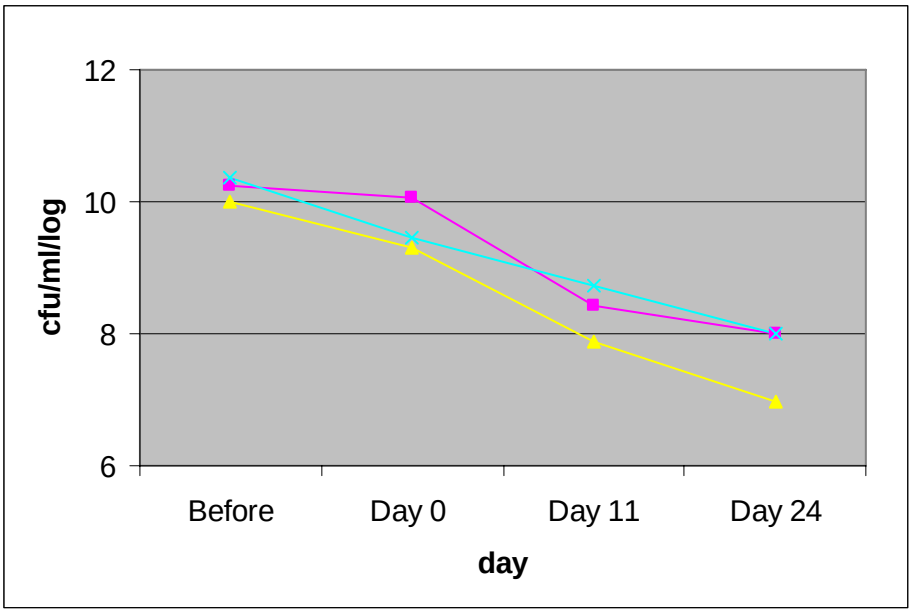


Figure 4.1. Log reduction of *Bifidobacterium longum* B6 (B6) when freeze-dried with Sucrose-Soymilk (■), Trehalose-Soymilk (▲) and Sucrose-Trehalose-Soymilk (x), and stored for 24 days at room temperature.

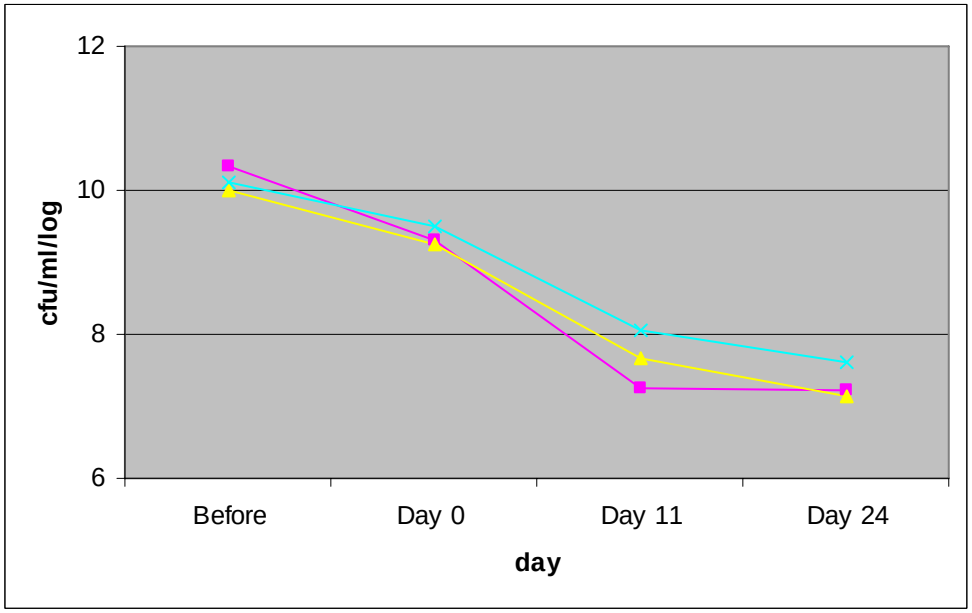


Figure 4.2. Log reduction of *Lactobacillus paracasei* (LP) when freeze-dried with Sucrose-Soymilk (■), Trehalose-Soymilk (▲) and Sucrose-Trehalose-Soymilk (x) and stored for 24 days at room temperature.

Analysis of variance was also determined to see which effects were significant.

Table 4.4. ANOVA table for the statistical significance of effects contributing to the determination of an optimal cryoprotectant.

Effect	**Pr > F**
Bacteria	0.0011
Treatment	0.004
Bacteria*Treatment	0.0185
Time	<.0001
Bacteria*Time	0.0777
Treatment*Time	0.1965
Bacteria*Treatment*Time	0.5006

4.4 Shelf Life of Probiotic Fortified Isolated Soy Protein Powder

Based on the results above, the cryoprotectant solution, STS, was added to B6 and LP, which were then freeze-dried. The freeze-dried bacteria were added to isolated soy protein powder (SPI) which was vacuum sealed and stored in the dark at either room temperature or 4^{o}C. Bacterial numbers (cfu/g) were determined before addition to the SPI (h 0) and after addition to the SPI on days 0, 15, 30, 45, 60, 75 and 90. Figure 3 shows that, except for an increase in numbers on day 75, B6 freeze-dried with soymilk only (SM) and stored at room temperature was consistently reduced more than the same stored at 4^{o}C (6.27 cfu/g and 6.79 cfu/g, respectively). The same results occurred for LP

(SM) stored at room temperature versus those at 4°C (5.2 cfu/g and 7.18 cfu/g, respectively) as bacterial numbers were reduced more overall (Figure 4). For the combined freeze-dried cultures, B6 + LP (SM), growth was again supported better at 4°C (Figure 5). Final counts of bacteria were 5.39 cfu/g and 7.82 cfu/g for room temperature and 4°C.

The cryoprotectant solution, STS, was shown to support growth of all probiotics better at 4°C than at room temperature (Figure 4.6-4.8). Final counts of *B. longum* B6 freeze-dried with STS were 7.55 cfu/g at room temperature and 8.48 cfu/g at 4°C. *L. paracasei* (STS) at 90 days resulted in counts of 6.26 and 8.48 cfu/g. At 90 days, *B. longum* B6 and *L. paracasei* survived better at 4°C when compared to room temperature (7.12 and 8.3 cfu/g).

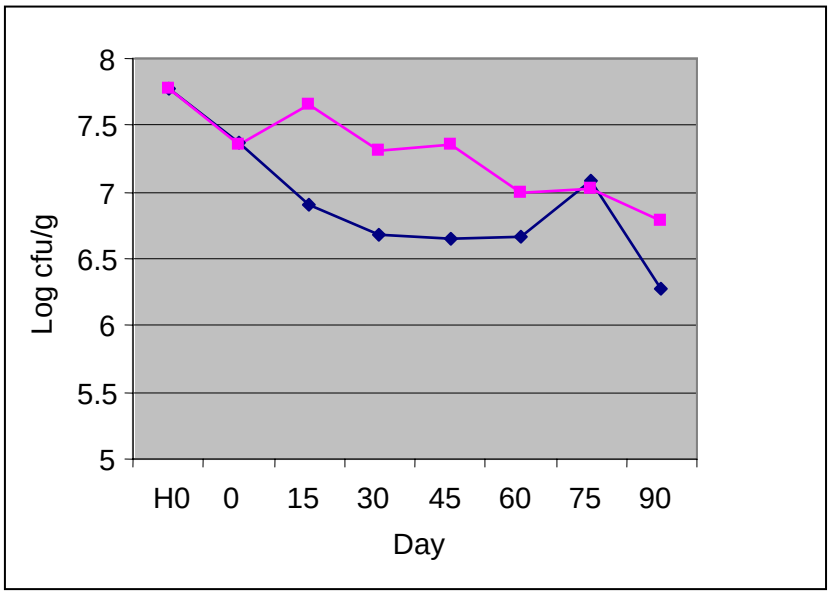


Figure 4.3. Log reduction of B6 when freeze-dried with Soymilk and stored at room temperature (♦) and 4°C (■). Sampling occurred before addition to SPI and after addition on days 0, 15, 30, 45, 60, 75, and 90.

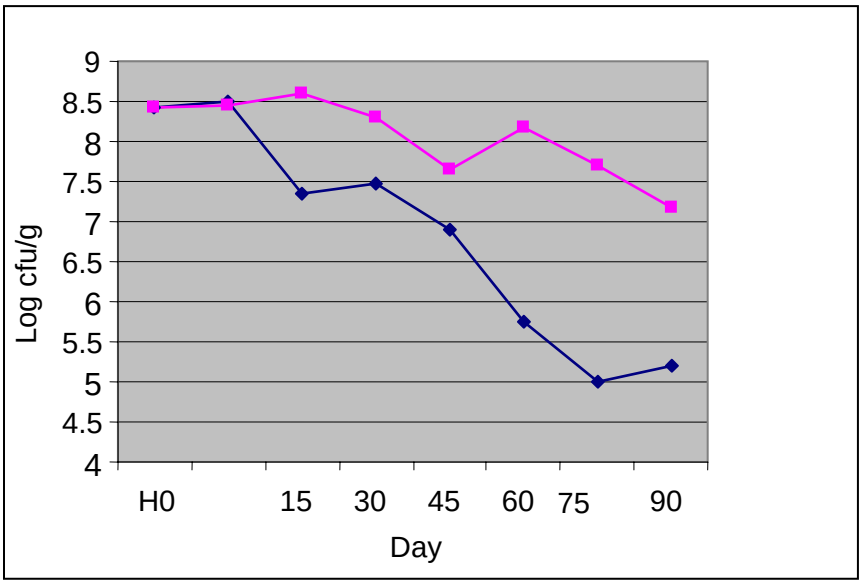


Figure 4.4. Log reduction of LP when freeze-dried with Soymilk and stored at room temperature (♦) and 4°C (■). Sampling occurred before addition to SPI and after addition on days 0, 15, 30, 45, 60, 75, and 90.

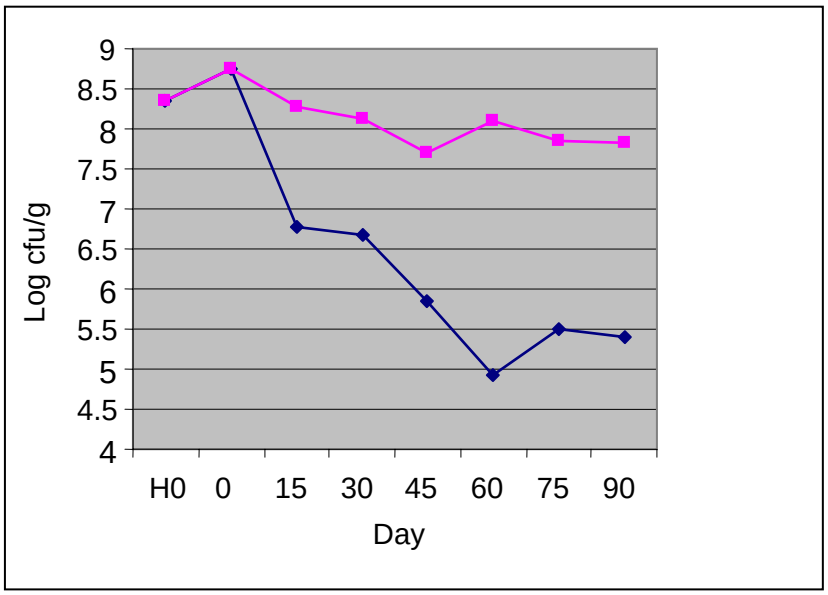


Figure 4.5. Log reduction of B6+LP when freeze-dried with Soymilk and stored at room temperature (♦) and 4°C (■). Sampling occurred before addition to SPI and after addition on days 0, 15, 30, 45, 60, 75, and 90.

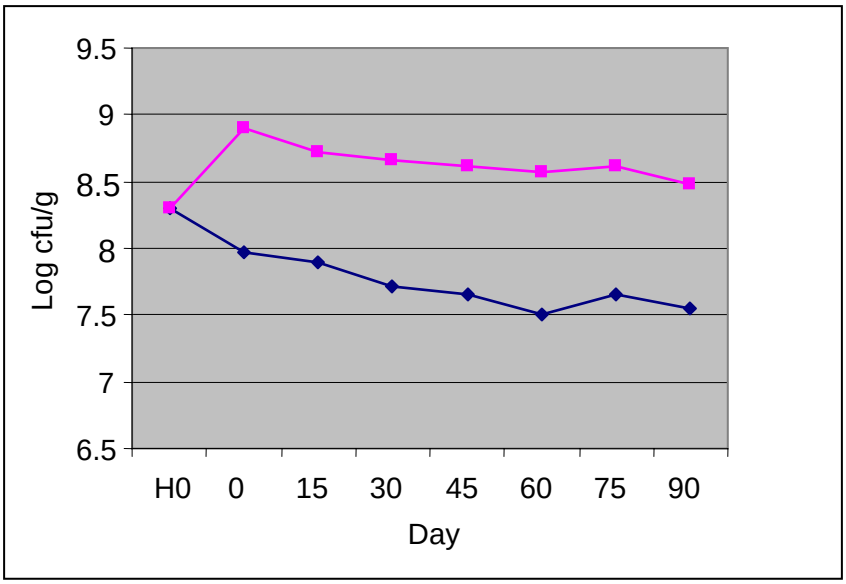


Figure 4.6. Log reduction of *B. longum* B6 when freeze-dried with the cryoprotectant solution, Sucrose-Trehalose-Soymilk, and stored at room temperature (♦) or 4°C (■). Sampling occurred before and after addition to SPI on days 0, 15, 30, 45, 60, 75, and 90.

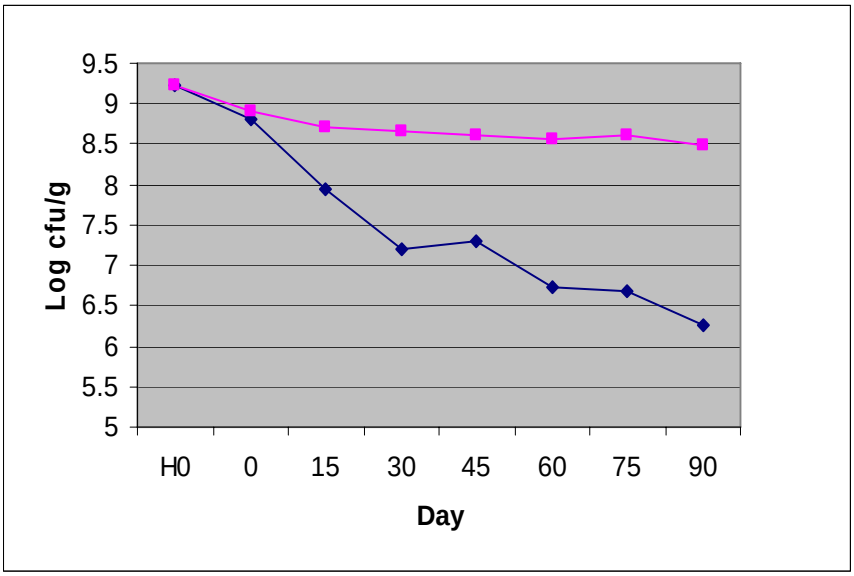


Figure 4.7. Log reduction of *L. paracasei* when freeze-dried with the cryoprotectant solution, Sucrose-Trehalose-Soymilk, and stored at room temperature (♦) or 4°C (■). Sampling occurred before and after addition to SPI on days 0, 15, 30, 45, 60, 75, and 90.

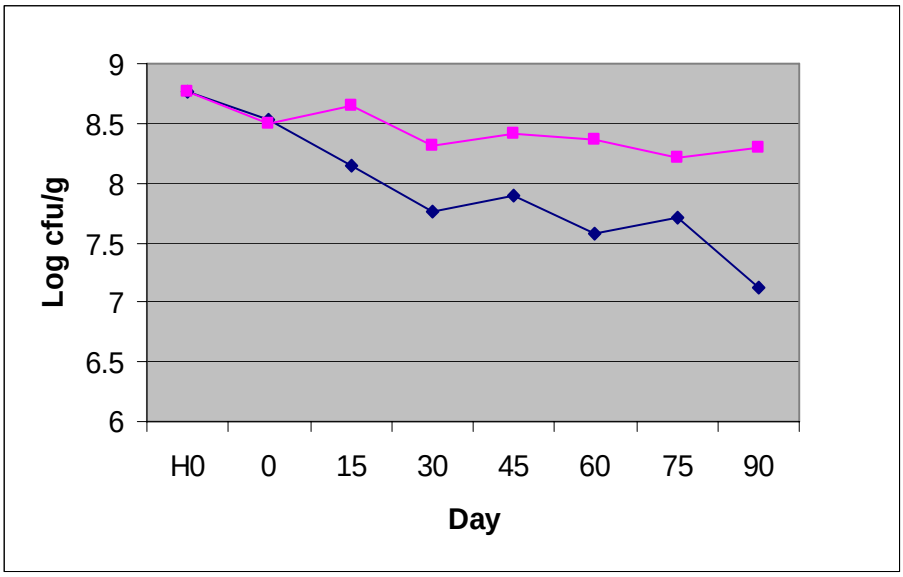


Figure 4.8. Log reduction of *B. longum* B6 and *L. paracasei* when freeze-dried with the cryoprotectant solution, Sucrose-Trehalose-Soymilk, and stored at room temperature (♦) or 4°C (■). Sampling occurred before and after addition to SPI on days 0, 15, 30, 45, 60, 75, and 90.

The bacteria stored at 4°C and treated with STS survived in higher numbers than those stored at 4°C and treated with soymilk. At day 90, *B. longum* B6 in STS survived in the amount of 8.48 cfu/g versus *B. longum* B6 in SM at 6.79 cfu/g. *L. paracasei* showed similar results. A final bacterial count of 8.48 cfu/g for the probiotic treated with STS was higher than that treated with only soymilk (SM) at 7.18 cfu/g. The mixture of B6 and LP was no exception to the others in that STS better supported growth at 8.3 as compared to 7.82 cfu/g by SM (Table 4.5.).

Table 4.5. Bacterial counts, in cfu/g, remaining after reduction.

Bacteria-Treatment-Temperature	Day 0 cfu/g	Day 15 cfu/g	Day 30 cfu/g	Day 45 cfu/g	Day 60 cfu/g	Day 75 cfu/g	Day 90 cfu/g
B6 SM - RT	2.6×10^7	8.5×10^7	4.8×10^6	4.7×10^6	6.4×10^6	3.6×10^7	2.4×10^6
B6 SM - 4°C	2.4×10^7	4.8×10^7	2.1×10^7	2.6×10^7	1.0×10^7	1.1×10^7	6.5×10^6
LP SM - RT	4.1×10^8	2.4×10^7	1.8×10^8	1.1×10^7	5.6×10^5	3.2×10^5	3.3×10^5
LP SM - 4°C	4.1×10^8	4.2×10^8	2.2×10^8	6.3×10^7	1.6×10^8	5.1×10^7	2.5×10^7
B6+LP SM - RT	6.5×10^8	6.0×10^6	4.7×10^6	1.9×10^6	4.9×10^5	3.5×10^5	3.8×10^5
B6+LP SM - 4°C	6.4×10^8	1.9×10^8	1.5×10^8	1.9×10^8	1.8×10^8	6.9×10^7	6.9×10^7
B6 STS – RT	1.0×10^8	7.8×10^7	5.1×10^7	4.6×10^7	3.6×10^7	4.5×10^7	3.7×10^7
B6 STS - 4°C	1.7×10^8	1.1×10^8	1.3×10^8	1.5×10^8	7.5×10^7	6.7×10^7	8.9×10^7
LP STS – RT	9.3×10^8	3.2×10^8	1.3×10^8	7.4×10^7	2.3×10^7	3.3×10^7	3.4×10^7
LP STS - 4°C	$9. \times 10^8$	8.4×10^8	4.6×10^8	4.6×10^8	4.6×10^8	4.6×10^8	3.9×10^8
B6+LP STS –RT	4.3×10^8	1.7×10^8	6.7×10^7	9.5×10^7	5.5×10^7	5.1×10^7	2.3×10^7
B6+LP STS - 4°C	2.7×10^9	4.8×10^9	2.5×10^8	2.6×10^8	2.8×10^8	2.1×10^8	2.0×10^8

In order to create an even baseline of the bacterial counts for statistical analysis, original numbers were set at 100% and subsequent reduction was calculated in percentage as well (Appendix 2). Use of the mixed procedure in SAS to determine analysis of variance of the percentages showed significant differences for bacteria, treatment*bacteria, and time ($P \leq 0.05$). There was no significance found for other interactions of two, three and four factors. Analysis of variance can be seen in Table 4.6. Therefore, further examination of the treatment*bacteria interaction was conducted and can be seen in Table 4.7.

Table 4.6. ANOVA table for the statistical significance of effects contributing to the shelf life of probiotic fortified isolated soy protein powder.

Effect	**Pr > F**
Bacteria	0.0032
Treatment	0.7146
Treatment*Bacteria	0.0418
Temperature	0.9198
Temperature*Bacteria	0.8507
Treatment*Temperature	0.9463
Treatment*Temperature*Bacteria	0.3486
Time	<.0001
Time*Bacteria	0.7990
Treatment*Time	0.8627
Treatment*Time*Bacteria	0.6564
Time*Temperature	0.6271

Time*Temperature*Bacteria	0.7753
Treatment*Time*Temperature	0.4210
Treatment*Time*Temperature*Bacteria	0.7171

Table 4.7. Statistical significance of a bacteria*treatment interaction.

	Least squared means		
Treatment	B6	B6+LP	LP
STS	62.92^{Aa}	58.40^{Aa}	40.65^{Aa}
SM	83.36^{Aa}	27.18^{Ab}	46.47^{Ab}

Means in the same column followed by different uppercase letters are significantly different at $P \leq 0.05$.
Means in the same row followed by different lowercase letters are significantly different.

Treatment by itself was not significant but an interaction of bacteria and treatment was. B6 STS was not significantly different from B6+LP STS and LP STS. B6 SM was significantly different from B6+LP SM and LP SM.

Analysis of variance did not find significance for a bacteria*temperature interaction but examination of the differences of the least squared means showed some significant differences. B6 at room temperature was significantly different from B6+LP at room temperature. A significant difference was also found for B6 at room temperature and LP at 4°C. B6 at 4°C was significantly different than B6+LP at room temperature and 4°C. A significant difference was also found between B6 at 4°C for LP at room temperature and 4°C.

Temperature was not significant and further analysis of the least squared means showed no significant differences between a treatment*temperature interaction.

An interaction of bacteria*treatment*temperature was not statistically significant but B6 STS room temperature was significantly different from B6+LP SM at room temperature. B6 STS at 4°C was significantly different than B6+LP SM at room temperature. B6 SM at room temperature was also significantly different from B6+LP SM at room temperature and B6+LP SM at 4°C, and LP SM STS at room temperature and LP SM at 4°C. Additionally, B6 SM at 4°C was significantly different from B6+LP SM at room temperature and B6+LP SM at 4°C. A significant difference was also found between B6 SM 4°C and LP STS at room temperature and LP STS 4°C. B6 SM at 4°C was significantly different than LP SM at 4°C. B6+LP STS at room temperature was significantly different than B6+LP none at room temperature. Comparisons of other least squared means are in Appendix 3.

Figures 4.9-4.12 show the percent reduction of B6, LP, and B6+LP freeze-dried with soymilk or sucrose-trehalose-soymilk solutions. Overall, there was a greater percent reduction of bacteria freeze dried with soymilk and stored at room temperature (RT) or 4°C (4).

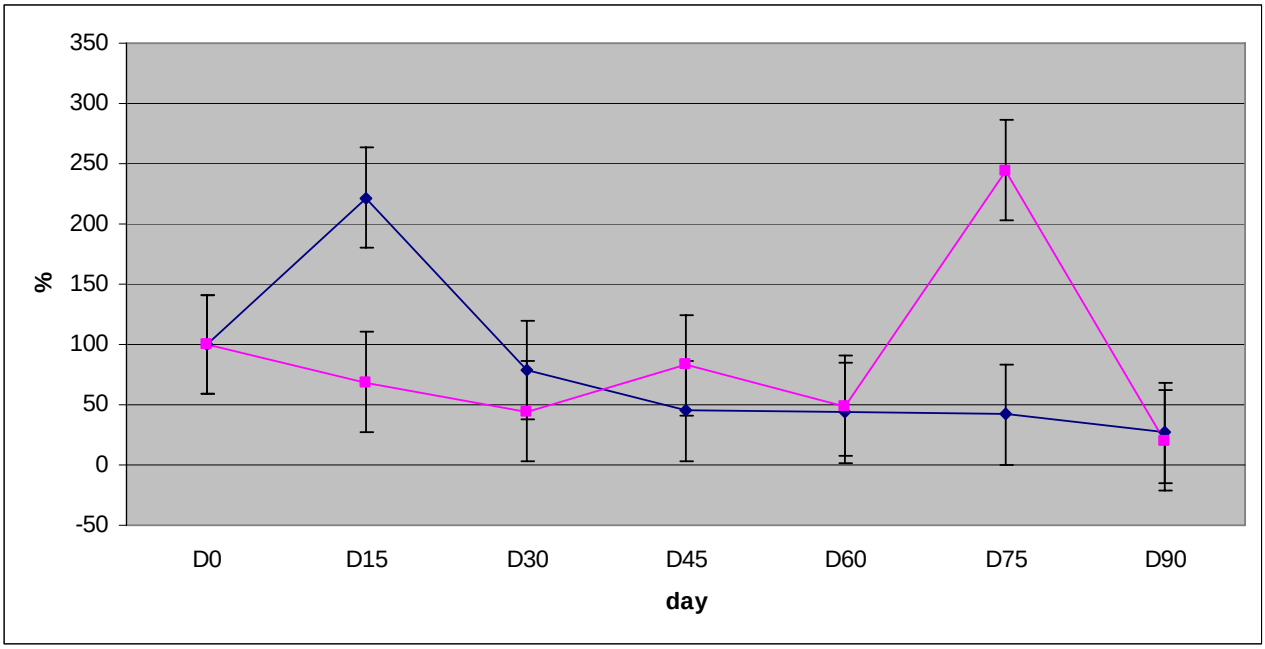


Figure 4.9. Percent reduction of B6 when freeze-dried with Soymilk and stored at room temperature (♦) and 4°C (■). Sampling occurred before addition to SPI and after addition on days 0, 15, 30, 45, 60, 75, and 90.

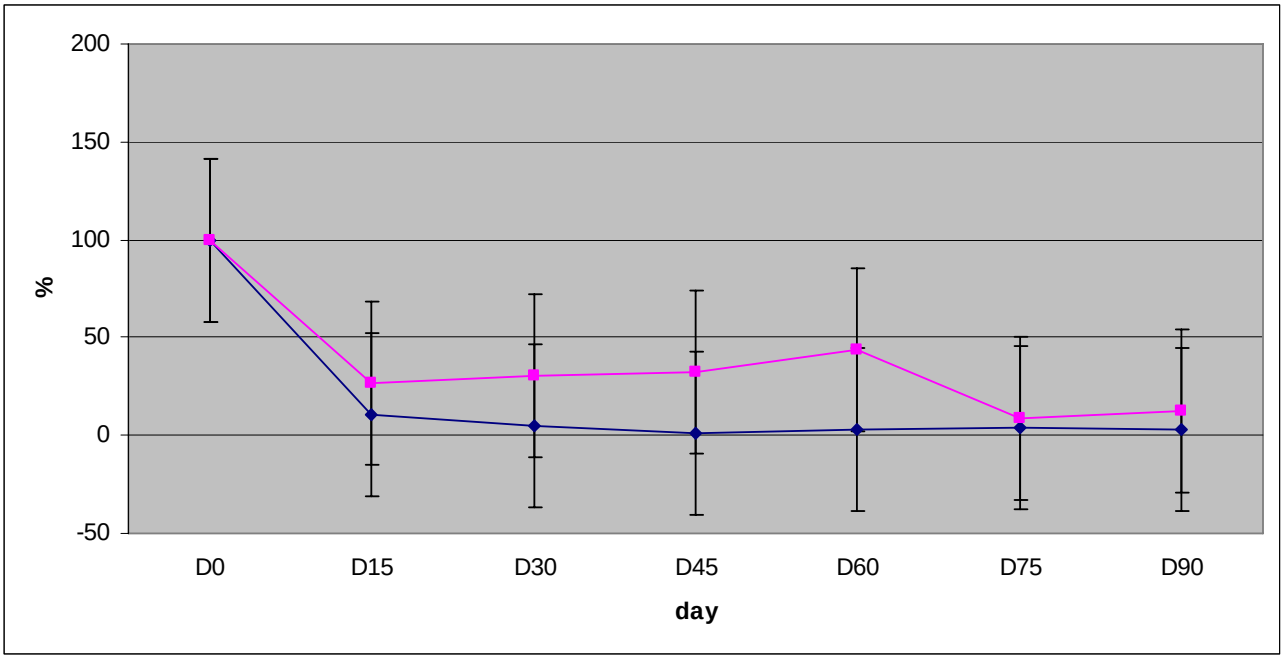


Figure 4.10. Percent reduction of LP when freeze-dried with Soymilk and stored at room temperature (♦) and 4°C (■). Sampling occurred before addition to SPI and after addition on days 0, 15, 30, 45, 60, 75, and 90.

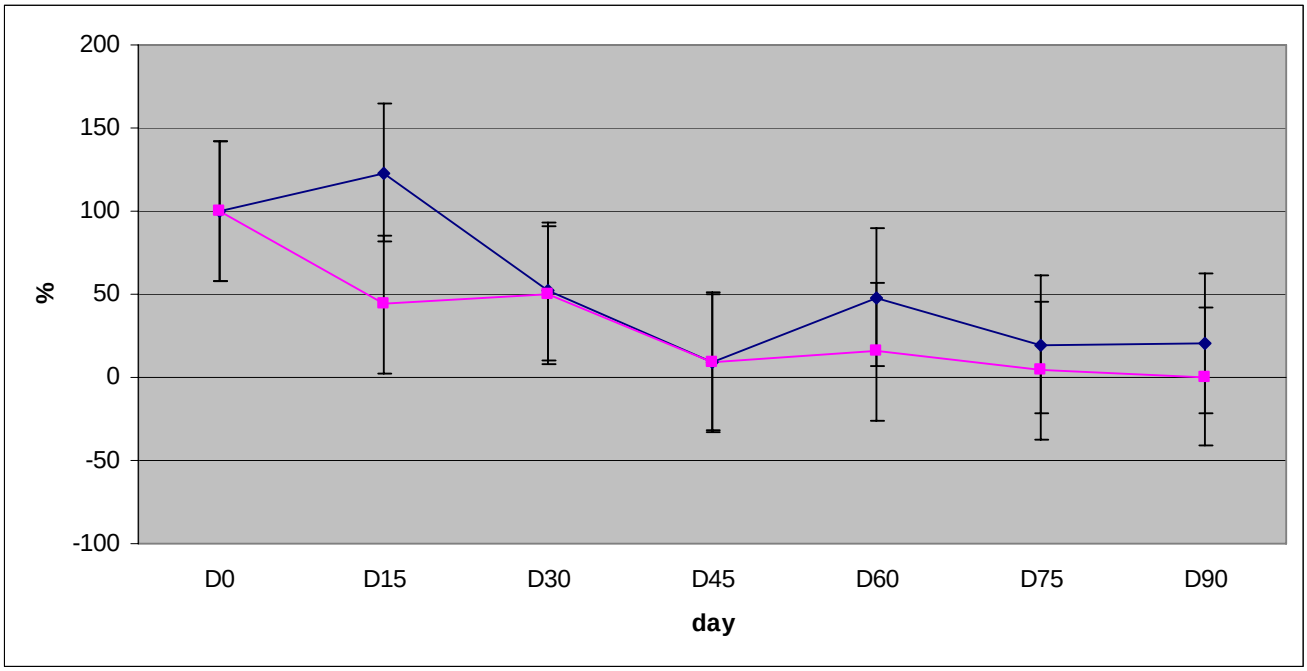


Figure 4.11. Percent reduction of B6+LP when freeze-dried with Soymilk and stored at room temperature (♦) and 4°C (■). Sampling occurred before addition to SPI and after addition on days 0, 15, 30, 45, 60, 75, and 90.

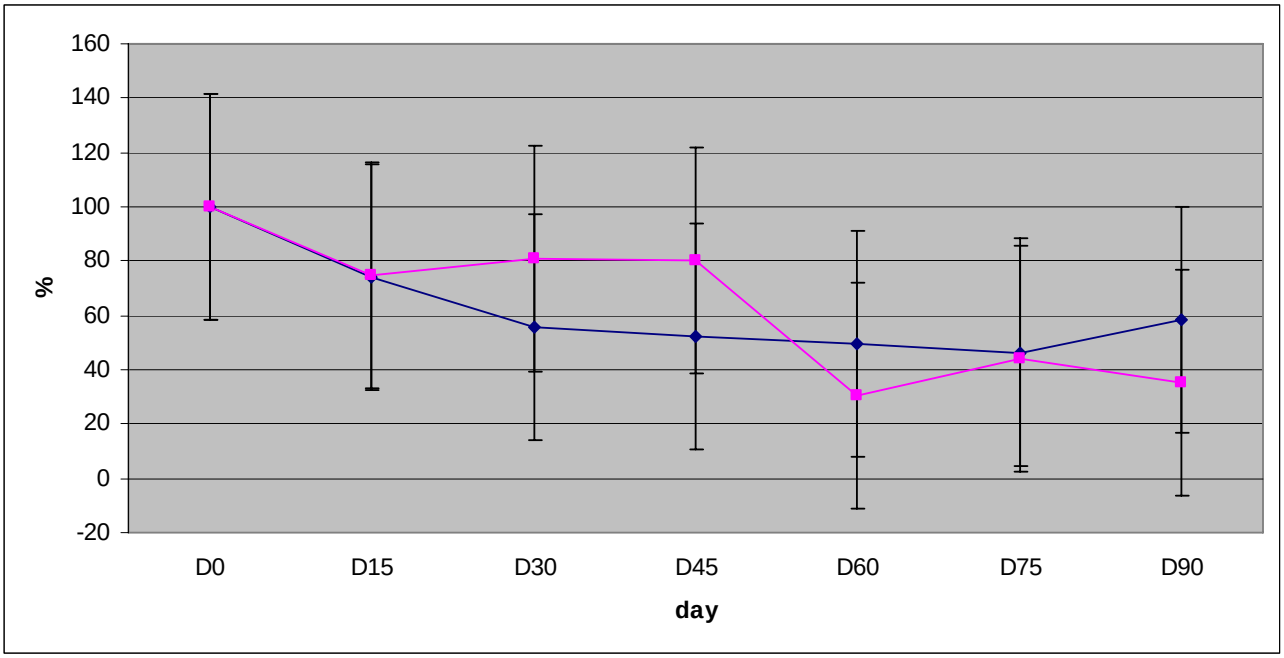


Figure 4.12. Percent reduction of B6 when freeze-dried with the cryoprotectant solution, Sucrose-Trehalose-Soymilk, and stored at room temperature (♦) or 4°C (■). Sampling occurred before and after addition to SPI on days 0, 15, 30, 45, 60, 75, and 90.

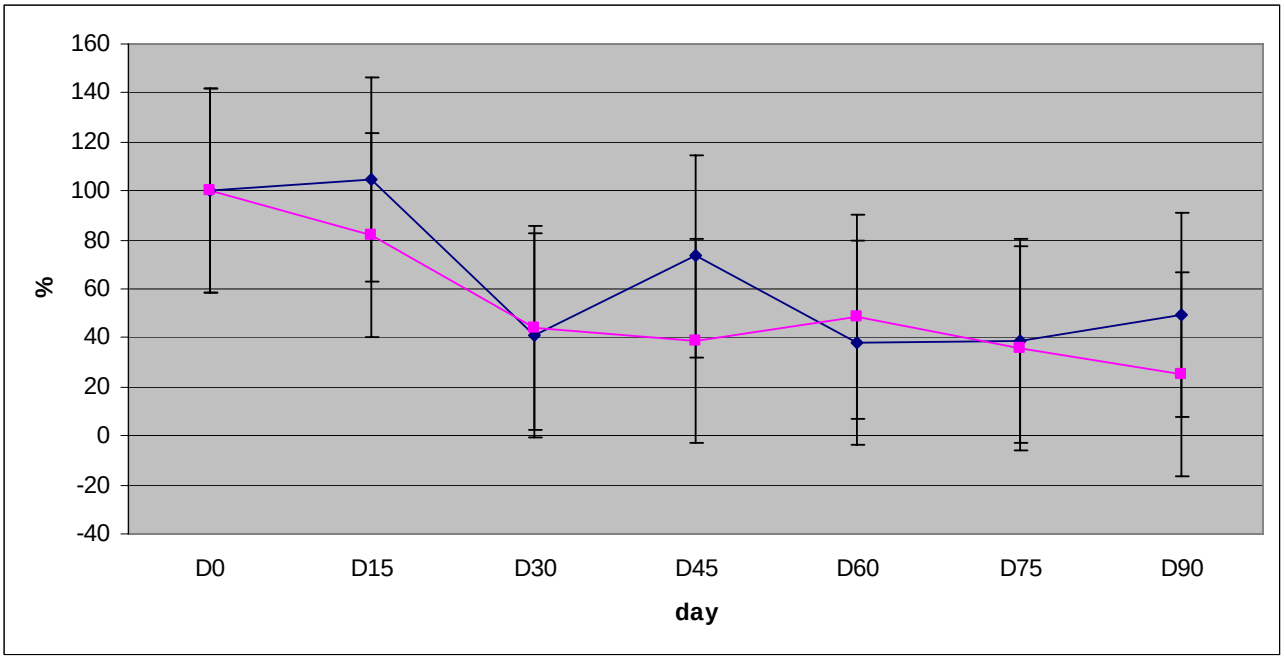


Figure 4.13. Percent reduction of LP when freeze-dried with the cryoprotectant solution, Sucrose-Trehalose-Soymilk, and stored at room temperature (♦) or 4°C (■). Sampling occurred before and after addition to SPI on days 0, 15, 30, 45, 60, 75, and 90.

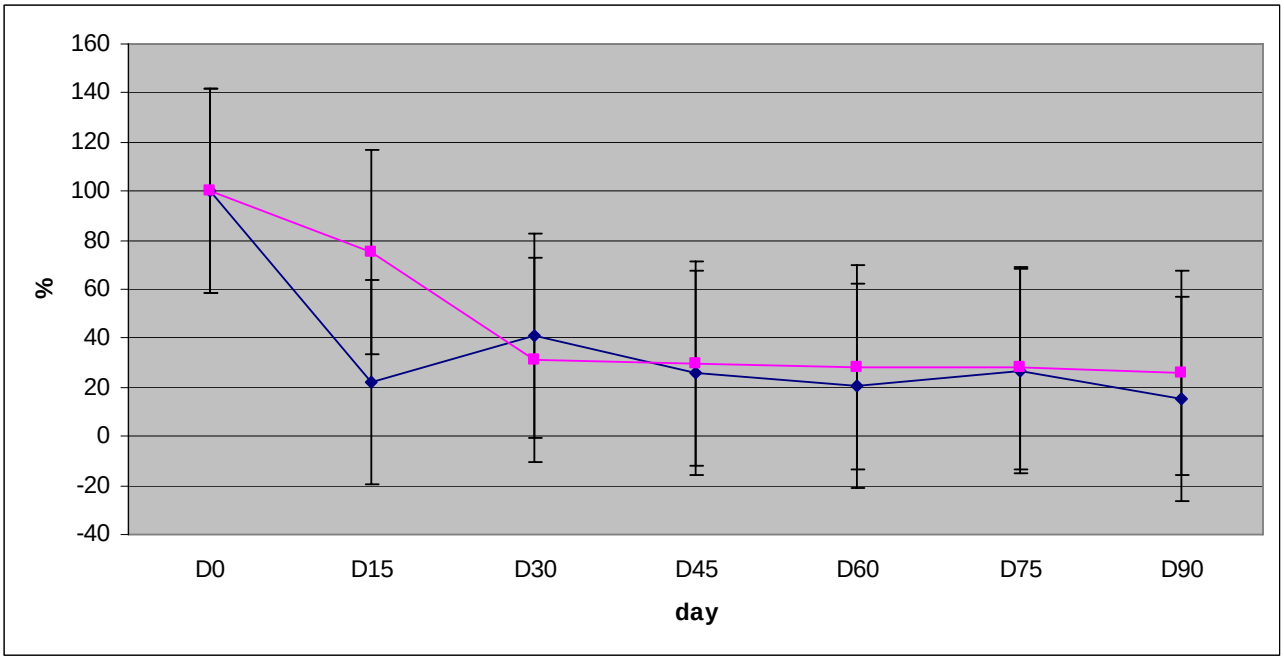


Figure 4.14. Percent reduction of B6+LP when freeze-dried with the cryoprotectant solution, Sucrose-Trehalose-Soymilk, and stored at room temperature (♦) or 4°C (■). Sampling occurred before and after addition to SPI on days 0, 15, 30, 45, 60, 75, and 90.

CHAPTER 5
DISCUSSION

5.1. Bacterial Enumeration

It was important to have bacterial numbers in sufficient amounts so as to create a thick pathogenic lawn for the well diffusion assay. A thick lawn provided a backdrop so that inhibition by probiotics could be clearly distinguished. All the probiotic organisms tested grew similarly in thick lawns. The numbers of the probiotic bacteria ranged from 1.5 x 10^9 to 1.5 x 10^{10} cfu/ml which was sufficient to inhibit growth of pathogens in the well diffusion assay.

5.1.1 Effect of Probiotics

The way probiotics inhibit pathogens and other bacteria is by the production of lactic acids. Probiotics, including *Lactobacillus* (Beck, 1961; Trammer and Feresu, 1966) and *Bifidobacterium* (Gibson and Wang, 1994), are known to have inhibitory effects against intestinal pathogens. Short chain fatty acids, such as acetic and lactic acids, which are produced by lactic acid bacteria are considered to be responsible for their antimicrobial activity against *Escherichia coli* in the intestine (Sinha, 1986). In the case of Shiga toxin-producing *E. coli* O157:H7, the bactericidal effect of *Lactobacillus* on the organism depends on its lactic acid production and pH reductive effect (Ogawa et al., 2001). Lactic acid produced by *Lactobacillus* was also shown to inhibit *Salmonella* Typhimurium as well as to halt an invasion into intestinal cells (Makras et al., 2006).

Bifidobacteria have been shown to inhibit gram-negative pathogens by a number of ways. These include a decrease in the local pH by the production of organic acids, the inhibitory action of undissociated organic acid molecules, competition for nutrients, competition for adhesion

sites, stimulation of the host's immunity, and production of specific antibacterial substances (Ballongue, 1998; De Vuyst et al., 2004; Fuller, 1989). Makras and De Vuyst (2006) found that bifidobacteria inhibited *Salmonella* Typhimurium SL1344 and *E. coli* C1845. The inhibitory mechanism was shown to be dependent on lowering of the pH of the medium and the production of acetic and lactic acids. These aforementioned modes of action for inhibition are probably responsible in this study.

5.2. Well Diffusion Assay

A well diffusion assay tested 100 species of lactic acid bacteria against pathogens including *Salmonella* Enteritidis, *Salmonella* Typhimurium, *E. coli*, *Listeria monocytogenes* and *Bacillus cereus* (Ham et al., 2003). None of the strains demonstrated activity against *S.* Enteritidis, whereas 27 strains showed activity against *S.* Typhimurium, six against *E. coli*, 31 against *L. monocytogenes* and 10 against *B. cereus.* These results are similar to this study, however *S.* Enteritidis was inhibited by all of the probiotics and it was difficult to see any inhibition with *Listeria*.

5.2.2. Effect of Cell-Free Extracts

Makras and De Vuyst (2006) examined the cell-free culture supernatants of *Bifidobacterium*. All strains produced acetic and lactic acids, while four of the strains tested produced formic acid as well. The cell-free extracts of seven *Bifidobacterium* strains inhibited the growth of *Lactobacillus*, indicating production of a bacteriocin. The cell-free extract of *B. longum* BB536 was also active against *E. coli*. When the pH of the cell free extracts or the

control medium was adjusted to pH 6.5, no killing effect was observed, suggesting that the antibacterial activity of the bifidobacterial cultures is pH-dependent.

The supernatants of *Lactobacillus* species isolated from cheese were shown to inhibit *Clostridium* species in well diffusion assays (Christiansen, et al., 2005). Contrary to that finding, our study showed no inhibition of *Clostridium* by *Lactobacillus* cell-free extract.

5.3. Determination of an Optimal Cryoprotectant

The results show that the best cryoprotectants were Soymilk-Sucrose (SS) or Soymilk-Trehalose-Sucrose (STS), while Soymilk-Trehalose (ST) showed a reduced number of bacteria at 24 days. These results are in concordance with those of Zayed and Roos (2004) which showed that a solution of sucrose, trehalose and skim milk best supported the survival of *Lactobacillus salivarius*. It was also shown that a solution of just sucrose and trehalose protected the cells. However, trehalose and skim milk performed better than sucrose and skim milk, which was opposite of the results of the sugars and soymilk solutions.

The use of soymilk as a cryoprotectant has not been widely investigated. Studies have been conducted to determine the action of skim milk, and how and why it protects. Perhaps the proteins form a protective coating on the cell wall proteins and, along with calcium, increase cell survival after freezing or freeze-drying, as with skim milk (King and Su, 1993; Carvalho et al., 2004). Skim milk solids prevent cellular injury by stabilizing cell membranes (Castro et al., 1995). When a cryoprotectant solution is considered, the amount of phosphates is important to stabilize the pH (Modler and Villa-Garcia, 1993). Soymilk contains such solutes as calcium phosphate and dipotassium phosphate. As noted by Zayed and Roos (2004), the addition of skim

milk in a solution resulted in easier drying and more end products. It is possible that soymilk behaves in a way similar to skim milk and therefore makes it a good cryoprotectant.

Trehalose and sucrose are good cryoprotectants because they can protect membranes and proteins in intact bacteria. Leslie et al. (1995) showed that *E. coli* DH5α and *Bacillus thuringiensis* HD-1 were increasingly tolerant to freeze-drying when treated with these two sugars. The sugars have the ability to lower the temperature of the membrane phase transition and maintain protein structure in the dry state. For these two organisms, and perhaps for the probiotic organisms, the addition of the two sugars lessened the breakdown of protein stability that occurs during freeze-drying (Leslie et al., 1995). In order to protect cytosolic proteins, the sugars must access the inside of the cells. Trehalose can flow down a concentration gradient into the cells when they are cooled (Leslie et al., 1995). Sucrose also enters the cells as they enter into a phase transition (Leslie, et al., 1995). It also has the ability to prevent injurious freezing of cell fluids by trapping salts in a highly viscous or glass-like phase (Hubalek, 2003). Drying with sucrose and trehalose prevents the damaging effects of freeze-drying by maintaining the membrane lipids in a liquid crystalline phase when stored at room temperature (Crowe et al., 1988).

5.4. Shelf Life of Probiotic Fortified Isolated Soy Protein Powder

Castro et al. (1995) published that in order to prevent or reduce lipid oxidation of the cell membrane during storage, dried powders should be stored under vacuum. This may have helped to contribute to the relatively high numbers of surviving bacteria at the end of 90 days.

Another contributing factor appears to be the storage temperature. Low temperatures have been shown to protect dry probiotic powders. King and Su (1993), Champagne et al., (1996) and Wang et al., (2004) noted that powdered *Lactobacillus* and *Bifidobacterium* preparations survived better at refrigerated temperatures compared with room temperature. Similarly, the *Lactobacillus* and *Bifidobacterium* investigated in this study maintained survival consistently better at 4^{o}C. A higher percent of survival was also noted for *S. thermophilus* and *B. longum* when a dried fermented soymilk was stored at 4°C than 25°C after 4 months of storage (Wang et al., 2006). A mixture of the probiotics, *L. acidophilus* 33200, *L. casei* 279, *B. longum* 536 and *L. rhamnosus* GG, in freeze dried yogurt was shown to maintain a higher cfu/g at 4^{o}C over six months compared to storage at 21^{o}C and 37^{o}C (Capela et al., 2006).

Differences in the survival rates between the two probiotic species may be attributed to differences in cell wall and membrane composition (Carvalho et al., 2004).

CONCLUSIONS AND FURTHER DIRECTIONS

Treatment, bacteria and time were all significant factors in the survival of freeze dried probiotic bacteria added to isolated soy protein. Based on bacterial numbers, in percent and actual counts, these bacteria would best survive in isolated soy protein after being freeze dried with sucrose-trehalose-soymilk and stored at 4^{o}. At the end of 90 days, numbers were sufficient to possibly colonize and exert anti-pathogenic activity.

Further directions could include, but are not limited to, sensory studies after the addition of flavors and testing the survival of these bacteria inside a simulated colon and subsequent animal models.

APPENDIX 1

Optical density of pathogens when used in well diffusion assay, after 18-22 h growth. - Chapter 4

Pathogen	OD_{600}	Count (cfu/ml)	Pathogen	OD_{600}	Count (cfu/ml)
Control (TSB broth)	-.152		Control (BHI broth)	-.172	
Salmonella Typhimurium 14028	.410	1.56×10^9	*Shigella flexneri* 12022	.5	1.1×10^8
Salmonella Binza	.846	1.33×10^9	*Shigella sonnei* 9890	.757	1.8×10^8
Salmonella Arizona	.425	1.68×10^9	*Escherichia coli* O157:H7 G5310	.979	2×10^9
Shigella dysentery 29028	.425	5.4×10^8	*Escherichia coli* O157:H7 3055-93	1.424	1.09×10^9
Salmonella Pullorum	.395	2.3×10^7	*Escherichia coli* O157:H7 C7927	1.022	3.8×10^8
Salmonella Seftenberg	.417	1.2×10^9	*Escherichia coli* O157:H7 505 B	5.417	4.2×10^9
Salmonella Tennessee 214	.455	8.9×10^8	*Escherichia coli* O157:H7 H2439	1.117	3.1×10^9
Salmonella	.373	1.53×10^9	*Escherichia*	.584	1.27×10^9

cholerae			*coli* O157:H7 3178-95		
Salmonella Berta 18	1.276	5.3×10^9	*Salmonella enteritidis* 13076	1.356	3.66×10^9
Salmonella Rubislaw 21	.291	1.1×10^9	*Staphylococcus aureus* 12600	.979	2.0×10^9
Yersinia entercolitica 35664	1.751	3.3×10^9	*Listeria monocytogenes* 7644	1.020	4.68×10^8
Bacillus cereus	1.986	4.2×10^9	*Listeria monocytogenes* Scott A	.74	1.65×10^7
Clostridium perfringens 12195	1.609	2.6×10^9	*Clostridium perfringens* UNL	1.405	2.1×10^9

APPENDIX 2

Bacterial counts, in percent, remaining after reduction - Chapter 4

Bacteria-Treatment-Temperature	Day 0 %	Day 15 %	Day 30 %	Day 45 %	Day 60 %	Day 75 %	Day 90 %
B6 SM - RT	100^{Aa}	221.53^{Aa}	78.82^{Aa}	45.21^{Aa}	43.2^{Aa}	42.12^{Aa}	27.2^{Aa}
B6 SM - 4^{o}	100^{Aa}	68.05^{Ba}	44.37^{Aa}	82.91^{Aa}	48.76^{Aa}	244.2^{Aa}	20.09^{Aa}
LP SM - RT	100^{Aa}	122.91^{Aa}	51.79^{Aa}	8.82^{Aa}	47.95^{Aa}	19.64^{Aa}	20.52^{Aa}
LP SM - 4^{o}	100^{Aa}	43.9^{Aa}	49.54^{Aa}	9.24^{Aa}	15.75^{Aa}	4.25^{Aa}	$.37^{Aa}$
B6+LP SM - RT	100^{Aa}	10.6^{Aa}	4.9^{Aa}	1.56^{Aa}	2.9^{Ba}	4.22^{Aa}	2.61^{Aa}
B6+LP SM - 4^{o}	100^{Aa}	26.47^{Aa}	30.67^{Aa}	32.17^{Aa}	43.58^{Aa}	8.47^{Aa}	12.59^{Aa}
B6 STS RT	100^{Aa}	73.94^{Aa}	55.8^{Aa}	52.01^{Aa}	49.56^{Aa}	46.56^{Aa}	58.35^{Aa}
B6 STS 4^{o}	100^{Aa}	74.9^{Aa}	80.86^{Aa}	80.45^{Aa}	30.31^{Aa}	44.01^{Aa}	35^{Aa}
LP STS RT	100^{Aa}	21.82^{Aa}	40.82^{Aa}	25.94^{Aa}	20.78^{Aa}	26.7^{Aa}	15.23^{Aa}
LP STS 4^{o}	100^{Aa}	75.09^{Aa}	31.48^{Aa}	29.9^{Aa}	29.1^{Aa}	27.05^{Aa}	26.22^{Aa}
B6+LP STS RT	100^{Aa}	104.62^{Aa}	41.18^{Aa}	73.3^{Aa}	37.71^{Aa}	38.58^{Aa}	49.07^{Aa}
B6+LP STS 4^{o}	100^{Aa}	81.78^{Aa}	43.82^{Aa}	39.02^{Aa}	48.84^{Aa}	35.72^{Aa}	25.14^{Aa}

Means in the same column followed by different uppercase letters are significantly different at $P \leq 0.05$.

Means in the same row followed by different lowercase letters are significantly different.

APPENDIX 3

Comparison of Least Squared Means for a Variety of Interactions

Comparison of Least Squared Means for time - Chapter 4

Sampling Time	Least Squared Means
D0	100^{A}
D15	77.13^{A}
D30	46.17^{BC}
D45	39.98^{BC}
D60	34.80^{BC}
D75	45.11^{BC}
D90	24.37^{BC}

Means in the column followed by different uppercase letters are significantly different at $P \leq 0.05$.

Comparison of Least Squared Means for a bacteria-time interaction-Chapter 4

	Least squared means		
Sampling Time	B6	B6+LP	LP
D0	100^{Aa}	100^{Aa}	100^{Aa}
D15	109.6^{Aa}	55.86^{ACb}	65.93^{ACab}
D30	64.96^{ACa}	30.14^{BCa}	43.41^{BCa}
D45	65.14^{ADa}	36.31^{BCa}	18.47^{BCa}
D60	42.95^{BDEa}	33.28^{BCa}	28.14^{BCa}
D75	94.19^{ADEFa}	21.75^{BCb}	19.41^{BCb}
D90	35.16^{BCDEGa}	22.36^{BCa}	15.58^{BCa}

Means in the same column followed by different uppercase letters are significantly different at $P \leq 0.05$.

Means in the same row followed by different lowercase letters are significantly different.

Comparison of Least Squared Means for a treatment-time interaction - Chapter 4

	Least Squared Means	
Sampling time	STS	None
D0	100^{Aa}	100^{Aa}
D15	72.02^{ACa}	82.24^{ACa}
D30	48.99^{BCa}	43.35^{BCa}
D45	50.10^{BCa}	29.85^{BDa}
D60	35.88^{BCa}	33.71^{BDa}
D75	36.42^{BCa}	53.81^{BCa}
D90	34.83^{BCa}	13.90^{BDa}

Means in the same column followed by different uppercase letters are significantly different at $P \leq 0.05$.

Means in the same row followed by different lowercase letters are significantly different.

Comparison of a bacteria-treatment-time interaction - Chapter 4

Least Squared Means						
Sampling time	B6 STS	B6 SM	B6+LP STS	B6+LP SM	LP STS	LP SM
D0	100^{Aa}	100^{Aa}	100^{Aa}	100^{Aa}	100^{Aa}	100^{Aa}
D15	74.42^{Aa}	144.79^{Aa}	93.20^{Aa}	18.53^{Bab}	48.5^{Aab}	83.4^{Aa}
D30	68.33^{Aa}	61.59^{ACa}	42.5^{Aa}	17.78^{Ba}	36.15^{Aa}	50.67^{Aa}
D45	66.23^{Aa}	64.06^{ACa}	56.16^{Aa}	16.42^{Ba}	27.92^{Aa}	9.03^{Ba}
D60	39.93^{Aa}	45.98^{ACa}	43.27^{Aa}	23.29^{Ba}	24.44^{Ba}	31.85^{Aa}
D75	45.23^{Aa}	143.16^{ADEFb}	37.15^{Aac}	6.34^{Bac}	26.87^{Aac}	11.94^{Bac}
D90	46.67^{Aa}	23.64^{BCFGa}	37.10^{Aa}	7.63^{Ba}	20.72^{Ba}	10.44^{Ba}

Means in the same column followed by different uppercase letters are significantly different at $P \leq 0.05$.

Means in the same row followed by different lowercase letters are significantly different.

Temp-time interaction - Chapter 4

	Least Squared Means	
Sampling Time	Room Temperature	4 °C
D0	100^{Aa}	100^{Aa}
D15	92.57^{Aa}	61.70^{Aa}
D30	45.55^{Ba}	46.79^{BCa}
D45	34.34^{Ba}	45.61^{BCa}
D60	33.70^{Ba}	35.89^{BCa}
D75	29.62^{Ba}	60.61^{ACa}
D90	28.84^{Ba}	19.90^{BCa}

Means in the same column followed by different uppercase letters are significantly different at $P \leq 0.05$.

Means in the same row followed by different lowercase letters are significantly different.

Bacteria-Temperature-Time Interaction - Chapter 4

Least Squared Means						
Sampling Time	B6 Room Temp	B6 4 °C	B6+LP Room Temp	B6+LP 4 °C	LP Room Temp	LP 4 °C
D0	100^{Aa}	100^{Aa}	100^{Aa}	100^{Aa}	100^{Aa}	100^{Aa}
D15	147.73^{Aa}	71.42^{Ab}	57.61^{Ab}	54.12^{Ab}	72.36^{Aa}	59.49^{Ab}
D30	67.31^{ABa}	62.61^{Aa}	23.04^{Ba}	37.24^{Aa}	46.31^{Aa}	40.51^{Aa}
D45	48.61^{ABa}	81.68^{Aa}	37.04^{Aa}	35.59^{Aa}	17.38^{Ba}	19.57^{Ba}
D60	46.38^{ABa}	39.53^{ABa}	20.36^{Ba}	46.21^{Aa}	34.36^{Aa}	21.93^{Ba}
D75	44.29^{ABa}	144.10^{ACb}	21.40^{Bac}	22.09^{Bac}	23.17^{Bac}	15.65^{Bac}
D90	42.77^{ABa}	27.54^{ADa}	25.87^{Ba}	18.86^{Ba}	17.87^{Ba}	13.29^{Ba}

Means in the same column followed by different uppercase letters are significantly different at $P \leq 0.05$.

Means in the same row followed by different lowercase letters are significantly different.

Treatment-Temp-Time Interaction - Chapter 4

Least Squared Means				
Sampling Time	STS Room Temp	STS 4 °C	SM Room Temp	SM 4 °C
D0	100^{Aa}	100^{Aa}	100^{Aa}	100^{Aa}
D15	66.78^{Aa}	77.25^{Aa}	118.35^{Aa}	46.14^{Aab}
D30	45.93^{Aa}	52.05^{Aa}	45.17^{ACa}	41.52^{Aa}
D45	50.41^{Aa}	49.79^{Aa}	18.27^{BCa}	41.44^{Aa}
D60	36.01^{Ba}	35.75^{Ba}	31.38^{BCa}	36.03^{Ba}
D75	37.24^{Ba}	35.59^{Ba}	21.99^{BCa}	85.64^{Aab}
D90	40.88^{Aa}	28.73^{Ba}	16.80^{BCa}	11.01^{Ba}

Means in the same column followed by different uppercase letters are significantly different at $P \leq 0.05$.

Means in the same row followed by different lowercase letters are significantly different.

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