CULTURE ENUMERATION, LACTOSE HYDROLYSIS AND SENSORY CHANGES IN STORED FROZEN YOGURT FERMENTED WITH TWO CULTURE SYSTEMS

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(Abstract)

The objective of this study was to compare products fermented with two culture systems to two endpoints for the following characteristics: survival of the culture bacteria, changes in protein, lactose and galactose concentrations and sensory changes. Frozen yogurt was produced using a standard lowfat ice cream mix formulation, fermented with supplemented and traditional culture systems, and stored for 11 weeks at -20°C.

Three methods of recovery were employed: Bifid Glucose Agar with the Repair Detection System and Roll Tubes, Bifid Glucose Agar with the Repair Detection System on plates incubated in an anaerobe jar with a GasPak™, and Maltose/Galactose Reinforced Clostridial Agar incubated in an anaerobe jar with a GasPak™.

Statistical analysis indicated that the Repair Detection System provided significantly ($p<.05$) enhanced recovery of *Bifidobacterium longum*. Recovery of *B. longum* on BGA Plates and M/G RCA plates was approximately one-half log lower than recovery on BGA in roll tubes.

Culture bacteria in both systems survived at approximately $5x10^6$ cfu/mL during frozen storage. Lactose and protein levels showed no significant changes or differences

between the two culture systems. Generally, galactose levels were significantly higher ($p<.05$) in the traditional culture system fermented to pH 5.6 compared to the supplemented system fermented to the same endpoint.

The manufactured products (supplemented and traditional) were not different from the commercial product with respect to flavor intensity of yogurt flavor, vanilla, sweetness and freshness. Acid flavor was usually more intense when product was fermented to a pH of 5.6. The commercial product was more smooth than the manufactured products. Consumers indicated a "like slightly" to "like moderately" response for the supplemented and traditional inoculated frozen yogurts.

The study concluded that the culture bacteria do survive the environment well enough to meet proposed standards of identity for frozen yogurt. The presence of probiotic bacteria in the supplemented system seemed to cause little to no difference in such attributes as protein and lactose levels, and sensory evaluation.

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CHAPTER I

INTRODUCTION

Frozen yogurt is a very successful dairy product. For example, frozen yogurt sales have increased eight-fold since 1986 and seen a 68% increase in sales between 1989 and 1990 (Kimbrell et al., 1991). Unfortunately, without a standard of identity, defining frozen yogurt is difficult. A variety of frozen dairy products, all called frozen yogurt, are currently manufactured; however, the relationship to the defined product, yogurt, may be vague. Frozen yogurt has no standard of identity, so the inferred relationship, by virtue of the name, may not exist. The importance of bacterial levels in frozen yogurt has become a controversial topic in the dairy industry, but the importance of culture-bacteria in frozen yogurt is well-defined.

For yogurt cultures containing the traditional strains, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus salivarius* ssp. *thermophilus*, the environment of frozen yogurt may cause a significant decrease in viable numbers of bacterial cells. Adjunct bacteria such as *Lactobacillus acidophilus* and *Bifidobacterium longum,* which are sometimes added to frozen yogurt, may also have difficulty surviving in the frozen product (Hekmat and McMahon, 1992).

The advantage of maintaining viable culture and adjunct bacteria in the product is related to nutrition and health (Rogers, 1991). The bacteria have a lactose-cleaving enzyme, beta-galactosidase, which is present only if bacterial cells are viable. Action of this enzyme hydrolyzes lactose to its component monosaccharides, glucose and galactose

(Fennema, 1985). These monosaccharides are more readily digested by many people than the disaccharide, lactose. Other potential benefits, such as decreased risk of certain types of cancer, and a hypocholesterolemic effect, can only occur when cells in the product are viable and active (Dellaglio and Torriani, 1992).

Another important factor in frozen yogurt is the perception of certain sensory attributes by the consumer. The high-acid flavor of the fermented frozen yogurt products manufactured in the 1970's was unacceptable to most consumers and product sales declined after the initial burst of success (Lieb, 1988). Other flavor characteristics developed in fermented yogurt may not be compatible with a variety of flavoring options. The presence of the active cultures cause an alteration of certain flavor attributes and the perception of these changes is important.

There were several objectives to this research. First, three methods of recovering cells of *B. longum* from a frozen yogurt product fermented with a mixed culture containing *L. acidophilus* and other culture bacteria were compared. Second, recovery of the three strains of bacteria found in the mix in addition to *B. longum* were also compared. Third, changes in lactose, galactose and protein concentration were monitored during the frozen-storage period. Fourth, evaluation of sensory changes in the frozen yogurt during frozen-storage was conducted, as well as an evaluation of consumer response to the products.

CHAPTER II

REVIEW OF LITERATURE

Based on the large increase in frozen yogurt sales, the number of new frozen yogurt products and outlets, no one can dispute the popularity of frozen yogurt. The national chain TCBY (The Country's Best Yogurt), opened new shops at the rate of one per day between the years 1986 and 1990 (Kimbrell et al, 1990.). Many ice cream manufacturers have entered the frozen yogurt market due to the huge popularity and high profitability of the snack. Ingredient supply companies have facilitated this option by providing complete flavoring and stabilization systems for the manufacturer. Some companies utilize a process known as the 'total yogurt program' offered by the BlankeBaer & Boweykrinko Corporation of Fenton, Missouri. An ice cream manufacturer who wishes to utilize this program to produce frozen yogurt is supplied with fruit bases, stabilizers, flavors, and formulations for the frozen yogurt mix. BlankeBaer & Boweykrinko also provides technical and plant production assistance as well as promotional and marketing support material (Anonymous, 1991). Other methods of marketing and selling frozen yogurt have evolved. The concept of selling frozen yogurt in glass-fronted vending machines has been tested (Levandoski, 1993). Initial trials with vending machines in the United States have been successful. Trends indicate that by 1997, the number of such vending machines will increase from 200, in 1993 to 20,000 and will generate $221 million in annual sales. The machines will be located in schools, hospitals, colleges, airports and factories.

The reasons for the popularity of frozen yogurt are varied. Frozen yogurt has less fat than ice cream, yet still provides a sweet, pleasant-tasting snack. Also, frozen yogurt contains, or supposedly contains, bacteria which are considered to be healthful to the consumer. Still some consumers believe that the key to longevity is the regular consumption of yogurt, whether it be frozen or otherwise.

One aspect which is questioned among those who consume this product is, "Exactly what is frozen yogurt?" Frozen yogurt currently has no definition because it has no standard of identity. Development of a standard of identity for frozen yogurt is needed to insure safety and uniformity. Standards of identity are designed to bring integrity to the marketplace (Dryer, 1988). Unfortunately, federal agencies responsible for developing a standard have not been able to reach a consensus. The National Yogurt Association (NYA) and the International Ice Cream Association (IICA) have presented the FDA with criteria they consider important for the definition and identity of frozen yogurt. While the suggested criteria from these two organizations are not identical, they are somewhat similar. Both groups identify levels and types of bacteria which should be present and active in order for the product to be called frozen yogurt. The term "active" is a very important stipulation. In order for bacteria to be beneficial to the consumer, they must be living organisms. Such aspects as anti-tumor activities, increased immune-response and other suggested, but unsubstantiated, health claims, require live frozen yogurt bacteria (Rogers, 1991; Ishibashi, 1993). Pasteurization or heat-treatment of

yogurt after the fermentation process will cause bacterial injury and death, and result in loss of the presumed health-benefits of frozen yogurt.

The need for a federal standard of identity is important so that, " we {may} put a halt to the bastardization of yogurt and its standard of identity" (Dryer, 1988). Although there is no federal standard of identity, many states have adopted standards for frozen yogurt. However, incompatibility of standards for frozen yogurt between the states have resulted in some problems regarding the sale of frozen yogurt between states with differing standards (Childs, 1994).

History of Frozen Yogurt

Since the early 1970's when frozen yogurt was first introduced to the American marketplace, it has undergone many changes. Frozen yogurt made its debut in Boston in 1972 when H. P. Hood formulated a soft-serve product for a Massachusetts health-food restaurant (Lieb, 1988). The product was considered trendy and many people did not like its tart and tangy taste. During the seventies, frozen yogurt was generally regarded as a fad which never really caught on. Furthermore, it was reported that frozen yogurt was either too gummy or too icy and did not taste enough like ice cream.

Many different forms of frozen yogurt entered the marketplace in the 1970's. A product known as yogglace was developed by Aries International as an alternative form of frozen yogurt. Natural enzymes were used in the place of culture bacteria, to hydrolyze milk proteins and milk sugar and provide emulsifying and stabilizing effects

without additives. Yogglace, was a product which contained less than 1% milk fat, 8% sucrose, and an overrun which approaching 100% (Anonymous, 1978). Frozen yogurt has also been sold with a relatively high degree of success in the form of Bon Bons in California. The dessert consists of frozen strawberry yogurt surrounded by a chocolate coating (Anonymous, 1977).

Ironically, the frozen yogurt produced twenty years ago contained higher numbers of bacteria than the frozen yogurt produced today. Generally, however, it was not received well by consumers (Vedamuthu, 1991).

Frozen Yogurt Today

Manufacturers have strived to provide the health-conscious public with a dessert they can feel good about eating Frozen yogurt manufacturers maintain that the low-fat qualities of the frozen yogurt, combined with the activities of the starter culture, provide a dessert which is not only enjoyable, but also healthful. Frozen yogurt sales increased eight-fold between 1986 and 1991 and realized a 68% sales increase between 1989 and 1990 (Kimbrell et al., 1991). In 1993 frozen yogurt sales reached $2.8 billion and accounted for over 26% of all frozen dessert sales (Anonymous, 1994).

Frozen yogurt can be divided into three major categories: soft-frozen, hard-frozen and mousse yogurt (Tieszen et al., 1989; Tamime, 1985). Soft-frozen yogurt usually contains an 80% yogurt base (cold), 20% fruit syrup base and a stabilizer/emulsifier. The mix is frozen in an ordinary ice-cream freezer with an outlet temperature of -6°C.

The product is then packaged and stored at 0 to -6°C. Hard-frozen yogurt usually contains 65% yogurt base (cold), 35% fruit-syrup base and stabilizer/emulsifier. The mix is frozen in an ice-cream freezer and stored at -25°C. Mousse yogurt involves mixing the yogurt with a hot mousse-base mixture (skim milk, sugar and stabilizer/emulsifier). The mix is cooled, whipped in an ice-cream freezer, packaged and stored below 0°C.

An Identity for Frozen Yogurt

While it is true that a federal standard of identity for frozen yogurt is non-existent at the present time, regular, non-frozen yogurt does have a standard of identity. The main ingredient is specified as cow's milk and all countries who have a standard agree that a compulsory fermentation by simultaneous addition of fermenting bacteria to the milk is necessary. The Code of Federal Regulations (21 C.F.R. S 131.200) states: "yogurt is the food produced by culturing one or more of the optional dairy ingredients specified below with a characterizing bacterial culture that contains the lactic acid-producing bacteria, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. Yogurt, before the addition of bulky flavors, contains not less than 3.25% milkfat and not less than 8.25% milk-solids-not fat, and has a titratable acidity of not less than 0.9%, expressed as lactic acid" (Hui, 1993). In the United States, two strains of bacteria must be used for the fermentation process, *L. bulgaricus* and *S. thermophilus.*

The Search for a Standard

Frozen yogurt lacks a federal standard of identity. This allows manufacturers to produce what could, essentially, be called yogurt- supplemented ice milk and market the product as frozen yogurt. For example, nineteen commercially available brands of frozen yogurt were tested for bacterial levels as well as fat, protein, total solids and ash (Tieszen, 1989). Fourteen of the nineteen samples tested were well below the advised levels of bacteria ($5x10^6$-$7x10^6$ cfu/gm) as recommended by the NYA and IICA. Levels of fat and other attributes also varied significantly among brands tested.

In another study, seventeen of twenty commercial brands of frozen yogurt were found to contain lactic organisms at a concentration greater than $1x10^6$ cfu/gm (Whitehead et al., 1993). The titratable acidity of all fruit-flavored frozen yogurt was found to be greater than 0.4%; however, only 43% of frozen yogurts which were not fruit-flavored had a titratable acidity greater than 0.3%. The results indicate that, of the brands tested, all contained levels of viable bacteria and had titratable acidities which were in compliance with proposed standards of identity for frozen yogurt in the state of Oregon (Whitehead et al., 1993). However, without a federal standard, compliance between states, which can have differing standards, can be a difficult task for manufacturers.

Many manufacturers are striving to produce frozen yogurt with viable bacteria counts of 100,000 to 200,000 per milliliter (Honer, 1991). Some products incorporate only traditional yogurt cultures such as *S. thermophilus* and *L. bulgaricus*. Other

manufacturers incorporate additional bacteria, such as *L. acidophilus* and *Bifidobacterium,* into the cultures. The fermentation process only requires the presence of *S. thermophilus* and *L. bulgaricus. B. longum* and *L. acidophilus* are included as adjunct organisms for the perceived health benefits and are not required for fermentation.

The titratable acidity of frozen yogurt varies widely among many brands, as previously described. Also, some manufacturers add lactic or other organic acids to provide the characteristic acidity and to increase the developed titratable acidity (Honer, 1991).

Specific standards proposed by the IICA for identification of a product as frozen yogurt are as follows:

-product must be frozen under agitation

-must contain "safe and suitable" ingredients

-contain live S. thermophilus and *L. bulgaricus*

-titratable acidity of .30% in mix, or a .15% increase due to action of bacteria

-food-grade acids may not be used for the purpose of meeting titratable acidity level

-must not undergo chemical preservative addition, which reduces live culture fermentation

-contain 3.25% milkfat, 8.25% milk solids, 1.3 lbs. total solids/gallon, and weigh 4 lbs./gallon

-contain 0.5-2% milkfat to be called low-fat

-contain <.05% milkfat to be called no-fat

(Kimbrell et al., 1990.)

This standard, and one of a similar nature, has been proposed to FDA for consideration by IICA and NYA. No indication has been given as to if, or when, a standard will be accepted for federal regulation of frozen yogurt.

The Starter Culture

The starter culture may be defined as a mixture of microorganisms having a physiological relationship and dependence on one another, (Driessen, 1992) which also inhibits the growth of any microorganism which isn't a starter organism. It also has specific characteristics responsible for defining the final fermented product (Driessen, 1992). The starter culture makes yogurt unique by fermenting the milk mix and causing numerous changes in the physical make-up of the milk (Speck, 1983). The bacteria contained in the starter culture usually include *S. thermophilus* and sometimes *L. acidophilus* and/or a human strain of *Bifidobacterium*. Species of *Bifidobacterium* are separated into human and animal categories. Those in the human category include: *B. bifidum, B. infantis, B. longum, B. breve* and *B. adolescentis* (Ishibashi and Shimamura, 1993).

The starter culture imparts certain characteristics into the yogurt which allow consumers to identify it as yogurt. For example, *L. bulgaricus* and *S. thermophilus* are

able to produce a polysaccharide from galactose which can act as a thickener. This attribute can eliminate the need to add such stabilizers as gelatin and alginates which may act to reduce the overall quality of the product. *L. bulgaricus* also produces acetaldehyde which gives yogurt its characteristic fermented flavor (Keogh, 1978).

The starter culture used in frozen yogurt manufacture also has the ability to inhibit the growth of such food-borne pathogens as Salmonella and Listeria. Live bacteria can also limit the time that these disease-causing bacteria can survive in the intestine, thereby limiting the pathogen's ability to cause disease symptoms (Anonymous, 1989). Specifically, *L. acidophilus* has also been found to suppress pathogenic bacterial growth and growth of *Escherichia coli* in the human intestine (Keogh, 1978).

Various bacterial strains are chosen to be used in the starter culture based on their ability to coagulate milk and improve milk-product taste (Hunger and Pietersen, 1992). The bacteria in the starter culture mix use certain nutritive aspects of the milk to survive and form a symbiotic relationship. Nitrogen is obtained from the milk protein. *L. bulgaricus* alters the globular structure of casein sufficiently so that *S. thermophilus* can initiate early growth. Alteration of the protein by the starter culture allows for easier digestion by humans (Speck, 1983). *S. thermophilus* also produces formic acid which is a factor required for growth by *L. bulgaricus* (Keogh, 1978). Lactose or milk sugar is also used as an energy source by the bacteria.

The Fermentation Process

Fermentation of lactose, which is found in milk, is used for energy production by lactic acid bacteria (Tamime and Robinson, 1985). Actual catabolism of the lactose takes place inside the cell, so a transport mechanism is necessary to bring the lactose into the cell. The enzyme galactoside permease is thought to be responsible for this transport in *L. bulgaricus* and *S. thermophilus.*

Once inside the cell, the lactose is cleaved into its monosaccharides, D-glucose and β-D-galactose. The specific enzyme responsible for this cleavage is β-galactosidase(β-gal)which has been found to be present in *L. bulgaricus,* or βPgal which is not present in the *L. bulgaricus* but is present in *Lactobacillus lactis*. Regardless of which enzyme is responsible, glucose, with the help of the hexokinase enzyme becomes glucose-6-phosphate, which is then transformed to glyceraldehyde-3-phosphate. Glyceraldehyde-3-P then enters the Embden-Myerhof Parnas Pathway where, in the presence of lactate dehydrogenase, it becomes lactic acid (Tamime and Robinson, 1985).

The exact pathway of galactose catabolism is not as well understood as that of glucose. Three theories predominate: the first hypothesizes that galactose, in the presence of galactokinase, becomes glucose 1-6 and subsequently glucose 1-6 phosphate, where it will follow the same pathway as glucose for metabolism to lactic acid. The second theory is that lactose is cleaved by β-Pgal to D-glucose and galactose-6-P; galactose-6-P will enter the D-tagatose-6-P pathway and become dihydroxyacetone-P which will be further metabolized to glyceraldehyde-3-P which can then be metabolized

to lactic acid. The final theory is that the galactose is not always metabolized by the cells because the D-tagatose-6-P pathway is suppressed by the presence of a more readily fermentable sugar such as glucose. In all cases, the sugar is fermented to lactic acid (Tamime and Robinson, 1985).

The importance of lactic acid cannot be overstated in the manufacture of frozen yogurt. Lactic acid converts the colloidal calcium/phosphate complex, contained in the micelle, to a soluble calcium/phosphate fraction, diffusible into milk's soluble phase. Further, the depletion of the micelles of calcium, results in a coagulation of casein at pH 4.6-4.7 and the subsequent formation of the yogurt gel (Tamime and Robinson, 1985)

Health Claims

The value of the starter culture goes beyond the physical and chemical changes it causes in the milk mix. The bacteria, themselves, supposedly have many healthful benefits (Ishibashi and Shimamura, 1993). Studies have revealed that diet-induced changes in the intestinal micro flora of human beings may play an important role in carcinogenesis of the colon (Bartram et al., 1994). For example, *L. bulgaricus* produces glycopeptides which have antitumor activity against Sarcoma 180 (Speck, 1983). Other strains which are sometimes used in starter cultures are human strains of *Bifidobacterium*, including *B. longum.*

Bifidobacteria

Bifidobacterium is a Gram-positive, bifurcated rod which is strictly anaerobic. It degrades glucose by the fructose-6-phosphoketolase shunt, produces acetic and lactic acid in a 3:2 molar ratio, produces no CO_2, is beta and alpha-galactosidase positive and fructose-6-phosphoketolase positive (Dellaglio and Torriani, 1992). Since it was first isolated in 1899 by Tissier, *Bifidobacterium* has become the subject of interest and study pertaining to its perceived health benefits. *Bifidobacterium* is one of the first bacterial groups to establish itself in the colon. Babies who are breast-fed appear to have a one-log advantage in intestinal bifid numbers compared to bottle-fed babies (Ishibashi and Shimamura, 1993). This is not due to a nutritional aspect of human milk, but rather human milk has a lower protein content and less buffering capacity than bovine milk (Hoover, 1993).

Bifidobacterium levels decrease with age, due to diet, stress and other environmental factors. Other bacteria such as coliforms, some enteropathogens and Clostridia begin to replace bifidobacteria and the presence of these other bacteria may present a threat to colonic health (Hoover, 1993).

Bifidobacterium is considered probiotic by meeting the following criteria:

-normal inhabitant of the intestinal tract

-survives passage through the upper digestive tract

-is capable of surviving and growing in the intestine

-produce beneficial effects when in the intestine

-maintain viability and activity in the carrier food prior to consumption

(Gilliland, 1989)

Bifidobacterium has been found to have an antidiarrheal effect in children (Nishihara, 1969). The bacterium seems to have other beneficial effects. A study by Takano (1986) indicates that *B. longum* is responsible for an increase in total IgA antibody level in mice, which may inhibit bacterial infection in the mice. Another benefit is a possible hypocholesterolemic effect in humans, thus making the bacterium useful to those with problems maintaining a safe level of blood-serum cholesterol (Dellaglio and Torriani, 1992).

At the present time *Bifidobacterium* is not used widely in this country. Although the bacterium has been linked to many possible health benefits, an insufficient number of controlled and definitive studies have been done to prove or disprove the usefulness of this bacterium in human gut health (Rogers, 1991). The organism is receiving much attention in Japan where the Japan Bifidus Foundation was established in 1981 to serve as a central organization of bifidus research. Research includes examining the biochemistry, ecology, taxonomy, physiological effects and other factors. Another focus of the foundation is to support health claims with in vivo, in vitro studies, or clinical tests and use these tests to determine what role these bacteria play in human health (Ishibashi and Shimamura, 1993).

The effects of consuming *B. longum*-enriched yogurt (500 mL/day for 3 weeks) on various factors within the colon of 12 healthy volunteers was reported by Bartram et

al.(1994). The stool of the subjects indicated an obvious increase in excretion of *B. longum* after consumption of the yogurt; however, counts of intestinal aerobes and anaerobes did not change significantly. Breath hydrogen levels in the subjects showed an increase after consumption of the test yogurt, and mouth-to-caecum transit time was accelerated. Generally, the results indicate good stability of the intestinal micro flora after the use of a dietary bacterial adjunct and only a slight increase in gas production due to lactose-fermenting bacteria in the colon (Bartram et al., 1994).

Another study using a double-blind placebo-control was completed to determine the effect of ingestion of mixtures of lactic acid bacteria and *Bifidobacterium* on gut micro flora (Nielsen et al., 1994). The effects were determined by microbiological examination of fecal samples and jejunal aspirates. Sixteen females and eight males, aged 19-59 years, who were considered to be in good health, were given either a placebo, a preparation containing *Enterococcus faecium* and *B. longum* (6.4×10^8 cfu/day) or a preparation containing *L. acidophilus*, *B. longum*, *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* (9×10^9 cfu/day). Upon examination of jejunal aspirates by microbiological methods, it was determined that microbiological counts of most species of bacteria were below the detectable limit. The preparation which contained the *E. faecum* and *B. longum* significantly ($P=.03$) reduced the anaerobe:aerobe ratio in the colon by a factor of three during initial treatment and then increased it by a factor of 30 during the following week ($P<.02$). The study concluded that the oral administration of bacteria

such as those found in frozen yogurt may have a distinct effect on distal intestinal micro flora.

Fujiwara et al. (1990)conducted a study to observe the immunopotentiating effects of *B. longum* SBT 2928 in animals which had experimental tumors. The specific strain of *B. longum* (BL2928) used was selected by mitogenic assay as a potent immunomodulating strain. The study found that when administered systemically, BL2928 showed significant anti-tumor activity in both allogeneic and syngeneic tumor models (Fujiwara et al., 1990).

Lactobacillus acidophilus

L. acidophilus is a bacterium which is sometimes added to the starter culture for frozen yogurt. *L. acidophilus* is a Gram-positive, microaerophilic, lactic acid-producing rod of the family *Lactobacillaceae* (Tamime and Robinson, 1985). Many studies have determined that a wide variety of potential health benefits exist due to consumption of frozen yogurt bacteria, in general, and *L. acidophilus*, specifically. Some important possible health benefits which have been documented are improved lactose digestion due to the presence of the lactase enzyme in cells of *L. acidophilus* and decrease in serum cholesterol levels. Also, an increase in vitamin B content of food and maintenance of enterohepatic circulation of bile acids through deconjugation reactions have been documented. The bacteria has also been shown to increase weight-gain in lab animals, presumably due to improved utilization of nutrients at the cellular level (Welch, 1987).

Starter Culture Survival in Frozen Yogurt

In order for the bacteria to exert effects on the colon or other systems within the body, it must first survive in the frozen yogurt itself. The environment of frozen yogurt can be extremely harsh to the starter culture. Since frozen yogurt is, by definition, frozen this extreme in temperature can cause injury to the cells of the starter culture, thus reducing their numbers and thereby decreasing their healthful attributes. After two weeks of frozen storage at -28.9°C, levels of *L. bulgaricus* dropped an average of one-and-one-half logs and levels of *S. thermophilus* decreased an average of one-half log (Miles and Leeder, 1981). This study indicated that frozen storage had little effect on the maintenance of high numbers of bacteria. Other variables, such as varying levels of dextrose, corn syrup solids, milk solids non-fat and Tween 80, were tested in this study. The average decrease in levels of bacteria remained relatively consistent no matter what the variable, with only Tween 80 appearing to make *L. bulgaricus* more cold-tolerant (Miles and Leeder, 1981).

Another study examined the effects of cold-storage (-29°C) on cells of *L. acidophilus* and *B. longum* in ice cream. The standard ice cream mix was fermented with the two bacteria, frozen in a batch freezer and stored at -29°C. Both species of bacteria were found to exhibit some loss in viable numbers after seventeen weeks of cold-storage. A two log loss was noted for the acidophilus and a one log loss was observed for the bifid population. β-galactosidase activity was also monitored and the activity decreased from 1800 to 1300 units/mL (Hekmat and McMahon, 1992).

Holcomb et al.(1991) studied the effects of cold-storage and the environment of frozen yogurt on *L. acidophilus* and *B. longum* survival. The frozen yogurt containing these bacteria was produced and then frozen at -5°C for six hours. Upon thawing of the frozen yogurt, the bacteria showed no evidence of freeze injury. Freezing did not alter the bile or acid tolerance of either culture. A study was also conducted simultaneously to determine the stability of the microorganisms to the acidic environment of the stomach, i.e. holding for 2 hr in 0.01 HCl. The *L. acidophilus* strain was able to survive these conditions; however, *B. longum* was unable to survive the acidic conditions (Holcomb et al.,1991).

The growth abilities of *B. longum* in a frozen yogurt environment with low and high levels of developed acidity was tested by Modler and Villa Garcia (1993). A whey-based medium was initially produced for the large-scale growth of *B. longum*. Medium was prepared by reconstituting demineralized whey powder to 6% solids, concentrating to approximately 30% solids and then frozen. The medium was then thawed, diluted to approximately 11% solids and growth of *B. longum* (ATCC 15707) was monitored in the medium. Growth seemed to improve when .05% L-cysteine and .23% yeast extract were added to the growth medium (Modler and Villa Garcia, 1993).

Modler and Villa Garcia(1993) also determined that *B. longum* grown in whey-based medium showed poor survival in high-acid yogurt which had a developed acidity of 0.45% (pH 4.47). However, when the bacteria were incorporated into yogurt which had low-titratable acidity (0.215%) and a pH of 5.85, the loss of bacteria over an eleven

week cold-storage period was only one log. Upon re-freezing, an additional one log decline in the numbers of bacteria was observed. An attempt was made to protect the bacteria from freeze-death by milkfat encapsulation; however, results indicated that encapsulation did not protect or improve the survival of the bacteria (Modler and Villa Garcia, 1993).

Another problem with frozen yogurt, with respect to *B. longum* survival, is the oxygen content of the mix when overrun is added. Oxygen is particularly detrimental to *B. longum,* since this organism is strictly anaerobic, and it tends to injure the cells of *B. longum.* Cells are considered injured when they will no longer grow on medium which is selective for them (Arany, 1992). Fortunately, if given the proper environmental conditions, the injured cells are able to repair their injury as they might do in the colon and become viable cells again (Arany, 1992).

One final hurdle, which the bacteria must overcome, is the harsh environment of the human stomach. In order to exert health-benefits, bacteria must survive a wide range of stomach pH depending on whether or not the person is fasting (pH 1.5) or has just eaten (pH 4.5). The bacteria will probably spend one to two hours in the stomach so cells must be acid-tolerant or survive in sufficient numbers to exert beneficial effects. Once past the stomach, the bacterium must then be able to adhere to the epithelial cells of the small intestine using adhesins. Also, bifids need vitamins and O_2 removal in order to enhance their growth in the intestine (Hunger and Pieterson, 1992).

Hydrolysis of Lactose by β-Galactosidase

Lactose is a disaccharide consisting of the monosaccharides D-glucose and D-galactose (Fennema, 1985) and is only found in products of milk origin. For most people, the consumption of lactose-containing products poses no digestive problems. However, for people who lack the enzyme lactase, or β-galactosidase, which is found in cells of the small intestine, the presence of lactose in the diet can lead to such unpleasant symptoms as flatulence, bloating and abdominal pains (Lerebours, 1989). These physical symptoms occur because the lactose, which is not cleaved into its constituent sugars, reaches the large intestine where it is fermented by bacteria which inhabit the colon. Fermentation of lactose in the large intestine leads to the production of lactic and acetic acids which bind water in the colon and cause diarrhea, which is another symptom of lactose malabsorption (Fennema, 1985).

In addition to observation of physical symptoms, another way to determine if a person is a lactose malabsorber is to measure breath hydrogen levels. When uncleaved lactose is fermented by the bacteria present in the colon, this fermentation leads to production of hydrogen gas, which is absorbed into the blood stream and released into the lungs, where it may be expelled during exhalation. The exhaled air may be analyzed for increased hydrogen levels to determine lactose malabsorption (Welch, 1987).

In dairy products, the enzyme is found only in products that contain active bacterial cultures. *S. thermophilus* and *L. bulgaricus* produce beta-galactosidase endogenously and the enzyme is exported from the cell. *S. thermophilus* produces three

times as much beta-galactosidase as *L. bulgaricus* grown under identical conditions (Khedkar et al., 1994). Frozen yogurt containing active and viable strains of bacteria, contains the enzyme. Activity of this enzyme hydrolyzes the β-1,4 linkage of lactose to its component monosaccharides which can then be used by the bacterial cells for energy. The importance of cultures in hydrolysis of lactose and the associated benefits to lactose malabsorbers is well-documented. One study tested the effects on subjects, previously identified as lactose-malabsorbers, who consumed either eighteen grams of lactose in milk or eighteen grams of lactose in yogurt. The consumption of lactose in the yogurt resulted in only 1/3 as much breath hydrogen release as the lactose in milk. Also, fewer subject reports of diarrhea and flatulence were noted, presumably due to bacterial enzymatic hydrolysis of the lactose (Khedkar et al, 1994). This study provides compelling evidence of the value of the lactase enzyme found in fermented dairy foods to lactose malabsorbers.

Another study by Lin et al. (1991) observed the effects of *S. thermophilus, L. acidophilus,* and *L. bulgaricus* present in a non-fermented dairy product. The purpose of the study was to determine if the bacteria present in the non-fermented milk would still be able to exert beneficial effects on the lactose malabsorber. Two percent lowfat milk inoculated with $1x10^7$ or $1x10^8$ of the aforementioned bacteria (hereafter referred to as "yogurt milk") was refrigerated immediately after inoculation, and then 400 mL aliquots of the different milk samples were consumed. The yogurt milk which contained $1x10^8$ cfu/mL of bacteria showed significantly high levels of lactase. This level remained

stable for up to 14 days at refrigeration temperatures. The measured breath hydrogen levels after consumption of the yogurt milk showed a delayed increase. The increase that eventually did occur was significantly lower than control values. Furthermore, all physical symptoms of lactose intolerance were eliminated. Yogurt milk containing levels of $1x10^7$ cfu/mL showed intermediate breath hydrogen levels, which were marginally significantly different from control breath hydrogen levels. These results indicate a correlation between the level of bacteria present and the level of positive effects generated from the lactase enzyme.

Studies (Lin et al., 1991; Khedkar et al., 1994) have shown that fermentation of foods can improve the nutritional value of food products. Fermentation of the food increases the availability, digestibility and assimilability of certain nutrients. β-galactosidase, whose production increases during fermentation of yogurt, is responsible for the increased digestibility of yogurt for people who lack the enzyme. Lactase activity seems to peak after incubating food for four hours. A study which reached this conclusion regarding rates of lactase production in fermented foods also determined that in vitro digestion enhanced the quantity of lactase released from the yogurt culture (Khedkar et al., 1994) .

The presence and activity of β-galactosidase in frozen yogurt bacteria is of obvious importance to those who are lactose intolerant. However, the effects of cold-storage on the β-galactosidase enzyme are not fully known. Speck and Geoffrion (1979) studied the effects of cold storage and heat treatment on activity of β-galactosidase. The

study involved the heat treatment of market samples of yogurt for various times and temperatures. The lactase enzyme was found to be appreciably inactivated in three min at 60°C and was completely inactivated in two minutes at 65°C and one minute at 70°C. No reactivation of the enzyme was noted. Unheated yogurt, stored at 1°C for eight weeks showed a decrease in β-galactosidase activity.

Nielsen and Gilliland (1992) studied the activity of β-galactosidase obtained from *L. acidophilus* NCFM, *L. acidophilus LA-1*, and *L. acidophilus* RAM-1 grown in peptonized milk nutrient broth. The enzyme was obtained from the bacteria by lysing the cells with lysozyme. Purification of the enzyme was carried out by ammonium sulfate precipitation and ion exchange chromatography. Activity of the enzyme was determined by measurement of the rate of o-nitrophenyl-β-D-galactopyranoside (oNPG) hydrolysis. The maximum rate of enzyme activity was observed when the enzyme is incubated with magnesium ions and mercaptoethanol at pH 6.6. The molecular weight of the β-galactosidase enzyme is 5.7×10^5 (daltons) when measured by gel filtration methods. Also, the study determined that the enzyme is activated by the presence of lactose and galactose, but not by glucose. Storage of the enzyme at 5°C for 12 days resulted in greater than 50% retention of the enzyme's original activity. *L. acidophilus* RAM-1 cells, which were lysed, had a significantly higher measured β-galactosidase activity value than the other two strains evaluated (Nielsen and Gilliland, 1992).

Sensory Characteristics of Frozen Yogurt

The presence of live and active bacteria in frozen yogurt, which are capable of metabolizing and producing end-products, causes readily- perceptible changes within the product. The active bacteria produce lactic acid, which is an end-product of lactose metabolism. The lactic acid gives the yogurt its characteristic sharp, acidic or otherwise "nutty" and/or "aromatic" flavor (Tamime and Robinson, 1985).

All of the products produced by the starter culture can be divided into four main categories: 1) non-volatile acids: lactic, pyruvic, oxalic or succinic; 2) volatile acids: formic, acetic, propionic or butyric; 3) carbonyl compounds: acetaldehyde, acetone, acetoin or diacetyl; 4) miscellaneous compounds: certain amino acids and/or constituents formed by thermal degradation of protein, fat or lactose (Tamime and Robinson, 1985). Many studies indicate that acetaldehyde, which is produced primarily by *L. bulgaricus,* is the most important aroma compound in yogurt and that the level of acetaldehyde should be relatively low to produce a "best or high" rating by a sensory panel (Tamime and Robinson, 1985).

The chemical reaction which produces acetaldehyde is as follows:

$$CH_3CHOHCHNH_3{}^{+}COO \xrightarrow{\quad} - \quad CH_3CHO \quad + \quad CH_2NH_3{}^{+}COO^{-}$$

Threonine	Pyroxidal phosphate	Acetaldehyde	Glycine

This reaction illustrates that increased levels of threonine can lead to an increase in the acetaldehyde levels (Tamime and Robinson, 1985).

Kneifel et al., (1992) studied 47 commercially available yogurts and starter cultures and determined that the acetaldehyde levels ranged from 5.5-20.7 ppm. Other compounds such as diacetyl, ethanol, butanone and acetone were also present, but at lower levels. Sensory evaluation of the yogurts resulted in classification of the all of yogurts into one of three categories, mild-sourish, sour or very sour. Thus, the acidity of frozen yogurt, which would contribute to the sourness, was deemed the most important attribute of yogurts in this study.

Speck (1983) evaluated frozen yogurts flavored with vanilla, chocolate or coffee at various acidities. Sensory evaluation of the products was performed by three professional judges. The judges determined that frozen yogurts with low titratable acidity (0.28-0.38%) had the highest overall quality, while high acidity frozen yogurts (.76-1.24%) were rated the lowest. The study concluded that all of the flavors tested would receive higher sensory quality scores when the acidity was kept relatively low.

Steinsholt and Abrahamsen(1978) tested different production methods to determine which would enhance the recovery of *L. bulgaricus* and *S. thermophilus*. Organoleptic evaluation of final products was also completed and conclusions suggested that a final pH of 4.7 was preferred to other samples which had lower pH. The exact value of the "lower" pH values were not supplied in this article.

Kankare and Antila (1978) evaluated a protein-enriched frozen yogurt organoleptically. A frozen yogurt with a pH of 5.17 received a high organoleptic rating

(4.15/5) for texture and 4.3 for flavor. The sample was not compared to any other sample.

Hauge et al. (1981) tested 33 flavoring ingredients used in frozen yogurt by using organoleptic assessment panels. The panels found the following: coffee, black currant, apple, cloudberry, grapefruit, pear and banana/vanilla flavors were considered to be unsuitable. Peach flavoring received the highest overall organoleptic score, with plain, cherry and strawberry following The relationship between the suitability of certain flavors over others to the pH was not established by this study.

The study which is being conducted will contribute to knowledge regarding the recovery of bacterial cells in a frozen yogurt environment. Also, important biochemical data will be collected which can aid in determination of enzyme activity in frozen yogurt bacteria. Sensory evaluation of the product will provide information regarding sensory effects of bacterial activity. Finally, a consumer study will allow a determination to be made regarding overall liking of the products.

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CHAPTER III

COMPARISON OF THREE METHODS FOR RECOVERY OF *BIFIDOBACTERIUM LONGUM* FROM FROZEN YOGURT IN A MIXED, PROBIOTIC-ENHANCED CULTURE

ABSTRACT

The objective of this study was to compare three methods of recovery for *Bifidobacterium:* a roll-tube method using anaerobic modified bifid glucose agar (MBGA) with the Repair Detection System, anaerobic plates using MBGA with the Repair Detection System, and plates with Maltose/Galactose Reinforced Clostridial Agar. The Repair Detection Roll Tube System was used to aid in recovery of injured cells. All methods were used to recover the bacteria on a biweekly basis for 11 weeks. Analysis of bacterial counts from each method were compared to determine the method best-suited for recovery of all metabolically-active cells. The roll-tube method with MBGA and the Repair Detection System provided significantly ($p<.05$) enhanced recovery of *B. longum*. The BGA plates and M/G RCA plates recovered *B. longum* at statistically similar levels and approximately one-half log less than recovery of anaerobic roll tubes, indicating that anaerobic roll tubes were probably the reason for the enhanced recovery.

INTRODUCTION

Bifidobacterium longum is a Gram-positive, anaerobic, bifurcated rod. It has been suggested that this organism aids in the maintenance of intestinal and general health of humans (Dellaglio and Torrianai, 1992; Ishibashi and Shimamura, 1993).

Bifidobacterium is part of the normal microflora of the intestine. When a human is an infant, levels of *Bifidobacterium* are at their highest; however, levels begin to drop as age increases. The levels of *Bifidobacterium* in the intestine decrease with age due to diet, stress and other environmental factors. As numbers of *Bifidobacterium* decrease, bacteria including enteropathogens, coliforms and Clostridia begin to proliferate and may pose a threat to colonic health (Hoover, 1993). *Bifidobacterium* had been found to have and an antidiarrheal effect in children (Nishihara, 1969). Takano (1986) determined that *B. longum* is responsible for an increase in total IgA intibody level in mice, and Dellagio and Torriani (1992) found that *B. longum* may have a hypocholesterolemic effect in humans.

The effects which have been documented only occur when bacteria are viable and active. Manufacturing or storage processes that cause death to *Bifidobacterium* cells will eliminate the benefits; however, injury to cells may not have as great a detrimental impact. *Bifidobacterium* is frequently supplemented into the diet in frozen yogurt. Unfortunately, since the bacterium is strictly anaerobic, the aerobic environment of frozen yogurt, in combination with the freezing process, can cause injury which can affect recovery. Injured bacteria are unable to grow on media which are selective for

them, even though they are metabolically active (Arany, 1992). However, if given adequate time and proper conditions they can repair their injury. Recovery of injured cells is important in frozen yogurt to determine the number of cells which may be available to inhabit the intestine.

A method of recovery, known as the Repair Detection System, in combination with the Roll Tube Method has been developed to aid in recovery of injured cells (Holdemann et al., 1977). The method involves anaerobic inoculation of a non-selective layer of medium, followed by a two hour repair period and then anaerobically overlaying with a selective layer of the same medium containing selective agents. Arany (1992) demonstrated that injured *Bifidobacterium* could be recovered from water and melted frozen yogurt inoculated with *Bifidobacterium* and *L. acidophilus* by this method. This method provides an environment for cells to repair injury and can be used as an alternative to conventional methods of recovery which do not account for injured cells.

MATERIALS AND METHODS

Source of Bacteria

A starter culture, which contained selected strains of *Bifidobacterium longum, Lactobacillus acidophilus, Streptococcus salivarius* ssp. *thermophilus* and *Lactobacillus delbrueckkii* ssp. *bulgaricus* was used. This mix, sold under the name BATL-1 (Sanofi Bio-Industries, Waukesha, Wisconsin) was used in conjunction with another culture mix, BA-61 (Sanofi Bio-Industries, Waukesha, Wisconsin) which contained *B. longum*-6 and

L. acidophilus-1 only. Together, these culture mixes were used to produce the frozen yogurt which was tested during this study. The cultures were shipped on dry ice and stored at -80°C in an ultra-low temperature freezer. The samples were stored for no more than three weeks prior to use in yogurt production.

Frozen Yogurt Manufacture

Raw milk, obtained from the bulk tank at Virginia Polytechnic Institute and State University (VPI&SU) dairy farm, was separated into cream and skim phases in a pilot plant-scale separator (Elecrem Separator Model 1G, Bonanza Industries, Calgary, Canada). Lowfat ice cream mix (4% fat) was manufactured with 1157.7 g cream, 1198.6 g non-fat dry milk, 12076.4 g skim milk, 1847.8 g granulated sucrose, 1539.1 g corn syrup, and 140.7 g stabilizer. The ice cream mix was mixed until no clumps were present, batch pasteurized at 55°C for 30 min, cooled to 3.3°C and stored for approximately 24 hr.

Cooled mix was heated to 40°C in a hot-water bath prior to inoculation. While mix was heating, containers of both yogurt cultures were removed from the freezer and thawed in 25°C water baths until thoroughly liquefied. When the mix reached the desired temperature of 40°C, four 3246.1 g aliquots were weighed into chlorine-sanitized, stainless steel cans. Yogurt cultures were added to two of the containers (BATL-1 Culture and BA-61, .2% of mix weight and .02% of mix weight, respectively) and thoroughly mixed. The containers were then placed in a 40°C incubator until

titratable acidity of one of the containers was .15% greater than the initial titratable acidity value (endpoint 1). The second container was incubated until the pH was 5.6 (endpoint 2).

Frozen yogurt was manufactured in a pilot plant batch freezer (Emory Thompson Freezer 2HSC A, Emory Thompson Machine and Supply Co., New York) with an overrrun of 55-60%. Product was frozen and stored at -20°C until all analyses were completed.

Enumeration of Bacteria

Bifidobacterium was enumerated after the product reached the first and second endpoints and was frozen, and then on a biweekly basis from frozen storage. Two media were used. The first method used a modified Mara and Oragui's, (Bifid Glucose Agar, BGA) medium (Arany, 1992). This medium contained (g/L distilled water): glucose 10, polypeptone 10, yeast extract 20, casamino acids 8, sodium chloride 3.2, and L-cysteine 0.5. Dyes were added in the following amounts: 3 mL methylene blue, 40 mL phenol red. The medium was dispensed into 25 x 142 mm roll tubes in 7 and 10 mL aliquots using anaerobic roll tube equipment (Bellco, Inc., Vineland, New Jersey) following procedures outlined in the Anaerobe Laboratory Manual (Holdeman, et al., 1977). Granulated agar (.02 g/mL) was dispensed into each roll tube (Bellco, Inc., Vineland, New Jersey) before the medium was added. Medium pH was adjusted to 7.1 (+/- 0.1),

using 1 N NaOH, prior to autoclaving and tubes were subsequently stored in the dark. Prepared media was used within two weeks.

A solution of selective agents was prepared (g/10 mL distilled water) nalidixic acid (.056), kanamycin monosulfate (.085) and polymixin B (.00215) for use with the Repair Detection System. The solution was filter-sterilized using a Gelman Sciences Sterile 0.2 micrometer Acrodisc and a 30cc syringe. The sterilized solution was then placed in the freezer at -4°C until used. Prior to use, the solution was placed in the refrigerator (4°C) for 24 hr to allow proper thawing.

The second medium, maltose/galactose reinforced clostridial agar was also used to enumerate *B. longum*. The ingredients were as follows (g/L distilled water): Trypticase peptone 10, beef extract 10, galactose 5, maltose 5, sodium chloride 5, yeast extract 3, sodium acetate 8, starch 1, L-cysteine .5, granulated agar 13.5. The medium pH was adjusted to 5.4 (+/- 0.1) using a 1 N HCl, and dispensed into 150 mL screw-cap bottles and autoclaved (121°C, 20 psi, 20 min). Medium was stored in the dark until used.

Dilution Blanks

Two types of dilution blanks were produced. The first was an aerobically-produced pre-reduced peptone dilution blank. The dilution blanks contained .01% (w/v) peptone (Difco-Bacto Peptone, Difco Laboratories) and .05% L-cysteine (Sigma Cell Culture Reagents). To 500 mL of distilled, deionized water, .50 g of peptone and .25 g

of L-cysteine were weighed and added. Solution pH was adjusted to 7.0 using 1N NaOH. Nine mL aliquots were dispensed into 15 mL screw-cap tubes using an Oxford dispenser (Monoject, Inc.). Tubes were then capped, sterilized (121°C, 20 psi, 20 min), cooled and stored in the dark until used. These dilution blanks were used in conjunction with M/G RCA.

A second set of dilution blanks were used with the Roll Tube Method and Repair Detection System. The ingredients were identical to aerobically-produced blanks; however, methylene blue (.3 mL/500mL) was added as an oxygen indicator. The blanks were produced following the VPI Anaerobe Manual instructions (Holdemann et al., 1977). The methodology used to produce the anaerobic blanks involved the injection of CO_2 into the tubes to facilitate the removal of the oxygen. The solutions were dispensed into small roll tubes which were stoppered as opposed to screw-capped.

Repair-Detection Procedure with Roll-Tubes

The Roll Tube Method used in this study followed procedures outlined in the Anaerobe Laboratory Manual (Holdemann et al., 1977). Modified BGA was prepared without the addition of selective agents, according to the VPI Anaerobic Manual (Holdemann et al., 1977) and dispensed into 25 x 142 mm roll tubes (Bellco, Inc., Vineland, New Jersey) in 10 and 7 mL aliquots. The seven ml. tubes were used to inoculate the dilution of frozen yogurt and the ten ml. tubes were used as the overlay. Tubes were autoclaved and stored for no longer than two weeks prior to use. Prior to

inoculation, tubes were steamed (100°C, 10 min) to facilitate melting of media and tempered in a 47°C water bath. Anaerobic dilution blanks were unstoppered under CO_2 canula and serial dilutions were made to the highest dilution required. Non-selective (7mL) tubes were unstoppered under CO_2 canula and inoculated from the anaerobic dilution blank corresponding to desired dilution. Tubes were then stoppered, spun until solid, inverted and then incubated for 2 hr at 37°C to allow injured cells to repair. Once the incubation time passed, the tubes were re-opened and placed under the canula. A 10 mL tube of tempered MBGA was then opened under CO_2 canula, .1 mL selective agent was added, and the contents of the tube were added to the unstoppered 7mL tube. Overlayed tube was then stoppered, spun until solid, inverted and incubated, at 37°C for up to 96 hr.

Repair Detection System with Plates

MBGA was also used with plates which were inoculated using anaerobic dilution blanks. Tubes which contained the medium were steamed and tempered as described above. The 7 mL tubes were then unstoppered under canula, inoculated and poured onto plates (non-selective layer), allowed to solidify and placed in an anaerobe jar with a GasPak and an indicator strip. The jar was then incubated at 37°C for 2 hr to allow repair of injured cells. After incubation, the jar was removed from the incubator and opened. Ten (10) ml MBGA tubes were unstoppered under canula, .1ml selective agent was added and the contents was poured over the inoculated plates (selective overlay),

allowed to solidify, placed in anaerobe jar with a fresh GasPak and indicator strip and returned to the incubator for up to 96 hours.

Maltose/Galactose Reinforced Clostridial Agar

Bottles of media were steamed (100°C, 10 min) and tempered at 46°C prior to use. Serial dilutions were made using liquefied frozen yogurt in aerobic dilution blanks. The plates were then inoculated using 1 ml of dilution and the pour-plate method, i.e. 1 mL of dilution was placed on the plate, and tempered media was poured over it until the bottom of the plate was covered, approximately 10 mls of media. The plate was slowly swirled to facilitate mixing, media was then allowed to solidify, plates were inverted and then placed in an anaerobe jar with a GasPak and indicator strip. The jar was then placed in a 40°C incubator for 72 hours and colonies were counted subsequent to removal of plates/tubes from the incubator.

Statistical Analysis

Data analysis was conducted on log transformation of bacterial counts. A model was developed to evaluate differences in recovery of *B. longum* from frozen yogurt fermented to two endpoints by three recovery methods. Analysis of variance on SAS (Statistical Trend Analysis System, Cary, N.C.) was used to analyze the data.

RESULTS AND DISCUSSION

The Repair Detection Roll-Tube Method (RDRT) offers enhanced recovery of *B. longum* ($p<.05$) compared to the BGA plates and M/G RCA plates at both endpoints (Figure 3-1). The RDRT system recovered cells at levels from one-quarter to one-half log higher than the BGA and M/G RCA plates.

Recovery of *B. longum* on BGA Plates and M/G RCA plates was not significantly different ($p>.05$)for both endpoints each week, with the exception of Endpoint 1, Week 5 data. Furthermore, no decrease in bacterial numbers was observed over the storage period for any of the three methods of recovery. The Repair Detection System provided the cells which were injured an environment and opportunity to repair their injury, thus maximizing bacterial counts. The ingredients in the medium may or may not have been responsible for this increase in recovery; but rather, the method itself may be the reason for enhanced recovery due to the constant anaerobic environment, i.e. from dilution blanks to inoculation.

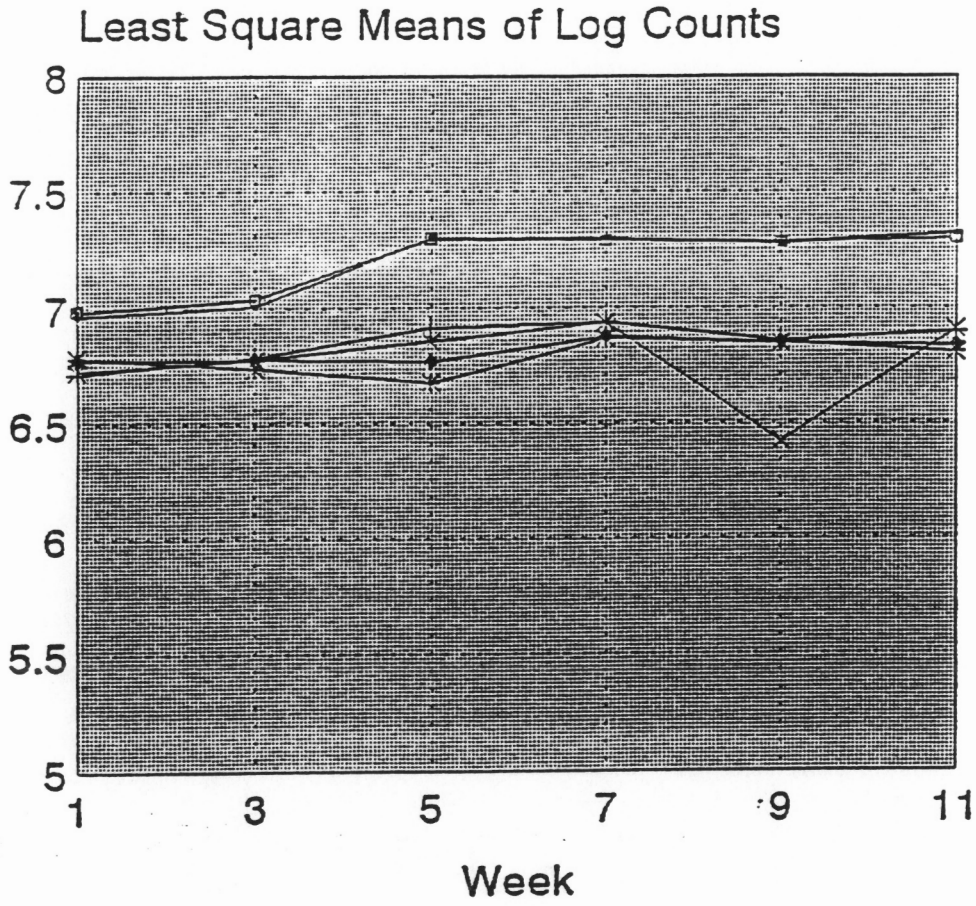

Figure 3-1. Comparison of methods of recovery of *B. longum* from frozen yogurt fermented with to two endpoints and stored for an eleven week period. -·-·- BGA roll tube method, endpoint 1; -|-|- BGA plate method, endpoint 1; -*-*- Maltose/galactose RCA plate method, endpoint 1; -□-□- BGA roll tube method, endpoint 2; -x-x- BGA plate method, endpoint 2; -♦-♦- Maltose/galactose RCA plate method; endpoint 2.

CONCLUSIONS AND FUTURE RESEARCH

The results of this study indicate that the use of the MBGA in conjunction with the Roll Tube Method and Repair Detection System, provides significantly improved bacterial recovery compared to other methods tested. Arany (1992) also determined that the Repair Detection System with Roll Tubes provided superior recovery, in terms of bacterial counts, for enumeration of *Bifidobacterium* ssp. in water and frozen yogurt.

The BGA plates and the M/G RCA plates showed statistically similar results. This indicated that the use of the Repair Detection System without the Roll Tube Method may not provide enhanced recovery of injured cells. Furthermore, these results indicated that the MBGA medium may not be the reason for enhanced recovery of injured cells since no significant difference was observed between MBGA and M/G RCA plates. The most logical conclusion would be that the Roll Tube Method with the Repair Detection System was the key to enhanced recovery of injured cells of *B. longum*. The fact that the MBGA plates and the M/G RCA plates recovered the same number of cells indicates that the method of enumerating cells, itself, may be the reason for cell injury. This may be concluded because the Repair Detection System without roll tubes provided similar recovery results as the M/G RCA without the repair detection system, which may indicate there were no injured cells to recover.

The results of this study indicate that the Repair Detection System in conjunction with the Roll Tube Method was vital to enhanced recovery of injured cells of *B. longum*. However, one area of future study could be the evaluation of M/G RCA medium with the

Roll Tube Method and Repair Detection System for comparison to BGA Roll Tubes and the Repair Detection System. This would allow us to determine if differences in the contents of each medium is the cause of enhanced recovery. This knowledge could shed light on a type of medium which could provide the maximum recovery possible for injured bacterial cells.

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CHAPTER IV

RECOVERY OF BACTERIA, BIOCHEMICAL ANALYSIS AND SENSORY EVALUATION OF FROZEN YOGURT

ABSTRACT

Frozen yogurt was produced using a standard lowfat ice cream mix formulation, fermented with supplemented and traditional starter culture systems and stored for 11 weeks at -20°C. The traditional culture system contained the strains, *Streptococcus salivarius* ssp. *thermophilus* and *Lactobacillus delbrueckkii* ssp. *bulgaricus*. The supplemented system contained the traditional cultures in addition to *Bifidobacterium longum* and *Lactobacillus acidophilus*. The objective of this study was to compare products fermented with the two culture systems to two endpoints for the following characteristics: survival of the culture bacteria, changes in protein, lactose and galactose concentrations and sensory changes.

Culture bacteria in both systems survived well in the yogurt during frozen storage. Lactose and protein levels showed some significant changes or differences between the two culture systems; however, the trends indicated no overall change in levels for products fermented with either culture system during storage. Generally, the galactose levels were significantly higher ($p<.05$) in the traditional culture system fermented to pH 5.6 compared to the supplemented system fermented to the same endpoint.

The manufactured products (supplemented and traditional) were not different from a commercially produced frozen yogurt with respect to flavor intensity of yogurt flavor, vanilla, sweetness and freshness. Acid flavor was usually more intense when product was fermented to pH 5.6. The commercial product was smoother than the manufactured products. The consumer study indicated a "like slightly" response for the supplemented and traditional inoculated frozen yogurts.

The study concluded that the culture bacteria do survive the processing and freezing environment well enough to meet proposed standards of identity for frozen yogurt. The presence of probiotic bacteria in the supplemented system seemed to cause little or no difference in such attributes as protein and lactose levels, and sensory characteristics.

INTRODUCTION

Since it was re-introduced to the marketplace, frozen yogurt has become an immensely popular food. In 1993, for example, sales of frozen yogurt reached $2.8 billion and accounted for over 26% of all frozen dessert sales (Anonymous, 1994). However, without a federal standard of identity, there is no requirement that frozen yogurt have active bacterial cultures.

Traditional yogurt cultures, *Lactobacillus bulgaricus* and *Streptococcus thermophilus,* can be found in frozen yogurt. These bacteria may be present for fermentation of the mix or as an addition to the mix to meet a characteristic of traditional yogurt. Other strains of bacteria, such as *Lactobacillus acidophilus* and *Bifidobacterium longum* are sometimes added to potentially increase the healthful aspects of the product.

All health benefits related to the consumption of yogurt (frozen or otherwise) are not known; however, it is known that if any health benefits can be derived from the food, the bacteria must be viable and active. This makes the accurate recovery of bacterial cells within the yogurt extremely important, since proposed standards of identity call for levels of bacteria at or above $5x10^6$-$7x10^6$ (Tieszen, 1989). The fermentation process itself may be responsible for decreasing bacterial numbers, as was shown by Modler and Villa-Garcia (1993), who demonstrated that the developed acidity (pH 4.47) of frozen yogurt decreases viable numbers of B. *longum*.

Active bacterial cells cause an increase in lactic and acetic acids which denature the quaternary, globular structure of milk protein. Active bacteria which may be present

in frozen yogurt also contain the enzyme β-galactosidase. This enzyme is able to hydrolyze the lactose before the product is consumed, thereby eliminating the unpleasant symptoms associated with lactose malabsorption.

Activity of yogurt bacteria causes specific changes in the chemistry of the product which affect sensory characteristics of the product. Specific compounds such as lactic and acetic acid and acetaldehyde are considered to be very important to certain sensory attributes in frozen yogurt (Tamime and Robinson, 1985). The presence of these products can cause sensory changes which must be evaluated. Lactic acid gives the yogurt its characteristic sharp, acidic or otherwise "nutty" and/or "aromatic" flavor (Tamime and Robinson, 1985). Acetaldehyde, which is produced primarily by *L. bulgaricus,* is the most important aroma compound in yogurt and the level of acetaldehyde should be relatively low to produce a "best or high" rating by a sensory panel (Tamime and Robinson, 1985).

Finally, acceptability of the product to the consumer is another important concept. If the product is not acceptable to the consumer, all production efforts are in vain, since the product will probably never sell. In this study, two culture systems, a system with traditional yogurt cultures and a probiotic-enhanced system, were used to ferment lowfat ice cream mix to two endpoints. The objectives of the project were to evaluate culture survival, lactose hydrolysis resulting from culture activity, and sensory changes in the products stored over 11 weeks at -20°C. Consumer acceptability of the two products fermented to the first endpoint were also determined. Products were not

tested at the second endpoint because this pH would probably be considered to acidic by consumers.

MATERIALS AND METHODS

Source of Bacteria

A culture system containing selected strains of *Bifidobacterium longum, Lactobacillus acidophilus, Streptococcus salivarius* ssp. *thermophilus,* and *Lactobacillus delbrueckki* ssp. *bulgaricus* (BATL-1, Sanofi Bio-Industries, Waukesha, Wisconsin), was used to ferment the frozen yogurt (supplemented product). This system was supplemented with a second culture system (BA-61 Sanofi Bio-Industries, Waukesha, Wisconsin), which contained *Bifidobacterium longum*-6 and *Lactobacillus acidophilus*-1 only, to increase levels of these probiotic bacteria. A third culture system containing only the traditional yogurt cultures, *Lactobacillus delbrueckki* ssp. *bulgaricus* and *Streptococcus salivarius* ssp. *thermophilus* (UltraGro, Sanofi Bio-Industries, Waukesha, Wisconsin) was used to ferment the control product (traditional). Cultures were shipped on dry ice and stored at -80°C in an ultra-low temperature freezer. Cultures were stored for no more than three weeks prior to use in yogurt manufacture.

Frozen Yogurt Manufacture

Raw milk, obtained from the bulk tank at Virginia Polytechnic Institute and State University (VPI&SU) dairy farm, was separated into cream and skim phases in a pilot

plant-scale separator (Elecrem Separator Model 1G, Bonanza Industries, Calgary, Canada). Lowfat ice cream mix (4% fat) was manufactured with 1157.7 g cream, 1198.6 g non-fat dry milk, 12076.4 g skim milk, 1847.8 g granulated sucrose, 1539.1 g corn syrup, and 140.7 g stabilizer. The ice cream mix was mixed until no clumps were present, batch pasteurized at 55°C for 30 min, cooled to 3.3°C and stored for approximately 24 hours.

Cooled mix was heated to 40°C in a hot-water bath prior to inoculation. While mix was heating, containers of yogurt cultures were removed from the freezer and thawed in 25°C water baths until thoroughly liquefied. When the mix reached the desired temperature of 40°C, four, 3246.1 g aliquots were weighed into chlorine-sanitized, stainless steel cans. Mix in two of the containers was inoculated with BATL-1 Culture and BA-61 at .2% of mix weight and .02% of mix weight, respectively and thoroughly mixed. Mix in the remaining two containers was inoculated with the traditional culture system (UltraGro) at .2% of mix weight. All containers were then placed in a 40°C incubator until titratable acidity of mix in a container from each culture treatment was .15% greater than the initial titratable acidity value (endpoint 1). The mix in the remaining two from each culture treatment container was incubated until the pH was 5.6 (endpoint 2).

Frozen yogurt was manufactured in a pilot plant batch freezer (Emory Thompson Freezer 2HSC A, Emory Thompson Machine and Supply Co., New York) with an

overrrun of 55-60%. Product was frozen and stored at -20°C until all analyses were completed.

Enumeration of Bacteria

S. thermophilus was enumerated using M-17 agar. Agar was made of the following (g/l distilled water): phytone peptone 5, polypeptone 5, lactose 5, beef extract 5, yeast extract 2.5, L-ascorbic acid .5, sodium glycerophosphate 19, $MgSO_4*7H_2O$ 1 M Solution 1mL, agar 12. Media was adjusted to pH 5.4, dispensed into 150 mL screw-cap bottles, sterilized (20 psi, 20 min) and stored in the dark until used.

L.. bulgaricus was enumerated using Reinforced Clostridial Agar. The media was pre-prepared in granulated form (BBL Company, Cockeysville, MD) and rehydrated in 1000 mL of distilled water. The media was adjusted to pH 5.4, dispensed into 150 mL screw-cap tubes, sterilized (121°C, 20 psi, 20 min) and stored in the dark until used.

L. acidophilus was enumerated on MRS Broth (BBL Becton-Dickinson Microbiological Systems, Cockeysville, MD) and 1.2% agar. The media was adjusted to pH 5.6, dispensed into 150 mL screw-cap bottles, sterilized (20 psi, 20 min) and stored in the dark until used.

Please refer to Chapter 3, Materials and Methods, for a detailed discussion of *B. longum* enumeration materials.

Biochemical Analysis

The protein content of the frozen yogurt was determined spectrophotometrically (Spectronic 1001 Split Beam Spectrophotometer, Milton Roy Company, Rochester, NY) based on the Bradford method of the Bio-Rad protein assay (Bio-Rad, Hercules, CA). See Appendix A of this thesis for a detailed procedure.

Lactose and D-Galactose levels in the frozen yogurt were determined spectrophotometrically (Spectronic 1001 Split Beam Spectrophotometer, Milton Roy Company, Rochester, NY) based on the methodology for examination of lowfat ice cream in the Boehringer-Mannheim Lactose/D-Galactose Test Kit (Appendix B).

Sensory Evaluation

Panelist Training: Seven panelists, consisting of students and staff at Virginia Polytechnic Institute and State University from the Food Science and Technology Department, were selected based on willingness to participate in this project. Panelists participated in six one-half hour training sessions. During these sessions, product descriptors were selected and defined. Training in identification and intensity ratings of each descriptor was completed. Six attributes (acidity, smoothness, yogurt flavor, freshness, vanilla flavor and sweetness) were selected and defined by group discussion (Appendix C).

Prior to initiating the product evaluation, panelists were tested for the ability to distinguish attributes and repeat measurements. Panelists independently evaluated three

different samples of laboratory-manufactured frozen yogurt products. Two of the products were the same. This was used to determine if panelists could replicate their own judgement. One-ounce samples in plastic souffle cups with lids were coded with 3-digit random numbers. Samples were presented such that each panelist received all samples, one at a time, in a random order. Each sample appeared in every position an equal number of times across all panelists. Samples were stored at -10°C in a counter-top freezer (Arctic Star of Texas model AS3, Arlington, TX) in order to maintain sample integrity during sensory analyses. Panelists evaluated one sample, then waited at least 30 seconds before presentation of the next sample. Panelists rated each product for intensity of each attribute, and marked the perceived intensity, with a hash mark, on 15 cm unstructured line scales with anchor terms (Appendix D). Distance (cm) from the origin to the hash mark was used as a measurement of intensity. Evaluations were completed in individual booths under fluorescent white light. Panelists were instructed to rinse the palate between samples and wait for one min before tasting the next product.

Product Evaluation: Descriptive analyses were completed on five frozen yogurt products. Panelists evaluated each of the four samples of manufactured product (i.e. two endpoints of each of two treatments) and a commercial brand of frozen yogurt (Crowley's Vanilla Frozen Yogurt) for intensity of each of the six attributes. All panelists received the same set of samples, in random order, during one session. Products were presented for evaluation as previously described. Products were

evaluated within 48 hr after manufacture and at two week intervals for an eleven week period.

Consumer Evaluation: Flavor acceptability of vanilla frozen yogurt fermented with supplemented or traditional cultures to the first endpoint was determined. A consumer population (n=50), recruited by the intercept method in the student center at VPI & SU, evaluated both products using a 9-point hedonic scale (1=dislike extremely and 9=like extremely). Consumers were asked to evaluate the product based on how well they liked the flavor. Each participant was seated at an individual table isolated from the instruction area, during the evaluation. Each participant also completed a demographic questionnaire which included several questions regarding knowledge and attitude toward frozen yogurt.

Statistical Analysis

The complete experiment was replicaed twice. Data analysis was conducted on least square means of the values using a general linear model procedure. A model was developed to evaluate differences in bacterial recovery, biochemical analysis and sensory values from frozen yogurt fermented to two endpoints. A complete block design was used for analysis using SAS (Statistical Analysis System, Cary, N.C.) to analyze the data.

RESULTS AND DISCUSSION

Bacterial Survival

Assessment of bacterial survival in frozen yogurt is important for the implications this may have on product quality, meeting proposed regulations for frozen yogurt identity, and the potential health aspects. Comparisons were made to determine the effect of fermentation to two different endpoints by a traditional culture system (*Lactobacillus bulgaricus* and *Streptococcus thermophilus)* and a culture system with traditional cultures supplemented with *Bifidobacterium longum* and *Lactobacillus acidophilus*. The fermentation proceeded to the suggested minimum for proposed standards of identity, i.e. increase of titratable acidity of .15% above the initial titratable acidity, and to the second endpoint of pH 5.6. The second endpoint was established as a reference to determine effects of increased acidity, beyond proposed standards, on bacterial survival and other attributes. Figures 4-1 and 4-2 compare frozen yogurt fermented with the two culture systems on recovery of the traditional fermentation organisms, *L.bulgaricus* and *S. thermophilus*, respectively, found in both culture systems. Week seven recovery of *L.bulgaricus* was the only week which showed a significant difference in the results. Week five data was not significantly different due to a high degree of variation of the counts which caused overlap of the standard errors. The supplemented system had significantly higher recovery of this bacteria during week seven at the first endpoint only. The *S. thermophilus* supplemented system had a significantly higher level of recovered cells during weeks 5-11 for both endpoints.

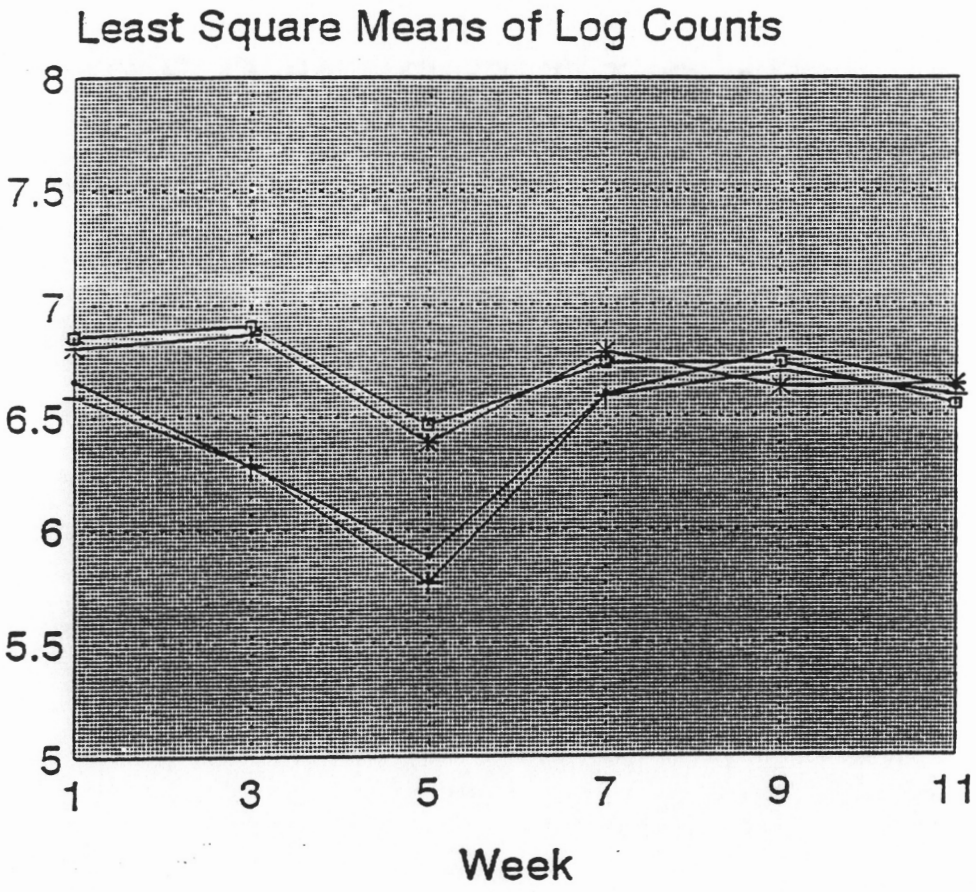

Figure 4-1. Recovery of *Lactobacillus bulgaricus* from frozen yogurt fermented with two different culture systems to two endpoints and stored for an eleven week period. -·-·- Traditional system, endpoint 1; -|-|- Traditional system, endpoint 2; -*-*- Supplemented system, endpoint 1; -□-□- Supplemented system, endpoint 2.

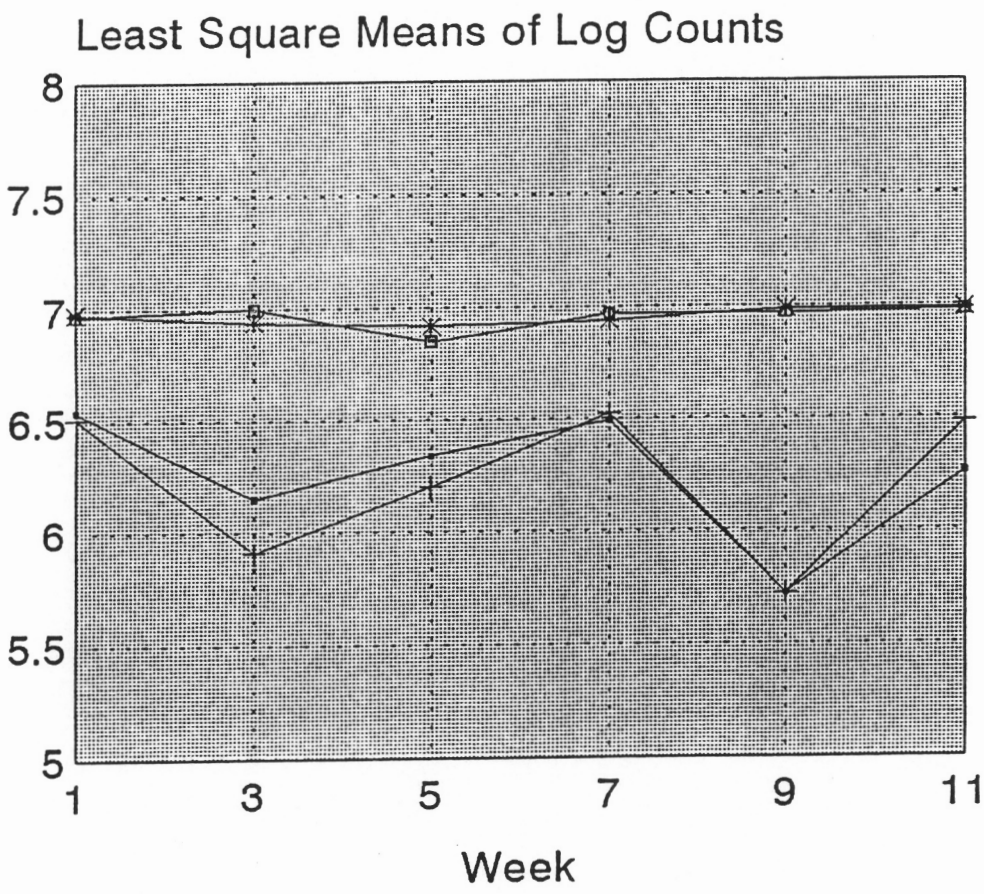

Figure 4-2. Recovery of *Streptococcus thermophilus* from frozen yogurt fermented with two different culture systems to two endpoints and stored for an eleven week period. -·-·- Traditional system, endpoint 1; -|-|- Traditional system, endpoint 2; -*-*- Supplemented system, endpoint 1; -□-□- Supplemented system, endpoint 2.

Thompson and Mistry (1994) studied the effects of cold-storage on survival of *L. bulgaricus* and *S. thermophilus* in frozen yogurt and found no significant decrease in bacterial numbers. In contrast, Miles and Leeder (1981) found that *L. bulgaricus* and *S. thermophilus* levels decreased an average of one-and-one-half and one-half logs, respectively, when stored for two weeks at -28.9°C in frozen yogurt.

Increased acidification, when fermentation was allowed to proceed to the second endpoint, had no significant effect of the recovery or survival of *L. bulgaricus* (Fig. 4-1), *S. thermophilus* (Fig. 4-2) *or L. acidophilus* (Figure 4-3). Hekmat and McMahon (1992) found that *L. acidophilus,* inoculated in a standard ice cream mix and allowed to ferment, exhibited a two log decrease when stored at -29°C. Holcomb (1991) observed no evidence of freeze injury to *L. acidophilus* when exposed to -5°C temperatures for six hr.

There was no difference ($p>.05$) in log counts of *Bifidobacterium* in the frozen yogurt fermented with the supplemented culture system to the two endpoints (Figure 3-1). Hekmat and McMahon (1992) observed a one log loss in *B. longum* cells in frozen yogurt when stored at -29°C for seventeen weeks. Modler and Villa-Garcia (1993) reported a two log loss in *B. longum* levels due to acidification of frozen yogurt due to fermentation and re-freezing Finally, Holcomb et al., (1991) observed no decrease in *B. longum* levels in frozen yogurt which was frozen at -5°C for six hr.

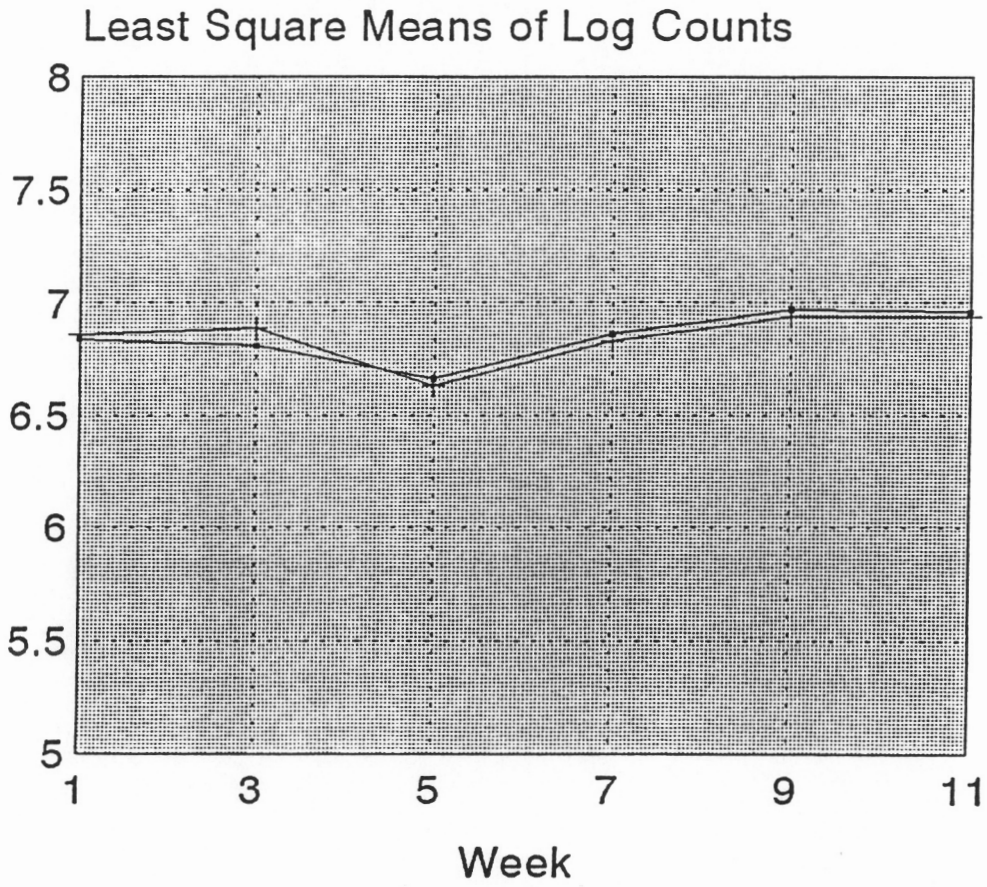

Figure 4-3. Recovery of *Lactobacillus acidophilus* from frozen yogurt fermented with two different culture systems to two endpoints and stored for an eleven week period. -·-·- Supplemented system, endpoint 1; -|-|- Supplemented system, endpoint 2.

Biochemical Changes

Culture bacteria utilize lactose as an energy source. Lactose is hydrolyzed to its component monosaccharides, glucose and galactose, which can then be used for energy by the bacterial cells. The cleavage of lactose makes this sugar digestible to those who are lactose intolerant, which means that they lack the β-galactosidase enzyme. Inability to cleave lactose leads to unpleasant physical symptoms for those affected (Lerebours, 1989).

Lactose levels were monitored in all products to determine if additional organisms (supplemented culture) increase lactose hydrolysis (Figure 4-4). Lactose levels were also evaluated to determine if the increase in acidity (i.e.: two endpoints) would cause a significant change in the lactose levels in the frozen yogurt samples. Differences between the traditional and supplemented systems were observed during weeks five and nine of storage. Generally, however, there were no significant difference due to the difference in levels of acidity. Thompson and Mistry (1994) observed no significant changes in lactose levels in frozen yogurt mix when frozen-stored for one and three months. Gilliland and Kim (1984) found that lactose levels decreased from 6.26% in uninoculated yogurt mix, to 4.23% in inoculated. It appears that the initial activity of yogurt bacteria in fermentation is what causes a decrease in lactose levels.

Galactose is an end product of lactose hydrolysis. Much greater levels of galactose were observed at the second endpoint in frozen yogurt fermented with the

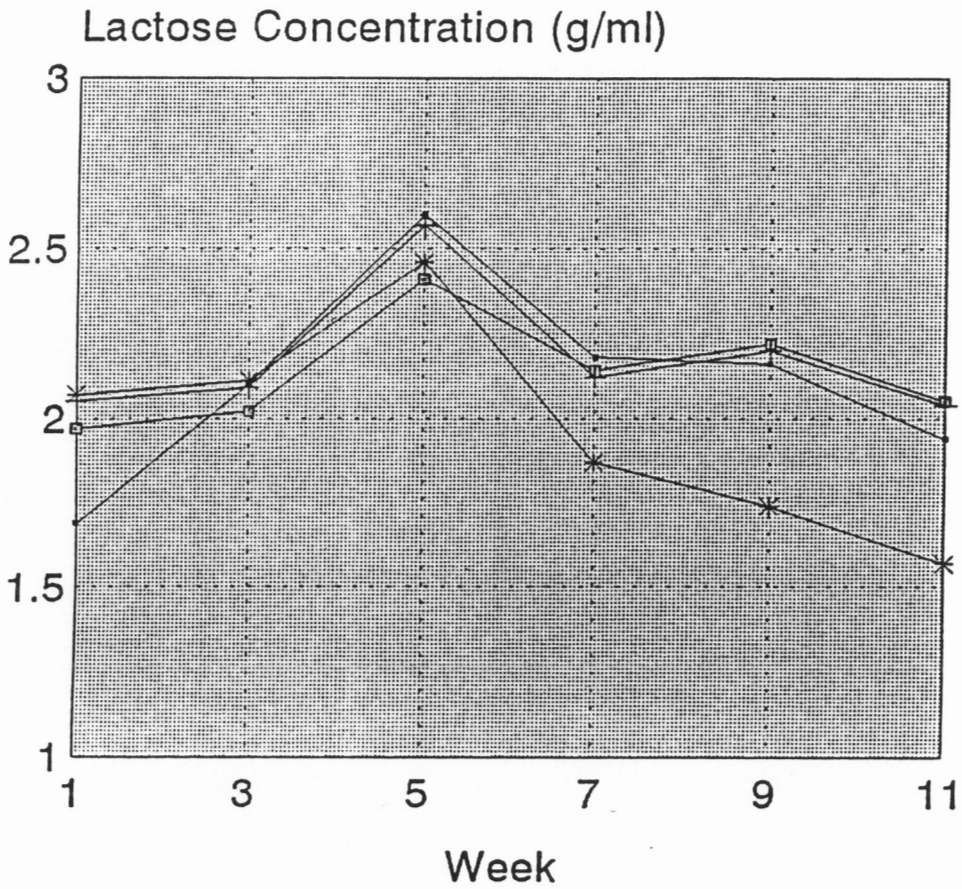

Figure 4-4. Lactose concentration in frozen yogurt fermented with two different culture systems to two endpoints and stored for an eleven week period. -·-·- Supplemented system, endpoint 1; -|-|- Supplemented system, endpoint 2; -*-*- Traditional system, endpoint 1; -□-□- Traditional system, endpoint 2.

traditional culture system (Figure 4-5) than the supplemented system for weeks five, seven and nine at endpoint two.

Protein levels were also evaluated using the same variables as galactose and lactose (Figure 4-6). The results indicated no significant differences in the protein levels when comparing culture systems at the same endpoint or the same culture system at both endpoints.

Sensory Evaluation of Frozen Yogurt

The fermentation process produces certain compounds such as acetaldehyde and lactic acid which produce different sensory characteristics. The attributes which were selected for evaluation were based on those attributes suspected to be influenced by the treatments. Therefore a complete sensory description was not obtained.

The commercially-produced brand of frozen yogurt received significantly higher scores for smoothness (Figure 4-7). The two culture systems compared to each other, at both endpoints, had scores which were not significantly different from each other. The manufactured products had mean values near the middle of the scale whereas the mean for smoothness of the commercial product was approaching the "very smooth" end of the scale. Although not statistically analyzed, the manufactured product did not generally change in perceived smoothness over time. The pilot plant facility where production took place did not have a blast freezer. Since the frozen yogurt had to be frozen slowly

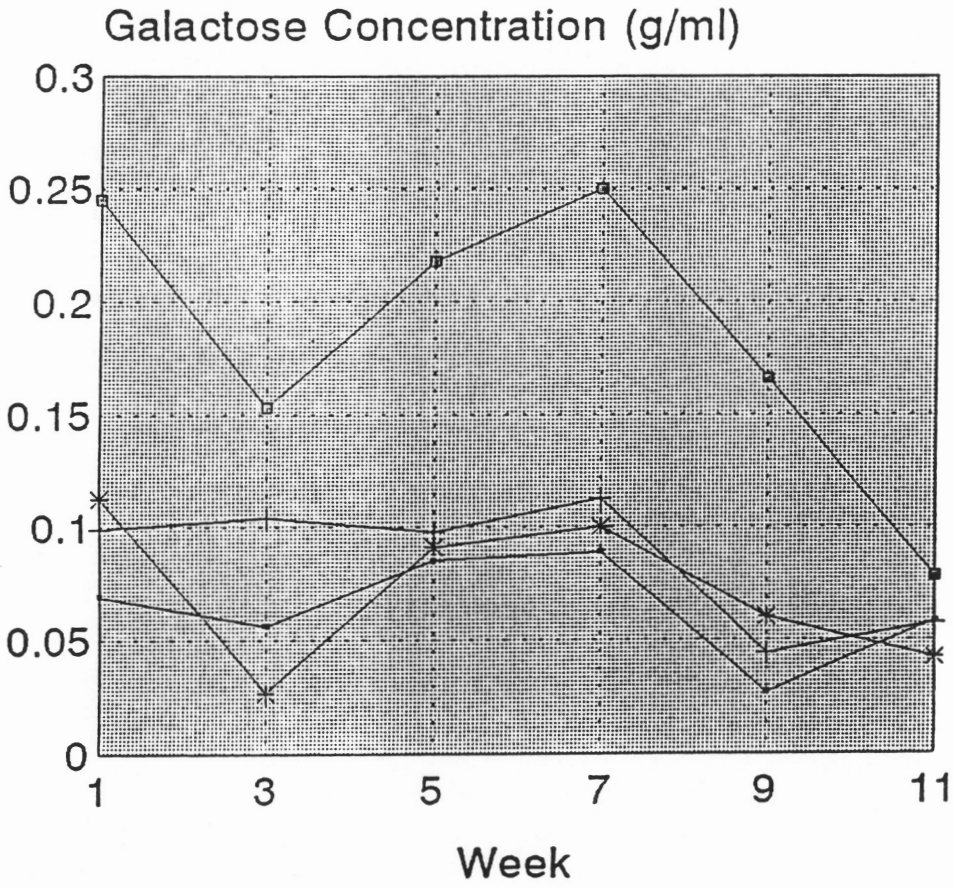

Figure 4-5. Galactose concentration in frozen yogurt fermented with two different culture systems to two endpoints and stored for an eleven week period. -·-·- Supplemented system, endpoint 1; -|-|- Supplemented system, endpoint 2; -*-*- Traditional system, endpoint 1; -□-□- Traditional system, endpoint 2.

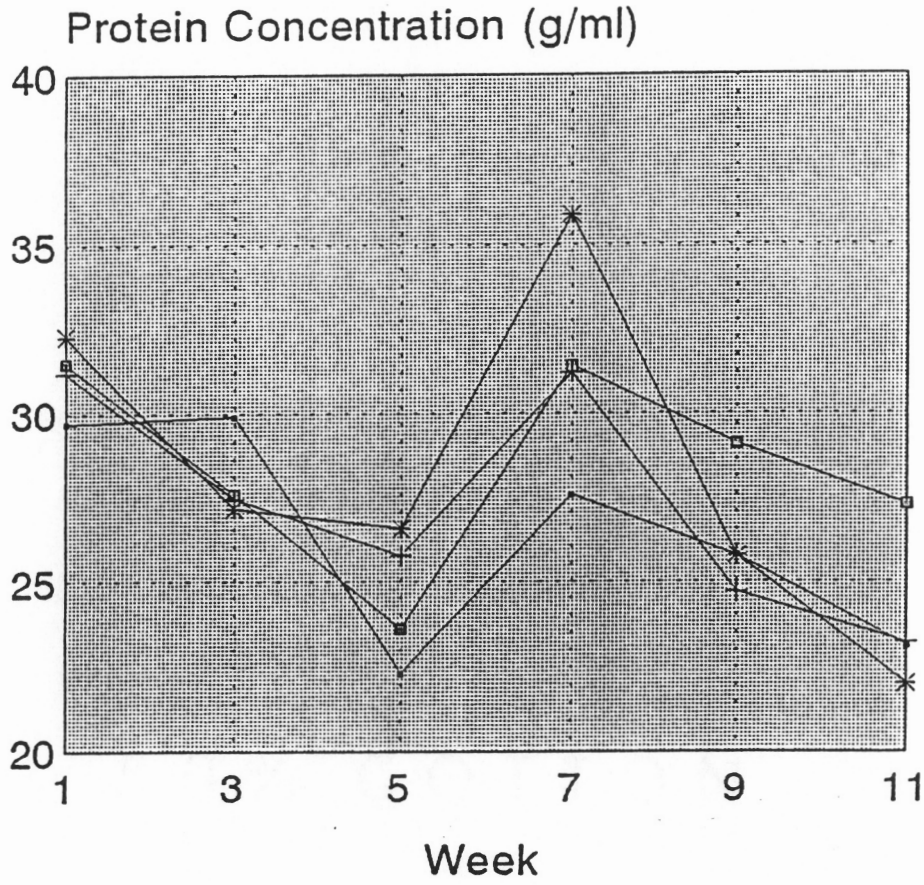

Figure 4-6. Protein concentration in frozen yogurt fermented with two different culture systems to two endpoints and stored for an eleven week period. -·-·- Supplemented system, endpoint 1; -|-|- Supplemented system, endpoint 2; -*-*- Traditional system, endpoint 1; -□-□- Traditional system, endpoint 2.

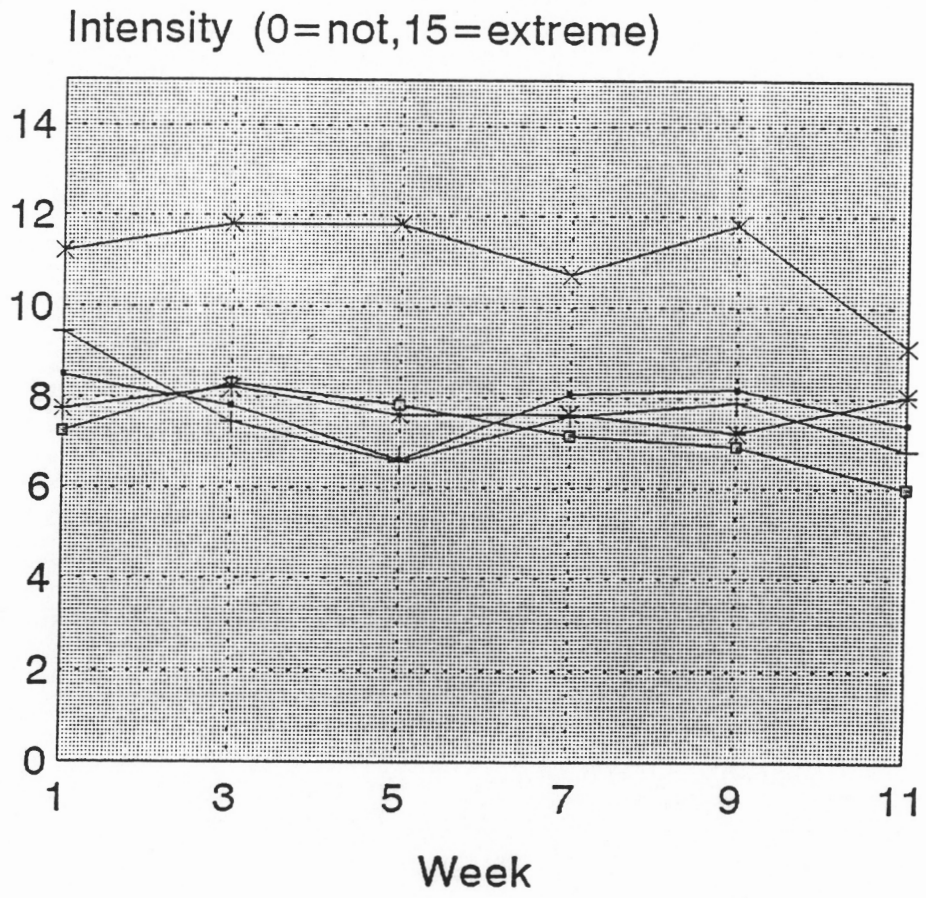

Figure 4-7. Smoothness intensity in frozen yogurt fermented with two different culture systems to two endpoints and stored for an eleven week period. -·-·- Supplemented system, endpoint 1; -|-|- Supplemented system, endpoint 2; -*-*- Traditional system, endpoint 1; -□-□- Traditional system, endpoint 2; -x-x- Commercial product.

this would account for some of the ice crystal formation which resulted in lower smoothness scores.

There were no differences ($p>.05$) in perceived intensity of vanilla flavor between treatments each week (Figure 4-8). The scores were generally in the middle of the scale for overall intensity of this attribute. In general, the scores appeared to decrease slightly over the course of this study. The commercially-produced product showed the largest decrease in perceived intensity of vanilla flavor.

There were no significant differences in sweetness when intensity of manufactured and commercial products were compared (Figure 4-9). Perceived acidity in the products corresponded to the development of acidity during the fermentation (Figure 4-10). However, the second endpoint of the supplemented system showed significantly higher scores, approaching "extreme", compared to the other treatments. The second endpoint of the traditional treatment also had higher acidity scores than the products fermented to the first endpoint, although statistical significance was observed only on a few occasions. The products fermented to the first endpoint had low to moderate acid flavor. The commercially produced frozen yogurt was consistently evaluated to be the lowest-acid product.

There were no differences in perceived intensity of yogurt flavor among products (Figure 4-11). Generally, however, the product fermented with supplemented cultures and the commercial product had less yogurt flavor than the other products. The product

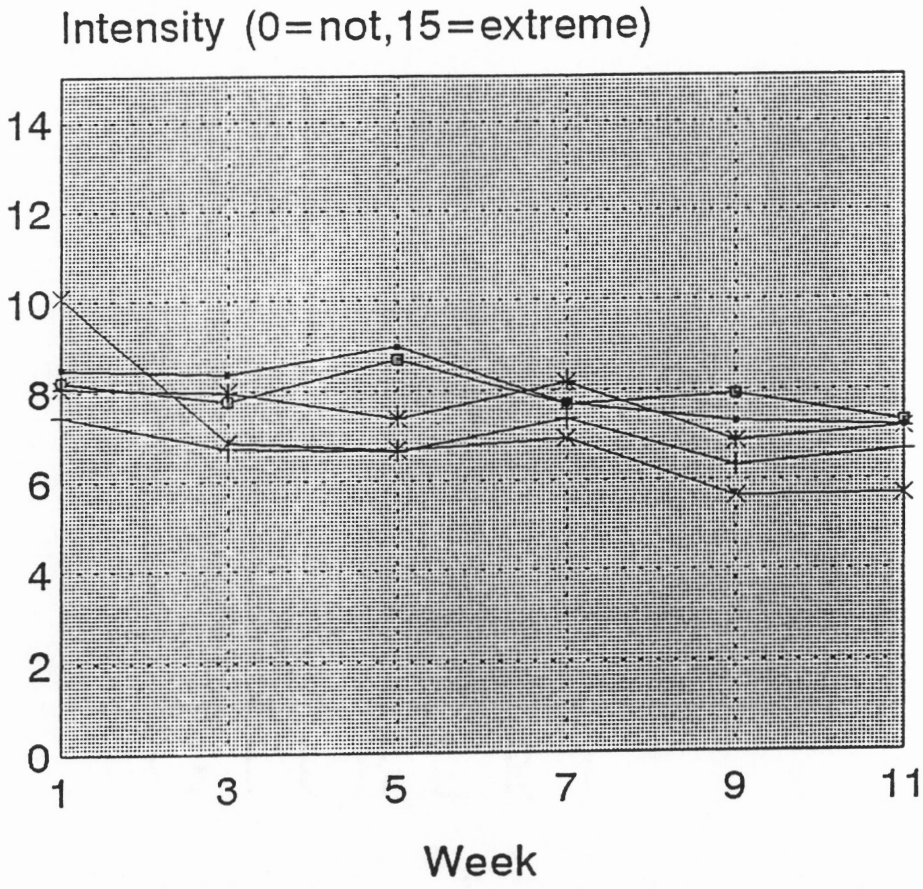

Figure 4-8. Vanilla flavor intensity in frozen yogurt fermented with two different culture systems to two endpoints and stored for an eleven week period. -·-·- Supplemented system, endpoint 1; -|-|- Supplemented system, endpoint 2; -*-*- Traditional system, endpoint 1; -□-□- Traditional system, endpoint 2; -x-x- Commercial product.

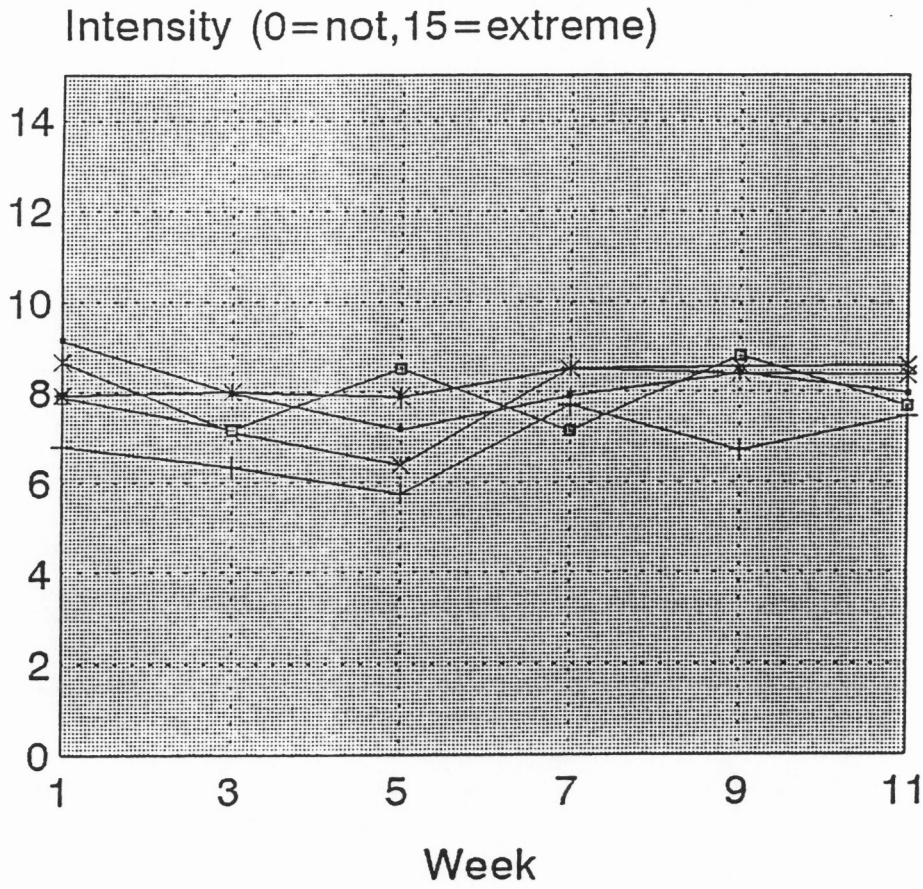

Figure 4-9. Sweetness intensity in frozen yogurt fermented with two different culture systems to two endpoints and stored for an eleven week period. -·-·- Supplemented system, endpoint 1; -|-|- Supplemented system, endpoint 2; -*-*- Traditional system, endpoint 1; -□-□- Traditional system, endpoint 2; -x-x- Commercial product.

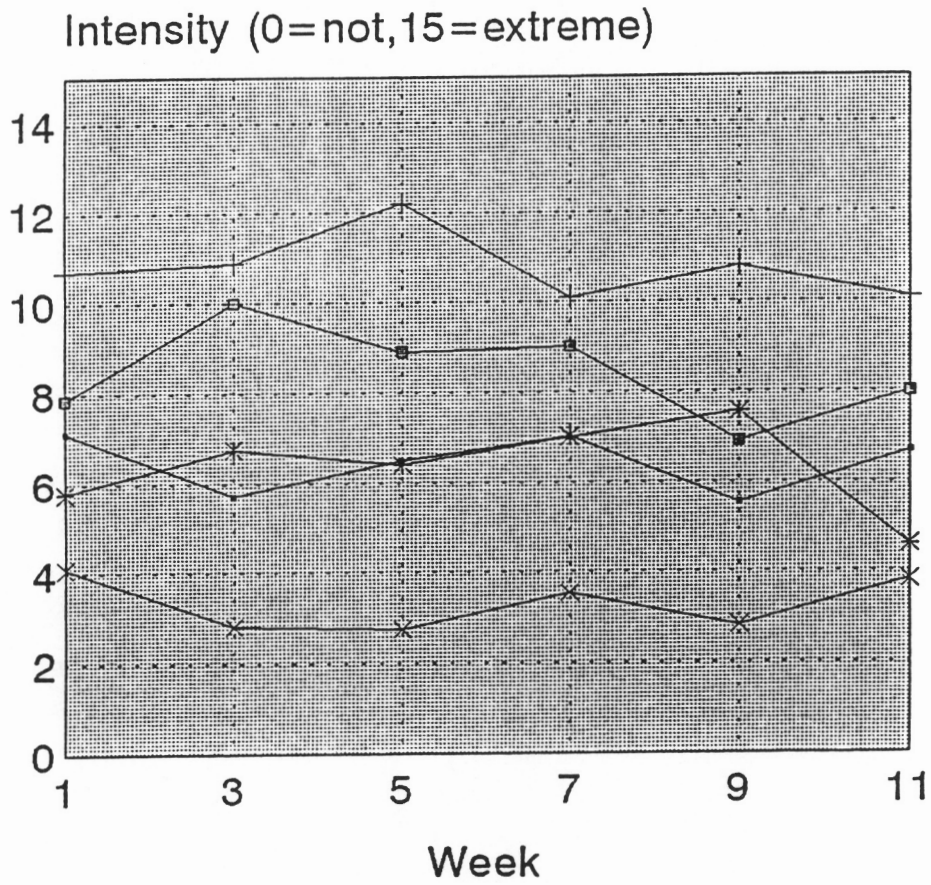

Figure 4-10. Acid flavor intensity in frozen yogurt fermented with two different culture systems to two endpoints and stored for an eleven week period. -·-·- Supplemented system, endpoint 1; -|-|- Supplemented system, endpoint 2; -*-*- Traditional system, endpoint 1; -□-□- Traditional system, endpoint 2; -x-x- Commercial product.

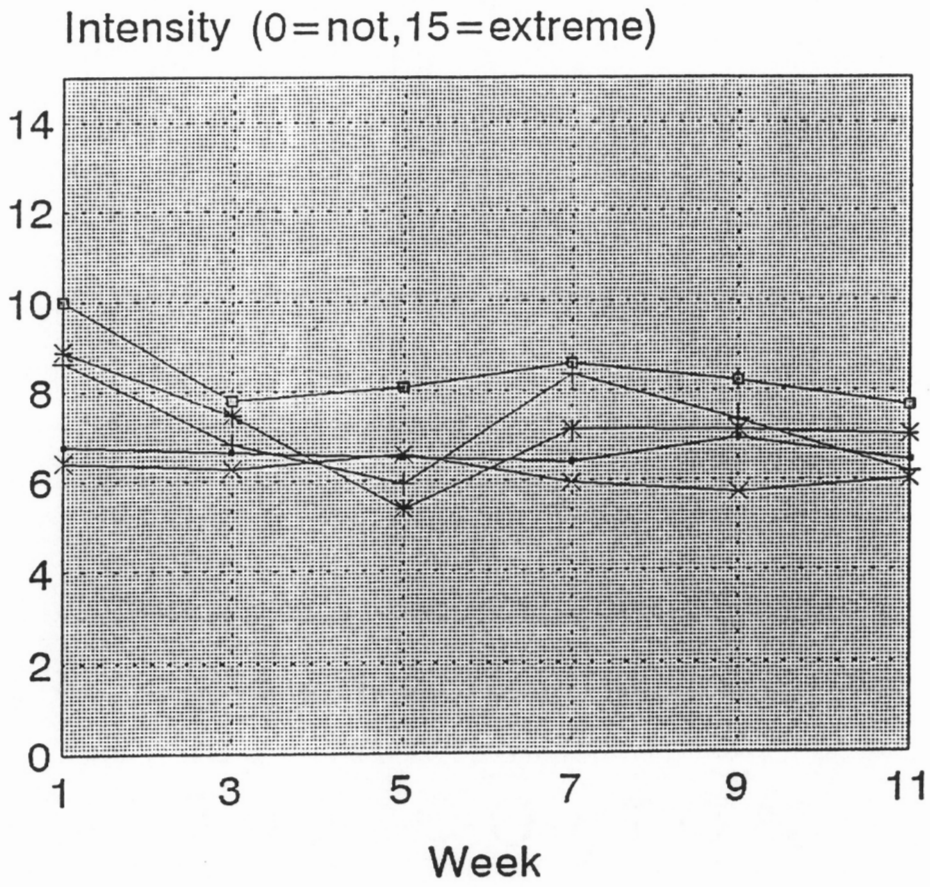

Figure 4-11. Yogurt flavor intensity in frozen yogurt fermented with two different culture systems to two endpoints and stored for an eleven week period. -·-·- Supplemented system, endpoint 1; -|-|- Supplemented system, endpoint 2; -*-*- Traditional system, endpoint 1; -□-□- Traditional system, endpoint 2; -x-x- Commercial product.

fermented with the traditional culture to the second endpoint had consistently higher levels of yogurt flavor.

Freshness scores showed no significant difference between the treatments (Figure 4-12). However, the freshness scores did show a general decline over the course of evaluation. The commercially-produced yogurt experienced the largest decline in overall freshness, and the second endpoint of the traditional culture system had the smallest decrease in scores. The decline in freshness of commercial product may be explained by the product being reused from the same container throughout the course of the study.

Kneifel (1992), studied 47 commercially available yogurts and starter cultures using a sensory panel. Sensory evaluation resulted in acidity being rated the most important attribute, in terms of perceived flavors, in frozen yogurt.

Speck (1983) determined that frozen yogurts with the lowest titratable acidity (.28%-.38%) received that highest overall quality scores. The study also determined that of all flavors tested, vanilla, chocolate and coffee, all would receive higher quality scores if the acidity was kept relatively low.

Hauge (1981) found that of 33 flavor ingredients tested using organoleptic assessment panels, coffee, black currant, apple, cloudberry, grapefruit, pear and banana/vanilla flavors were found to be unsuitable in frozen yogurt.

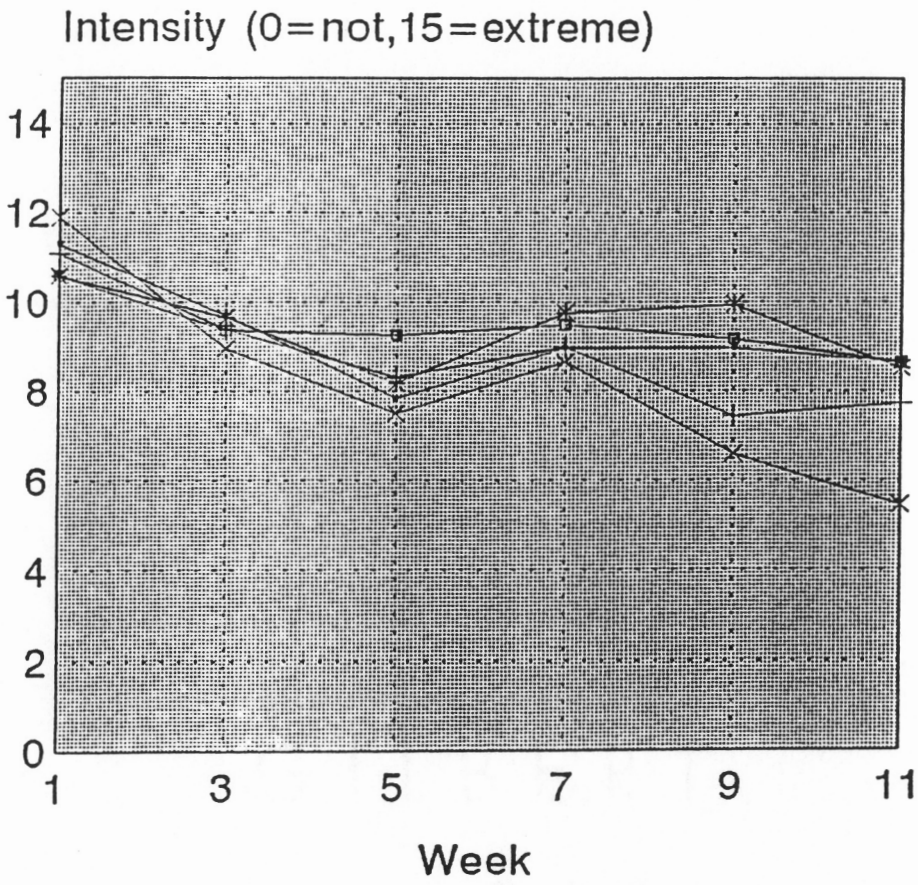

Figure 4-12. Freshness intensity in frozen yogurt fermented with two different culture systems to two endpoints and stored for an eleven week period. -·-·- Supplemented system, endpoint 1; -|-|- Supplemented system, endpoint 2; -*-*- Traditional system, endpoint 1; -□-□- Traditional system, endpoint 2; -x-x- Commercial product.

Consumer Study

The results of the consumer study indicate that, of those who sampled both types of frozen yogurt, no significant difference was noted in overall preference. Both frozen yogurt samples received scores of "like slightly". The reported scores for the supplemented system fermented to the first endpoint averaged to 6.76 +/-1.92 and the traditional system fermented to the first endpoint scored 6.75 +/-1.72 on a 9-point hedonic scale (9=like extremely, 1=dislike extremely).

The demographics indicated that most of the panelists were collegiate males under the age of 21. Most consume frozen yogurt once a month, are not familiar at all with types and quantities of bacteria in frozen yogurt today, do not consider the presence of active bacteria in frozen yogurt to be important at all, and would not be affected by the knowledge that the frozen yogurt they consumed met proposed standards for bacterial levels (Table 4-1).

Table 4-1.Panelist[1] Responses to Questionnaire to Determine Consumption Frequency, Knowledge of Frozen Yogurt Bacteria and Perceived Importance of Bacterial Presence in Frozen Yogurt

Question: How often do you consume/purchase frozen yogurt?

More than once a week	Once a week	Once a month	Once a year	Never
2%	14%	59%	18%	6%

[1] Of those who sampled products and answered the questionnaire, n=49

Question: How familiar would you consider yourself to be with the types and/or quantities of bacteria contained in frozen yogurt produced today?

Extremely familiar	Moderately familiar	Not familiar at all
0%	10%	90%

[1] n=49

Question: How important do you consider the presence of live bacteria to be in frozen yogurt?

Extremely important	Moderately important	Not important at all
21%	38%	40%

[1] n=47

Question: How would the knowledge that frozen yogurt contained live bacteria, at levels which met proposed government standards for frozen yogurt, affect how often you consumed/purchased frozen yogurt?

Effect strongly	Effect moderately	Effect slightly	Would have no effect at all
9%	28%	23%	40%

[1] n=47

CONCLUSIONS AND FUTURE RESEARCH

The observed effects of cold-storage on the survival of the bacteria appeared to be minimal. The fermentation process, itself, seemed to cause a decrease in the levels of bacteria, initially. The effects of increased acidification appeared to be minimal for all strains of bacteria, with the exception of *Streptococcus salivarius* ssp. *thermophilus* which appeared to survive at higher levels in the supplemented system. The exact reason for this is unknown; however, the supplemented probiotics may have a "growth stimulating" effect on this particular strain of bacteria due to some biochemical interactions. The results also imply that the *Bifidobacterium* and *Lactobacillus* are capable of surviving a frozen storage period of up to three months.

The results of the biochemical tests indicate that the lactose and galactose levels are highly variable. The reason for this is unclear; however, human and test kit error cannot be ruled out. The lactose levels should have decreased over the course of the study; they did not. Also, the galactose levels should have increased due to hydrolysis of the lactose. Some trends were noted; however, the results generally indicated that the galactose levels reported may be unreliable.

Protein levels did show a general decrease during the course of the study, which would be expected since *Lactobacillus bulgaricus* provides *Streptococcus thermophilus* with essential amino acids through proteolysis of casein.

Overall, the manufactured products were not different from the commercial product with respect to flavor intensity of yogurt flavor, vanilla, sweetness and freshness.

Acid flavor was usually more intense when product was fermented to a pH of 5.6. The commercial product was more smooth than the manufactured product. College-age consumers liked the product moderately well but were generally not concerned with the health-related aspects associated with live bacteria in frozen yogurt.

Frozen storage of the yogurt, fermented at levels suggested to promote health, seems to cause little change in biochemical or sensory quality of the product. Furthermore, the activity of the traditional and/or probiotic organisms does not seem to have a deleterious effect on either biochemical or sensory quality.

The results of this study indicate a need to monitor the activity of the ß-galactosidase enzyme more thoroughly and carefully in order to determine the accuracy of certain tests designed to directly or indirectly monitor its activity. The survival of the bacteria could also be monitored again to determine the statistical significance of some of the findings in this study.

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Appendix A

Bio-Rad Protein Assay

Materials:

Frozen Yogurt Sample

Concentrated Dye Reagent
Protein Standard- Bovine Serum Albumin

Methods:

1. Dilute dye reagent 1:4 with distilled water.

2. Filter dye reagent through moistened Whatman's #1 filter paper.

3. Filtrate will be used for the assay

4. Number test tubes- 0, .2, .4, .6, .8 standards.

5. Prepare standard curve dilutions according to quantity of protein in the BSA used. The numbers in step 4 correspond to final protein concentrations in units of mg/mL

6. Prepare frozen yogurt sample after it has thawed by placing 1mL of the sample in a 10mL volumetric flask. Fill the flask up with distilled water to the fill line. This is a 1:10 dilution.

7. Take .5 mL of the sample from the volumetric flask and mix it with 4.5 mL of distilled water in a test tube. This gives a 1:100 dilution.

8. Place 0.1 mL of the diluted samples and standard curve solutions into labeled test-tubes.

9. Add 5mL of filtered dye reagent to each test-tube, cap and vortex.

10. Incubate for 5 min to one hour at room temperature. (Absorbance will increase over time.)

11. Measure absorbance at 595 nm.

Appendix B

Boehringer-Mannheim Assay

Materials:

-12% Trichloracetic acid
-610 mg lyophilisate consisting of citrate buffer, pH 6.6; NAD, --35 mg; magnesium sulfate; stabilizers
-1.7 mL beta-galactosidase suspension
-potassium diphosphate buffer, pH 8.6
-1.7 galactose dehydrogenase suspension
-1 Normal Sodium Hydroxide solution

Methods:

1. 2 mL of liquid yogurt sample were added to 20 mL of 12% Trichloracetic acid.

2. Samples were mixed and placed in large centrifuge tubes.

3. Tubes were spun at 6000 RPM in a Sorvall Refrigerated Superspeed Centrifuge for 20 min. 10 mL of clear supernatant from each sample were placed in separate 50 mL beakers

5. Samples were adjusted to pH of approximately 7.0 +/- .2 using 1 normal and 1/10 normal sodium hydroxide solution.

6. Once pH was adjusted, the samples were brought up to a volume of 25 mL using distilled water.

7. This sample was used in the Boehringer-Mannheim assay.

8. See photocopied assay outline.

Appendix C

Definitions of Attributes

Acidity: early taste which is noticed on the sides, tip and back of tongue. May produce a "dry" feeling on the tongue. A sour flavor

Smoothness : lack of granular texture, mouth-feel is of even consistency, with no "crunching" if the sample is chewed rather than allowed to melt in the mouth.

Yogurt Flavor: fermented flavor; "cheese-cake" taste

Sweetness: candy-like flavor, sensation is like cake-icing sweetness

Freshness: doesn't have "freezer" flavor; lacks stale-air taste; "cardboard", "old" flavor.

Vanilla: has flavor like vanilla beans, can be easily tasted and smelled. "Alcohol" type taste can be sensed when excessive amounts of vanilla are used.

Appendix D

Sample Scorecard

Date_____

Panelist Name _____

Sample Number _____

Please taste the product presented to you. Evaluate it for the attributes listed. Mark the intensity of each attribute by placing a hash mark at the appropriate location on the line. Continue until all attributes have been scored. You may retaste the sample as many times as necessary.

ACIDITY

_____

none extreme

SMOOTH

_____

None extreme

YOGURT FLAVOR

_____

none extreme

SWEETNESS

_____

none extreme

FRESHNESS

_____

not extreme

VANILLA FLAVOR

_____

none extreme

Pass the sample and scorecard through the hatch and wait for the next sample. Rinse your palate with water during this time. Please rest at least 1 min between each sample. You have ____ more samples to taste today.
Additional comments about the product:

Appendix E

Babcock Test for Fat

Materials:
-Garver Babcock centrifuge
-Hot water bath
-Concentrated sulfuric acid
-Cream sample
-Garver Babcock Bottle Shaker

Methods:

1. Turn centrifuge, water bath warm water source on 45 min prior to use.

2. Weigh out 9 grams of sample into a weigh boat. Add 9 grams distilled water.

3. Measure 17.5 mL of concentrated sulfuric acid into beakers.

4. Add the sulfuric acid into the Babcock bottles containing the cream. It is important to add the sulfuric acid to the sample in a stepwise fashion; i.e. add half the volume of the acid to the sample, point beaker away from yourself and swirl gently, then add half of the remaining sample of sulfuric acid and repeat until all sulfuric acid is added. Note: It is important to add all of the sulfuric acid within 30 seconds.

5. Place sample bottles in the Garver shaker for 5 min.

6. Place Babcock tubes into Garver Babcock Centrifuge. Be sure to place bottles on directly opposite ends of the centrifuge and place in the outside row. Centrifuge for 5 min.

7. Remove from centrifuge. Add warm water until volume of mixture reaches the bottom of the neck of the Babcock tube.

8. Centrifuge for 2 min

9. Add warm water until 3/4 of neck of bottle is full.

10. Centrifuge for 1 min

11. Place in water bath for 5 min

12. Measure fat content percent using the measuring calipers.

VITAE

Richard Harry Davidson, Jr. was born on the 3rd day of April, 1970 in Trenton, New Jersey. He graduated from North Hunterdon Regional High School in Annandale, New Jersey, June 1988. During the Fall of 1988, he began studies at Virginia Polytechnic Institute and State University in Blacksburg, Virginia and graduated in December of 1992 with a B.S. in Biology. In January of 1993, he began work towards a Master of Science degree in Food Science at Virginia Polytechnic Institute and State University.