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FACTORS THAT INFLUENCE VIABILITY
OF BIFIDOBACTERIA IN MILK

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FACTORS THAT INFLUENCE VIABILITY OF BIFIDOBACTERIA IN MILK

BY

Han-Seung Shin

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ABSTRACT

FACTORS THAT INFLUENCE VIABILITY OF BIFIDOBACTERIA IN MILK

By

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The overall goal of this research was to investigate the viability of bifidobacteria in several commercial dairy products and enhance growth and viability of bifidobacteria in milk to be consistent with clinical studies on health benefits. In the first part of the study, viability of bifidobacteria in commercial A/B milk (containing both bifidobacteria and *Lactobacillus acidophilus*) and two brands of yogurt was investigated. The viability of bifidobacteria in these products maintained at 10^6 cfu/ml or g during refrigerated storage.

The second part of this study involved investigating the effect of oligosaccharides and inulin on growth and viability of selected bifidobacteria strains. Effect of *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* in lowering redox potential to enhance growth and viability of bifidobacteria co-cultured with these organisms was also studied. FOS was most effective ($p<0.05$) in enhancing growth and viability of bifidobacteria investigated whereas inulin was the least effective. Growth of bifidobacteria was more affected by the pH of the system than redox potential.

The third part of this study involved conditions identified above in the manufacture of yogurt. The initial bifidobacteria counts in yogurt were well above $>10^8$ cfu/g. The viability of bifidobacteria in yogurt manufactured with our condition were higher ($p<0.05$) than yogurt manufactured according to the manufacturer directions with two commercial starter culture blends.

Dedicated to My parents, Dr. Hyosun Shin and Dr. Yangja Yoo

and my sisters, Haeseung and Yeonseung

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I. INTRODUCTION

Bifidobacteria are inhabitants of the human intestine and well adapted for metabolism in the gastrointestinal tract of humans. Bifidobacteria were first isolated from feces of breast-fed infants in 1899 by Tissier at the Pasteur Institute in Paris (Tamine *et al.*, 1995). Bifidobacteria has received much attention recently due to studies on their health promoting effects. Thus, there is an increasing interest in incorporating bifidobacteria into foods, particularly dairy products. Currently, There are more than 70 dairy products containing bifidobacteria produced world wide (Ventling and Mistry, 1993).

Some health benefits of bifidobacteria include maintaining a normal intestinal microflora balance (Yoshioka *et al.*, 1983), improving lactose tolerance of milk products (Gilliland, 1989), promoting anti-tumorigenic activity (Fernandes and Shahani, 1990), reducing serum cholesterol levels (Homma, 1988), and synthesis of β-complex vitamins (Hughes and Hoover, 1991).

Dairy products have been used as a medium to reintroduce viable populations of bifidobacteria into the GI tract of both children and adults (Hughes and Hoover, 1991). Maintaining viability of bifidobacteria in the carrier food prior to consumption is thought to

be necessary for their health promoting effect (Samona and Robinson, 1991).

Several clinical studies have shown significant benefits, which were observed upon ingestion of approximately 10^9-10^{10} organisms/d (Sanders *et al.*, 1996). However, viability of bifidobacteria in dairy products has been very variable. Because of the low pH of fermented dairy product and aerobic conditions of production of dairy products, viability of bifidobacteria in dairy products has not been satisfactory (Dinakar and Mistry, 1994). Many published studies on viability of bifidobacteria were not done using commercial strains, or strains that have been shown to provided health benefits. In addition, several growth promoting factors investigated for bifidobacteria are not food grade or approved for use in dairy products and they can not be incorporated into commercial dairy products. Thus, these studies are not directly relevant to the dairy industry. The purpose of my study was to investigate the viability of bifidobacteria in commercial dairy products and determine factors that enhance and maintain viability that are commercially feasible using strains of bifidobacteria and lactic acid bacteria that may have positive health benefits.

II. LITERATURE REVIEW

2.1 Fermented dairy products and lactic acid bacteria

Milk is an excellent medium to support the growth of many microorganisms and to produce numerous fermented dairy products. Fermented milks, like yogurt, were available thousands of years ago, and recently there has been increasing interest in consumption of fermented dairy foods (Mutukumira, 1995). Fermented milk products have several important advantages, such as a means of preserving food, providing better taste, increasing digestibility, allowing for production of a variety of foods, and providing several health benefits (Kroger *et al.*, 1989; Marshall, 1993). Milk from domestic mammals such as cows, buffalo, sheep, goats, horses, camels, and yaks has been used to make traditional fermented milk products around the world, which include a variety of cheeses, butter milk, kefir, yogurt, kumiss, taette, acidophilus milk, tarhana and other products (Driessen and de Boer, 1989). Since Mechnikoff (1908) at the Pasteur Institute proposed that ingestion of fermented milks offer health benefits and longevity in humans, fermented dairy products have been the subject of much speculation (Hoover, 1993). Tamine and Robinson (1988) reported

that consumption of fermented milk per capita in the U.S. was 3.8 kg in 1987; consumption has rapidly increased.

Production of dairy products such as buttermilk, sour cream, yogurt and cheese require controlled fermentation. The starter culture used in dairy products is important in determining product type, character, and quality. Fermentation of milk is primarily accomplished by lactococci and lactobacilli, which breakdown lactose to lactic acid. In addition, other parallel or post-fermentation reactions produce compounds distinctive of fermented foods. Two groups of lactic organisms have been used typically in fermented dairy foods. Thermophilic organisms which include *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus salivarius* subsp. *thermophilus*, *Lactobacillus helveticus*, and *Lactobacillus acidophilus* have an optimum temperature of growth around 37-45°C. These organisms are used for manufacture of products such as yogurt, Bulgarian buttermilk, and skyr. Mesophilic organisms, which include *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Leuconostoc* sp. *Lactobacillus kefir*, and *Lactobacillus casei* have an optimum temperature of around 30°C. These organisms are used for manufacture of products such as cheeses, cultured butter milk, and fermented milks (Marshall, 1993). Mesophilic organisms such as *Leuconostoc* sp. metabolize

citrate to diacetyl, acetate, and CO_2, which are responsible for the flavor or aroma of fermented dairy products (Tamine and Robinson, 1988).

Yogurt is the traditional and one of the most popular fermented dairy products in many countries. Standard of identity require that all yogurts in the U.S. are manufactured using *S. salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus* as starter cultures (Tamine and Deeth, 1980). *S. salivarius* subsp. *thermophilus* is a gram-positive and nonmotile organism. It has been classified as a facultative anaerobic microorganism and its optimum growth temperature is 40°C to 45°C. *S. salivarius* subsp. *thermophilus* produces lactic acid and small quantities of volatile acids such as formic, acetic, propionic, butyric, isovaleric, and caproic acids (Marshall, 1993; Tamine and Robinson, 1988; Tamine and Deeth, 1980).

L. delbrueckii subsp. *bulgaricus* is a gram-positive, nonmotile organism; it has a slender rod shape with rounded ends. It has been classified as a facultative anaerobic microorganism and has an optimum growth temperature of 40°C to 43°C. *L. delbrueckii* subsp. *bulgaricus* produces lactic acid and small quantities of carbonyl compounds and ethanol. The most important carbonyl compounds include acetaldehyde, acetone, butanone-2 and trace of acetoin.

Both *L. delbrueckii* subsp. *bulgaricus* and *S. salivarius* subsp. *thermophilus* have been classified as homofermentive bacteria, which use the glycolytic pathway for glucose fermentation (Jay, 1992). The homolactic bacteria possess enzymes such as aldolase and hexose isomerase but lack phosphoketolase, which is found in heterolactic bacteria. In homolactic fermentation, glucose results in 2 moles of lactic acid and a net gain of 2 ATP per mole glucose consumed (Jay, 1992; Tamine and Deeth, 1980). Tamine and Deeth (1980) reported a symbiotic relationship between *L. delbrueckii* subsp. *bulgaricus* and *S. salivarius* subsp. *thermophilus* during their growth. Mixed cultures of *L. delbrueckii* subsp. *bulgaricus* and *S. salivarius* subsp. *thermophilus* in milk produced more acid than single strain cultures (Marshall, 1987; Tamine and Deeth, 1980). *L. delbrueckii* subsp. *bulgaricus* provided essential growth requirements such as glycine and valine for stimulation of *S. salivarius* subsp. *thermophilus* (Tamine and Robinson, 1988). *S. salivarius* subsp. *thermophilus* stimulated growth of *L. delbrueckii* subsp. *bulgaricus* by removing oxygen, which lead to production of toxic hydrogen peroxide (Tamine and Deeth, 1980).

Organisms such as *L. acidophilus*, *L. casei*, *L. delbrueckii* subsp. *lactis*, and *Lactococcus lactis* subsp. *cremoris* have been used in various commercial dairy products as adjunct cultures (Driessen

and de Boer, 1989; Kim, 1988). They are normal inhabitants of the human intestinal tract, they would not disturb the normal intestinal flora (Martin and Chou, 1992). Recently, *Bifidobacterium* sp. was recognized as possible dietary adjunct and several researchers have reported on their beneficial effects on human health (Laroia and Martin, 1990; Hughes and Hoover, 1991). Although requirement for an adjunct cultures has not been clearly established, it is suggested that they should be normal inhabitants of the intestinal tract, they should be metabolically active and maintain viability in the carrier food and should survive the gastrointestinal tract (Tamine and Robinson, 1988).

2.2 Bifidobacteria: Discovery and introduction

Bifidobacteria are gram-positive, nonspore-forming, and nonmotile cells that often stain irregularly with methylene blue (Scardovi, 1986). Although the organism is classified as being anaerobic, some species can tolerate low levels of O_2. The optimum growth temperature for bifidobacteria is 37°C to 41°C, and the optimum pH for growth is 6.5 to 7.0 (Tamine *et al.*, 1995).

Bifidobacteria produces both acetic and lactic acid via an unusual glucose-metabolizing system that results in a ratio of 3:2 acetate:lactate as the primary metabolites (Scardovi and Trovatelli,

1965). In addition, small amounts of formic acid and ethanol are often produced by bifidobacteria fermentation (Scardovi, 1986). In the 8th edition of "Bergey's Manual" (Scardovi, 1986), twenty-four species of bifidobacteria have been defined. Those species of *Bifidobacterium* typically colonize the human GI tract are *B. bifidum, B. infantis, B. breve, B. longum, B. adolescentis, and B. catenulatum.* The other species occurring in the intestinal tract of various animals and insects (Scardovi, 1981).

The taxonomy of bifidobacteria has changed since bifidobacteria were first isolated. Many of the species groupings are heterogeneous, and the entire genera are being reexamined using several methods. Currently, there is no test that allows the determination of the origin and classification based on the taxonomy of the strains. Many bifidobacteria species are isolated from both animal and human flora, with human bifidobacteria species have been more extensively studied than the animal species (Scardovi, 1986).

2.3 Physiology and metabolism of bifidobacteria

The pathway for the metabolism of carbohydrates by *Bifidobacterium* sp. differs from that of homo and hetero-fermentative lactic acid bacteria. Figure 1 shows the metabolic

pathway used by *Bifidobacterium* sp. Hexoses are degraded by the fructose-6-phosphate pathway (Modler *et al.*, 1990). Fructose-6-phosphate phosphoketolase (F6PPK) is found in *Bifidobacterium* sp., whereas there is no fructose-1,6-bisphosphate aldolase, which is present in homofermentive lactic acid bacteria (Scardovi, 1986). The fermentation of two moles of glucose leads to the production of three moles of acetate and two moles of lactate when pyruvate is converted to lactate by L(+) lactate dehydrogenase. The pathway involves the splitting of pyruvate to form formate and acetyl phosphate, which is reduced to form ethanol (Tamine *et al.*, 1995). Oxygen dissimilation by *Bifidobacterium* sp. is shown in Figure 2. Oxygen toxicity to *Bifidobacterium* sp. results from metabolism of various compounds such as superoxide and hydrogen peroxide. The degree of tolerance to oxygen by bifidobacteria depends on the species and the culture medium (Tamine *et al.*, 1995). Some strains grow in the presence of oxygen without accumulating hydrogen peroxide, whereas other strains exhibit limited growth and accumulation of hydrogen peroxide. Also, some strains appear to be intolerant to oxygen and grow only when the redox potential is lowered. The presence of oxygen prevents growth because of the

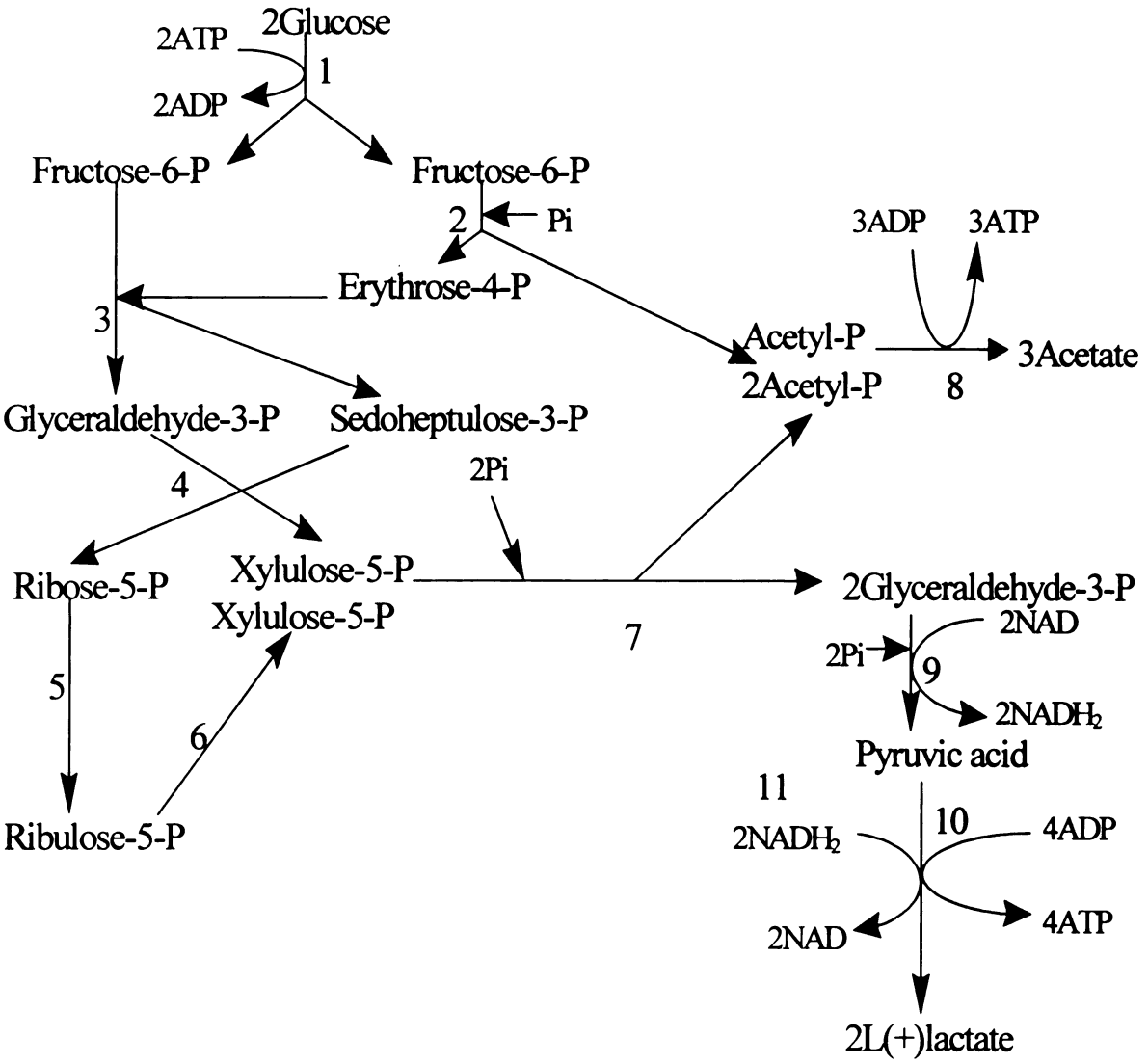

Figure 1. Metabolic pathway of *Bifidobacterium* sp. 1 = hexokinase and glucose-6-phosphate isomerase; 2 = fructose-6-phosphate phosphocetolase; 3 = transaldolase; 4 = transketolase; 5 = ribose-5-phosphate isomerase; 6 = ribulose-5-phosphate epimerase; 7 = xylulose-5-phosphate phosphocetolase; 8 = acetate kinase; 9 = homofermentative pathway enzymes; 10 = L(+) lactate dehydrogenase; 11 = phosphoroclastic enzyme (Tamine *et al.*, 1995).

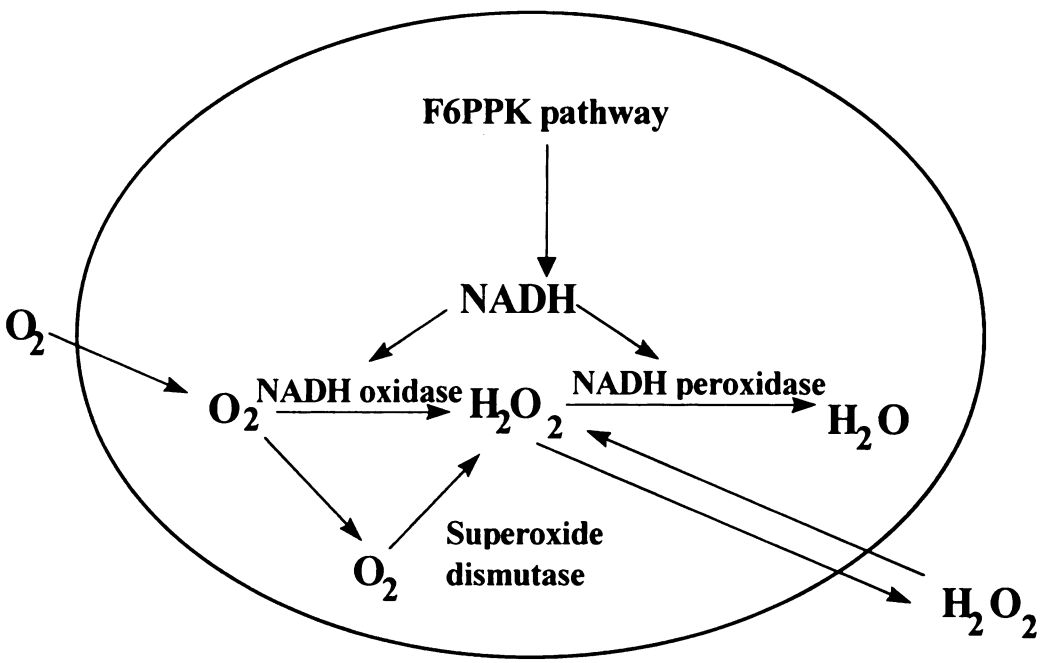

Figure 2. Oxygen dissimilation in *Bifidobacterium* sp. (Ballongue, 1993).

difficulty in establishing a suitable redox potential (Klaver *et al.*, 1993). NADH oxidase and NADH peroxidase are enzymes involved in oxygen utilization. The strains that are sensitive to oxygen have low NADH oxidase and NADH peroxidase activity, resulting in an accumulation of toxic hydrogen peroxide.

2.4 Therapeutic effects of bifidobacteria

Bifidobacteria have received much attention recently due to data accumulating on their health promoting effects (Hoover, 1993). Bifidobacteria account for 92% of the intestinal flora of breast-fed infants, but only 20% in bottle-fed or weaned infants (Hori, 1984). The beneficial effects of bifidobacteria on human health are summarized in Figure 3. Bifidobacteria have been reported to be effective inhibitors of gut pathogens due to their ability to produce acetate, lactate and small amounts of formate from carbohydrate catabolism. These organic acids and the lowering pH inhibit gram-negative microorganisms such as *Escherichia coli*, *Staphylococcus aureus*, *Shigella dysenteriae*, and *Samonella typhi* (Samona *et al.*, 1996).

Bifidin is a compound isolated from *B. bifidum*; it has been reported to have antibacterial activity against *Micrococcus flavus* and *Staphylococcus aureus* (Kanbe, 1992).

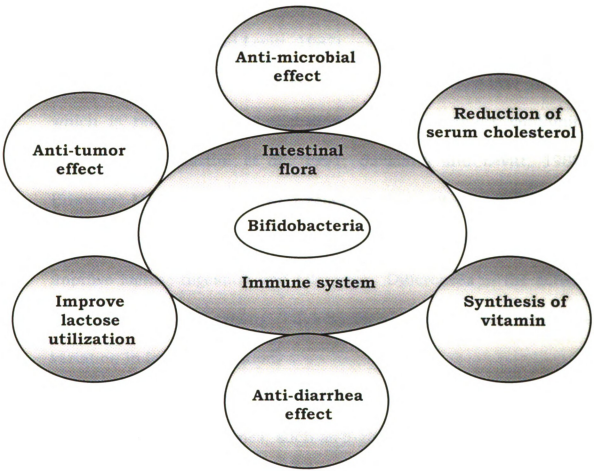

Figure 3. Beneficial effects of bifidobacteria on human health.

The improvement of lactose tolerance of milk products is another important health benefit of bifidobacteria.

Lactose intolerance is the result of insufficient amounts of β-galactosidase in the small intestine (Martini *et al.*, 1991; Modler *et al.*, 1990; Savaiano and Levitt, 1987).

Bifidobacteria possess high levels of β-galactosidase activity, which is released during digestion of dairy products containing bifidobacteria (Martini *et al.*, 1991; Savaiano and Levitt, 1987). However, Martini *et al.* (1991) reported that not all bifidobacteria strains provided sufficient microbial β-galactosidase activity to improve lactose digestion and tolerance. Differences in the activity of β-galactosidase among bifidobacteria strains may cause a variation in lactose digestion.

It has been reported that bifidobacteria reduced the source of procarcinogens or enzymes, such as β-glucuronidase, azoreductase, and nitroreductase, that lead to their formation (Hawkins, 1993; Tamine *et al.*, 1995). Sekine *et al.* (1985) reported that *B. infantis* ATCC 15697 significantly suppressed tumor growth in mice. Also, Koo and Rao (1991) reported that proliferation of liver tumors significantly decreased when *B. longum* was present in the intestinal flora.

Reduction of serum cholesterol levels is another benefit of bifidobacteria. The role that bifidobacteria cultures may play in lowering serum cholesterol is not yet understood. In rat models, serum cholesterol was lowered by feeding bifidobacteria in a mechanism that may involve HMG-CoA reductase (Homma, 1988). Gilliland and Walker (1990) reported that bifidobacteria produce HMG, which inhibits HMG-CoA reductase involved in the synthesis of cholesterol. Jaspers *et al.* (1984) reported that both orotic acid and uric acid produced during fermentation of cultured dairy products lowered the serum cholesterol levels in humans.

Synthesis of vitamins is another benefit of bifidobacteria. Hawkins (1993) reported that bifidobacteria produced thiamine, riboflavin, vitamin K and vitamin B_6. Deguchi *et al.* (1985) also observed that *B. longum* produced vitamin B_6 and B_2. Thus, dairy foods containing bifidobacteria help in meeting the requirements for B-vitamin consumption (Hawkins, 1993).

Anti-diarrhea effect of bifidobacteria has been reported as beneficial effect of bifidobacteria. Hotta *et al.* (1987) showed that normal functions of children suffering from diarrhea were restored more rapidly after the ingestion of milk fermented with *B. breve*. Tojo *et al.*, (1987) reported that feeding of bifidobacteria containing dairy products has been used to treat diarrhea infections in

Japanease children. Homma (1988) also reported that ingestion of milk fermented with *B. longum* helped prevent diarrhea in humans.

2.5 Bifidobacteria in dairy products

Because of the health benefits discussed above, there is increasing interest in incorporating *Bifidobacterium* sp. into fermented dairy products as a medium to reintroduce viable populations of bifidobacteria into the GI tracts of children and adults. Currently, more than 70 different dairy products containing bifidobacteria are produced worldwide, including sour cream, butter milk, yogurt, powered milk, cultured milk, ice-cream, cheese, and other frozen desserts (Ventling and Mistry, 1993). The most commonly employed bifidobacteria strains in dairy products are *B. bifidum, B. infantis, and B. longum.* Because these species are of human origin, they have an affinity to colonize the human intestine (Ishibashi and Shimamura, 1993). Before 1980s, the use of bifidobacteria in food products was largely limited to foods and beverages intended for therapeutic treatment (Hawkins, 1993). In the 1970s, technology began to catch up with the objective of delivering viable bifidobacteria to commercial dairy products (Driessen and de Boer, 1989). Today, dairy products containing bifidobacteria have become as common in the U.S. as they are in

Japan and Europe. The incorporation of bifidobacteria into dairy products in addition to the nutritional benefits also provides for better taste, milder, less sour and bitter products.

Manufacturing problems do occur in cultured dairy products containing bifidobacteria more often than they do in traditional cultured dairy products. These problems occur because cultivation of bifidobacteria in milk is more difficult than with other microorganisms. More aseptic working conditions are needed because of longer incubation times and slower acidification. Also, bifidobacteria are classified as anaerobic microorganisms, although some species are able to tolerate oxygen. Thus, oxygen toxicity is an important and critical problem in commercial dairy processing. Inoculum and starter culture amounts are much larger, and bifidobacteria lose their viability more quickly at low pH values during storage (Hoover, 1993; Ishibashi and Shimamura, 1993). For the development of dairy product containing bifidobacteria, not only the health benefits and taste, but also the viability of bifidobacteria is very important.

2.6 Viability of bifidobacteria in dairy products

Sanders (1993); Salminen and Deighton (1992) recently reviewed the various clinical benefits of consuming viable

bifidobacteria. Although scientific opinions regarding the significance of viability in the therapeutic efficacy of lactic acid bacteria and bifidobacteria remain divided, the public expects fermented dairy products to contain viable organisms at the time of consumption. Clinical studies have shown significant clinical benefits are observed upon ingestion of approximately 10^9 - 10^{10} organisms/d (Sanders *et al.*, 1996). Viability of bifidobacteria in commercial dairy products is not consistent with clinical data available. The National Yogurt Association has established standards for lactic acid bacteria, which is at least 10^9 cfu/g for refrigerated yogurt and 10^7 cfu/g for frozen yogurt at the time of manufacturing for the Seal Program in order to promote the importance of live and active cultures. No standards have been established for bifidobacteria. France has regulations on viable culture numbers in fermented dairy products, requiring $\geq 1 \times 10^8$ cfu/ml. Japan, South Korea, and Poland have regulations pertaining to viability of cultures in fermented dairy products, which is $\geq 1 \times 10^6$ - 1×10^7 cfu/ml of viable cultures (Orihara *et al.*, 1992). In the U. S., states such as California and Oregon have already adopted regulations pertaining to viability of cultures in dairy foods, which is 2×10^6 cfu/ml of viable lactic cultures (Sanders *et al.*, 1996). Other states are also expected to adopt regulations regarding viability of

cultures in fermented foods. Increasing viability of lactic acid bacteria and bifidobacteria in dairy products has been the focus of much research (Hekmat and McMahon, 1992; Ibrahim and Bezkorovainy, 1994; Medina and Jordano, 1994; Poch and Bezkorovainy, 1988). It requires skill, knowledge, and advanced technologies to maintain a satisfactory level of viable bifidobacteria in dairy products for their probiotic effects (Kurmann *et al.*, 1992). There are many factors, which influence the viability of bifidobacteria. These include the strain of bifidobacteria used in milk, milk solids content, pH, storage temperature, presence and content of sugars, culturing conditions, and individual manufacturing conditions.

Medina and Jordano (1994) reported on the bifidobacteria count of fermented milk produced in Spain stored at 7°C. They observed a 92.6% decrease in bifidobacteria count when the product was expired. Biavati *et al.* (1992) observed that the viability of bifidobacteria in skim milk at pH 4.0 and 4°C decreased by more than 90% after 15 days. Modler *et al.*, (1990) reported that the viability of bifidobacteria is affected by a low pH environment however some strains of bifidobacteria showed an acid tolerance at pH 4.0. Blanchette *et al.* (1996) reported on the viability of *Bifidobacterium infantis* ATCC 27920 in creamed cottage cheese.

They observed a decrease of 2-4 log cycles during 15 days of storage at 4°C. Hekmat and McMahon (1992) reported that ice-cream may serve as a good vehicle for delivering viable bifidobacteria. Their study indicated that ice-cream mix fermented with *L. acidophilus* and *B. bifidum* maintained viable cell counts after 17 weeks of storage at -29°C. After fermented mix was frozen, *L. acidophilus* and *B. bifidum* counts were 1.5×10^8 cfu/ml and 2.5×10^8 cfu/ml, respectively. Seventeen weeks after frozen storage, counts of *L. acidophilus* and *B. bifidum* in the ice-cream were decreased by two log cycles to 4×10^6 cfu/ml and by one log cycle to 1×10^7 cfu/ml, respectively. Shah *et al.* (1995) reported on initial bifidobacteria counts in five brands of commercial yogurt. In two of the five brands of yogurt, counts were 10^6-10^7 cfu/g, and in the ramaining three they were $<10^3$ cfu/g, indicating significant variability in counts in similar products. They also reported that all products showed a constant decline in bifidobacteria and lactic acid bacteria counts during storage.

2.7 Growth factors of bifidobacteria

Research also has been conducted to optimize growth conditions for the various strains of bifidobacteria used in dairy products (Desjardins and Roy, 1990; Dinakar and Mistry, 1994;

Dubey and Mistry, 1996; Hughes and Hoover, 1995). Growth-promoting factors have been investigated to increase the viability of bifidobacteria for significant clinical benefits (Driessen, 1988; Hidaka *et al.*, 1986; Ibrahim and Bezkorovainy, 1994; Poch and Bezkorovainy, 1988). Some growth-promoting factors that have been tested include *N*-acetylglucosamine (Jao *et al.*, 1978), casein (Nicholas *et al.*, 1974), carrot juice (Rasic and Kurmann, 1983), porcine mucine (Modler *et al.*, 1990), lactulose (Nagendra *et al.*, 1995), oligosaccharides (Yun, 1996), and inulin (Yamazaki and Dilawri, 1990). To enhance the growth of bifidobacteria, trace elements (Bezkorovainy *et al.*, 1986) and vitamins (Deguchi *et al.*, 1985) also have been studied as nutrient requirements.

N-acetylglucosamine has been identified as a required substance for growth of *B. bifidum* var. *pennsylvanicus* and has been named "bifidus factor I" (Tamine *et al.*, 1995). *N*-acetylglucosamine is found in human and cow's milk (Jao *et al.*, 1978). Casein also has been shown to have growth-promoting activity and is known as "bifidus factor II". Casein from human milk enhanced the growth of various strains of bifidobacteria without any treatment and became much more effective after treatment with chymosin or pepsin (Azuma *et al.*, 1984).

Carrot root extract also has been found to contain a bifidus factor identified as a precursor of coenzyme A which is water soluble and heat resistant (Rasic and Kurmann, 1983; Tamine *et al.*, 1995). Various studies have reported that growth of bifidobacteria was promoted more in human milk than cow's milk (Hidaka *et al.*, 1991; Homma, 1988; Petschow and Talbott, 1990). Lower buffering capacity in human milk (Bullen *et al.*, 1977), because of its lower protein and mineral contents and the presence of lactoferrin and transferrin (Roberts *et al.*, 1992), could be responsible for the better growth of bifidobacteria in human milk. Human milk also contains nucleotides such as cytidine-5-phosphate that are not found in cow's milk. These nucleotides have been considered as bifidus factors, which promote the establishment of bifidobacteria in the human intestinal tract.

Lactulose is a disaccharide composed of one molecule each of galactose and fructose, and its ingestion has been reported to stimulate growth of bifidobacteria in the large intestine (Azuma *et al.*, 1984; Rasic and Kurmann, 1983; Roy and Goulet, 1991). However, other studies have shown that several strains of other intestinal bacteria can also utilize lactulose, but to a lesser extent than bifidobacteria (Azuma *et al.*, 1984; Roy and Goulet, 1991).

A number of studies (Crittenden and Playne, 1996; McKellar and Modler, 1989; Yazawa and Tamura, 1982; Yazawa *et al.*, 1978) have been undertaken to identify oligosaccharides that can be utilized by bifidobacteria. Fructooligosaccharides, *trans*-galactosyl-oligosaccharides, 4'-galactosyl-lactose and other oligosaccharides also have been reported as bifidogenic factors, resulting in the proliferation of human intestinal bifidobacteria (Hidaka *et al.*, 1986). *B. breve* and *B. infantis* selectively use raffinose, stachyose, and inulin, but these sugars are not used by *E. coli, L. acidophilus*, and *S. faecalis*. Oligosaccharides that are not digested in the small intestine reach the large intestine where they become available for degradation by the indigenous bifidobacteria in the colon (Yun, 1996). Yazawa *et al.* (1978) reported on various ingestible oligosaccharides useful for increasing the number of intestinal bifidobacteria. Generation times of *B. breve* and *B. infantis* have been found to be as rapid with these sugars as with glucose or lactose (Hidaka *et al.*, 1991, Hayakawa *et al.*, 1990). Fructooligosaccharide consists of polymers of D-fructose linked by a $\beta(2\rightarrow1)$ bond and terminated with a D-glucose linked to fructose by a $\alpha(1\rightarrow2)$ linkage. Fructooligosaccharides with a degree of polymerization (DP) between 3 and 5 have been synthesized from

sucrose. Inulin is polymers with a DP more than 30 (McKellar and Modler, 1989; Yun, 1996).

McKellar and Modler (1989) reported that *B. adolescentis* ATCC 15703, *B. longum* ATCC 15707, and *B. thermophilum* ATCC 25525 have the ability to metabolize short chain fructooligosaccharide with a DP between 3 and 5. Yazawa and Tamura (1982) reported that inulin was selectively utilized by *B. infantis*. Yazawa and Tamura (1982) and Yazawa *et al.* (1978), reported that fructooligosaccharide and inulin stimulated the growth of bifidobacteria. Although bifidobacteria have been shown to metabolize single and complex sugars, growth factors must be added to milk to promote their growth (Crittenden and Playne, 1996; Yun, 1996).

Oligosaccharides are water soluble and 0.3 to 0.6 times as sweet as sucrose. However, sweetness of oligosaccharides is affected by their chemical structure, molecular weight, and the levels of mono and disaccharides in the oligosaccharides. The low sweetness of oligosaccharides may be desirable in dairy products because they enhance flavors, increase solids thus may improve texture and lower water activity (Crittenden and Playne, 1996; Spiegel *et al.*, 1994; Yun, 1996).

2.8 Redox potential and bifidobacteria

Another significant factor affecting the viability of bifidobacteria is oxygen. It has been known that microorganisms show different degrees of sensitivity to redox potential (oxidation-reduction potential; Eh) of their growth medium (Jay, 1992). Redox potential is a measure of the tendency of a given system to donate electrons or to accept electrons. The redox potential of a given system is determined by measuring the electrical potential difference between that system and a standard hydrogen electrode (Singleton, 1987). Redox potential (Eh) can be calculated using the following equation:

$$Eh = E_0 - \frac{RT}{nF} \log_e \frac{\text{Activity of reduced species}}{\text{Activity of oxidized species}}$$

E_0 = Constant characteristic potential

R = Gas constant (8.31 J/mol/degree abs.)

T = Absolute temperature

n = Number of electrons

Redox potential, Eh is measured using a platinum electrode and is expressed in millivolts (mv). Strict aerobic organisms require a high positive Eh, between 300 and 500 mv for their growth. Microaerophilic organisms require an Eh between 100 and 300 mv. Facultative anaerobic organisms such as *Bifidobacterium* require a

Eh between –200 and 200 mv for their growth (Jay, 1992). Oxygen toxicity is an important and critical problem because *Bifidobacterium* sp. are facultative anaerobes (Kim, 1988). Reuter (1989) suggested applying a *S. salivarius* subsp. *thermophilus* strain with high oxygen consuming ability to enhance the viability of bifidobacteria in yogurt type products. *S. salivarius* subsp. *thermophilus* is a homofermentative facultative anaerobic microorganism. During anaerobic fermentation, *S. salivarius* subsp. *thermophilus* catabolize 1 mol of glucose to 2 mol of lactate through the glycolytic pathway, but in aerobic metabolisms glucose or pyruvate leads to the formation of acetate, α-acetolactate, acetoin, and diacetyl in addition to lactic acid production. The following equation shows the aerobic metabolism of *S. salivarius* subsp. *thermophilus* associated with consumption of oxygen:

$$\text{NADH} + H^+ + O_2 \xrightarrow{\text{NADH oxidase}} \text{NAD} + H_2O_2$$

$$\text{Pyruvate} + 1/2O_2 \xrightarrow{\text{Pyruvate oxidase}} \text{Acetate} + CO_2$$

NADH generated in the aerobic metabolism is consumed in the reaction catalyzed by NADH oxidase. Aerobic O_2 uptake has also been observed with lactobacilli, but to a lesser extent (Teraguchi *et al.*, 1987; Tinson *et al.*, 1982). Okonogi *et al.* (1986), in a patent,

reported that due to the high oxygen uptake of *Streptococcus salivarius* subsp. *thermophilus*, it provided a suitable environment for bifidobacteria and greatly enhanced viability of bifidobacteria. Developing or selecting oxygen and acid resistant bifidobacteria strains would be another possible means of improving their viability.

The purpose of the following study was to determine the viability of bifidobacteria in commercial dairy products and investigate the role of food grade oligosaccharides, inulin, and redox potential in enhancing viability of bifidobacteria that may have enhanced health benefits. The final part of this study will involve manufacture of a yogurt product that is consistent with clinical studies.

III. MATERIALS AND METHODS

3.1 Viability of bifidobacteria in commercial dairy products.

3.1.1 Sampling of commercial dairy products.

Commercial A/B milk (containing *L. acidophilus* and bifidobacteria) and two brands of yogurt (containing *Lactobacillus delbrueckii* subsp. *bulgaricus, Streptococcus salivarius* subsp. *thermophilus* and bifidobacteria) were obtained from retail outlets in the Michigan area and stored at 5°C. All products claimed to contain viable bifidobacteria and lactic acid bacteria. Milk was evaluated 9, 6 and 3 days prior to its expiration date, at its expiration date and 3, 6, and 9 days after its expiration date. Whereas, yogurts were evaluated 3, 2 and 1 week prior to their expiration dates, at their expiration date and 1, 2 and 3 weeks past the expiration date. Samples were mixed well and aseptically removed from each container and diluted by mixing 1ml of milk or 1g yogurt with 99ml of 0.1% (w/v) bacto peptone (Difco, Detroit, MI) and subsequent serial dilutions were made. Another sample was collected for pH measurements. The pH of the products was determined at each sampling point. Unopened A/B milk and two brands of yogurt were used each sampling point for the enumeration and pH determination.

3.1.2 Enumeration of bifidobacteria and lactic acid bacteria.

The first part of this research involved screening selective media to enumerate bifidobacteria in commercial dairy products. Various media were screened for selective enumeration of bifidobacteria. Brain heart infusion agar, modified Columbia agar, RCA and MRS agar containing 5% (w/v) lactose and 5% (v/v) NPNL antibiotic solution were evaluated. MRS agar containing 5% (w/v) lactose and 5% (v/v) NPNL antibiotic solution was most successful in enumerating bifidobacteria and inhibiting all other lactic acid bacteria. Thus, in the following studies bifidobacteria were enumerated using MRS agar (Difco) containing 5% (w/v) lactose and 5% (v/v) NPNL antibiotic solution. NPNL was prepared by mixing 60g of LiCl (Sigma, St. Louis, MO), 4g of paromomycin sulphate (Sigma), 2g of neomycin sulphate (Sigma), 0.3g of nalidixic acid (Sigma) in 1 liter of demineralized water. The mixture was filter-sterilized (0.22um) prior to adding to MRSL. The inoculated plates were incubated anaerobically at 37°C for 48hr using Gas Pak® (Becton Dickinson Co., Cockeysville, MD). Lactic acid bacteria were enumerated using MRS agar containing 5% (w/v) lactose. The inoculated plates were incubated aerobically at 37°C for 72hr. The colonies were counted using a Quebec colony counter (Fisher Scientific, Pittsburgh, PA). Bifidobacteria and lactic acid bacteria

count were determined by phenotype characteristics as presented in the 8th edition of Bergey's Manual of Determinative Bacteriology (Scardovi, 1986).

3.2 Effect of oligosaccharides and inulin on growth and viability of bifidobacteria in fermented milk.

3.2.1 Culture preparation.

Commercial strains of *Bifidobacterium* Bf-1 and Bf-6 from Sanofi Bio-Industries (Waukesha, WI) were selected in this research because they have been shown to stimulate immune function via altered cytokine secretion by leukocytes within the gastrointestinal immune compartment in *in vitro* studies (Marin *et al.*, 1997). Each bifidobacteria culture was cultured and subcultured anaerobically in MRS medium (Difco) containing 5% (w/v) lactose (MRSL) at 37°C for 48hr using Gas Pak® (Becton Dickinson Co.). Cultures were centrifuged 15 min at 1000 × *g* at 4°C and resuspended in 12% (w/v) pasteurized (70°C, 30 min) non-fat dry milk (NDM; Difco) at a 5% (v/v) level.

3.2.2 Effect of oligosaccharides and inulin on growth of bifidobacteria.

Frutooligosaccharide (FOS) and inulin were supplied from Rhone-Poulenc Inc. (Cranbury, NJ). Galactooligosaccharide (GOS)

was supplied from Samyang Genex Co., Ltd. (Seoul, Korea). They were added at 0.5, 1, 3, and 5% (w/v) level to 12% (w/v) NDM. The controls had no oligosaccharides or inulin added. Each sample was pasteurized at 70°C for 30min. Tubes inoculated with the cultures prepared above were incubated anaerobically as described previously at 37°C for 48hr. A sample was taken at 6hr intervals and diluted (1:10, v/v) with 0.2% EDTA (pH 12.0) and turbidity was measured at 640 nm as described by Hughes and Hoover (1995). Uninoculated NDM was used as the blank for turbidity measurement. Specific growth rate (μ) for each culture was calculated using the following equation (Roy and Goulet, 1991):

$$\mu = \frac{\ln X_2 - \ln X_1}{t_1 - t_2}$$

X_2 and X_1 are the cell density at time t_2 and t_1. Mean doubling time (T_d) was calculated as:

$$T_d = \frac{\ln 2}{\mu}$$

Another sample was collected for pH measurements. The pH of culture samples was also monitored at 6hr intervals for 48hr.

3.2.3 Effect of oligosaccharides and inulin on viability of bifidobacteria during refrigerated storage.

Each bifidobacteria sample was cultured anaerobically at 37°C for 48 hr with or without oligosaccharides and inulin as previously described. The samples were stored at 5°C for 4 weeks. One ml each of the bifidobacteria sample was diluted with 99ml of 0.1% (w/v) bacto peptone (Difco) and subsequent serial dilutions were made. Bifidobacteria were enumerated using MRSL agar. The inoculated plates were incubated anaerobically at 37°C for 48hr using Gas Pak® (Becton Dickinson Co.). The colonies were counted as described previously. Percent viability of each culture sample was calculated as follows:

% viability = (cfu at 4 week storage) / (initial cfu before storage) × 100

3.2.4 HPLC assay.

Culture activity was determined by end products of fermentation (lactic acid and acetic acid) using HPLC (High Performance Liquid Chromatography). The HPLC system (Waters Associates, Inc., Milford, MA) available in our laboratory consist of a

M-45 solvent delivery system, a 486 UV/Vis tunable absorbance detector and a 730 data module. The UV detector, set at 220nm, was used for quantification of organic acids. An Aminex HPX-87H Column (300 mm × 7.8 mm, Bio-Rad Laboratories, Richmond, CA) and guard column with disposable cartridges H^+ (Bio-Rad Laboratories) maintained at 65°C was used for the analysis. A mobile phase of 0.009N H_2SO_4 filtered through a 0.45 μm membrane filter (Millipore Corp., Bedford, MA) and degassed by vacuum was used at a flow rate of a 0.6 ml/min.. The wavelength for the detection of organic acid set at 220 nm was optimized and organic acid was quantitated (Bouzas *et al.*, 1991). Standard solution of organic acids (lactic acid and acetic acid; Sigma) was prepared to establish elution times and calibration curves.

NDM fermented with two strains of bifidobacteria in 5% of FOS, GOS, or inulin were prepared for the HPLC analysis using the method described by Dubey and Mistry (1996). One hundred microliters of 15.8N HNO_3 and 14.9ml of 0.009N H_2SO_4 were added to 1.5ml of sample and centrifuged at 5000 × *g* for 10 min. The supernatant was filtered using Whatman #1 filter paper, 0.22 μm membrane filter (Millipore Corp., Bedford, MA) and eluted through a reversed phase Supelclean tube (Supelco Inc., Bellefonte, PA) and stored in HPLC vials at -20°C until the HPLC analysis. Figure 4

shows a typical HPLC chromatogram of lactic acid and acetic acid produced by bifidobacteria cultured in 12% NDM with 5% FOS, GOS, and inulin.

3.2.5 Standard curves and HPLC chromatograms of acetic acid and lactic acid.

Acetic acid had a retention time of 12 min (Figure 4). A standard curve was made using five different standard acetic acid solutions (2.5, 5, 10, 20 and 40 mmol/L). Figure 5 shows the standard curve for acetic acid determination (R^2 = 0.99). Concentration of acetic acid (mmol/L) in the samples was calculated using the following relationship:

Peak area = -56254.5 + (22021.6 × acetic acid (mmol/L)).

Lactic acid had a retention time of 10 min (Figure 4). A standard curve was made using five different standard acetic acid solutions (2.0, 2.2, 3.0, 3.7 and 4.4 mmol/L). Figure 6 shows the standard curve for lactic acid determination (R^2 = 0.99). The concentration of lactic acid (mmol/L) was in the samples calculated using the following relationship:

Peak area = -95772 + (79723.8 × lactic acid (mmol/L)).

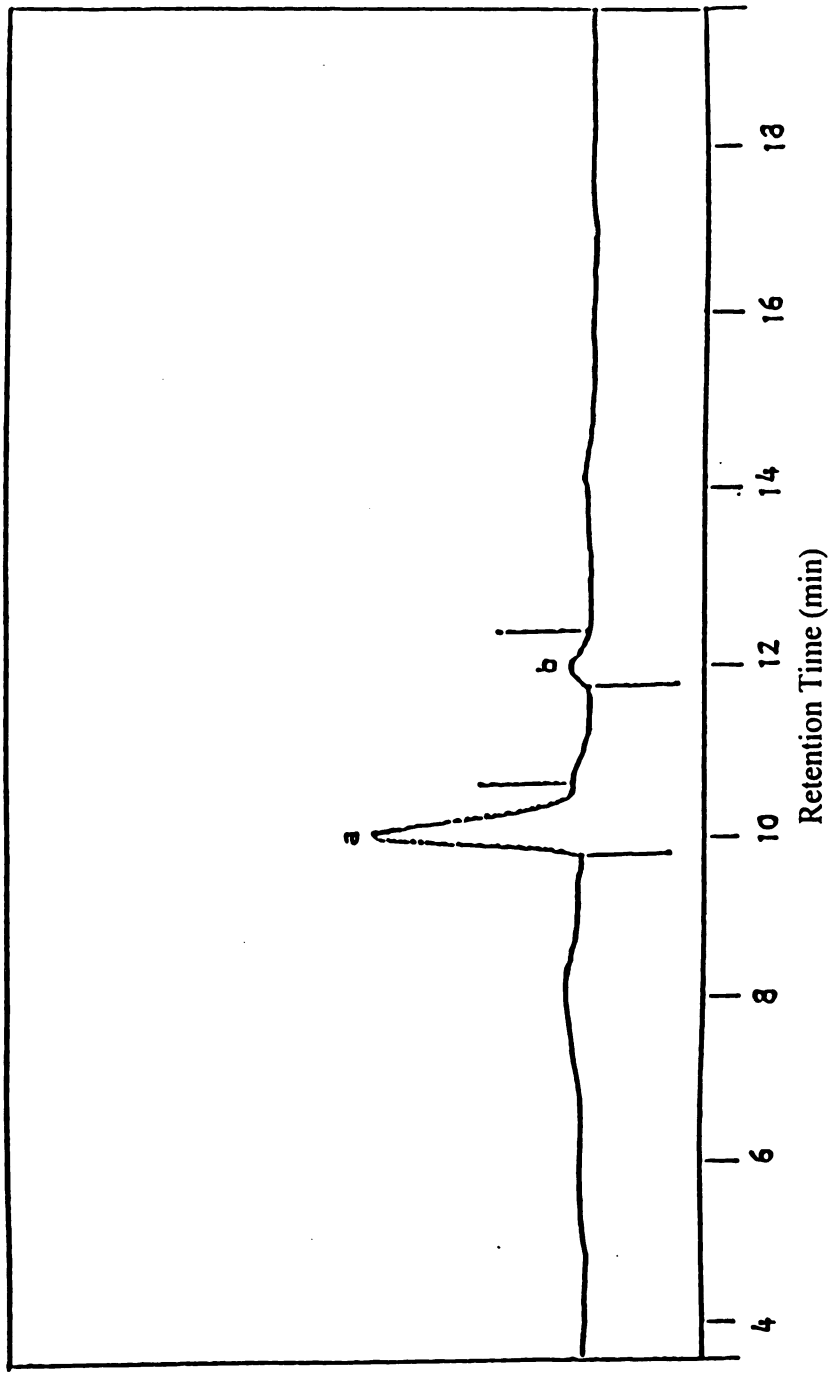

Figure 4. Typical HPLC chromatogram of lactic acid and acetic acid produced by bifidobacteria cultured in 12% NDM with fructooligosaccharide, galactooligosaccharide and inulin: (a) lactic acid and (b) acetic acid.

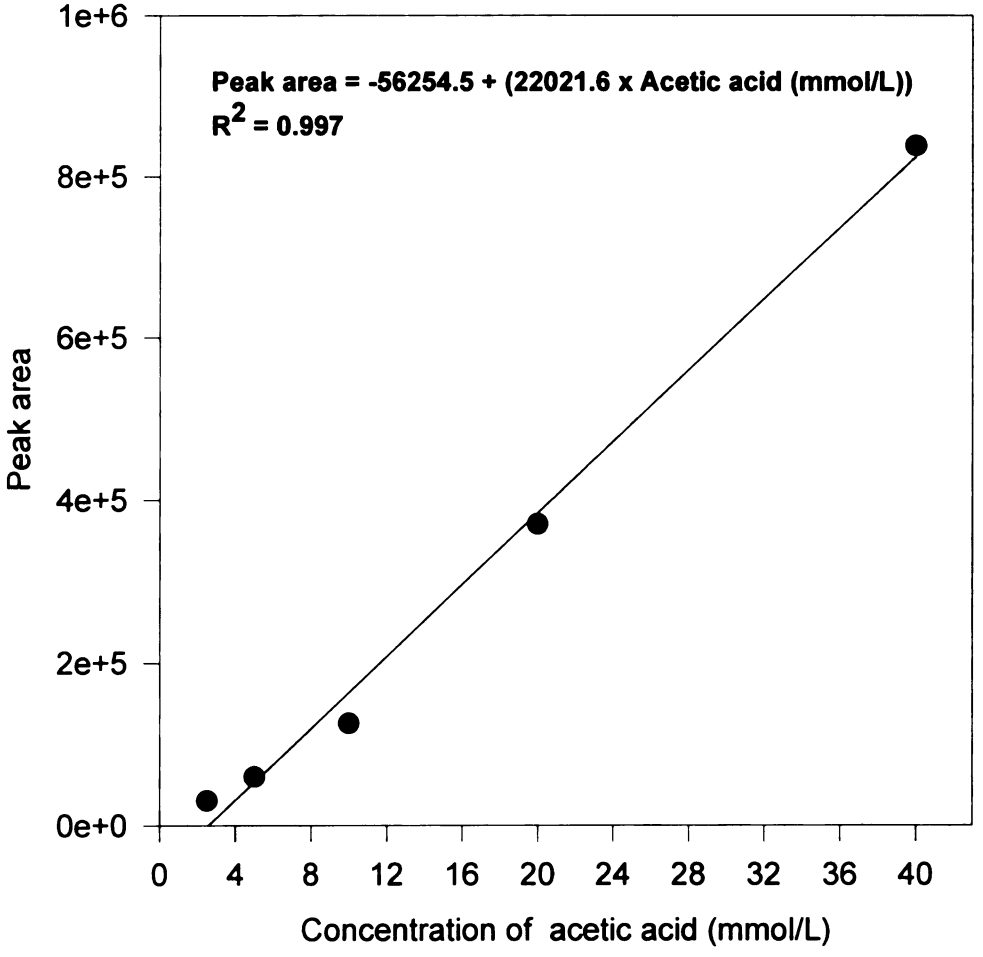

Figure 5. Acetic acid standard curve using different standard acetic acid solutions (2.5, 5, 10, 20 and 40 mmol/L).

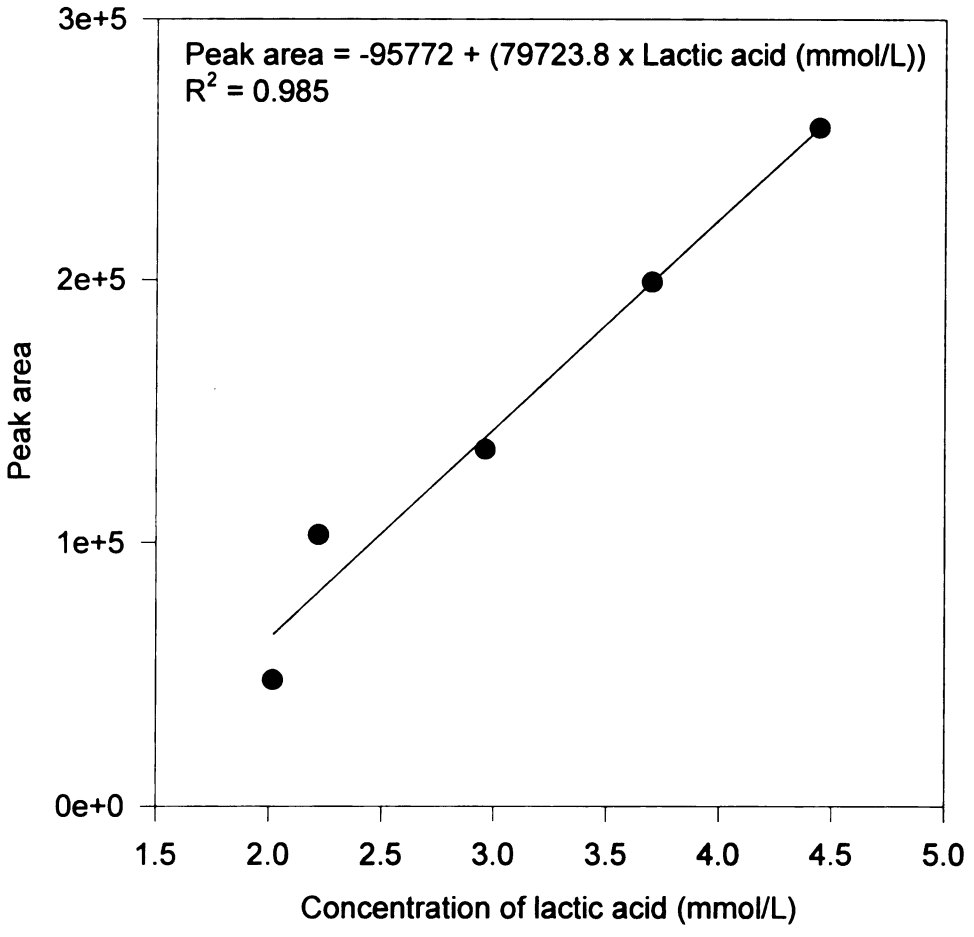

Figure 6. Lactic acid standard curve using different standard lactic acid solutions (2.0, 2.2, 3.0, 3.7 and 4.4 mmol/L).

3.3 Redox potential of NDM cultured with *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* and its effect on growth of bifidobacteria.

3.3.1 Selective medium for lactic acid bacteria

The first part of this research involved screening selective media to enumerate the specific lactic acid bacteria of interest in this study. Various media were screened for selective enumeration of *S. salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. Lee's agar, Streptosel agar, Azide dextrose agar, ST agar and M17 agar were evaluated for selective enumeration of *S. salivarius* subsp. *thermophilus*. MRS agar containing oxgall and modified RCA (Reinforced Clostridial agar) were evaluated for selective enumeration of *L. delbrueckii* subsp. *bulgaricus*. RCA was modified by supplementing with 2% bacto agar (Difco) and adjusting pH to 5.0 after autoclaving. M17 agar was shown to be the most successful in enumerating *S. salivarius* subsp. *thermophilus* and modified RCA for selective enumeration of *L. delbrueckii* subsp. *bulgaricus*.

3.3.2 Screening *L. delbrueckii* subsp. *bulgaricus* and *S. salivarius* subsp. *thermophilus*.

Initially, *S. salivarius* subsp. *thermophilus* and *L. delbrueckii*

subsp. *bulgaricus* were screen for their ability to provide optimum pH and redox potential conditions for growth of bifidobacteria. *S. salivarius* subsp. *thermophilus* St-52, St-113, St-133, St-134, *L. delbrueckii* subsp. *bulgaricus* Lr-28, Lr-78, Lr-79 from Sanofi Bio-Industries (Waukesha, WI) and NCK231 from North Carolina State University were screened. *S. salivarius* subsp. *thermophilus* St-133 was selected because of its ability to reduce redox potential faster than other strains. *L. delbrueckii* subsp. *bulgaricus* NCK 231 was selected because it produced the most suitable amount of acid in NDM.

3.3.3 Culture preparation.

Bifidobacterium Bf-1 and *S. salivarius* subsp. *thermophilus* St-133 was provided from Sanofi Bio-Industries (Waukesha, WI). *L. delbrueckii* subsp. *bulgaricus* NCK 231 was supplied by North Carolina State University and *B. adolescentis* M 101-4 was provided from Japan Bifidus Foundation (Tokyo, Japan). *S. salivarius* subsp. *thermophilus* St-133 and *L. delbrueckii* subsp. *bulgaricus* NCK231 were cultured and subcultured in MRS medium (Difco) containing 5% (w/v) lactose at 37°C for 48hr. *Bifidobacterium* Bf-1 and *B. adolescentis* M 101-4 were cultured and subcultured anaerobically in MRS medium (Difco) containing 5% (w/v) lactose at 37°C for 48hr

using Gas Pak® (Becton Dickinson Co.). Cultures were centrifuged 15min at 1000 × *g* at 4°C and resuspended in 12% (w/v) pasteurized (90°C, 10 min) NDM (Difco).

3.3.4 Determining redox potential.

Twelve percent (w/v) pasteurized NDM cultured with or without *S. salivarius* subsp. *thermophilus* St 133 and/or *L. delbrueckii* subsp. *bulgaricus* NCK 231 was inoculated with *Bifidobacterium* Bf-1 or *B. adolescentis* M101-4 to a final ratio of 1:1, 1:2, 2:1, 1:1:1, 1:2:1, 1:1:2, or 2:1:1 (based on cfu). These ratios were obtained after determining cells counts of each pure cultures cultured in MRSL and appropriate volumes were transferred into NDM to have a total inoculum at a 5% (v/v) level. Redox potential of NDM samples was monitored at 12h intervals for 48h using a platinum electrode (Corning Incorp., New York). Each sample was equilibriated at 25°C for 10 min prior to determining redox potential. The electrode was submerged into the sample and mV value was measured. The Eh_7, the redox potential of system standardized to pH 7 and 25°C, was calculated by a formula from Montville and Conway (1982) by substituting the correction factor 59mV/pH at 25°C (Montville and Conway, 1982). Thus, the formula derived was: Eh_7 = Eh measured - 59(7.00 - pH measured).

At each interval, a separate sample was collected for the pH measurement.

3.3.5 Growth of bifidobacteria co-cultured with lactic acid bacteria.

Growth of bifidobacteria co-cultured with lactic acid bacteria was monitored at 12h intervals for 48h. Inoculated samples were incubated at 37°C for 48h. Bifidobacteria were enumerated using anaerobic incubation of MRS agar containing 5% (w/v) lactose and 5% (v/v) NPNL antibiotic solution. *S. salivarius* subsp. *thermophilus* was enumerated using M17 agar, and *L. delbrueckii* subsp. *bulgaricus* was enumerated using modified RCA as described previously.

3.4 Viability of bifidobacteria in yogurt manufactured using commercial yogurt starter cultures and selected condition.

S. salivarius subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus* used in this section were selected for pH and redox potential conditions for growth of bifidobacteria as described previously. *S. salivarius* subsp. *thermophilus* St-133 and *L. delbrueckii* subsp. *bulgaricus* NCK 231 were cultured and subcultured as described previously. *Bifidobacterium* Bf-1 and Bf-6

were cultured and subcultured anaerobically as described previously. Cultures were centrifuged 15 min at 1000 × *g* at 4°C and resuspended in 12% (w/v) pasteurized (90°C, 10 min) NDM (Difco) containing 5% (w/v) fructooligosaccharide which previously have been determined to be optimum concentration of fructooligosaccharide to enhance the growth and viability of bifidobacteria. Twelve percent (w/v) pasteurized NDM containing fructooligosaccharide cultured with *S. salivarius* subsp. *thermophilus* St-133 and *L. delbrueckii* subsp. *bulgaricus* NCK 231 was inoculated with *Bifidobacterium* Bf-1 to a final ratio of 4:1:2 (*Bifidobacterium*: *L. delbrueckii* subsp. *bulgaricus*: *S. salivarius* subsp. *thermophilus*; based on cfu) which previously have been determined to be terms of redox potential and acid production. Inoculated samples were incubated at 37°C for 8hr and stored at 5°C for 15 days. Viability of bifidobacteria was monitored at 5 days intervals for 15 days. Yogurt containing bifidobacteria manufactured using commercial yogurt starter cultures obtained from Chr. Hansen's Laboratories Inc. (Milwaukee, WI) was used as a control.

3.5 Statistical analysis.

In 3.1., three batches of milk and yogurt were purchased at three different times. In 3.2, 3.3 and 3.4 analysis were conducted

in triplicates. All experiments were replicated three times in a randomized design. Statistical analysis was done using Sigma Stat 1.0 (Jandel Corp., San Rafael, CA). Appropriate comparisons were made using Student-Newman-Keuls test for multiple comparisons. A $p< 0.05$ was considered statistically significant.

IV. RESULTS & DISCUSSION

A. Viability of bifidobacteria in commercial dairy products.

4.1 Evaluation of media for selective enumeration of bifidobacteria.

Table 1 shows the evaluation of various media for selective enumeration of bifidobacteria. Brain heart infusion agar did not inhibit *S. salivarius* subsp. *thermophilus* and Columbia agar did not inhibit *L. delbrueckii* subsp. *bulgaricus*. RCA inhibited both bifidobacteria and *S. salivarius* subsp. *thermophilus*. It was concluded that they would not be suitable for selective enumeration of bifidobacteria from dairy products such as yogurt. MRS agar containing 5% (w/v) lactose and 5% (v/v) NPNL antibiotic solution was the most successful in inhibiting growth of lactic acid bacteria and selective enumeration of bifidobacteria. Wijsman *et al.* (1989) reported that NPNL agar gave the highest recovery of bifidobacteria in dairy products compare to MSB agar and *Bifidobacterium* medium. Bifidobacteria grown on selective medium were more irregular shaped compare to when grown on non-selective medium. Samona and Robinson (1991) suggested that presence of inhibitors such as antibiotics caused alterations in morphology of bifidobacteria. MRSL + NPNL were used for selective enumeration of bifidobacteria in the following studies.

Table 1. Evaluation of MRSL+NPNL, brain heart infusion agar, columbia agar, and RCA media for selective enumeration of bifidobacteria.

Strains	Media			
	MRSL+NPNL[1]	Brain heart infusion agar	Columbia agar	RCA[2]
	(cfu/ml)[3]			
B. adolescentis ATCC 15703	7.3×10^7	5.0×10^7	5.0×10^7	$<10^2$
B. bifidum ATCC 29521	4.5×10^7	3.8×10^7	4.0×10^7	$<10^2$
L. delbrueckii subsp. *bulgaricus* NCK 231	$<10^2$	2.0×10^8	1.0×10^8	3.0×10^8
L. delbrueckii subsp. *bulgaricus* Lr-28	$<10^2$	9.5×10^7	8.0×10^7	1.2×10^8
S. salivarius subsp. *thermophilus* St-133	$<10^2$	$<10^2$	$<10^2$	$<10^2$
S. salivarius subsp. *thermophilus* St-113	$<10^2$	$<10^2$	$<10^2$	$<10^2$

[1]MRSL+NPNL is MRS agar containing 5% lactose and 5% NPNL antibiotic solution
[2]RCA is Reinforced Clostridial Agar
[3]Mean of duplicates for all treatments.

4.2 Viability of bifidobacteria and lactic acid bacteria in commercial A/B milk

Figure 7 shows the viability of bifidobacteria and lactic acid bacteria in commercial A/B milk. Although, a significant decrease ($p< 0.05$) in the bifidobacteria counts were observed at 3 days prior to product expiration, viability of bifidobacteria in commercial A/B milk remained above 10^6 cfu/ml until the product expired. Six days after the expiration date, bifidobacteria counts dropped below 10^6 cfu/ml. Lankaputhra *et al.* (1996) observed that viability of *B. infantis* in 12% skim milk at pH 4.3 were decreased by 30% after 12 days of storage at 4°C and more than 82% after 24 days at the same temperature. Medina and Jordano (1994) reported on the bifidobacteria count of fermented milk produced in Spain that was stored at 7°C. They observed a 92.6% decrease in bifidobacteria count when the product was expired. In our study, we observed a 70.7% reduction in bifidobacteria count at the time of expiration. Comparing to Medina and Jordano's study, our product was not a fermented milk product. The pH of this product remained at 6.6 or above during the investigation (Table 2). Thus, acid injury to the organism was prevented. *L. acidophilus* counts had decreased by 37.0% in the A/B milk at expiration time and the numbers were

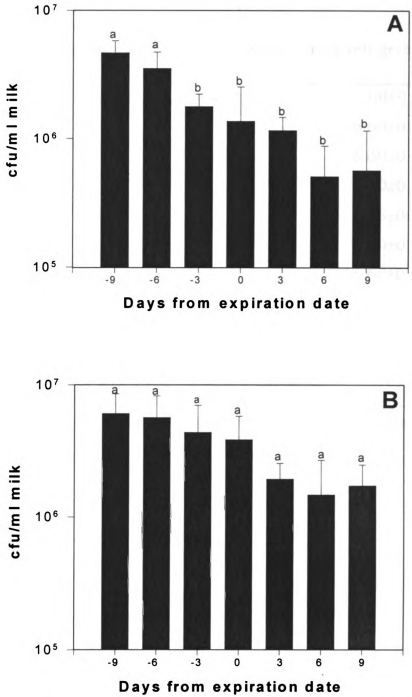

Figure 7. Viability of (A) bifidobacteria and (B) lactic acid bacteria in commercial A/B milk during 18 days of refrigerated storage at 5°C. 0 = Expiration day, -9, -6, -3 = days prior to product expiration, 3, 6, 9, = days past expiration. Bars with different letters are significantly different ($p < 0.05$)

Table 2. The pH of commercial A/B milk during refrigerated storage.

Days from product expiration[1]	(pH[2])
-9	6.60±0.01[a]
-6	6.60±0.02[a]
-3	6.60±0.08[a]
0	6.58±0.04[a]
3	6.60±0.04[a]
6	6.60±0.01[a]
9	6.56±0.01[a]

[1]0 = Product expiration day, -9, -6, and -3 = days prior to product expiration, 3, 6, and 9 = days past expiration.
[2]Means with the same superscript are not significantly different (p>0.05). Means with standard deviations; n=3 for all treatment.

above 10^6 cfu/ml, however this change was not significantly during the duration of this study (Figure 7).

4.3 Viability of bifidobacteria and lactic acid bacteria in commercial yogurt

Figure 8 shows the viability of bifidobacteria and lactic acid bacteria in commercial brand A yogurt. Although a significant decrease ($p<0.05$) was observed one week past product expiration day, viability of bifidobacteria in this product remained above 10^6 cfu/g, 2 weeks past the product expiration. It was only 3 weeks after the product expired that the counts were below 10^6 cfu/g. Lactic acid bacteria counts were maintained above 10^7 cfu/g during the duration of the study although a significant decline ($p<0.05$) was observed on the expiration day of the product. In brand A yogurt, bifidobacteria and lactic acid bacteria counts decreased 64.3% and 87.0%, respectively when the product expired. The pH of brand A yogurt during refrigerated storage is shown in Table 3.

Figure 9 shows the viability of bifidobacteria and lactic acid bacteria in commercial brand B yogurt. Viability of bifidobacteria in this product steadily declined during refrigerated storage. This decline was significant ($p<0.05$) at 1 week prior to product expiration and again at the date of expiration. However,

bifidobacteria counts remained above 10^6 cfu/g until 2 weeks past product expiration. Lactic acid bacteria count in brand B yogurt although declined significantly ($p<0.05$) at 1 week prior to product expiration the counts were above 10^6 cfu/g during the duration of the study. Bifidobacteria and lactic acid bacteria counts decreased 88.0% and 65.0%, respectively on the expiration day. The pH of brand B yogurt during refrigerated storage is shown in Table 4. Brand B yogurt had lower lactic acid bacteria counts than brand A. Difference in results may be due to different volume of inoculum, different processing environment, and differences in lactic acid bacteria strains between the two brands of yogurt.

Laroia and Martin, (1991) reported that viability of *B. bifidum* was very poor in low-pH (3.9-4.6) frozen fermented dairy desserts. However, Modler et al (1990) reported that some strains of bifidobacteria showed acid tolerance at pH 4.0. Blanchette *et al.* (1996) reported on manufacturing creamed cottage cheese with *B. infantis* ATCC 27920G and count of *B. infantis* in cottage cheeses was 10^7 cfu/g after the cream dressing was fermented by *B. infantis*. Viability of bifidobacteria in creamed cottage cheese was decreased 2-4 log cycles during storage for 15 days at 4°C. Hekmat and McMahon (1992) reported that ice cream may serve as a good vehicle for delivering bifidobacteria having potential health benefits

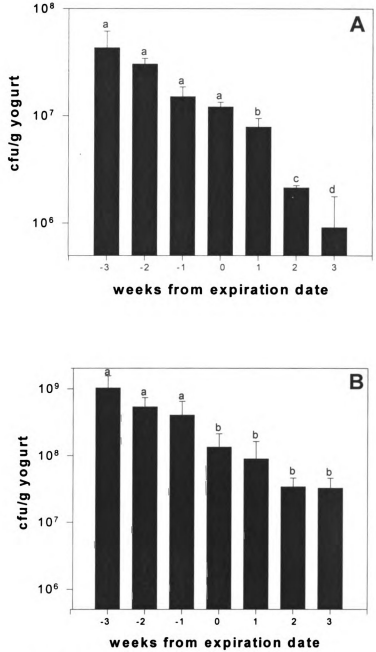

Figure 8. Viability of (A) bifidobacteria and (B) lactic acid bacteria in commercial brand A yogurt during 6 weeks of refrigerated storage at 5°C. 0 = Expiration day, -3, -2, -1 = weeks prior to product expiration, 1, 2, 3, = weeks past expiration. Bars with different letters are significantly different ($p< 0.05$)

Table 3. The pH of commercial brand A yogurt during refrigerated storage.

Weeks from product expiration[1]	(pH[2])
-3	4.23±0.02[a]
-2	4.23±0.01[a]
-1	4.21±0.02[a]
0	4.23±0.02[a]
1	4.21±0.01[a]
2	4.18±0.01[b]
3	4.17±0.01[b]

[1]0 = Product expiration day, -3, -2, and -1 = weeks prior to product expiration, 3, 2, and 1 = weeks past expiration.
[2]Means with different superscripts are significantly different ($p<$ 0.05). Means with standard deviations; n=3 for all treatment.

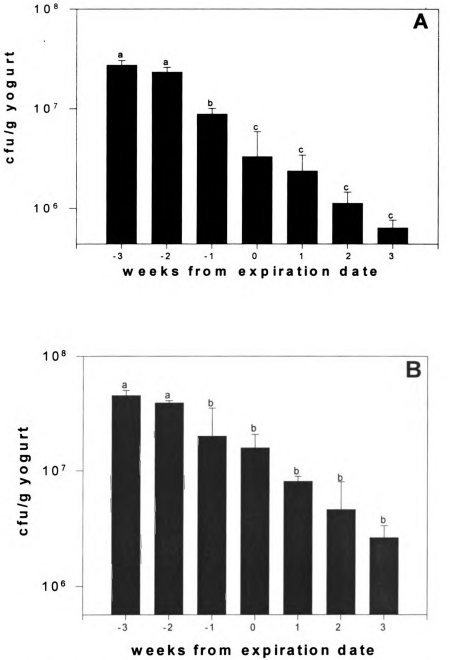

Figure 9. Viability of (A) bifidobacteria and (B) lactic acid bacteria in commercial brand B yogurt during 6 weeks of refrigerated storage at 5°C. 0 = Expiration day, -3, -2, -1 = weeks prior to product expiration, 1, 2, 3, = weeks past expiration. Bars with different letters are significantly different ($p<0.05$)

Table 4. The pH of commercial brand B yogurt during refrigerated storage.

Weeks from product expiration[1]	(pH[2])
-3	4.20±0.01[a]
-2	4.20±0.01[a]
-1	4.19±0.00[a]
0	4.19±0.00[a]
1	4.17±0.01[b]
2	4.17±0.01[b]
3	4.16±0.01[b]

[1]0 = Product expiration day, -3, -2, and -1 = weeks prior to product expiration, 3, 2, and 1 = weeks past expiration.
[2]Means with different superscripts are significantly different ($p<$ 0.05). Means with standard deviations; n=3 for all treatment.

to humans. Their study indicated that ice cream mix fermented with *L. acidophilus* and *B. bifidum* had higher numbers of viable cell after 17 weeks of storage at -29°C. After freezing of the fermented mix, *L. acidophilus* and *B. bifidum* count were 1.5×10^8 cfu/ml and 2.5×10^8 cfu/ml, respectively. Seventeen weeks after frozen storage, counts of *L. acidophilus* and *B. bifidum* were decreased by two log cycle to 4×10^6 and by one log cycles to 1×10^7 cfu/ml, respectively.

Shah *et al.* (1995) reported on the initial bifidobacteria counts to be 10^6-10^7 cfu/g in two of five brands of yogurt they studied and other three brands of yogurt had counts $<10^3$ cfu/g. Samples of five brands of commercial yogurt were obtained directly from the processors within two to three days of production. They also reported that all products showed a constant decline in the bifidobacteria and lactic acid bacteria counts during storage. There appears to be a significant variation in counts among products.

Although significance of viability in receiving health benefits of lactic acid bacteria have not been clearly established in the scientific literature, U.S.A., France, Japan, South Korea and Poland already have regulation for viable culture numbers in fermented dairy product which range $\geq10^6$ - 10^8 cfu/ml. In the U.S. these standards are used for the Seal Program to promote live and active cultures. Individual states such as California and Oregon state have also

adopted specific regulations pertaining to viability of cultures in dairy products consistent with the national standard (Sanders *et al.*, 1996). There appears to be a great variation in viability of cultures among products. Sanders *et al.*, (1996) reported that clinical studies have shown significant clinical benefits are observed upon ingestion of approximately 10^9 - 10^{10} organisms/d. Viability of cultures in dairy products may or may not be consistent with clinical studies. Thus, next part of this research focused on enhancing growth of bifidobacteria in dairy foods and maintaining its viability during refrigerated storage.

4.4 Effect of oligosaccharides and inulin on growth of bifidobacteria.

Table 5 shows the mean doubling times of *Bifidobacterium* sp. Bf-1 and Bf-6 in 12% NDM in the presence of various concentrations of oligosaccharides and inulin. Mean doubling time was used as a measure of specific growth rate for each culture. FOS showed the highest growth-promoting activity ($p<0.05$) on both strains of bifidobacteria when ≥1% was added as evident by the mean doubling time. Growth of *Bifidobacterium* sp. Bf-1 and Bf-6 was stimulated ($p<0.05$) when ≥3% of GOS was added to NDM prior to inoculation. In case of inulin, growth of *Bifidobacterium* sp. Bf-1

Table 5. Effect of oligosaccharides and inulin on growth of commercial *Bifidobacterium* sp. in 12% NDM.

Treatment	Conc. (%)	Mean doubling time[1](min)	
		Bifidobacterium sp. Bf-1	*Bifidobacterium* sp. Bf-6
12% skim milk	0	237±10	242±13
Fructooligosaccharide	0.5	215±16	227±10
(FOS)	1	200±12*	196±15*
	3	166±9*	171±8*
	5	132±15*	125±10*
Galactooligosaccharide	0.5	218±8	225±12
(GOS)	1	213±10	221±17
	3	184±12*	189±6*
	5	148±5*	140±17*
Inulin	0.5	230±7	234±10
	1	217±19	226±12
	3	211±20	220±18
	5	179±14*	170±15*

[1]Mean doubling time (T_d) = ln 2 / μ (Specific growth rate) and μ= ln X_2 – ln X_1 / t_1 – t_2
*Indicates significantly different (p<0.05). Comparisons are made only with the control. Means ± standard deviations; n=3 for all treatments.

and Bf-6 was stimulated ($p<0.05$) only when ≥5% of inulin was added. Bifidobacteria strains utilized inulin slowly, which deficient in inulinase necessary for their metabolism (Yamazaki and Dilawri, 1990). Inulinase is an enzyme which splits off fructose moieties from certain sugars displaying a fructose unit at the terminal β-2,1 position (Vandamme and Derycke, 1983). Fructooligosaccharide can be characterized as polymers of D-fructose joined by β(2→1) linkages and terminated with a D-glucose molecule linked to fructose by an α(1→2) bond as in sucrose (Kosaric *et al.*, 1984). Hidaka *et al.* (1986) reported that some bifidobacteria produce enzymes, which hydrolyzed fructooligosaccharide (FOS) efficiently. This is consistent with our study in that FOS was utilized most effectively with the two strains of bifidobacteria studied and showing the highest growth-promoting activity on bifidobacteria. However, the degree of polymerization (DP) of the fructooligosaccharide is also important. DP of fructooligosaccharide used in this study was between 2 and 7. Gibson and Roberfroid (1995) reported that maximum activity was obtained with short chains of fructooligosaccharide with DP of between 3 and 5. However, Dubey and Mistry (1996) reported that 0.5% of FOS did not stimulated the growth of bifidobacteria in infant formulas. Difference in results may be due to different composition of infant formulas and NDM,

Table 6. Effect of oligosaccharides and inulin on viability of commercial *Bifidobacterium* sp. in 12% NDM after 4 weeks of refrigerated storage.

Treatment	Conc (%)	% Viability[1]	
		Bifidobacterium sp. Bf-1	*Bifidobacterium* sp. Bf-6
Control	0	11.6±1.57[a]	9.3±1.70[a]
Fructooligosaccharide	.5	16.4±4.32[a]	10.1±0.40[a]
(FOS)	1	17.5±2.60[a]	13.4±2.54[a]
	3	41.6±3.67[b]	28.7±3.89[b]
	5	67.3±7.44[bc]	44.6±3.90[b]
Galactooligosaccharide	.5	14.1±1.30[a]	9.7±1.84[a]
(GOS)	1	15.4±2.52[a]	11.5±1.93[a]
	3	32.1±2.15[b]	24.9±3.23[b]
	5	52.2±4.76[c]	39.3±3.48[b]
Inulin	.5	12.1±2.30[a]	9.5±1.80[a]
	1	14.3±2.60[a]	10.2±2.74[a]
	3	17.9±5.46[a]	14.6±3.87[a]
	5	31.5±4.76[b]	18.9±5.19[a]

[a-c]Means with different superscripts are significantly different ($p<0.05$). Comparisons are made only within the same column. Means ± standard deviations; n=3 for all treatments.
[1]% viability = (cfu after 4 week storage / initial cfu before storage).

different concentration of FOS used, and differences in bifidobacteria strains between the two studies.

4.5 Effect of oligosaccharides on viability of bifidobacteria during refrigerated storage.

Table 6 shows the viability of two commercial bifidobacteria after 4 weeks of refrigerated storage. The initial viability of bifidobacteria was calculated to be 100% for both strains of bifidobacteria. *Bifidobacterium* sp. Bf-1 and Bf-6 in control 12% NDM exhibited marked drop in viability of approximately 90% after 4 weeks of refrigerated storage at 5°C. Only 11.6 ± 1.6% and 9.3 ± 1.7% of *Bifidobacterium* sp. Bf-1 and Bf-6 respectively remaining viable, after 4 weeks of refrigerated storage at 5°C. The viability of both strains of bifidobacteria was enhanced significantly ($p<0.05$) when ≥3% of FOS or GOS was added. However, concentration of 5% was needed for inulin to show a significant effect ($P<0.05$) on viability of *Bifidobacterium* sp. Bf-1. Inulin had no effect on viability of *Bifidobacterium* sp. Bf-6. The preparation of freshly autoclaved 12% skim milk as a medium, followed by inoculation with a large number of cells, incubation under anaerobic condition, and presence of good carbon sources, may have contributed to enhancing growth and maintaining good viability through refrigerated storage. Viabilities observed in this study was better

than those reported by Lee *et al.* (1996), probably due to commercial strains used in this study, which probably had better acid and oxygen tolerance, and perhaps better utilization of oligosaccharides and inulin. Modler *et al.* (1990) reported that FOS had no effect on viability of bifidobacteria in ice cream. In their study, FOS was mixed into ice cream and stored at -17°C. Bifidobacteria probably didn't have the opportunity to utilize FOS.

4.6 Effect of oligosaccharides and inulin on the production of acetic acid and lactic acid by *Bifidobacterium* sp.

Culture activity of commercial bifidobacteria was determined by measuring fermentation end products (lactic acid and acetic acid) by HPLC. Table 7 shows the production and ratio of acetic and lactic acid by *Bifidobacterium* sp. Bf-1 and Bf-6. Acetic acid production ranged from 6.2 mmol/L (in 12% NDM; control) to 28.7 mmol/L (FOS) for *Bifidobacterium* sp. Bf-1. For *Bifidobacterium* sp. Bf-6, acetic acid production ranged from 10.3 mmol/L (in 12% NDM; control) to 15.8 mmol/L (FOS). Lactic acid production on the other hand ranged from 4.9 mmol/L (in 12% NDM; control) to 15.5 mmol/L (FOS) for *Bifidobacterium* sp. Bf-1. For *Bifidobacterium* sp. Bf-6, lactic acid production ranged from 5.7 mmol/L (in 12% NDM; control) to 11.7 mmol/L (FOS). Acetic acid and lactic acid

Table 7. Effect of oligosaccharides and inulin on acetic acid and lactic acid production by *Bifidobacterium* sp. in 12% NDM.

	Bifidobacterium sp.					
	Bifidobacterium sp. Bf-1			*Bifidobacterium* sp. Bf-6		
Treatment	AA[1]	LA[2]	Ratio[3]	AA	LA	Ratio
	(mmol)			(mmol)		
Skim milk (control)	6.2±0.5[a]	4.9±0.5[a]	1.27±0.1[a]	10.3±1.0[a]	5.7±0.4[a]	1.80±0.1[a]
5% FOS	28.7±4.8[d]	15.5±0.9[c]	1.85±0.1[b]	15.8±2.6[d]	11.6±1.2[b]	1.36±0.1[a]
5% GOS	10.9±1.6[c]	7.0±0.9[b]	1.55±0.1[ac]	14.2±0.5[cd]	11.7±3.8[b]	1.28±0.2[a]
5% Inulin	8.2±0.5[b]	5.5±0.2[a]	1.49±0.1[ac]	11.3±0.8[ab]	9.5±2.2[ab]	1.24±0.1[a]

1 = Acetic acid
2 = Lactic acid
3 = Acetic acid concentration/ Lactic acid concentration
[a-d]Means with different superscripts are significantly different ($p<0.05$). Comparisons are made only within the same column. Means ± standard deviations; n=3 for all treatments.

production was stimulated significantly ($p<0.05$) in *Bifidobacterium* sp. Bf-1 and Bf-6 when FOS or GOS were added to NDM. Whereas, inulin only stimulated ($p<0.05$) acetic acid production in *Bifidobacterium* sp. Bf-1 and did not stimulate lactic acid production in either strain of bifidobacteria. The theoretical ratio of acetic acid to lactic acid by bifidobacteria is reported to be 3:2 (Roy and Goulet, 1991; Tamine *et al.*, 1995). Organic acid in dairy products is important in monitoring culture activity, understanding microbial metabolism, and in determining quality of milk products (Bouzas *et al.*, 1991). Fermented dairy products have various proportions of lactic acid ranging from 0.9 - 6%, which has been reported to develop the characteristic flavor and texture, and the inhibition of certain pathogenic bacteria in dairy products (Driessen and de Boer, 1989; Fernandez-Garcia and McGregor, 1994). Acetic acid in fermented dairy products also has antimicrobial effects (Samona *et al.*, 1996). However, high acetic acid concentrations in dairy products are not typically desirable from a quality stand point. The mean ratio of acetic acid and lactic acid in this study ranged from 1.24 to 1.85, and no difference in the ratios were observed between the treatments except for *Bifidobacterium* sp. Bf-1 grown with FOS (Table 7). Samona *et al.* (1996) reported that an imbalanced ratio of acetic acid and lactic acid could contribute to risk of a vinegar over lactic acid

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Table 8. Screening media for selective enumeration of *L. delbrueckii* subsp. *bulgaricus* and *S. salivarius* subsp. *thermophilus*.

Strain	Media						
	Lee's agar	ST agar	M17 agar	Streptosel agar	Azide dextrose agar	MRS agar + oxgall	RCA
	(cfu/ml)						
Bf M101-4[2]	$<10^2$	5.5×10^5	$<10^2$	$<10^2$	$<10^2$	4.0×10^8	$<10^2$
Bf-1[3]	$<10^2$	4.4×10^5	$<10^2$	$<10^2$	$<10^2$	3.2×10^8	$<10^2$
NCK 231[4]	5.3×10^8	3.2×10^8	$<10^2$	7.0×10^7	3.5×10^7	8.0×10^8	7.5×10^8
Lr-28[5]	1.0×10^8	8.5×10^7	$<10^2$	9.0×10^7	6.8×10^8	4.5×10^8	4.3×10^8
St-133[6]	1.3×10^8	2.7×10^8	5.4×10^8	4.8×10^8	2.0×10^8	5.5×10^8	$<10^2$
St-113[7]	8.6×10^7	1.8×10^8	4.0×10^8	3.5×10^8	3.5×10^8	3.3×10^8	$<10^2$

[1]Mean of duplicates for all treatments.
[2]Bf M101-4 is *B. adolescentis* M101-4
[3]Bf-1 is *Bifidobacterium* sp. Bf-1
[4]NCK 231 is *L. delbrueckii* subsp. *bulgaricus* NCK 231
[5]Lr-28 is *delbrueckii* subsp. *L. bulgaricus* Lr-28
[6]St 133 is *S. salivarius* subsp. *thermophilus* St-133
[7]St 113 is *S. salivarius* subsp. *thermophilus* St-113

M17 agar was shown to be the most successful in enumerating of *S. salivarius* subsp. *thermophilus* and modified RCA was shown to be the most successful for the selective enumeration of *L. delbrueckii* subsp. *bulgaricus* (Table 8).

4.8 Screening *L. delbrueckii* subsp. *bulgaricus* and *S. salivarius* subsp. *thermophilus*.

S. salivarius subsp. *thermophilus* St-52, St-113, St-133, St-134, *L. delbrueckii* subsp. *bulgaricus* Lr-28, Lr-78, Lr-79, NCK231 were screened for their ability to provide optimum pH and redox potential conditions for growth of bifidobacteria. *S. salivarius* subsp. *thermophilus* St 133 showed the ability to reduce redox potential faster than other strains (Table 9). It has been shown that some strains of *S. salivarius* subsp. *thermophilus* have high oxygen consuming ability thus reducing redox potential suitable for growth of bifidobacteria (Okonogi *et al.*, 1986; Reuter, 1989; Tinson *et al.*, 1982; Teraguchi *et al.*, 1987). *L. delbrueckii* subsp. *bulgaricus* is typically an acid producer. *L. delbrueckii* subsp. *bulgaricus* NCK 231 was selected because it produced the most suitable amount of acid in 12% NDM (Table 10). Most strains of bifidobacteria are very sensitive to the low pH (≤ 4.6) however, Rasic and Kurmann (1983) reported that some strains of *B. bifidum* showed acid tolerance to

Table 9. The redox potential (Eh_7) of 12% NDM cultured with *S. salivarius* subsp. *thermophilus*.

Strain	$(Eh_7)^1$			
	0hr	8hr	16hr	24hr
S. salivarius subsp. *thermophilus* St-52	314	217	128	26
S. salivarius subsp. *thermophilus* St-113	309	206	112	27
S. salivarius subsp. *thermophilus* St-133	311	183	82	-10
S. salivarius subsp. *thermophilus* St-134	311	193	90	6

All data are mean of duplicates
[1]Eh_7 = Eh measured – 59(7.00 – pH measured).

Table 10. The pH of 12% NDM cultured with *L. delbrueckii* subsp. *bulgaricus* or *S. salivarius* subsp. *thermophilus*.

Strain	(pH)			
	0hr	8hr	16hr	24hr
S. salivarius subsp. *thermophilus* St-52	6.30	5.58	5.02	3.88
S. salivarius subsp. *thermophilus* St-113	6.31	5.71	5.17	4.00
S. salivarius subsp. *thermophilus* St-133	6.30	5.95	5.40	4.20
S. salivarius subsp. *thermophilus* St-134	6.32	5.80	5.31	4.08
L. delbrueckii subsp. *bulgaricus* Lr-28	6.28	5.45	4.48	3.65
L. delbrueckii subsp. *bulgaricus* Lr-78	6.30	5.52	4.77	3.78
L. delbrueckii subsp. *bulgaricus* Lr-79	6.28	5.45	4.50	3.66
L. delbrueckii subsp. *bulgaricus* NCK 231	6.28	5.50	4.56	3.70

All data are mean of duplicates

pH 4.0. Although, acid tolerance varies from strain to strain, pH 4.6 – 4.0 might be the lowest pH value to prevent acid injury to growth of bifidobacteria.

4.9 Growth of bifidobacteria co-cultured with *L. delbrueckii* subsp. *bulgaricus* NCK 231 and *S. salivarius* subsp. *thermophilus* St-133.

Table 11 and 12 show the EH_7, redox potential of the NDM standardized to pH 7 and 25°C that has been co-cultured with *L. delbrueckii* subsp. *bulgaricus* NCK 231, *S. salivarius* subsp. *thermophilus* St-133 and *Bifidobacterium* sp. Bf-1 or *B. adolescentis* M101-4, respectively over a 24 hr incubation period. EH_7 of NDM cultured with *S. salivarius* subsp. *thermophilus* St-133 alone was lowest ($p<0.05$) both at 12 and 24 hr compared to Eh_7 of NDM, NDM cultured with *L. delbrueckii* subsp. *bulgaricus* NCK 231 or bifidobacteria alone. Co-culturing bifidobacteria with *S. salivarius* subsp. *thermophilus* St-133 was more effective in lowering redox potential ($p<0.05$) than co-culturing with *L. delbrueckii* subsp. *bulgaricus* NCK 231. When co-cultured, a ratio of 1:2 (bifidobacteria to *S. salivarius* subsp. *thermophilus* St-133) provided the lowest ($p<0.05$) Eh_7 after 24 hr of incubation in both strains of bifidobacteria (Table 11 and 12). When either strains of

bifidobacteria was co-cultured with *L. delbrueckii* subsp. *bulgaricus* NCK 231 and *S. salivarius* subsp. *thermophilus* St-133, the most effective ratio for lowering Eh_7 was 1:1:2 (when *S. salivarius* subsp. *thermophilus* St-133 was inoculated at twice the concentration of *L. delbrueckii* subsp. *bulgaricus* NCK 231 and bifidobacteria). The Eh_7 at 36 and 48h were similar to that of 24h for all treatments. Thus, these results are not provided in the tables. Okonogi *et al.* (1986) reported that novel strain of *S. salivarius* subsp. *thermophilus* has high oxygen utilization and this ability can enhance viability of bifidobacteria. This patent strains of *S. salivarius* subsp. *thermophilus* M-8202, M-8203, M-8204, and M-8205, which have high oxygen uptake ability could be utilized to reduce fermentation cost of bifidobacteria in commercial culture processing. During anaerobic fermentation, *S. salivarius* subsp. *thermophilus* catabolizes 1mol of glucose to 2mol of lactate through the glycolytic pathway, but in aerobic metabolism glucose or pyruvate leads to the formation of acetate, α-acetolactate, acetoin, and diacetyl in addition to lactic acid production. NADH generated in aerobic metabolism is consumed in the reaction catalyzed by NADH oxidase. Aerobic O_2 uptake was also observed with lactobacilli but to a lesser extent (Tinson *et al.*, 1982; Teraguchi *et al.*, 1987). Bifidobacteria are classified as anaerobes thus, certain precautions

Table 11. Redox potential (Eh_7) and pH of NDM co-cultured with *Bifidobacterium* sp. Bf-1, *L. delbrueckii* subsp. *bulgaricus* NCK231 and *S. salivarius* subsp. *thermophilus* St-133.

Treatments	(Eh_7)[4]			(pH)		
	0 hr	12 hr	24 hr	0 hr	12 hr	24 hr
12% NDM (Control)	329±43[a]	314±35[d]	314±43[i]	6.47±.04[d]	6.39±.03[f]	6.35±.08[e]
Bf-1[1]	328±45[a]	278±54[c]	190±45[gh]	6.39±.03[c]	5.73±.03[c]	3.68±.04[d]
LB[2]	313±40[a]	228±63[bc]	76±27[cd]	6.26±.04[a]	5.43±.06[a]	3.50±.07[a]
ST[3]	295±50[a]	141±40[a]	-43±39[a]	6.28±.06[b]	5.50±.05[ab]	3.55±.06[ab]
Bf-1:LB 1:1	302±37[a]	251±34[c]	149±40[fg]	6.28±.05[b]	5.51±.03[ab]	3.55±.02[ab]
Bf-1:LB 1:2	300±46[a]	248±45[bc]	140±37[fg]	6.28±.05[b]	5.50±.03[ab]	3.54±.03[ab]
Bf-1:LB 2:1	311±35[a]	252±50[b]	154±25[fg]	6.32±.04[bc]	5.52±.03[ab]	3.57±.02[abc]
Bf-1:ST[3] 1:1	269±60[a]	181±45[b]	50±34[b]	6.38±.03[c]	5.65±.03[b]	3.60±.04[bc]
Bf-1:ST 1:2	262±47[a]	162±55[ab]	-32±29[a]	6.36±.04[bc]	5.62±.03[b]	3.60±.02[bc]
Bf-1:ST 2:1	277±50[a]	232±42[bc]	97±37[de]	6.38±.01[c]	5.67±.03[b]	3.61±.04[bc]
Bf-1:LB:ST 1:1:1	275±45[a]	246±40[c]	100±46[ef]	6.34±.04[bc]	5.50±.03[ab]	3.56±.02[abc]
Bf-1:LB:ST 1:2:1	278±50[a]	246±50[c]	111±32[ef]	6.26±.02[a]	5.42±.03[a]	3.52±.02[a]
Bf-1:LB:ST 1:1:2	269±65[a]	194±35[b]	74±30[c]	6.34±.01[bc]	5.57±.02[b]	3.61±.02[bc]
Bf-1:LB:ST 2:1:1	295±50[a]	240±55[c]	148±42[f]	6.37±.03[bc]	5.62±.09[bd]	3.62±.03[c]

[1]Bf-1 is *Bifidobacterium* sp. Bf-1.
[2]LB is *L. delbrueckii* subsp. *bulgaricus* NCK 231.
[3]ST is *S. salivarius* subsp. *thermophilus* St-133.
[4]Eh_7 = Eh measured –59 (7.00 – pH measured).
[a-i] Means with different superscripts are significantly different ($p<0.05$). Comparisons are made only within the same column. Means ± standard deviations; n=3 for all treatments.

Table 12. Redox potential (Eh_7) and pH of NDM co-cultured with *B. adolescentis* M101-4, *L. delbrueckii* subsp. *bulgaricus* NCK231 and *S. salivarius* subsp. *thermophilus* St-133.

Treatments	(Eh_7)[4]			(pH)		
	0 hr	12 hr	24 hr	0 hr	12 hr	24 hr
12% NDM (Control)	334±11[a]	322±13[c]	311±10[i]	6.50±.10[a]	6.43±.07[e]	6.34±.08[c]
M[1]	333±10[a]	268±15[bc]	178±17[gh]	6.53±.09[a]	5.97±.10[c]	4.55±.15[b]
LB[2]	330±13[a]	235±10[b]	77±6[de]	6.49±.08[a]	5.60±.08[a]	3.81±.10[a]
ST[3]	332±15[a]	148±12[a]	-59±7[a]	6.52±.13[a]	5.70±.08[ab]	3.97±.07[ab]
M:LB 1:1	332±10[a]	246±16[b]	131±15[gh]	6.52±.12[a]	5.71±.05[ab]	3.94±.02[a]
M:LB 1:2	332±10[a]	243±6[b]	122±10[gh]	6.52±.10[a]	5.69±.05[ab]	3.89±.04[a]
M:LB 2:1	332±13[a]	246±12[b]	140±12[gh]	6.50±.10[a]	5.72±.05[ab]	4.01±.05[a]
M:ST 1:1	329±10[a]	198±10[a]	25±5[b]	6.51±.11[a]	5.88±.10[b]	4.47±.12[b]
M:ST 1:2	327±10[a]	159±12[a]	-27±8[a]	6.49±.11[a]	5.86±.10[b]	4.43±.10 [b]
M:ST 2:1	335±11[a]	228±8[a]	55±4[cd]	6.51±.09[a]	5.90±.11[b]	4.49±.11[b]
M:LB:ST 1:1:1	333±17[a]	229±10[b]	81±7[e]	6.54±.13[a]	5.70±.06[b]	3.98±.09[a]
M:LB:ST 1:2:1	333±15[a]	232±12[b]	95±5[f]	6.53±.09[a]	5.65±.11[a]	3.94±.10[a]
M:LB:ST 1:1:2	332±12[a]	208±10[ab]	42±5[c]	6.54±.08[a]	5.77±.09[b]	4.05±.11[a]
M:LB:ST 2:1:1	335±15[a]	352±10[b]	128±10[g]	6.54±.11[a]	5.81±.03[bd]	4.09±.02[a]

[1]M is *B. adolescentis* M101-4.
[2]LB is *L. delbrueckii* subsp. *bulgaricus* NCK 231.
[3]ST is *S. salivarius* subsp. *thermophilus* St-133.
[4]Eh_7 = Eh measured –59 (7.00 – pH measured).
[a-i]Means with different superscripts are significantly different ($p<0.05$). Comparisons are made only within the same column. Means ± standard deviations; n=3 for all treatments.

are required to prevent the toxic effects of oxygen when *Bifidobacterium* sp. are cultivated for industrial application (De Vries and Stouthamer, 1989).

Table 11 and 12 also show the pH variation of 12% NDM when each bifidobacteria strain was co-cultured with both *S.salivarius* subsp. *thermophilus* St-133 or *L. delbrueckii* subsp. *bulgaricus* NCK 231. Tinson *et al.* (1982) reported that the rate of acid development by mixed starter cultures, *S. salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, was greater than that of single strain. In our study, pH was primarily influenced by the inoculum level of *L. delbrueckii* subsp. *bulgaricus*.

Table 13 and 14 show the growth of *Bifidobacterium* sp. Bf-1 and *B. adolescentis* M101-4 in 12% NDM when it was co-cultured with *L. delbrueckii* subsp. *bulgaricus* NCK 231 and *S. salivarius* subsp. *thermophilus* St-133. For both strains of bifidobacteria there were no differences observed in the cfu of bifidobacteria when cultured alone compared to bifidobacteria co-cultured with *L. delbrueckii* subsp. *bulgaricus* NCK 231 and *S. salivarius* subsp. *thermophilus* St-133. Co-culturing bifidobacteria with lactic acid bacteria although provided the initial low Eh_7 in NDM, this was not significant to enhance the growth of bifidobacteria due to the low pH of the system. It appears that growth of bifidobacteria is more

strongly influenced by pH than Eh_7. De Vries and Stouthamer (1989) reported that although the sensitivity to oxygen varies between strains of *Bifidobacterium* sp., redox potential is related to the growth of *Bifidobacterium* sp. Our results are consistent with Klaver *et al.* (1993) who reported that low redox potential but more importantly proper pH change in the early phase of incubation are required for good growth of bifidobacteria. Growth of *Bifidobacterium* sp. in NDM was related to pH more so than Eh_7.

Table 15 and 16 show the growth of *S. salivarius* subsp. *thermophilus* St-133 in 12% NDM when it was co-cultured with *L. delbrueckii* subsp. *bulgaricus* NCK 231 and *Bifidobacterium* sp. Bf-1 or *B. adolescentis* M101-4. There were no differences observed in the cfu of *S. salivarius* subsp. *thermophilus* St-133 when cultured alone compared to *S. salivarius* subsp. *thermophilus* St-133 co-cultured with *L. delbrueckii* subsp. *bulgaricus* NCK 231 and *Bifidobacterium* sp. Bf-1 or *B. adolescentis* M101-4. Table 17 and 18 show the growth of *L. delbrueckii* subsp. *bulgaricus* NCK 231 in 12% NDM when it was co-cultured with *S. salivarius* subsp. *thermophilus* St-133 and *Bifidobacterium* sp. Bf-1 or *B. adolescentis* M101-4. There were no differences observed in the cfu of *L. delbrueckii* subsp. *bulgaricus* NCK 231 when *L. delbrueckii* subsp. *bulgaricus* NCK 231 cultured alone compared to co-cultured with *S.*

Table 13. Growth of *Bifidobacterium* sp. Bf-1 co-cultured in 12% NDM with *L. delbrueckii* subsp. *bulgaricus* NCK 231 and *S. salivarius* subsp. *thermophilus* St-133.

Treatments	(cfu/g)				
	0 hr	12 hr	24 hr	36 hr	48 hr
Bf-1[1]	$8.7\times10^{6}\pm8.9\times10^{6}$	$1.9\times10^{7}\pm2.3\times10^{6}$	$5.5\times10^{7}\pm6.1\times10^{6}$	$1.6\times10^{8}\pm1.9\times10^{7}$	$1.8\times10^{8}\pm2.2\times10^{7}$
Bf-1:LB[2] 1:1	$8.7\times10^{6}\pm1.1\times10^{6}$	$2.5\times10^{7}\pm\times3.210^{6}$	$8.3\times10^{7}\pm1.0\times10^{6}$	$1.8\times10^{8}\pm2.2\times10^{7}$	$2.1\times10^{8}\pm2.7\times10^{7}$
Bf-1:LB 1:2	$8.7\times10^{6}\pm1.1\times10^{6}$	$2.4\times10^{7}\pm3.1\times10^{6}$	$8.3\times10^{7}\pm1.0\times10^{6}$	$1.8\times10^{8}\pm2.3\times10^{7}$	$2.0\times10^{8}\pm2.6\times10^{7}$
Bf-1:LB 2:1	$8.7\times10^{6}\pm1.1\times10^{6}$	$2.2\times10^{7}\pm2.8\times10^{6}$	$6.3\times10^{7}\pm7.5\times10^{6}$	$2.3\times10^{8}\pm2.3\times10^{7}$	$2.2\times10^{8}\pm2.7\times10^{7}$
Bf-1:ST[3] 1:1	$8.7\times10^{6}\pm1.1\times10^{6}$	$2.8\times10^{7}\pm3.6\times10^{6}$	$8.9\times10^{7}\pm1.1\times10^{6}$	$2.4\times10^{8}\pm2.9\times10^{7}$	$2.6\times10^{8}\pm3.4\times10^{7}$
Bf-1:ST 1:2	$8.7\times10^{6}\pm1.1\times10^{6}$	$3.1\times10^{7}\pm4.1\times10^{6}$	$1.1\times10^{8}\pm1.5\times10^{6}$	$2.1\times10^{8}\pm3.2\times10^{7}$	$2.8\times10^{8}\pm3.6\times10^{7}$
Bf-1:ST 2:1	$8.7\times10^{6}\pm1.1\times10^{6}$	$3.0\times10^{7}\pm3.8\times10^{6}$	$7.1\times10^{7}\pm8.6\times10^{6}$	$2.1\times10^{8}\pm2.7\times10^{7}$	$2.4\times10^{8}\pm3.0\times10^{7}$
Bf1:LB:ST 1:1:1	$8.7\times10^{6}\pm1.1\times10^{6}$	$2.8\times10^{7}\pm3.6\times10^{6}$	$8.1\times10^{7}\pm1.0\times10^{6}$	$2.1\times10^{8}\pm2.7\times10^{7}$	$2.3\times10^{8}\pm3.0\times10^{7}$
Bf1:LB:ST 1:2:1	$8.7\times10^{6}\pm1.1\times10^{6}$	$2.4\times10^{7}\pm3.1\times10^{6}$	$7.0\times10^{7}\pm8.7\times10^{6}$	$2.0\times10^{8}\pm2.6\times10^{7}$	$2.2\times10^{8}\pm2.8\times10^{7}$
Bf1:LB:ST 1:1:2	$8.7\times10^{6}\pm1.1\times10^{6}$	$3.0\times10^{7}\pm3.7\times10^{6}$	$1.0\times10^{8}\pm1.4\times10^{6}$	$2.3\times10^{8}\pm3.0\times10^{7}$	$2.6\times10^{8}\pm3.3\times10^{7}$
Bf1:LB:ST 2:1:1	$8.7\times10^{6}\pm1.1\times10^{6}$	$2.6\times10^{7}\pm3.3\times10^{6}$	$6.3\times10^{7}\pm7.5\times10^{6}$	$2.0\times10^{8}\pm2.6\times10^{7}$	$2.2\times10^{8}\pm2.7\times10^{7}$

Means of triplicates for all treatments.
[1]Bf-1 is *Bifidobacterium* sp. Bf-1.
[2]LB is *L. delbrueckii* subsp. *bulgaricus* NCK 231.
[3]ST is *S. salivarius* subsp. *thermophilus* St-133.
No statistical differences were detected between means ($p> 0.05$)

Table 14. Growth of *B. adolescentis* M101-4 co-cultured in 12% NDM with *L. delbrueckii* subsp. *bulgaricus* NCK 231 and *S. salivarius* subsp. *thermophilus* St-133.

Treatment	(cfu/g)				
	0 hr	12 hr	24 hr	36 hr	48 hr
M[1]	$1.9\times10^{6}\pm5.6\times10^{5}$	$5.4\times10^{6}\pm4.0\times10^{5}$	$1.7\times10^{7}\pm2.1\times10^{6}$	$4.0\times10^{7}\pm4.1\times10^{6}$	$4.8\times10^{7}\pm8.0\times10^{6}$
M:LB[2] 1:1	$2.3\times10^{6}\pm1.7\times10^{5}$	$6.2\times10^{6}\pm0.0$	$2.0\times10^{7}\pm0.0$	$4.3\times10^{7}\pm0.0$	$5.5\times10^{7}\pm2.9\times10^{6}$
M:LB 1:2	$2.3\times10^{6}\pm1.7\times10^{5}$	$6.0\times10^{6}\pm0.0$	$2.0\times10^{7}\pm0.0$	$4.5\times10^{7}\pm2.3\times10^{6}$	$5.4\times10^{7}\pm3.5\times10^{6}$
M:LB 2:1	$2.4\times10^{6}\pm2.9\times10^{5}$	$5.8\times10^{6}\pm2.9\times10^{5}$	$1.8\times10^{7}\pm2.9\times10^{6}$	$4.8\times10^{7}\pm2.3\times10^{6}$	$5.6\times10^{7}\pm2.8\times10^{6}$
M:ST[3] 1:1	$2.4\times10^{6}\pm2.8\times10^{5}$	$7.3\times10^{6}\pm2.9\times10^{5}$	$2.2\times10^{7}\pm2.9\times10^{6}$	$5.8\times10^{7}\pm2.9\times10^{6}$	$6.5\times10^{7}\pm2.8\times10^{6}$
M:ST 1:2	$2.4\times10^{6}\pm2.8\times10^{5}$	$7.3\times10^{6}\pm2.9\times10^{5}$	$2.3\times10^{7}\pm1.5\times10^{6}$	$6.0\times10^{7}\pm2.9\times10^{6}$	$6.9\times10^{7}\pm3.6\times10^{6}$
M:ST 2:1	$2.4\times10^{6}\pm2.8\times10^{5}$	$7.6\times10^{6}\pm2.8\times10^{5}$	$2.0\times10^{7}\pm3.6\times10^{6}$	$5.5\times10^{7}\pm2.9\times10^{6}$	$6.1\times10^{7}\pm3.0\times10^{6}$
M:LB:ST 1:1:1	$2.4\times10^{6}\pm2.8\times10^{5}$	$7.3\times10^{6}\pm2.8\times10^{5}$	$2.0\times10^{7}\pm2.0\times10^{6}$	$5.3\times10^{7}\pm2.9\times10^{6}$	$5.9\times10^{7}\pm2.9\times10^{6}$
M:LB:ST 1:2:1	$2.4\times10^{6}\pm2.8\times10^{5}$	$6.3\times10^{6}\pm3.1\times10^{5}$	$1.8\times10^{7}\pm3.7\times10^{6}$	$5.3\times10^{7}\pm2.9\times10^{6}$	$5.8\times10^{6}\pm2.8\times10^{6}$
M:LB:ST 1:1:2	$2.4\times10^{6}\pm2.8\times10^{5}$	$8.1\times10^{6}\pm3.7\times10^{5}$	$2.3\times10^{7}\pm1.4\times10^{6}$	$5.8\times10^{7}\pm3.0\times10^{6}$	$6.1\times10^{7}\pm3.3\times10^{6}$
M:LB:ST 2:1:1	$2.4\times10^{6}\pm2.8\times10^{5}$	$6.8\times10^{6}\pm3.3\times10^{5}$	$1.8\times10^{7}\pm2.5\times10^{6}$	$5.3\times10^{7}\pm2.6\times10^{6}$	$5.6\times10^{7}\pm2.7\times10^{6}$

Means of triplicates for all treatments.
[1]M is *B. adolescentis* M101-4.
[2]LB is *L. delbrueckii* subsp. *bulgaricus* NCK 231.
[3]ST is *S. salivarius* subsp. *thermophilus* St-133.
No statistical differences were detected between means ($p> 0.05$)

Table 15. Growth of *S. salivarius* subsp. *thermophilus* St-133 in 12% NDM co-cultured with *L. delbrueckii* subsp. *bulgaricus* NCK 231 and *Bifidobacterium* sp. Bf-1.

Treatment	(cfu/g)				
	0 hr	12 hr	24 hr	36 hr	48 hr
ST[3]	$9.3\times10^{6}\pm2.7\times10^{5}$	$4.4\times10^{7}\pm3.5\times10^{6}$	$9.3\times10^{7}\pm2.0\times10^{7}$	$7.0\times10^{8}\pm7.0\times10^{6}$	$8.3\times10^{8}\pm2.0\times10^{8}$
Bf-1[1]:ST 1:1	$9.3\times10^{6}\pm2.7\times10^{5}$	$4.3\times10^{7}\pm4.0\times10^{6}$	$9.5\times10^{7}\pm1.5\times10^{7}$	$7.0\times10^{8}\pm2.5\times10^{7}$	$8.5\times10^{8}\pm3.4\times10^{8}$
Bf-1:ST 1:2	$9.3\times10^{6}\pm2.7\times10^{5}$	$4.8\times10^{7}\pm2.5\times10^{6}$	$8.0\times10^{7}\pm1.0\times10^{7}$	$7.0\times10^{8}\pm5.5\times10^{7}$	$8.0\times10^{8}\pm3.6\times10^{8}$
Bf-1:ST 2:1	$9.3\times10^{6}\pm2.7\times10^{5}$	$4.6\times10^{7}\pm2.8\times10^{6}$	$8.0\times10^{7}\pm4.6\times10^{7}$	$6.5\times10^{8}\pm4.7\times10^{7}$	$8.5\times10^{8}\pm3.0\times10^{8}$
Bf-1:LB[2]:ST 1:1:1	$9.3\times10^{6}\pm2.7\times10^{5}$	$4.3\times10^{7}\pm4.1\times10^{6}$	$8.1\times10^{7}\pm2.0\times10^{7}$	$6.5\times10^{8}\pm1.7\times10^{7}$	$8.0\times10^{8}\pm3.0\times10^{8}$
Bf-1:LB:ST 1:2:1	$9.3\times10^{6}\pm2.7\times10^{5}$	$4.0\times10^{7}\pm2.9\times10^{6}$	$9.5\times10^{7}\pm1.5\times10^{7}$	$7.0\times10^{8}\pm5.8\times10^{7}$	$8.0\times10^{8}\pm2.8\times10^{8}$
Bf-1:LB:ST 1:1:2	$9.3\times10^{6}\pm2.7\times10^{5}$	$4.6\times10^{7}\pm2.8\times10^{6}$	$7.8\times10^{7}\pm1.5\times10^{7}$	$7.0\times10^{8}\pm1.0\times10^{8}$	$8.5\times10^{8}\pm3.3\times10^{8}$
Bf-1:LB:ST 2:1:1	$9.3\times10^{6}\pm2.7\times10^{5}$	$4.0\times10^{7}\pm3.5\times10^{6}$	$8.0\times10^{7}\pm2.5\times10^{7}$	$6.5\times10^{8}\pm6.6\times10^{7}$	$8.3\times10^{8}\pm2.7\times10^{8}$

Means of triplicates for all treatments.
[1]Bf-1 is *Bifidobacterium* sp. Bf-1.
[2]LB is *L. delbrueckii* subsp. *bulgaricus* NCK 231.
[3]ST is *S. salivarius* subsp *thermophilus* St-133.
No statistical differences were detected between means ($p > 0.05$)

Table 16. Growth of *S. salivarius* subsp. *thermophilus* St-133 in 12% NDM co-cultured with *L. delbrueckii* subsp. *bulgaricus* NCK 231 and *B. adolescentis* M101-4.

Treatment	(cfu/g)				
	0 hr	12 hr	24 hr	36 hr	48 hr
ST[3]	$9.3\times10^{6}\pm2.7\times10^{5}$	$5.3\times10^{7}\pm4.0\times10^{6}$	$9.0\times10^{7}\pm3.0\times10^{7}$	$7.3\times10^{6}\pm5.5\times10^{7}$	$8.3\times10^{8}\pm7.0\times10^{7}$
M[1]:ST 1:1	$9.3\times10^{6}\pm2.7\times10^{5}$	$5.0\times10^{7}\pm3.0\times10^{6}$	$9.0\times10^{7}\pm3.5\times10^{7}$	$7.5\times10^{8}\pm6.5\times10^{7}$	$7.5\times10^{8}\pm5.5\times10^{7}$
M:ST 1:2	$9.3\times10^{6}\pm2.7\times10^{5}$	$5.0\times10^{7}\pm2.5\times10^{6}$	$8.5\times10^{7}\pm2.0\times10^{7}$	$7.5\times10^{8}\pm6.0\times10^{7}$	$8.0\times10^{8}\pm1.0\times10^{8}$
M:ST 2:1	$9.3\times10^{6}\pm2.7\times10^{5}$	$5.5\times10^{7}\pm2.5\times10^{6}$	$8.0\times10^{7}\pm3.7\times10^{7}$	$6.0\times10^{8}\pm3.7\times10^{7}$	$7.5\times10^{8}\pm5.0\times10^{7}$
M:LB[2]:ST 1:1:1	$9.3\times10^{6}\pm2.7\times10^{5}$	$5.5\times10^{7}\pm4.5\times10^{6}$	$8.5\times10^{7}\pm3.0\times10^{7}$	$7.0\times10^{8}\pm1.0\times10^{8}$	$8.0\times10^{8}\pm8.0\times10^{7}$
M:LB:ST 1:2:1	$9.3\times10^{6}\pm2.7\times10^{5}$	$4.8\times10^{7}\pm2.5\times10^{6}$	$9.0\times10^{7}\pm3.5\times10^{7}$	$7.0\times10^{8}\pm5.5\times10^{7}$	$7.5\times10^{8}\pm1.0\times10^{8}$
M:LB:ST 1:1:2	$9.3\times10^{6}\pm2.7\times10^{5}$	$5.0\times10^{7}\pm2.0\times10^{6}$	$8.0\times10^{7}\pm2.5\times10^{7}$	$7.5\times10^{8}\pm6.0\times10^{7}$	$8.5\times10^{8}\pm7.8\times10^{7}$
M:LB:ST 2:1:1	$9.3\times10^{6}\pm2.7\times10^{5}$	$5.0\times10^{7}\pm3.5\times10^{6}$	$8.5\times10^{7}\pm3.5\times10^{7}$	$7.5\times10^{8}\pm6.5\times10^{7}$	$7.5\times10^{8}\pm2.0\times10^{8}$

Means of triplicates for all treatments.
[1]M is *B. adolescentis* M101-4.
[2]LB is *L. delbrueckii* subsp. *bulgaricus* NCK 231.
[3]ST is *S. salivarius* subsp *thermophilus* St-133.
No statistical differences were detected between means ($p> 0.05$)

Table 17. Growth of *L. delbrueckii* subsp. *bulgaricus* NCK 231 in 12% NDM co-cultured with *S. salivarius* subsp. *thermophilus* St-133 and *Bifidobacterium* sp. Bf-1.

Treatment	(cfu/g)				
	0 hr	12 hr	24 hr	36 hr	48 hr
LB[2]	$1.3\times10^{7}\pm4.5\times10^{6}$	$5.3\times10^{7}\pm6.0\times10^{6}$	$2.5\times10^{8}\pm1.0\times10^{7}$	$8.5\times10^{8}\pm5.5\times10^{7}$	$9.5\times10^{8}\pm2.0\times10^{8}$
Bf-1[1]:LB 1:1	$1.3\times10^{7}\pm4.5\times10^{6}$	$5.3\times10^{7}\pm1.1\times10^{7}$	$2.5\times10^{8}\pm4.9\times10^{7}$	$8.7\times10^{8}\pm5.0\times10^{7}$	$9.5\times10^{8}\pm4.5\times10^{8}$
Bf-1:LB 1:2	$1.3\times10^{7}\pm4.5\times10^{6}$	$5.0\times10^{7}\pm5.5\times10^{6}$	$2.5\times10^{8}\pm3.5\times10^{7}$	$8.2\times10^{8}\pm1.0\times10^{8}$	$9.0\times10^{8}\pm5.5\times10^{8}$
Bf-1:LB 2:1	$1.3\times10^{7}\pm4.5\times10^{6}$	$5.0\times10^{7}\pm7.0\times10^{6}$	$2.3\times10^{8}\pm1.7\times10^{7}$	$8.5\times10^{8}\pm2.0\times10^{8}$	$9.5\times10^{8}\pm4.0\times10^{8}$
Bf-1:LB:ST[3] 1:1:1	$1.3\times10^{7}\pm4.5\times10^{6}$	$4.7\times10^{7}\pm7.3\times10^{6}$	$2.5\times10^{8}\pm3.2\times10^{7}$	$8.2\times10^{8}\pm1.0\times10^{8}$	$9.0\times10^{8}\pm4.0\times10^{8}$
Bf-1:LB:ST 1:2:1	$1.3\times10^{7}\pm4.5\times10^{6}$	$5.0\times10^{7}\pm1.0\times10^{7}$	$3.0\times10^{8}\pm2.5\times10^{7}$	$8.5\times10^{8}\pm6.3\times10^{7}$	$9.0\times10^{8}\pm2.0\times10^{8}$
Bf-1:LB:ST 1:1:2	$1.3\times10^{7}\pm4.5\times10^{6}$	$5.3\times10^{7}\pm6.4\times10^{6}$	$2.5\times10^{8}\pm3.0\times10^{7}$	$8.5\times10^{8}\pm5.5\times10^{7}$	$9.3\times10^{8}\pm3.0\times10^{8}$
Bf-1:LB:ST 2:1:1	$1.3\times10^{7}\pm4.5\times10^{6}$	$5.3\times10^{7}\pm7.5\times10^{6}$	$3.0\times10^{8}\pm5.5\times10^{7}$	$8.7\times10^{8}\pm4.5\times10^{7}$	$9.0\times10^{8}\pm2.5\times10^{8}$

Means of triplicates for all treatments.
[1]Bf-1 is *Bifidobacterium* sp. Bf-1.
[2]LB is *L. delbrueckii* subsp. *bulgaricus* NCK 231.
[3]ST is *S. salivarius* subsp *thermophilus* St-133.
No statistical differences were detected between means ($p > 0.05$)

Table 18. Growth of *L. delbrueckii* subsp. *bulgaricus* NCK 231 in 12% NDM co-cultured with *S. salivarius* subsp. *thermophilus* St-133 and *B. adolescentis* M101-4.

Treatment	(cfu/g)				
	0 hr	12 hr	24 hr	36 hr	48 hr
LB[2]	$1.3\times10^{7}\pm4.5\times10^{6}$	$5.0\times10^{7}\pm5.5\times10^{6}$	$2.3\times10^{8}\pm1.0\times10^{7}$	$8.5\times10^{8}\pm5.5\times10^{7}$	$9.3\times10^{8}\pm5.0\times10^{8}$
M[1]:LB 1:1	$1.3\times10^{7}\pm4.5\times10^{6}$	$5.3\times10^{7}\pm1.1\times10^{7}$	$2.5\times10^{8}\pm4.9\times10^{7}$	$8.7\times10^{8}\pm5.0\times10^{7}$	$9.0\times10^{8}\pm4.5\times10^{8}$
M:LB 1:2	$1.3\times10^{7}\pm4.5\times10^{6}$	$5.0\times10^{7}\pm5.5\times10^{6}$	$2.5\times10^{8}\pm3.5\times10^{7}$	$8.2\times10^{8}\pm1.0\times10^{8}$	$9.0\times10^{8}\pm5.5\times10^{8}$
M:LB 2:1	$1.3\times10^{7}\pm4.5\times10^{6}$	$5.0\times10^{7}\pm7.0\times10^{6}$	$2.3\times10^{8}\pm1.7\times10^{7}$	$8.5\times10^{8}\pm2.0\times10^{8}$	$9.3\times10^{8}\pm4.0\times10^{8}$
M:LB:ST[3] 1:1:1	$1.3\times10^{7}\pm4.5\times10^{6}$	$4.7\times10^{7}\pm7.3\times10^{6}$	$2.5\times10^{8}\pm3.2\times10^{7}$	$8.2\times10^{8}\pm1.0\times10^{8}$	$9.0\times10^{8}\pm4.0\times10^{8}$
M:LB:ST 1:2:1	$1.3\times10^{7}\pm4.5\times10^{6}$	$5.0\times10^{7}\pm1.0\times10^{7}$	$3.0\times10^{8}\pm2.5\times10^{7}$	$8.5\times10^{8}\pm6.3\times10^{7}$	$9.5\times10^{8}\pm2.0\times10^{8}$
M:LB:ST 1:1:2	$1.3\times10^{7}\pm4.5\times10^{6}$	$5.3\times10^{7}\pm6.4\times10^{6}$	$2.5\times10^{8}\pm3.0\times10^{7}$	$8.5\times10^{8}\pm5.5\times10^{7}$	$9.5\times10^{8}\pm3.0\times10^{8}$
M:LB:ST 2:1:1	$1.3\times10^{7}\pm4.5\times10^{6}$	$5.3\times10^{7}\pm7.5\times10^{6}$	$3.0\times10^{8}\pm5.5\times10^{7}$	$8.7\times10^{8}\pm4.5\times10^{7}$	$9.3\times10^{8}\pm2.5\times10^{8}$

Means of triplicates for all treatments.
[1]M is *B. adolescentis* M101-4.
[2]LB is *L. delbrueckii* subsp. *bulgaricus* NCK 231.
[3]ST is *S. salivarius* subsp *thermophilus* St-133.
No statistical differences were detected between means ($p > 0.05$)

salivarius subsp. *thermophilus* St-133 and *Bifidobacterium* sp. Bf-1 or *B. adolescentis* M101-4. This was similar to the data on growth of bifidobacteria.

4.10 The conditions to enhance growth and viability of bifidobacteria in yogurt.

Based on the results discussed previously, in this study the conditions determine above were combined to enhance growth and viability of bifidobacteria in yogurt. The culture blend selected was a ratio of 4: 1: 2 of *Bifidobacterium* sp.: *Lactobacillus delbrueckii* subsp. *bulgaricus*: *Streptoccus salivarius* subsp. *thermophilus*. These cultures were inoculated into 12% NDM supplemented with 5% FOS. Two commercial yogurt culture blends containing bifidobacteria PY-3 and PY-58 were used as controls. The initial count of bifidobacteria under our conditions was 1.56×10^8 cfu/g. The initial counts of bifidobacteria manufactured using commercial yogurt starter cultures PY-3 and PY-58, were 6.67×10^6 cfu/g and 7.43×10^8 cfu/g, respectively.

To investigate the viability of bifidobacteria under our conditions, the percent viability of bifidobacteria during 15 days of refrigerated storage at 5°C was determined. After 5 days of storage, 74 ± 4.0% of bifidobacteria remained viable in the product

manufactured under our conditions. Whereas, in yogurts manufactured with PY-3 and PY-58, 61 ± 9.5% and 60 ± 6.5%, respectively of the organisms were viable. These difference, however, were not statistically significant (Table 19). After 10 days of refrigerated storage, the 47 ± 11.1% of the bifidobacteria were viable in the yogurt product produced under our conditions. This was higher ($p<0.05$) than 28 ±1.8 and 31 ± 3.1 % which was the viability of bifidobacteria remaining in yogurts manufactured with PY-3 and PY-58, respectively. After 15 days of cold storage, the viability of bifidobacteria in the yogurt product manufactured with our conditions was 35 ± 2.0%. This was more than double the viability observed in yogurts manufactured with PY-3 and Py-58, which had 15 ± 2.1% and 17 ± 3.6% viability respectively remaining (Table 19). The pH of our product remained higher ($p<0.05$) than pH of yogurt produced with the two commercial cultures (Table 20). Growth of bifidobacteria in dairy products can be greatly enhanced and viability maintained during refrigerated storage by proper selection of all strains of organisms, by proper their ratio and inoculum levels, by providing suitable growth factors in the milk and properly maintaining pH and redox potential in the milk medium.

Our results may be more consistent with the doses recommended to receive health benefit of these organisms.

Table 19. Viability of bifidobacteria in yogurt during refrigerated storage.

Storage time (Days)	% Viability[4]		
	T1[1]	PY-3[2]	PY-58[3]
5	74±4.0[a]	61±9.5[a]	60±6.5[a]
10	47±11.1[a]	28±1.8[b]	31±3.1[b]
15	35±2.0[a]	15±2.1[b]	17±3.6[b]

[1]T1 is 12% (w/v) pasteurized NDM containing fructooligosaccharide cultured with final ratio of 4:1:2 (*Bifidobacterium*: *L. delbrueckii* subsp. *bulgaricus*: *S. salivarius* subsp. *thermophilus*; based on cfu).
[2]PY-3 is PY-3 yogurt culture obtained from Chr. Hansen's Laboratories Inc.
[3]PY-58 is PY-58 yogurt culture obtained from Chr. Hansen's Laboratories Inc.
[4]% viability = (cfu at each storage time / initial cfu before refrigerated storage) × 100.
[a-b]Means with different superscripts within the same row are significantly different ($p<0.05$). Means ± standard deviations; n=3 for all treatments.

Table 20. The pH of yogurt during refrigerated storage.

Storage time (Days)	(pH)		
	T1[1]	PY-3[2]	PY-58[3]
0	4.55±0.05[a]	4.37±0.06[b]	4.38±0.06[b]
5	4.55±0.03[a]	4.35±0.05[b]	4.37±0.06[b]
10	4.53±0.03[a]	4.34±0.05[b]	4.35±0.05[b]
15	4.51±0.04[a]	4.31±0.05[b]	4.33±0.04[b]

[1]T1 is 12% (w/v) pasteurized NDM containing fructooligosaccharide cultured with final ratio of 4:1:2 (*Bifidobacterium*: *L. delbrueckii* subsp. *bulgaricus*: *S. salivarius* subsp. *thermophilus*; based on cfu).
[2]PY-3 is PY-3 yogurt culture obtained from Chr. Hansen's Laboratories Inc.
[3]PY-58 is PY-58 yogurt culture obtained from Chr. Hansen's Laboratories Inc.
[a-b]Means with different superscripts within the same row are significantly different ($p<0.05$). Means ± standard deviations; n=3 for all treatments.

V. CONCLUSIONS

<1> Our results show that the viability of bifidobacteria in commercial milk (pH ~ 6.6) and two brands of commercial yogurt (pH ~ 4.2) remained above 10^6 cfu/ml or g, respectively.

<2> The growth of *Bifidobacterium* sp. Bf-1 and Bf-6 in NDM were stimulated by FOS>GOS>Inulin. Both FOS and GOS had similar effects on maintaining viability of *Bifidobacterium* sp. in NDM during 4 weeks of refrigerated storage. Both FOS and GOS stimulated lactic acid and acetic acid production by *Bifidobacterium* sp. Among the carbon sources tested, inulin was the least effective in stimulating lactic acid and acetic acid production.

<3> Although *S. salivarius* subsp. *thermophilus* St-133 was effective at lowering Eh_7 of NDM media, the growth of *Bifidobacterium* sp. in skim milk was influenced more by pH than Eh_7.

<4> Growth and viability of bifidobacteria in milk was enhanced by proper conditions such as strain selection, optimizing inoculum levels, using suitable growth factors and proper monitoring of pH and redox potential.

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