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This is to certify that the
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INFLUENCE OF SWEETENER TYPE ON GROWTH, ACTIVITY, AND VIABILITY OF YOGURT CULTURES

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INFLUENCE OF SWEETENER TYPE ON GROWTH, ACTIVITY, AND VIABILITY OF YOGURT CULTURES

By

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ABSTRACT

INFLUENCE OF SWEETENER TYPE ON GROWTH, ACTIVITY, AND VIABILITY OF YOGURT CULTURES

By

Darclee Sidonia Popa

Three different floral sources of honey (sage, alfalfa, sourwood) were compared to sucrose, high fructose corn syrup (HFCS) and inulin in their ability to support growth, activity and viability of yogurt cultures: *Streptococcus salivarius* subsp. *thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus acidophilus*, and *Bifidobacterium bifidum*. The effect of yogurt ingredients on cultures was also investigated. A consumer panel determined sensory attributes of the product. Viability of lactic acid bacteria (LAB) and bifidobacteria in yogurt was investigated during 42 d of refrigerated storage. Each culture underwent three successive 24 h transfers at 37 °C in MRS or MRSL media. Subsequently, the cultures were resuspended in 12% non-fat dry milk and 5 or 10% (w/v) of each sweetener was added. Controls were devoid of sweetener. Each sample was inoculated with 5% (v/v) cultures listed above and incubated (37 °C/ 24 h). Overall, growth of LAB and bifidobacteria were enhanced in the presence of honey in a similar manner to HFCS particularly at 10% sweetener level. Yogurt ingredients had no inhibitory effect on growth of cultures. Sensory evaluation showed consumers' preference for sucrose and HFCS rather than honey. Viability of LAB and bifidobacteria was retained above 85% and 90% respectively for all treatments with the exception of yogurt sweetened with sourwood honey.

To my loving husband, Iuliano, and my wonderful son, Bogdan, for their love support and encouragement.

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INTRODUCTION

Consumption of functional foods has increased as consumers recognize their role in health and nutrition. Consumers' interest in the potential, health-promoting properties of functional foods is growing at a rate of 15-20% per year, based on purchasing trends, and it is claimed that the industry is worth $33 billion (Hilliam 2000). Functional foods are recognized as foods that provide health benefits or have disease-preventing effects beyond their natural nutritional values. There are many health-promoting products on the market placed in the category of functional foods: fermented milk and yogurt, sports drinks, baby foods, ice cream, confectionery, biscuits, snack foods, and calcium-fortified drinks (Stanton and others 2001). In an attempt to deliver specific health benefits to the targeted consumer, functional foods consist in the addition of active components such as: phytochemicals, bioactive peptides, dietary fibers, omega-3-polyunsaturated fatty acids, probiotics and prebiotics.

A major category of functional foods consists of fermented dairy products that contain probiotic bacteria, such as *Lactobacillus* and *Bifidobacterium* species (Shah 2000). An estimated two thirds of the functional foods available are associated with probiotic microorganisms (Holzapfel and Schillinger 2002). In the development of functional foods containing probiotics, a major challenge is to maintain viability and activity of these cultures during processing and refrigerated storage of the product. To exert the health benefits within consumer's body, probiotics must be able to grow and proliferate in the human intestine (Stanton and others 2001) and therefore it has been suggested that they be viable and possess the ability to survive passage through gastrointestinal tract. Hence, in the production of fermented dairy products selection of

strains of probiotics that meet these characteristics is critical. Probiotics selection is based on general microbiological criteria that refer to safety, processing, performance and health benefits (Klaenhammer and Kullen 1999). Probability of probiotic strains to survive in the product in relatively high viable cell number, to retain metabolic activity and to provide desirable organoleptic qualities (Holzapfel and others 1998) are also criteria that must be fulfilled in order to provide effective probiotic food products for general consumption.

The product most extensively marketed as containing probiotics is yogurt. The presence of probiotics in yogurt has historical reasons since Metchnikoff (1907) about a century ago suggested that lactobacilli present in yogurt to have health promoting effects. This suggestion was based on his observation that Bulgarian peasants that consumed large quantities of yogurt lived longer lives. Today, yogurt is growing in popularity and there are various types of yogurt on the market. Traditionally, to obtain yogurt, milk is fermented with *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. In recent years, the popularity of "bio-yogurts", which contain *Lactobacillus acidophilus* and species of *Bifidobacterium* in addition to the traditional yogurt organisms (Dave and Shah 1998) has increased significantly. Consequently, the trend is to use yogurt cultures as the main starter and probiotic bacteria as adjunct starter (Shah 2004). In addition to the concept of probiotics, the concept of prebiotics and synbiotics (a combination of probiotics and prebiotics) has become popular (Rastall and Maitin 2002). Prebiotics have the ability to resist digestive enzymes, due to their chemical structure, and therefore pass into the large intestine where they become available for fermentation by bifidobacteria (Roberfroid and others 1998).

Numerous studies have demonstrated the ability of some prebiotics such as fructooligosaccharides and oligosaccharides to stimulate the activity of certain species of *Bifidobacterium* (Gibson and others 1995; Shin and others 2000; Bruno and others 2002). There are also food sources of oligosaccharides. Oligosaccharides are naturally present in honey. Honey was found to be effective in stimulating the growth of commercial bifidobacteria in skim milk (Chick and others 2001) and intestinal bifidobacteria (Kajiwara and others 2002). Hence, the use of honey in a dairy product such as yogurt is of great interest due to its prebiotic potential by the presence of oligosaccharides in the chemical composition.

The rationale for this study was to compare the effects of honeys from three floral sources (sourwood, sage, and alfalfa) varying in oligosaccharides content with traditional sweeteners (sucrose and high fructose corn syrup) or inulin (another well known prebiotic) on promoting probiotics. Therefore, the objectives of this project were:

1) Determine the effect of sucrose, high fructose corn syrup (HFCS), honey or inulin, on growth and activity of *Streptococcus salivarius* subsp. *thermophilus* (St-133), *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lr-78), *Lactobacillus acidophilus* (La-7) and *Bifidobacterium bifidum* (Bf-1).
2) Determine the effect of ingredients (stabilizer, unsweetened strawberry puree) used in the manufacture of yogurt on the growth of microorganisms listed above.
3) Evaluate the yogurts manufactured above with different sweeteners for their sensory attributes.
4) Determine the viability of lactic acid bacteria and bifidobacteria in yogurt during typical shelf life of refrigerated yogurt.

CHAPTER 1

LITERATURE REVIEW

1.1 YOGURT AND PROPERTIES

Yogurt has been known as the dairy product with nutritional and potentially therapeutic value by much of the consuming public. Many researchers (Collins and Gibson 1999; Gardiner and others 1999; Adolfson and others 2004; Ried 2004) have reported on the beneficial effects of consuming fermented milk products. Yogurt is a fermented dairy product obtained from the action of two thermophilic lactic acid bacteria: *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* (Guven and Karaca 2002). The two yogurt bacteria exist either naturally in the milk or are introduced as pure cultures in a 1:1 ratio (Kosikowski and Mistry 1997) and are responsible for the fermentation of lactose into the desired yogurt product.

In addition to these two essential yogurt bacteria, secondary microflora can be added to satisfy the following objectives: a) contribute different organoleptic properties to yogurt (e.g. *Streptococcus lactis* subsp. *diacetylactis* or *Leuconostoc* are added for flavor and *Streptococcus lactis* for consistency); b) increase the nutritional value of yogurt using cultures which increase nutrients such as folic acid (B-complex vitamins); and c) increase the potential of health benefits by supplementing the yogurt flora with probiotics such as *Lactobacillus acidophilus* and bifidobacteria (Mareschi and Cueff 1989).

Almost 80% of the yogurt manufactured in the United States contains *L. acidophilus* (Dairy Management Inc. 2004). According to Shah (2004), a probiotic yogurt may contain only *L. acidophilus* or *L. acidophilus* and bifidobacteria, or it may

contain *L. acidophilus*, bifidobacteria and *Lactobacillus casei* as probiotic organisms, in addition to the traditional yogurt starter cultures. However, other potential probiotic cultures are widely used to increase the prophylactic and therapeutic characteristics of the final product: *Lactobacillus johnsonii*, *L. reuteri*, *L. rhamnosus*, *L. paracasei* ssp. *paracasei* biovar *shirota*, and *Enterococus faecium* and *E. fecalis* (Tamime and others 1995; Lourens-Hattingh and Viljoen 2001).

Yogurts produced in the U.S. have a standard of identity listed under the Code of Federal Regulations (CFR) title 21 of the Food and Drug Administration (FDA). In 2000, the National Yogurt Association (NYA) petitioned the FDA to modernize the 20-year-old "standard of identity" for yogurt and required a minimum level of live and active cultures, among other requirements (NYA 2003).

Yogurts with live and active cultures can be identified by the NYA's "Live and Active Culture" seal. According to the NYA (2003), the seal program criteria require that (1) the yogurt be fermented with both *L. delbrueckii* subsp. *bulgaricus* and *S. salivarius* subsp. *thermophilus*, (2) that the total viable count at the time of manufacture is 10^8 CFU/ gram, and (3) that the cultures be active at the end of the stated shelf life of the product as determined by the specific activity test. The seal can also be used on frozen yogurts containing 10^7 viable lactic acid bacteria / gram at time of manufacture.

The activity test requires analysis of a sample of yogurt that has been stored at temperatures between 0 and 7 °C (Robinson and others 2002). Furthermore, the activity test uses the following steps: pasteurization of reconstituted nonfat dry milk (12% solids) at 92 °C for 7 min, cooling to 43 °C, inoculation with the material under test at a level of 3%, and fermentation at 43 °C for 4h. Before and after fermentation in the test material

the total yogurt organisms are enumerated and the activity criteria are met if there is an increase of 10^1CFU/g or more during fermentation (Chandan 1999).

A variety of forms of cultured yogurt are commercially available worldwide: drinking, concentrated, pasteurized, and frozen yogurt. These can be divided into various categories, and the subdivisions are created on the basis of the following: existing or proposed legal standards (full, medium, or low-fat), method of production (set or stirred), post-incubation processing (heat treatment, freezing, drying, or concentration), and flavors (natural, fruit, or flavored) (Robinson and others 2002). Two types of flavored yogurt are available: sundae-style, in which fruit puree is layered at the bottom of the cup, and Swiss-style, in which plain yogurt is softly blended with fruit puree before packaging.

Three essential criteria that define yogurt have been proposed (Mareschi and Cueff 1989): the main ingredients, the fermenting agents, and the manufacturing process. Several steps are involved in the manufacture of yogurt: standardization of mix, homogenization, heat treatment, cooling to incubation temperature, and inoculation with yogurt cultures, incubation, cooling, and packaging. Sweeteners, fruit preparation, fruit flavors, and fruit purees enhance texture, and color, and add a very desirable flavor dimension to the taste of yogurt. Also, different packaging ideas (dual compartment cup, multipacks) provide the consumer with an assortment of flavors and multiple textures (Chandan 1999).

The demand for yogurts with therapeutic properties is growing as consumer exposure to the probiotic concept increases (Robinson and others 2002). Of the yogurts and fermented milk products to which probiotic cultures have been applied, "LC1"

(Nestlé), "Vifit" (Campina Melkunie), "Actimel" (Danone), and "Yakult" (Yakult) have emerged as market leaders (Stanton and others 2001). The trend is toward development of the synergistic effect of combining probiotics with prebiotics in dairy products to meet consumers' expectations.

1.2 LACTIC ACID BACTERIA AND BIFIDOBACTERIA AND THEIR USE IN FERMENTED DAIRY PRODUCTS

Lactic acid bacteria (LAB) (lactococci, lactobacilli, streptococci, enterococci, etc.) are an important group of starter cultures, traditionally defined by formation of lactic acid as sole or main end product of carbohydrate metabolism (Suskovic and others 2001). They occur as cocci or rods, generally lack catalase, and comprise a diverse group of Gram-positive, non-spore forming bacteria found in foods (dairy products, fermented meat, sourdough, fermented vegetables, silage, beverages). They are common on plants, in sewage, but also in the genital, intestinal and respiratory tracts of humans and animals (Suskovic and others 2001). In general, the classification of LAB is based on morphology, mode of glucose fermentation, growth at different temperatures, configuration of the lactic acid produced, ability to grow at high salt concentration, fatty acid composition, and acid or alkaline tolerance (Axelsson 1993). Modern classification, mainly based on comparative sequence analysis of 16S ribosomal ribonucleic acid (16S rRNA), defined lactic acid bacteria as a group of gram-positive bacteria with a DNA base composition of less than 50 mol % guanine plus cytosine (G+C), and bifidobacteria as a group with a DNA composition of higher than 50% (55-67%) mol G+C (Suskovic and others 2001, Klaenhammer and others 2004).

1.2.1 Carbohydrate fermentation by lactic acid bacteria and bifidobacteria

The LAB and bifidobacteria receive their energy requirements via fermentation of carbohydrates either through homofermentative or heterofermentative metabolic pathways. Generally, the term homofermentative LAB refers to those in the group that use the glycolytic pathway for glucose fermentation, which produces lactic acid and small amounts of by-products (Rasic and Kurmann 1983). Theoretically, homolactic fermentation of glucose results in 2 moles of lactic acid and a net gain of 2 ATP (adenosine triphosphate) per molecule glucose consumed. Heterofermentative LAB use the 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway, which leads to significant amounts of other end products (CO_2, ethanol) in addition to lactic acid (1 mole each of lactic acid, ethanol, and CO_2 and 1 ATP/glucose) (Axelsson 1993).

Lactose (glucose and galactose β 1-4 linked) is the main carbohydrate present in milk, and *S. salivarius* subsp. *thermophilus*, *L. delbrueckii* subsp. *bulgaricus* and *L. acidophilus* ferment it homofermantatively, while *Bifidobacterium* ssp. ferment the same carbohydrate heterofermentatively via the fructose 6-phosphate shunt (Rasic and Kurmann 1983). The main products of the bifido pathway are acetate and lactate in a 3: 2 ratio from 2 moles of glucose (Marshall and Tamime 1997).

The metabolism of lactose takes place inside the microbial cell (Tamime and Robinson 1999), and the first step of lactose utilization by LAB involves a transfer through the cell membrane (Loones 1989). This transfer of lactose from outside to the inside of the cell depends on the type of bacteria involved in the process; it can be finalized by the phosphoenolpyruvate: phosphotransferase (PEP: PTS) system or involves cytoplasmic proteins (permease) that translocate lactose without chemical modification

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(Marshall and Tamime 1997; Tamime and Robinson 1999). Lactose is phosporylated by PEP: PTS system and become lactose 6-phosphate. Next, lactose 6-phosphate is hydrolyzed into its monosaccharide components (galactose 6-phosphate and glucose) by the enzyme β-phosphogalactosidase. Both products of the reaction are at the same time catabolized: glucose to lactic acid via Embden-Meyerhof-Parnas (EMP) pathways and galactose 6-phosphate via the Tagatose (stereoisomer of fructose) pathway. If galactose 6-phosphate is dephoshporylated, galactose will remain unmetabolized and be excreted from the cell (Marshall and Tamime 1997). The PEP: PTS system is used by most mesophilic, homofermentative LAB (lactococci used as starter cultures for common cheese varieties).

Most of the yogurt starter cultures, such as *S. salivarius* subsp. *thermophilus*, *Lactobacillus* spp. (Hutkins 2001) as well as *Bifidobacterium* spp. (Tamime and Robinson 1999), transport lactose via lactose permease through the cell membrane. After entering the cell, lactose is split to glucose and galactose by the enzyme β-galactosidase (Greenberg and Mahoney 1982). Glucose and galactose are subsequently phosphorylated and metabolized via the EMP and Leloir pathways, respectively. However, free galactose will appear and accumulate in fermented dairy products made with thermophilic starter cultures containing *S. salivarius* subsp. *thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* subsp. *lactis* and *L helveticus* or other galactose nonfermenting strains (Hutkins 2001).

In *S. salivarius* subsp. *thermophilus*, lactose uptake is driven by galactose efflux. In other words, lactose transport is fueled by a proton motive force (PMF); permease not only binds and transports lactose in symport with a proton, but the transporter has

exchange or antiporter activity, so that lactose uptake can be driven by efflux of galactose (Hutkins 2001). Inside the cell, lactose is hydrolyzed into glucose and galactose by the enzyme β-galactosidase (Vaillancourt and others 2004). The glucose is then metabolized to pyruvate via the EMP pathway, and lactate dehydrogenase converts the pyruvate to lactic acid. Galactose accumulates in the extracellular medium and may appear in the final product, since most *S. salivarius* subsp. *thermophilus* strains do not synthesize galactokinase. Galactokinase is the first enzyme of the Leloir pathway that phosphorylates intracellular galactose to generate galactose 1-phosphate or galactose 6-phosphate, depending on the strain, and further metabolized into lactic acid (Robinson and others 2002). In yogurt, accumulated galactose is of little consequence of product quality (Hutkins 2001). Human health may be affected, particularly in individuals with galactosemia (Novelli and Reichardt 2000).

It has been proposed (Vaillancourt and others 2004) that the inability of *S. salivarius* subsp. *thermophilus* to grow on galactose may result from the inability to translate the *galK* mRNA (*S. salivarius* subsp. *thermophilus galK* gene), depriving the cells of suitable levels of galactokinase. They reported the potential of the recombinant galactose-positive (Gal^+) strain to grow on galactose. Although the *S. salivarius* subsp. *thermophilus* strain engineered for this study was not a food grade organism (the plasmid construct contained an antibiotic resistance gene), the data suggested that derivation of food grade equivalent strains might provide an advantage as starter cultures for manufacture of fermented dairy foods.

The optimum growth temperature for *S. salivarius* subsp. *thermophilus* is ~ 37 °C, and during the commercial production of yogurt at 42 °C it is able to grow together with

L. delbrueckii subsp. *bulgaricus* due to its thermophilic nature. The principal product of metabolism is L (+) lactic acid for *S. salivarius* subsp. *thermophilus* and D (-) lactic acid for *L. delbrueckii* subsp. *bulgaricus*; humans less metabolize the later form than the L (+) acid isomer (Robinson and others 2002).

Lactobacillus delbrueckii subsp. bulgaricus has an optimum growth temperature of 45 °C; this microorganism can utilize lactose and, glucose and some strains can use galactose. Previous studies (Hickey and others 1986) indicate that glucose is imported via PTS in some strains of *L. delbrueckii* subsp. *bulgaricus*. The possibility of the simultaneous consumption of glucose and lactose by *L. delbrueckii* subsp. *bulgaricus* has been reported. Sugar transporters might coexist in some strains, or one strain may have a single transporter for three sugars: glucose, mannose and fructose, as described for strain *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 (Chervaux and others 2000). However, in that study a chemically defined medium was used, called "milieu proche du lait" (MPL), that allowed a high growth rate for *L. delbrueckii* subsp. *bulgaricus*. An increasing commercial interest in the addition of probiotic bacteria (*L. acidophilus* and bifidobacteria) to fermented dairy products has been observed in recent years (Vinderola and others 2002b). *Lactobacillus acidophilus* is considered an important member of the probiotic lactobacilli, and has been reported (De Vuyst 2000) to utilize both glucose and fructose moieties of sucrose, whereas the galactose moiety of lactose cannot be metabolized to a significant degree. De Vuyst's observations are attributed to differences in the activity of two enzymes: fructofuranosidase, a constitutive enzyme, and galactosidase, which can be induced in *L. acidophilus*. The growth temperature of *L. acidophilus* is around 45 °C or higher, and the organism produce large amounts of acid,

mainly DL- lactic acid, as a result of lactose metabolism. Marshall and Tamime (1997) have shown that *L. acidophilus* and bifidobacteria do not produce acid at the same rate as *S. salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. Hence, most of the probiotics rely on the traditional yogurt organisms for the acidification of milk.

Bifidobacteria are generally characterized as Gram-positive, non-spore forming, nonmotile, and catalase negative (Rasic and Kurman 1983). The optimum growth temperature is 37-41 °C with maximum growth at 43-45°C (De Vuyst 2000). In general bifidobacterial growth is limited in milk, but is enchanced in rich synthetic media such as tryptone phytone yeast extract (TPY) and MRS (De Vuyst 2000). Bifidobacteria typically ferment hexose by the fructose-6-phosphate or "bifid" shunt, due to the presence of the enzyme fructose-6-phosphate phosphoketolase, which can be used as a distinguishing feature of bifidobacteria (Robinson and others 2002). Fermentation of glucose by this pathway yields acetic acid and L (+) lactic acid in a theoretical 3:2 molar ratio, although in practice this exact ratio may not be achieved (Scardovi 1989), for example in yogurt. A high ratio of acetic acid to lactic acid in dairy products is typically undesirable (Bruno and others 2002) because of the distinctive vinegar flavor that can be imparted to the product. In addition, all bifidobacteria of human origin are also able to utilize galactose, lactose, usually, fructose and in some instances complex carbohydrates as carbon sources (De Vuyst 2000). It is important to underline that the pathway used by a particular strain or culture may have a profound effect on flavor, texture, and overall quality of fermented dairy product (Hutkins 2001).

1.2.2. Interactions among traditional yogurt cultures

The production of lactic acid from lactose in yogurt is the result of a combination of growth of *S. salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus.* This combination is favorable to both strains and it is called symbiotic relationship or proto-cooperation (Frederickson 1977). This relationship often has a beneficial effect on bacterial growth and on the production of lactic acid and aroma compounds (Courtin and Rul 2003). This interaction is easily identified by comparing the production of lactic acid by pure cultures grown individually with that of mixed strain cultures of both species (Loones 1989). Milk fermented with *S. salivarius* subsp. *thermophilus* only is less acidic and has a buttery aroma while milk fermented with *L. delbrueckii* subsp. *bulgaricus* is quicker to set, has a lower pH and has a pronounced yogurt (acetaldehyde) aroma (Marshall and Tamime 1997). The amount of lactic acid produced by mixed strain culture is greater than the sum of acidities produced by each pure culture (Loones 1989).

Regardless of its protein-rich habitat, *S. salivarius* subsp. *thermophilus* displays limited proteolytic ability, and since some amino acids are not present in milk at levels sufficient to support the essential growth of *S. salivarius* subsp. *thermophilus*, the increase in cell numbers necessary to complete the yogurt fermentation depends on the absorption of short-chain peptides released by *L. delbrueckii* subsp. *bulgaricus* from casein, and hydrolysis of these to the constituent amino acids (Robinson and others 2002). In turn, *S. salivarius* subsp. *thermophilus* produces pyruvic acid, formic acid, and CO_2, which stimulates the growth of *L. delbrueckii* subsp. *bulgaricus* (Tamime and Robinson 1999). However, depending on the bacterial strains employed, the type of milk, the method used to heat the milk and the temperature of milk fermentation, this

association can be neutral or detrimental (Courtin and Rul 2003).

Courtin and Rul (2003) recently studied the impact of co-culturing *S. salivarius* subsp. *thermophilus* with *L. delbrueckii* subsp. *bulgaricus* on bacterial growth in milk and showed that *S. salivarius* subsp. *thermophilus* / *L. delbrueckii* subsp. *bulgaricus* association on growth of *S. salivarius* subsp. *thermophilus* was dependent on the proteolytic capacities of *L. delbrueckii* subsp. *bulgaricus* strains: a positive association was found with *L. delbrueckii* subsp. *bulgaricus* strain 1038, contrary to the association with *L. delbrueckii* subsp. *bulgaricus* strain 397. However, the bacterial association had no significant effect on *L. delbrueckii* subsp. *bulgaricus* growth, possibly due to an insufficient production of formic acid by the *S. salivarius* subsp. *thermophilus* strain (Courtin and Rul 2003). Vinderola and others (2002a) investigated the effect of cell-free supernatants of *L. delbrueckii* subsp. *bulgaricus* strains on the growth of *S. salivarius* subsp. *thermophilus* strains using a well-diffusion agar assay, and results of complete and weak inhibitions as well as absence of interaction were recorded. Van de Guchte and others (2001) examined the effect of the *L. delbrueckii* subsp. *bulgaricus* VII007 culture supernatant fluids on growth of *S. salivarius* subsp. *thermophilus* CNRZ 302. After six hours of incubation in *L. delbrueckii* subsp. *bulgaricus* VII007 culture supernatant fluid the number of colony forming units (CFU) of *S. salivarius* subsp. *thermophilus* CNRZ 302, established in a plate count assay, was diminished 100-fold. It was concluded from this study that *L. delbrueckii* subsp. *bulgaricus* VII007 produce at least three growth-inhibiting factors (H_2O_2, bacteriocidal molecule with a molecular weight greater than 50 kDa, and a third factor), other than lactic acid, when grown under microaerobic conditions in MRS broth, and that production of bactericidal factors might depend on the

growth medium and might be strain specific.

1.2.3. Lactic acid bacteria and bifidobacteria in dairy products

Two approaches incorporating probiotic cultures individually or in combination, into fermented milk products have been suggested: the application of a probiotic as a starter culture or as an adjunct to starter cultures (Gardiner and others 2002). The later approach is more favorable because of the inability of probiotic cultures to produce sufficient lactic acid in milk. Therefore, it has been suggested (Gomes and others 1998) the addition of growth-promoting supplements, such as cysteine, yeast extract, and casein hydrolysates. Sweet acidophilus milk and sweet AB milk that contain concentrated probiotic bacteria (*L. acidophilus* and *L. acidophilus* plus bifidobacteria) require intensive heat treatment before fermentation in order to have a successful fermentation (Heller 2001). In addition, the probiotic culture, as an adjunct, could take advantage of possible symbiotic relationships that exist between the strains, resulting in increased microbial growth rates and improved flavor of the finished product (Gardiner and others 2002).

Dairy products are considered excellent carriers of probiotic organism. Recently, probiotics have been incorporated into Cottage cheese. Heller (2001) stated two points for adding probiotics to Cottage cheese: either with the starter culture or with cream dressing and salt. The addition of probiotics with cream dresing it appears to be better because avoids: (1) the lost of bacterial cells from the coagulum during draining of the whey, and (2) the scalding temperatures ≤ 55 °C that may negatively affect survival of probiotic bacteria in the product.

Vinderola and others (2000a) studied the use of Argentinian Fresco cheese as a

carrier of probiotic bacteria. They reported that bifidobacteria, in combination with *L. acidophilus,* had satisfactory viability (<1 log decrease in 60 days) in the cheese as well as for the combination of bifidobacteria with *L. casei* (<1 log order in 60 days for bifidobacteria, but no decrease was detected for *L. casei*). Hekmat and McMahon (1992) demonstrated that ice cream is also a suitable vehicle for delivering probiotics such as *L. acidophilus* and *Bifidobacterium bifidum.* These researchers found that the bacteria, grown to high numbers (10^8 CFU/mL) in the ice cream mix, remain viable during frozen storage ($10^{6-}10^7$ CFU/mL) 17 weeks after freezing.

In the development of a marketable probiotic product, it has been established that probiotics must meet the following requirements: (1) they need to survive in sufficient number in the product; (2) they must be stable during the storage of the product; (3) they need to maintain their health promoting properties during manufacturing and storage; (4) they should not have adverse effects on the taste or aroma of the product; (5) they should not enhance acidification during the shelf life of the product; (6) methods of clearly identifying probiotic strains should be available (Heller 2001). The development of successful probiotic products will be dependent on both proof of probiotic effect and the development of foods that harbor high numbers of viable organisms at the time of consumption (Stanton and others 2001).

1.2.4 Lactic acid bacteria and bifidobacteria as probiotics

Metchnikoff (1907) was one of the first to propose the health benefits of ingesting dairy products fermented with lactic acid bacteria. He suggested in his book "The Prolongation of Life" that the reason Balkan peasants lived long lives was because they

drank milk fermented with *L. delbrueckii* subsp. *bulgaricus* and *S. salivarius* subsp. *thermophilus*, bacteria that would suppress putrefactive-type fermentation leading to better health and longevity. The great interest in the healthy role of gut microflora generated by Metchnikoff's ideas persists to this day.

The word "probiotic" is derived from Greek and means "pro life". Probiotics have been defined as "live microbes which transit the gastro-intestinal tract and in doing so benefit the health of the consumer" (Tannock and others 2000). Members of the genus *Bifidobacterium* and *Lactobacillus* are widely used as probiotic microorganisms and both are normal components of the intestinal flora throughout the life cycle. It is estimated that over 400 species of bacteria inhabit the human gastrointestinal tract, and the *Bifidobacterium* species belongs to the dominant anaerobic flora of the colon (Champagne and others 2005). There are numerous factors such as: changing lifestyle, changing dietary patterns, increasing stress and antibiotic consumption with harmful effect on the balance of the gut microflora (Mckinley 2005). These factors cause a shift away from probiotics (lactobacilli and bifidobacteria,) potentially health promoting, towards an increase in pathogenic microorganisms (Fooks and Gibson 2002) that will make the host more susceptible to infections.

The role of gastrointestinal tract microflora in resistance to disease and promoting normal intestinal functions is well known (Salminen and others 1998). Scientific evidence indicates that probiotics exert a positive effect in maintaining a healthy microbial population. Yogurt consumption has been associated with maintenance of intestinal flora balance, effectiveness against diarrhea, *Helicobacter pylori* infection and inflammatory bowel disease, improvement in lactose metabolism, reduction in serum

cholesterol, immune system stimulation, anticancer and allergy-lowering effect, all of which have been investigated (Ouwehand and others 2002; Saavedra and Tschernia 2002). Yogurt is known to decrease or suppress the symptoms of lactose intolerance (De Vrese and others 2001), to display antitumor activity that appears to be mediated via enhancement of immune response (Perdigon and others 1998).

The addition of *Lactobacillus* spp. and bifidobacteria, and the prominent members of the commensal intestinal flora (Soomro and others 2002), to fermented dairy products are known to have an inhibitory growth effect on a wide range of intestinal pathogens in humans as well as animals (Wang and others 2004). The presence of probiotic strain *Lactobacillus rhamnosus* GG (ATCC 53013) in fermented milk has been reported to reduce to about half duration of diarrhea in children with rotavirus diarrhea (Salminen and others 1998; De Roos and Katan 2000; McFarland 2000). There are also studies that used *Bifidobacterium lactis* Bb-12 or *Lactobacillus reuteri* as well as heat-inactivated *Lactobacillus acidophilus* LB1 and reported shortening of the duration of rotavirus diarrhea in children (De Roos and Katan 2000; Pochapin 2000). Supporting studies using *Lactobacillus* GG in the treatment of severe antibiotic-associated form of *Clostridium difficile* colitis indicate some beneficial aspects (Pochapin 2000; Marteau and others 2001). However, more controlled clinical studies in this specific area still needed. Chandan (1999) pointed out potential mechanisms by which probiotics may exert their beneficial effects: (1) competition with other microflora for nutrients; (2) production of acids with inhibitory effect to certain pathogens; (3) production of bacteriocin (such as short-chain fatty acids, hydrogen peroxide and antimicrobial peptides) or inhibitory metabolites; (4) immuno-modulation; (5) competition for adhesion to intestinal mucosa.

The inhibitory effect of *Lactobacillus acidophilus* (La5) and *Bifidobacterium lactis* (Bb12) against *Helicobacter pylori* was studied (Wang and others 2004). The results showed that Bb12 exerted an in *vitro* effect against *H. pylori* while La5 did not show any effect. Yogurt was manufactured from a mixture of both strains and consumed by 59 adults with *H. pylori* infection twice daily as a meal, for 6 weeks (Wang and others 2004). The organism *H. pylori* was suppressed only when yogurt was consumed regularly whereas *H. pylori* continued to increase in the subjects consuming placebo. Cholesterol-lowering and immunomodulatory properties of yogurt have recently been reviewed (Hosono and others 2002).

The nutritional value of yogurt is dependent on its composition, principally on the nutrient composition of the milk. Furthermore, changes in milk constituents that occur during lactic acid fermentation influence the nutritional and physiologic value of the finished yogurt product (Adolfsson and others 2004). Yogurt and milk have similar mineral and vitamin composition, with few exceptions, that depend on the bacteria used for fermentation. However, some minerals (e.g., calcium) are more bioavailable from yogurt than from milk because the compositional changes that occur as milk is converted into yogurt. Because of the lower pH of yogurt compared to that of milk, calcium and magnesium are present in yogurt in their ionic forms (Adolfsson and others 2004). Bronner and Pansu (1999) concluded that the acidic pH of yogurt facilitates intestinal calcium uptake, and it also may reduce the inhibitory effect of dietary phytic acid on calcium bioavailability. Studying the effect of yogurt-derived calcium on bone mineralization in animals (Pointillart and others 1986), it was suggested that the bioavailability of calcium in yogurt is greater, and yogurt may increase bone

mineralization more than does nonfermented milk products. However, there are no recent human studies to show a superior effect of yogurt on bone mineralization. Other nutritional changes in milk due to fermentation include a decrease in lactose and vitamins B-6 and B-12 and an increase in peptide, free amino acids, free fatty acids, folic acid, and choline contents (Meydani and Ha 2000).

1.3 VIABILITY OF PROBIOTICS IN DAIRY PRODUCTS

Numerous studies reported that after ingestion probiotics must overcome biological barriers that include acid in the stomach and bile in the intestine (Gilliland 1978; Lankaputhra and Shah 1995) and must implant in the intestinal tract in order to exert health-promoting effects there (Kailasapathy and Rybka 1997). Although it has been reported that nonviable forms of probiotic bacteria can adhere to intestinal mucus and have immunomodulatory effects (Pessi and others 1999), it is generally believed that, as a condition to produce therapeutic benefits, a sufficient number of viable microorganisms must be present throughout the entire shelf life of the product until ingestion of the product (Gueimonde and others 2004).

Various organizations worldwide have introduced standards requiring a minimum of 10^7 CFU/ml of *L. acidophilus* and 10^6 CFU/g of bifidobacteria in fermented milk products at the time of the sale to ensure that the efficacy of probiotic food products is maintained (Talwalkar and Kailasapathy 2003a). For example, Fermented Milk and Lactic Acid Beverages Association in Japan require at least 10^7 CFU/ml of viable bifidobacteria in fermented milk drinks (Lourens-Hattingh and Viljoen 2001). In U.S. the National Yogurt Association (NYA) require 10^8 CFU/g of lactic acid bacteria at the

time of manufacture for yogurt (NYA 2003).

Shah and Lankaputhra (1997) reported that a number of factors are responsible for the loss of viability for probiotics. These factors include: acidity of products, acid produced during refrigerated storage (postacidification), level of oxygen in products, oxygen permeation through the package, and sensitivity to antimicrobial substances produced by yogurt bacteria. Bifidobacteria are anaerobic species and generally considered more sensitive than *L. acidophilus* to the damaging effect of oxygen (Talwalkar and Kailasapathy 2003a). Since bifidobacteria lack catalase necessary for the decomposition of hydrogen peroxide, exposure to oxygen determine the accumulation of this toxic oxygen metabolite in the cell (oxygen toxicity), leading eventually to cell death (Condon 1987). Some studies reported changes of probiotics due to oxygen exposure such as: physiological changes (elongated with a rough surfaces cells) in the cellular fatty acid profiles of *B. longum* (Ahn and others 2001) or metabolic (decrease in lactate production) and biochemical changes of *Bifidobacterium* spp. and *L. acidophilus* as the oxygen concentration increased from 0 to 21% (Talwalkar and Kailasapathy 2003b). According to De Vuyst (2000) viability of probiotic bacteria is considerably influenced by the food matrix composition, the interactions and stability of the culture, the inoculum level, and the technological process conditions. It is believed that during the process of manufacture and through the polystyrene packaging high concentration of dissolved oxygen is introduced in yogurts with negative impact on viability of probiotics (Talwalkar and Kailasapathy 2003a). Hence, different techniques to protect probiotics in fermented dairy products have been suggested.

There are contradictory reports on the survival of probiotic bacteria in yogurts

during storage. Adequate viability results for probiotics have been reported throughout the shelf life of yogurts (Lourens and others 2000). However, some studies have shown low viability of probiotics in commercial products (Shah and others 1995; Shin and others 2000). Studying the viability of *Bifidobacterium infantis* in 12% skim milk, Lankaputhra and others (1996) observed that after 12 days of storage at 4°C and pH 4.3 the viability of *B. infantis* decreased by 30%. The counts of bifidobacteria decreased by more than 82% after 24 days at the same temperature. Shin and others (2000) observed an 88% reduction in the population of bifidobacteria and 65% reduction for LAB, over a storage period of 6 weeks, in two commercial yogurts containing probiotics.

Vinderola and others (2000b) investigated the survival (4 weeks at 5 °C), of *B. bifidum* (BBI) and *L. acidophilus* (LAI) in reduced-fat (liquid) and full-fat (set) yogurts manufactured with two commercial lactic starter cultures (SID and SISD) containing *S. salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. Also, the viability of the two probiotics was assayed (4 weeks at 5 °C) in milk (10% reconstituted skim milk) acidified with lactic acid at different pH values (5.5, 4.5 and 3.5). The results showed that the highest reduction in viable cell counts was found in full-fat yogurt (starter SISD) for each probiotic organism: 1.6 to 4.0 log reduction for *B. bifidum* (BBI) and 2.7 to 4.6 log reduction for *L. acidophilus* (LAI). In general, pH values of 4.5 or lower jeopardized the cell viability of probiotics in yogurt stored at 5°C.

Ability of inulin and oligosaccharides to enhance activity and viability of *Bifidobacterium* spp. has been reported. Shin and others (2000) studied the growth, activity and viability of two commercial *Bifidobacterium* spp. (Bf-1 and Bf-6) in 12% reconstituted skim milk containing fructooligosaccharides (FOS),

galactooligosaccharides (GOS), and inulin at different concentrations (0.5, 1.0, 3.0 or 5.0 %). From this study it was concluded that enhancement of growth, activity and viability of bifidobacteria were dependent on carbon sources and concentration as well as strain of bifidobacteria. The results showed high percent viability for both strains (67% for Bf-1 and 45% for Bf-6) grown and stored in the presence of 5% FOS in comparison with control devoid of oligosaccharide or inulin. Inulin was found to be the least effective in retaining viability of either strain.

Using four types of prebiotics (inulin, lactulose, raftilose and hi-maize corn starch powder) to determine the viability of five *Bifidobacterium* species, Bruno and others (2002) found the retention of viability during the 4 weeks storage significantly higher in comparison with control without any prebiotic. The most effective prebiotic in this study was hi-maize followed by lactulose, raftilose and inulin. Charalampopoulos and others (2003) studied the effect of malt, wheat and barley extracts and several dietary constituents (reducing sugars, free amino nitrogen) on the viability of *L. plantarum L.reuteri* and *L. acidophilus* under conditions that stimulate the gastric tract (exposure for 4 h in phosphate buffer acidified at pH 2.5). The viability of probiotics upon addition of cereal extracts was improved: for *L. plantarum* by ~ 4 log cycles (malt) and ~3 log cycles (barley), and for *L.reuteri* and *L. acidophilus* viability was increased by more than 1.5 and 0.7 log cycle. These results were attributed to the amount of sugar present in the cereal extracts.

Addition of an oxygen scavenger such as ascorbic acid, a common food additive, or elimination of oxygen from the yogurt headspace has been suggested to improve viability (Dave and Shah 1997b). Shah (2000) suggested changes in the yogurt

manufacturing process of using a two-step fermentation in which yogurt, before the final fermentation (second step) with traditional starter cultures, underwent, as a first step, 2h fermentation with probiotic strains. Another approach suggested was the addition of probiotic growth supplements, such as whey powder, whey protein concentrate, casein hydrolysates and cysteine (sulfur-containing amino acid) as they have been shown to increase the viability of probiotics (Dave and Shah 1998). Studying the growth and viability of probiotic bacteria in yogurt supplemented with 0, 50, 250 or 500 mg/L of L-cysteine Dave and Shah (1997c) reported that L-cysteine at 250 or 500 mg/L improved the counts of *L. acidophilus*. However, in the same study, these levels of L-cysteine were found to suppress the growth of *S. salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus* with negative impact on the textural and cultural properties of the yogurt.

Selection of more acid resistant species (Shah 2000) and the use of microencapsulation of bifidobacteria within a protective envelope of κ-carrageenan (Adhikari and others 2003) have been suggested as other alternatives. Microencapsulation is a technique reported to enhance the survival of probiotic bacteria in dairy foods (Shah 2000; Kailasapathy 2002). This technique consists in covering live cells within a shell material protecting the cells from the unfavorable environment and at the same time allowing the diffusion of nutrients in and out of the matrix supporting the viability of the cells (Talwalkar and Kailasapathy 2003a). Microencapsulated cells of *Bifidobacterium longum* ATCC 15696 were added to Cheddar cheese during milling of the curd (Dinakar and Mistry 1994) and the microorganism remained viable and well dispersed in the cheese matrix over a period of 24 wk. Additionally, no significant

contribution to the flavor profile was determined by sensory evaluation in comparison with control.

The effect of microencapsulation with κ-carrageenan on the viability of *Bifidobacterium longum* B6 and *B.* longum ATCC 15708 in set-type plain yogurt during 30 days of refrigerated storage was also studied (Adhikari and others 2000). The results of this study showed that microencapsulation increased the viability of bifidobacteria in yogurt. However, sensory evaluation results showed consumer preference for control and non-encapsulated over the encapsulated treatment.

Viability of bacteria is usually assessed by plate counting on a suitable growth medium (Auty and others 2001). An important factor in monitoring viable organisms is the ability to count probiotic bacteria differentially (Tharmaraj and Shah 2003). Various media have been proposed and used over time for the isolation, cultivation, and enumeration of probiotics from fermented milks. Ravula and Shah (1998) developed a medium (LC agar) for selective enumeration of *L. casei.* Several media for selective enumeration of *L. acidophilus* and *Bifidobacterium* spp. have been previously proposed (Laroia and Martin 1991; Dave and Shah 1996; Shah 2000). It is necessary to have a medium that selectively promotes the growth of bifidobacteia, whereas other bacteria are suppressed. De Man Rogosa Sharpe (MRS) medium, supplemented with neomycin, paromomycin, nalidixic acid, and lithium chloride, was recommended for a selective enumeration of bifidobacteria in dairy products (Roy 2001). This medium was successfully used for the enumeration of bifidobacteria under anaerobic incubation at 37 °C for 72 hours (Tharmaraj and Shah 2003).

Auty and others (2001) suggested new and modern techniques for enumeration of

probiotics: the use of a microscopic technique, which enabled the differentiation of live and dead bacteria or the rapid use of fluorescence microscopy. The results of their study using *in situ* LIVE/DEAD *Bac*Light bacterial viability staining in conjunction with confocal scanning laser microscopy (CSLM) demonstrated that microscopy viability counting of probiotic milk and fermented milk yielded consistently higher counts (up to 20-fold for milk) than plate counting. However, for cheese products and spray-dried cultures, microscopic counts were lower than plate counts, highlighting the need for further work to establish the effect of environmental factors such a pH, ionic profile, and water activity on viability staining.

1.4 SWEETENERS USED IN DAIRY PRODUCTS

The sugar (sucrose) most commonly used in food industry as a sweetening agent can be obtained in granulated or syrup form from cane or beet. Sucrose has a high solubility, thereby making it an ideal ingredient in food products (Papademas and Bintsis 2002). Apart from providing the required sweetness in a dairy product, sucrose contribute to the total solids of the product providing texture, body, viscosity and moisture retention. It aids in preventing syneresis in gels and denaturation of proteins (Vlitos 1974). Sucrose is known to assist the emulsification of fats, as well as to develop and modify flavors either by autolysis or by synergistic action with salt or citric acid (Pangborn 1963).

In the dairy industry, high fructose corn syrup (HFCS) is also widely used. High fructose corn syrup is a sweetener obtained during the enzymatic hydrolysis of starch that transforms dextrose (glucose) from cornstarch into a mixture of fructose and glucose.

Isomerization of glucose with glucose isomerase yields HFCS with 42 %(w/w) fructose, 50 % (w/w) glucose, and 8% (w/w) other saccharides (Lecomte and others 2002). A characteristic of HFCS is its dextrose equivalent (DE) value, which represents a measure of the reducing sugar content of the syrup calculated as dextrose and expressed as a percentage of the total dry weight. Several physical and functional characteristics vary according to the DE value; the solubility, sweeteners, hygroscopy, and compressibility increase with increasing DE, while the viscosity and the inhibition of crystallization of syrups decreases as the DE increases (Storz and Steffens 2004). High fructose corn syrup is considered to be a useful ingredient because of its sweetness and ability to blend with other food and beverage ingredients.

In yogurt, sweeteners are added in two ways: in the initial milk base or by the addition of fruit concentrate. One reason for adding sweetening compounds is to restrain the level of acidity produced, especially when high acid/low sugar content fruits, such as raspberry, are added to the cooled fermented base (Staff 1998). Staff (1998) reported that the amount of sweeteners added to yogurt depended on: the type, level and acidity of fruit used; the type of sweeteners used; consumer preference, economic consideration, legal requirements, and the inhibitory effects on starter organisms.

1.4.1 Inulin composition and properties

Inulin belongs to a class of carbohydrates known as fructans (Kaur and Gupta 2002) consisting of 1 molecule of glucose and $\leq$60 molecules of fructose, and is thus considered to be an "extended-sucrose" molecule (Bezkorovainy 2001). The number of monomers units in inulin (essentially fructose) represents the degree of polymerization (DP), and varies from 2 to more or less 60 units (Roberfoid 2002). Inulin type fructans consist of a linear β 2$\rightarrow$1 linked fructofuranosyl units (Kaur and Gupta 2002) and is

biologically present in a large variety of plants (Roberfoid 2002). Some important sources of inulin are: garlic, Jerusalem artichoke, dahlia tubers, chicory root (15-20% inulin), and asparagus root (10-15% inulin) (Gupta and Kaur 1997). However, only a limited number of species are suitable for industrial food and nonfood applications (Kaur and Gupta 2002). The plant species currently used by the food industry to produce inulin belongs to the Compositae family and are known as Jerusalem artichoke (*Helianthus tuberosus*), and chicory (*Cichorium intybus*) (Debruyn and others 1992).

Earlier interest in inulin was because of its ability to act as a fat or sugar replacer without negatively affecting flavor (Tungland 2000). The fat substituting property of inulin is based on its ability to stabilize the structure of the aqueous phase, which creates an improved "creaminess" feel in the mouth (El-Nagar and others 2002) and for this reason, inulin has been successfully used to replace fat in table spreads, baked goods, fillings, dairy products, frozen desserts, and salad dressings (Kaur and Gupta 2002).

According to Gibson and others (1994), inulin (manufactured as Raftiline® ST) is obtained industrially by hot water extraction of fresh chicory roots that are a concentrated source of inulin. Its composition includes 92% fructooligosaccharides with an average DP of 10 hexose units. Inulin is generally regarded as a safe (GRAS) status in the United States, and the average daily consumption has been estimated to be 1-4 g (Roberfoid 2002).

Recent studies emphasize the importance of inulin addition in food products due to its prebiotic activity. Prebiotics are known as carbohydrate, non-digestible food components that reaching the colon are selectively fermented by probiotics. The presence of the beta configuration of the anomeric C_2 in the fructose monomers that form

β 2→1 glycosidic linkages make inulin resistant to hydrolysis by human small intestinal digestive enzymes, which are specific for alpha glycosidic linkages (Roberfoid 2002). Because fructooligosaccharides (FOS) cannot be digested in the upper gut, they are able to reach the colon where they are selectively utilized by bifidobacteria, which produces the enzyme β-fructosidase that breaks down FOS (Gibson 1999).

A recent *in vitro* study (Sanz and others 2005) showed that the carbohydrate structure, more specifically different glycosidic linkages and monosaccharide compositions of disaccharides, have an influence on probiotic selectivity. The results obtained from an in *vitro* fermentation of fecal bacteria using 7 mg carbohydrates were compared with those obtained using pH-controlled batches with 1.5 and 150 ml carbohydrates. A prebiotic index (PI) was calculated for each disaccharide in order to compare the influence of disaccharides structures on the selectivity of fermentation. From this study, high PI score was obtained with disaccharides containing monomers glycosidic linked 1-2, 1-4 and 1-6 and low PI score for manose-containing disaccharides. This structure-function information may be utilized in predicting how specific structures are fermented by the gut microflora.

Using inulin, together with FOS and galactooligosaccharides (GOS), on growth and viability of commercial *Bifidobacterium* spp. in skim milk, Shin and others (2000) showed that the effects of oligosaccharides and inulin increased with increasing carbohydrate concentration. In another study in which humans ingested 15 g of FOS per day for two weeks showed a significant increase of beneficial bifidobacteria and a reduction of pathogenic clostridia and other species in the feces (Gibson and Roberfoid 1995). Furthermore, on the basis of the results of well-designed human studies that have

shown significant changes in the composition of human fecal flora (Gibson 1999; Roberfoid and others 1998), it can was concluded that inulin is a prebiotic having a bulking effect, as well as an increase in stool frequency because of the increase in microbial mass that resulted from its fermentation.

In addition to these properties and taking into account the role of inulin as a fiber in the diet, it has been shown that inulin induce interesting physiological/nutritional effects. These effects relay to improved calcium bioavailability, the reduction of the risk of developing precancerous lesions in the colon, and hypoinsulinemia in experimental models (Roberfoid 2002).

The use of inulin and oligofructose as bifidogenic agents has been shown in experimental studies as: stimulating the immune system, decreasing the levels of pathogenic bacteria in the intestine, reliving constipation, decreasing the risk of osteoporosis by increasing mineral (calcium) absorption, reducing the risk of atherosclerosis by lowering the synthesis of tryglicerides and fatty acids in the liver, and decreasing the level in serum (Kaur and Gupta 2002). The studies of Gibson and others (1995) showed that fructooligosaccharides and inulin significantly modified the *in vivo* composition of the microbiota by stimulating the growth of bifidobacteria. In a recent study Langlands and others (2003), using an *in vitro* model of the large bowel, showed that inulin and oligofructose selectively stimulate the growth of bifidobacteria in the surface associated flora. The researchers went on to feed these carbohydrates to healthy patients scheduled to have a colonoscopy and examined their effect on the mucosa-associated flora in all regions of the large bowel. The results obtained *in vitro* showed that prebiotics increased surface counts of bifidobacteria from 6.6 to 7.3 log CFU/slide.

In the feeding study prebiotics increased mucosal bifidobacteria and lactobacilli in both the proximal and distal colon. They concluded that prebiotics could change the composition of the mucosa-associated flora significantly. Ried (2004), studying the role of pro- and prebiotics in standard food, outlined the potential of "everyday standard" food items, such as cheese, to promote healthy gastrointestinal microflora and to prevent gastrointestinal illness such as diarrhea. The researcher emphasize that the regular consumption of cheese containing both probiotics and inulin as prebiotic has been associated with a reduction in the risk of *Campylobacter enteritis.* Since the role of probiotics and prebiotics is very important in terms of health benefits, there is a need to combine these two that will lead to the use of synbiotics, foods that contain both probiotic and prebiotic (Shah 2004). Prebiotics are used in dairy products, infant formulas, beverages, breakfast cereals, snack bars, and deserts.

1.4.2 Honey and its properties

The use of honey as a natural sweetener and as a healing agent has been acknowledged since ancient times. Numerous health-promoting and curative properties attributed to honey are the basis for traditional medical treatments that are used all over the world today (Miraglio and Nicholls 2003). According to Sanz and others (2004) the composition of honey depended on which plants were visited by the bees as that determined the production of nectar or honeydew, and also on the climatic and environmental conditions. The floral honey is produced from nectar, whereas honeydew honey is a product of bees that extract sugars from the living tissues of plants or fruits (Al-Qassemi and Robinson 2003).

Each variety of honey is a unique mixture of compounds that, depending on the

floral sources, season, and processing (Miraglio and Nicholls 2003) varies in composition, color, and flavor. In the United States there are more than 300 floral sources for honey, including clover, alfalfa, sage, sourwood, and buckwheat. Typically, placing the hive in a field in which a single type of plant is in bloom produces monofloral honey.

The three monofloral honey's used in this research were sage, alfalfa, and sourwood honeys. If the plant is *Medicago sativa*, alfalfa honey is produced, which is white or extra-light amber with a mild flavor and aroma similar to beeswax and is produced extensively throughout Canada and United States. White sage (*Salvia apiana*) honey is rich and light with a predominant sweet, clover-like flavor and floral aftertaste produced in California and in the southwest part of the United States. Another kind of honey comes from sourwood (*Oxydendrum arboreum*) and has a sweet, spicy, anise aroma and flavor, and is produced in the eastern areas of the United States (NHB 2003).

Honey is a carbohydrate-rich syrup that contains fructose (38.5%), glucose (31.3%), and water (17%). Other sugars in honey include maltose (7.2%), sucrose (1.5%), and various oligosaccharides (4.2%). In comparison to sucrose that contains 100 g of carbohydrate/100g, honey contains 82g of carbohydrate/100g and provides 304 calories/100g versus 400 calories for sucrose (Miraglio and Nicholls 2003). Honey also contains a variety of organic acids, such as acetic, butyric, citric, formic, gluconic, lactic, malic, pyroglutamic, and succinic acids (0.17 to 1.17%), which give the product an average pH of 3.9 (NHB 1996). Many authors have proposed the use of sugar composition to establish honey authenticity (Weston and Brocklebank 1998; Da Costa Leite and others 2000; Sanz and others 2004). Studying the carbohydrate composition of

artisanal honey from Madrid (Spain), Sanz and others (2004) detected and quantified 25 carbohydrates (glucose, fructose, 16 disaccharides, and seven trisaccharides) in 27 honey samples. The mean values of some of the sugar content (g/100g honey) were: 28.62 glucose, 33.71 fructose, and 0.07 sucrose. Four sugars (maltose, turanose, nigerose and an unknown second disaccharide) could not be resolved, and they were quantified together with a mean of 6.07 g/100g honey. Trisaccharides melezitose and erlose presented the highest values in honey samples, followed by panose: 0.70, 0.38 and 0.22 g/100g honey respectively. Another study (Da Costa Leite and others 2000) focused on the determination of oligosaccharides in Brazilian honey of different botanical origin. The researchers reported the levels of 10 oligosaccharides in 70 genuine Brazilian honeys of different floral types. The contents of sucrose and isomaltose were broad, ranging from mean values of 0.07-0.77 and 0.18-0.71 % respectively. Low amounts of melibiose (0.05-0.15%) and panose (0.03-0.08%) were found in Brazilian honey. Maltotriose, melezitose, and raffinose were determined with means values of 0.24-1.03, 0.21-0.37 and 0.10-0.25% respectively.

The effects of honey as a potential prebiotic have been reported. Kajiwara and others (2002) reported that oligosaccharides in honey might be responsible in promoting intestinal bifidobacteria thus serve as prebiotic. They used 5% (w/vol.) clover honey and compared to fructooligosaccharides (FOS), galactooligosaccharides (GOS) and inulin. They concluded that honey enhanced growth and acid production (lactic and acetic acid) of five intestinal *Bifidobacterium* spp. (*B. longum*, *B. adolescentis*, *B. breve*, *B. bifidum*, and *B. infantis*) in a similar manner to FOS, GOS and inulin. Chick and others (2001) reported that clover honey at 5% (w/w) was not inhibitory, but supported growth and acid

production by lactic acid bacteria (S. *salivarius* subsp. *thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, and *L. acidophilus*) and bifidobacteria (*B. bifidum*) in skim milk similar to fructose and sucrose. Shamala and others (2000) compared the effect of honey and sucrose on lactic acid bacteria *in vitro* and in rat gut. Feeding honey to rats resulted in a significant increase ($P < 0.05$) in counts of lactic acid bacteria in the small and large intestine over the control and sucrose-feed animals. The results obtained during this study concluded the beneficial effect of honey consumption on the physiological constitution of animals fed with honey. Furthermore, under *in vitro* conditions, the number of *Lactobacillus acidophilus* and *Lactobacillus plantarum* counts increased 10-100 fold in the presence of honey compared with sucrose in the same study.

The potential action of honey as a prebiotic represents a characteristic of a product that could be of interest. The role of yogurt as a probiotic carrier is well known, and the participation of this product to the increasing marketing of functional foods is a real challenge to many yogurt processors.

Therefore, the aim of this study was to compare the effects of honeys from three floral sources (sourwood, sage, and alfalfa) varying in oligosaccharides content with traditional sweeteners (sucrose and high fructose corn syrup) or inulin (another well known prebiotic) on promoting probiotics in low-fat yogurt, and to investigate if the product meets and/or exceeds the NYA live-and-active-culture seal criteria.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

Commercial strains of *Streptococcus salivarius* subsp. *thermophilus* (St-133), *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lr-78), together with the probiotic organisms *Lactobacillus acidophilus* (La-7) and *Bifidobacterium bifidum* (Bf-1), were obtained from System Bio-Industries (Waukesha, WI). Six types of sweeteners were used for growth and acid production: (1) sucrose (J. T. Baker Inc., Phillipsburg, NJ); (2) sage honey (Gene Brandi Apiaries, Los Banos, CA) (3) alfalfa honey (Gene Brandi Apiaries, Los Banos, CA), (4) sourwood honey (Haw Creek Honey, Asheville, NC); (5) high fructose corn syrup (HFCS) (MinTose^{TM} 3400-42 HFCS Minnesota Corn Processors; Marshall, MI) or (6) inulin (Rhone-Poulenc, Washington, PA). Test kits for D- and L-lactic acid determination as well as acetic acid determination were obtained from R-Biopharm (Marshall, MI).

For the manufacture of low-fat strawberry flavored yogurt for sensory analysis and viability experiments; sucrose was obtained from Michigan Sugar Company (Saginaw, MI). Alfalfa honey was from Golden Heritage Foods (Santa Fe, KA), and sourwood honey was from Georgia Honey Corporation (Perry, GA). The source of other ingredients was the same as listed above. Additional ingredients used in the low-fat strawberry flavored yogurt manufacture were: stabilizer (Continental Custom Ingredients, W. Chicago, IL), non-fat dry milk solids (Michigan Milk Producers Association, Ovid, MI), and unsweetened strawberry puree (Kraus & Co., Walled Lake, MI) used as flavoring agent. The culture used was a commercial yogurt and probiotic blend: YC-087

(Chr. Hansen Laboratories, Milwaukee, WI).

Nonfat dry milk (NDM), De Man, Rogosa, Sharpe (MRS) medium, lactose for MRSL, bacto agar and bacto-peptone were purchased from Difco Laboratories (Detroit, MI). The antibiotics used in the preparation of NPNL (neomycin sulfate, paramomycine, nalidixic acid and lithium chloride) solution were obtained from Sigma-Aldrich (St. Louis, MO).

2.2 INFLUENCE OF HONEY ON GROWTH AND ACTIVITY OF LACTIC ACID BACTERIA AND BIFIDOBACTERIA

2.2.1 Organisms and culture preparation

Each strain *S. salivarius* subsp. *thermophilus* (St-133), *L. delbrueckii* subsp. *bulgaricus* (Lr-78), *L. acidophilus* (La-7) and *B. bifidum* (Bf-1), underwent three successive 24 h transfers at 37 °C in MRS broth. Bifidobacteria were grown in MRS broth containing 5% (w/v) lactose (MRSL) and anaerobically incubated at 37 °C for 24 h, using Gas Packs® (BBL Microbiology Systems, Cockeysville, MD). All cultures were centrifuged for 5 min at 10,000 x g at 4 °C and resuspended in 12% (w/v) pasteurized NDM (70 °C, 15 min) to obtain approximately 10^8 CFU/mL.

2.2.2 Growth of lactic acid bacteria and bifidobacteria in the presence of different sweeteners

Honeys from three different monofloral sources were used in this study: sage (*Salvia apiana*), alfalfa (*Medicago sativa*), and sourwood (*Oxydendrum arboreum*). They varied in their carbohydrate and oligosaccharide composition and content. Their compositional analysis is reported in Table 2.1.

Table 2.1 Chemical composition of different monofloral honeys

Composition (%)	Sage	Alfalfa	Sourwood
Moisture	6.3 ± 0.2	7.7 ± 0.1	6.9 ± 0.1
Fructose	38.9 ± 3.6	38.4 ± 4.3	35.7 ± 4.1
Glucose	37.5 ± 5.3	35.1 ± 6.1	33.3 ± 3.3
Maltose	11.7 ± 1.4	10.2 ± 1.8	9.8 ± 1.5
Sucrose	1.6 ± 0.2	2.7 ± 0.3	3.1 ± 0.4
Oligosaccharides	3.8 ± 0.6	5.5 ± 1.0	10.9 ± 1.1
Ash	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.1

Source: Shin and Ustunol (2005)

A 12% (w/v) reconstituted NDM (Difco) solution was prepared and divided into thirteen portions. Sweeteners: sucrose, HFCS, sage honey, alfalfa honey, sourwood honey or inulin was added to each of the tubes at 5 or 10% level (w/v). Controls were devoid of sweetener. Next, the tubes were pasteurized at 70 °C for 15 min and cooled to 37 °C. Tubes with each sweetener level and the control were inoculated with 5% (v/v) of starter containing *S. salivarius* subsp. *thermophilus* (St-133), *L. delbrueckii* subsp. *bulgaricus* (Lr-78), *L. acidophilus* (La-7), or *B. bifidum* (Bf-1). All tubes were incubated aerobically at 37 °C for 24h except for *B. bifidum*, which was incubated anaerobically using Gas Packs®. Figure 2.1 provides a schematic diagram of the experimental design used in determining the effect of different sweeteners (sucrose, HFCS, honey or inulin) on growth and activity of lactic acid bacteria and bifidobacteria. Initially, and after 24 h incubation, for each of the treatments, one mL of each thoroughly mixed fermented milk sample was taken and serially diluted with 99 mL of sterile 0.1% (w/v) bacto-peptone to determine the numbers of lactic acid bacteria and bifidobacteria by plating using MRS containing 1.5% (w/v) bacto agar (lactic acid bacteria) or MRSL agar (bifidobacteria).

The plates were then incubated aerobically at 37 °C for 48h with the exception of bifidobacteria plates, which were incubated anaerobically using Gas Packs® under similar conditions. The colonies were counted using a Quebec colony counter (Fisher Scientific, Pittsburgh, PA). Initially and after 24h of incubation pH of the samples was also determined.

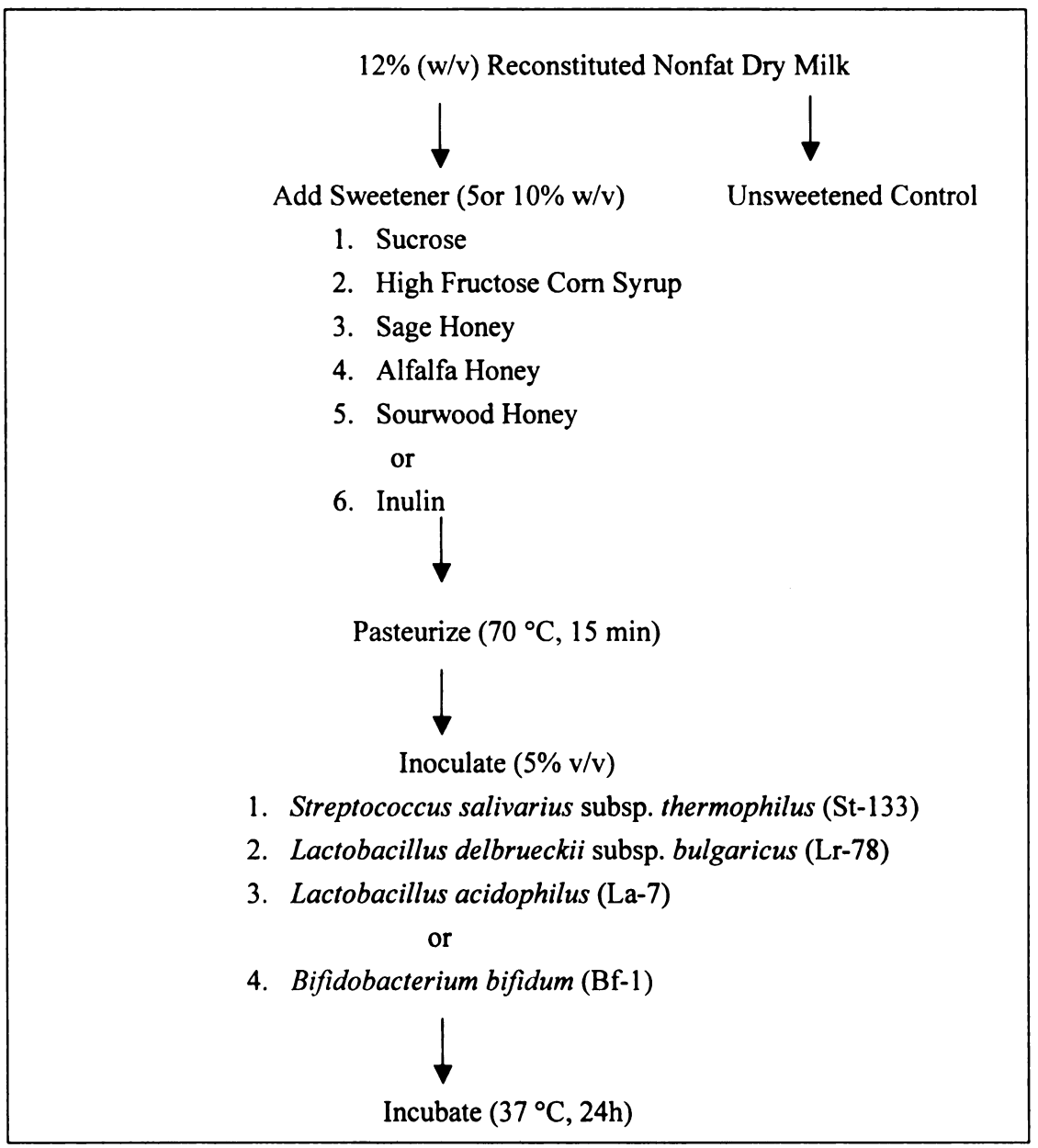

Figure 2.1 Schematic diagram for the experiment determining effect of sweeteners on growth and activity of lactic acid bacteria and bifidobacteria.

2.2.3 Lactic and acetic acid determination using the spectrophotometric method

Lactic acid production by *S. salivarius* subsp. *thermophilus* (St-133), *L. delbrueckii* subsp. *bulgaricus* (Lr-78), *L. acidophilus* (La-7), and *B. bifidum* (Bf-1) as well as acetic acid produced by *B. bifidum*, grown in the presence of different sweeteners (5% w/v): sucrose, HFCS, honey (sage, alfalfa, sourwood) or inulin were determined using test kits for D- and L-lactic and acetic acid. The total amount of lactic (D- and L-lactic acid) and acetic acid at 0 and 24 h was reported (g/L).

2.2.3.1 Lactic acid determination

Two grams of fermented milk sample were mixed with 98 mL redistilled water into a volumetric flask. The solution was homogenized for 1 minute using a Polytron PT 10.35 homogenizer with a PTA 20 TS homogenizing head (Tekmar Co., Cincinnati, OH) at speed 5 (1350 rpm). Next, the solution obtained was filtered through Whatman #1filter paper (Whatman Limited) and 0.100 mL of the filtrate was used for the assay. The assay was performed using the reagents for D- and L-lactic acid from the kit. Absorbance of blank and samples was measured at 340 nm using a spectrophotometer (Spectronic 1201Plus, Milton Roy, Rochester, NY).

Test principle is based on the presence of enzymes D-lactate dehydrogenase (D-LDH) and L-lactate dehydrogenase (L-LDH) that catalyze the oxidation of D-lactic and L-lactic, respectively, to pyruvate by nicotinamide-adeneine dinucleotide (NAD).

(1) D-Lactate + NAD^+ $<\xrightarrow{\text{D-LDH}}>$ pyruvate + NADH + H^+

(2) L-Lactate + NAD^+ $<\xrightarrow{\text{L-LDH}}>$ pyruvate + NADH + H^+

Because the equilibrium of the reactions lies on the side of lactate, the pyruvate is included in a subsequent reaction.

(3) Pyruvate + L-glutamate $<\overset{GPT}{\longrightarrow}>$ L-alanine + 2-oxoglutarate

The reaction is catalyzed by the enzyme glutamate-pyruvate transaminase (GPT) in the presence of L-glutamate and the equilibrium can be shifted in the favor of pyruvate and nicotinamide-adeneine dinucleotide reduced (NADH). The determination of D-lactic and L-lactic acid, is based on the amount of NADH formed that is stoichiometric to the amount of acid. The increase in NADH is determined by means of its light absorbance at 340 nm.

Briefly, the test procedure (Table 2.2) involved mixing solutions 1, 2, 3 plus sample and redistilled water in a 20 mL glass tubes (individual sets for each treatment) and incubation at room temperature (23 ± 2 °C) for 5 min.

Table 2.2 Reagents used in the determination of lactic acid in yogurt using the spectrophotometric method

Reagents	**Blank (mL)**	**Sample (mL)**
Solution 1 (glycylglycine buffer + L-glutamic acid)	1.000	1.000
Solution 2 (nicotinamide-adenine dinucleotide- NAD)	0.200	0.200
Suspension 3 (glutamate-pyruvate transaminase)	0.020	0.020
Sample solution	-	0.100
Redistiled water	1.000	0.900
Solution 4 (D-lactate dehydrogenase)	0.020	0.020
Solution 5 (L-lactate dehydrogenase)	0.020	0.020

Next, absorbance (A_1) was read for both blank and sample. The reaction was started by the addition of solution 4 and after 30 min incubation at room temperature the

second absorbance (A_2) was read. After the addition of solution 5 and 30 min incubation at room temperature (23 ± 2 °C), absorbance A_3 was determined and the assay was completed. The absorbance difference and lactic acid concentration was calculated using the following equations:

$\Delta A_{D\text{-lactic}} = (A_2\text{-}A_1)_{\text{sample}} - (A_2\text{-}A_1)_{\text{blank}}$

$\Delta A_{L\text{-lactic}} = (A_3\text{-}A_2)_{\text{sample}} - (A_3\text{-}A_2)_{\text{blank}}$

$C = [(V \times MW) / \varepsilon \times d \times v \times 1000] \times \Delta A$ [g/L]

where:

C = sample solution concentration (g/L)

V = final volume [mL]; 2.240 mL for D-lactic and 2.260 mL for L-lactic

v = sample volume [mL]; 0.100 mL

MW = molecular weight of the substance to by assayed [g/mol]; 90.1 g/mol

d = light path [cm]; 1.00 cm

ε = extinction coefficient of NADH (amount formed will be stoichiometric to the amount of D/L-lactic acid) at 340 nm; 6.3 [1 x $mmol^{-1}$ x cm^{-1}]

2.2.3.2 Acetic acid determination

Five grams of fermented milk sample were mixed with 50 mL of distilled water in a 100 mL volumetric flask and heated in a water bath at 50-60 °C for 20 min. The flask was shaken from time to time during heating. After the sample was cooled to room temperature(~23 °C), the volumetric flask was brought to 100 mL volume with distilled water. The solution was homogenized for 1 minute using a Polytron PT 10.35

homogenizer with a PTA 20 TS homogenizing head (Tekmar Co., Cincinnati, OH) at speed 5 (1350 rpm). Next, the solution was filtered through Whatman #1 filter paper, and 0.100 mL of the filtrate was used for the assay. The assay was performed using the acetic acid reagents from the kit. Absorbance of blank and sample was measured at 340 nm using a spectrophotometer (Spectronic 1201Plus, Milton Roy, Rochester, NY).

Test principle is based on the presence of enzyme acetyl-CoA synthetase (ACS) that catalyze the conversion of acetic acid to acetyl-CoA by adenosine-5'-triphosphate and coenzyme A (CoA).

(1) Acetate + ATP + CoA $\xrightarrow{ACS}$ acetyl-CoA + AMP + pyrophosphate

(2) Acetyl-CoA + oxaloacetate + H_2O $\xrightarrow{CS}$ citrate + CoA

(3) L-Malate + NAD^+ $\xleftarrow{L\text{-}MDH}$ oxaloacetate + NADH + H^+

Next, acetyl-CoA reacts with oxaloacetate (2), reaction is catalyzed by citrate oxalate (CS). In the last reaction (3) NAD is reduced to NADH in the presence of L-malate-dehydrogenase (L-MDH). The determination of acetic acid is based on the formation of NADH measured by the increase in light absorbance at 340 nm.

Table 2.3 Reagents used in the determination of acetic acid in yogurt using the spectrophotometric method

Reagents	**Blank (mL)**	**Sample (mL)**
Solution 1(triethanolamine buffer+L-malic acid+magnesium chloride)	1.000	1.000
Solution 2 (ATP+CoA+NAD)	0.200	0.200
Sample solution	-	0.100
Redistiled water	1.000	0.900
Solution 3 (L-malate dehydrogenase+citrate synthase)	0.010	0.010
Solution 4 (lyophilizate acetyl-CoA synthetase)	0.020	0.020

Briefly, the test procedure involved mixing solutions 1 and 2, plus sample and redistilled water, and reading the first absorbance (A_0) for blank and sample. Next, solution 3 was added, and after 3 min incubation at room temperature, the second absorbance was taken (A_1). The reaction was started by the addition of solution 4 and after 15 min incubation at room temperature third absorbance (A_2) was read. The absorbance difference and concentration of acetic acid was calculated using the following equations:

$$\Delta A_{acetic\ acid} = \{(A_2 - A_0)_{sample} - [(A_1-A_0)^2_{sample}/(A_2-A_0)_{sample}] - (A_2 - A_0)_{blank} - [(A_1-A_0)^2_{blank} /(A_2-A_0)_{blank}]\}$$

$$C = [(V \times MW) / \varepsilon \times d \times v \times 1000] \times \Delta A\ [g/L]$$

where:

C = sample solution concentration (g/L)

V = final volume [mL]; 3.230 mL

v = sample volume [mL]; 0.100 mL

MW = molecular weight of the substance to by assayed [g/mol]; 60.05 g/mol

d = light path [cm]; 1.00 cm

ε = extinction coefficient of NADH; 6.3 [1x$mmol^{-1}$ x cm^{-1}]

2.3 EFFECT OF YOGURT INGREDIENTS ON GROWTH OF LACTIC ACID BACTERIA AND BIFIDOBACTERIA

Figure 2.2 shows the experimental design used for the effect of different yogurt ingredients on growth and activity of lactic acid bacteria (*S. salivarius* subsp. *thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*) and *B. bifidum* (Bf-1).

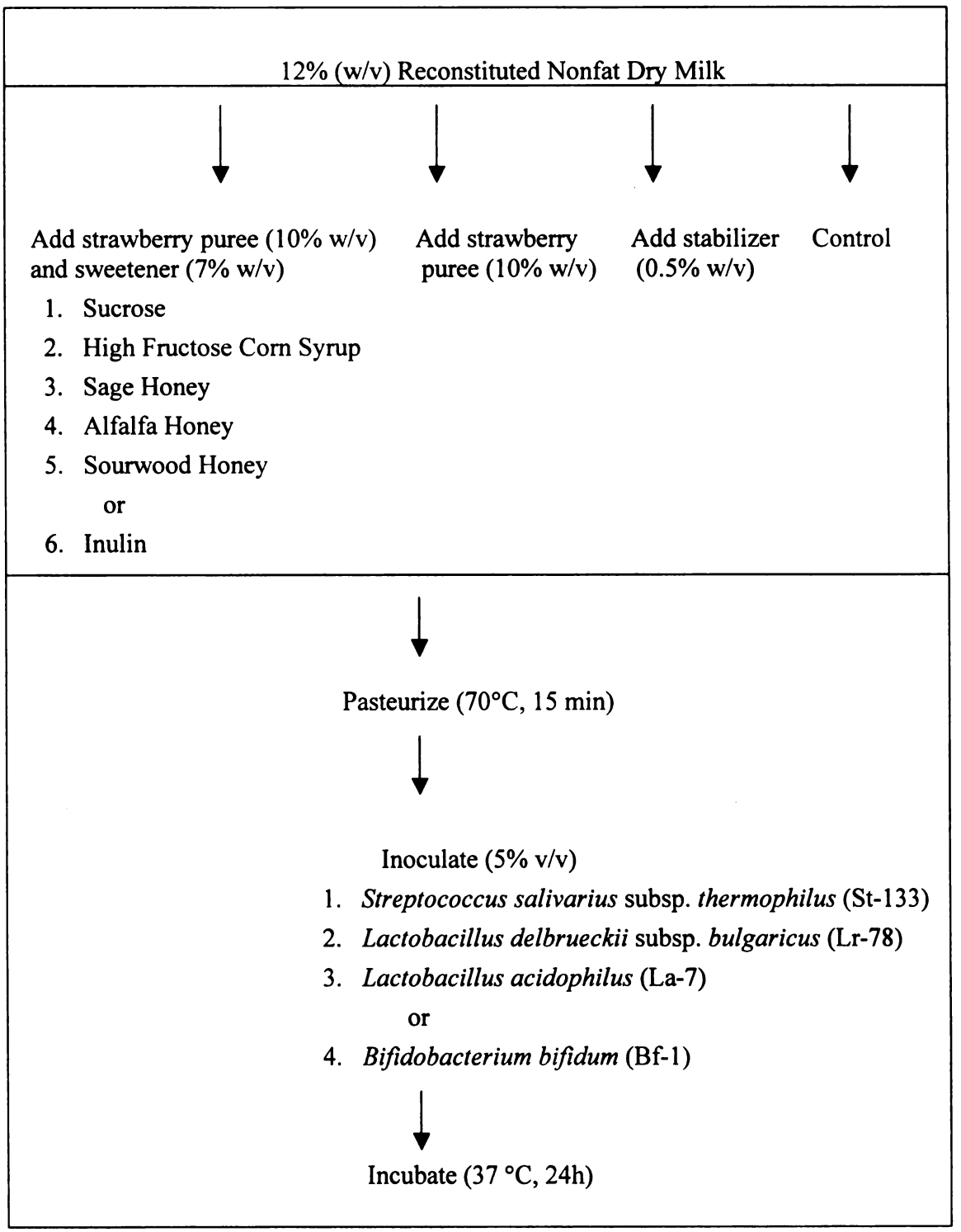

Figure 2.2 Schematic diagram for the experiment determining effect of ingredients on growth of lactic acid bacteria and bifidobacteria.

A 12% (w/v) reconstituted NDM (Difco) solution was prepared and divided into nine portions. Stabilizer (0.5% w/v) containing pectin, unsweetened strawberry puree (10% w/v) or a mixture of unsweetened strawberry puree (10% w/v) and sweetener (7% w/v) (sucrose, HFCS, sage honey, alfalfa honey, sourwood honey or inulin) was added to each tube. Control samples had no ingredients added. Subsequently, all the tubes were pasteurized at 70 °C for 15 min, and cooled to room temperature. Each tube was inoculated with 5% (v/v) starter containing *S. salivarius* subsp. *thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus* or *B. bifidum.* Next, the tubes were incubated aerobically at 37 °C for 24h except for *B. bifidum*, which was incubated anaerobically using Gas Packs®. Initially, and after 24 h incubation, for each of the treatments, one mL of each thoroughly mixed fermented milk sample was taken and serially diluted with 99 mL of sterile 0.1% (w/v) bacto-peptone to determine the numbers of lactic acid bacteria and bifidobacteria by plating using MRS containing 1.5% (w/v) bacto agar (lactic acid bacteria) or MRSL agar (bifidobacteria). The plates were then incubated aerobically at 37 °C for 48h with the exception of bifidobacteria plates, which were incubated anaerobically using Gas Packs® under similar conditions. The colonies were counted using a Quebec colony counter (Fisher Scientific, Pittsburgh, PA). Initially and after 24h of incubation pH of samples was also determined.

2.4 LOW FAT YOGURT FORMULATION AND MANUFACTURE

Strawberry flavored low-fat yogurt was manufactured in the Dairy Pilot Plant at Michigan State University from 2% fat milk. Table 2.4 provides the yogurt formulations used and Figure 2.4 shows the flow diagram for the manufacture process.

Table 2.4 Low - fat yogurt formulation

Ingredients (%)	Treatments					
	Sucrose	HFCS[2]	Sage	Alfalfa	Sourwood	Inulin
Milk	78.50	76.40	78.06	77.97	78.02	78.50
NDM[1]	4.00	4.00	4.00	4.00	4.00	4.00
Sweetener	7.00	9.10	7.44	7.53	7.48	7.00
Stabilizer	0.50	0.50	0.50	0.50	0.50	0.50
Strawberry puree	10.00	10.00	10.00	10.00	10.00	10.00
Total	100.00	100.00	100.00	100.00	100.00	100.00

[1]NDM = Nonfat dry milk

[2]HFCS = High fructose corn syrup

Commercial milk with 2% fat was blended together with 4 % (w/v) nonfat dry milk (NDM), 0.5% (w/v) stabilizer, and 7% (w/v) sweetener (sucrose, HFCS, sage honey, alfalfa honey, sourwood honey or inulin). In the formulation, moisture content of honey and HFCS was taken into account. Sucrose sweetened yogurt was the control. Milk bases were dual stage homogenized (2000 psi and 500 psi) (Homogenizer-200, Cherry Burrell Corp. Chicago, IL) at 60°C and batch pasteurized (85 °C, 30 min), cooled to 43 °C and inoculated with 0.5% (w/v) YC-087, a commercial yogurt and probiotic culture blend. Inoculated mixtures were incubated at 43 °C until pH 4.4 (0.9 to 1.2 percent titratable acidity) was attained. Subsequently, unsweetened strawberry puree (10% w/v) was blended into each of the yogurt treatments. Stirred yogurt was packaged into 8 oz containers and stored at 4°C for ten days, when the sensory evaluation was completed.

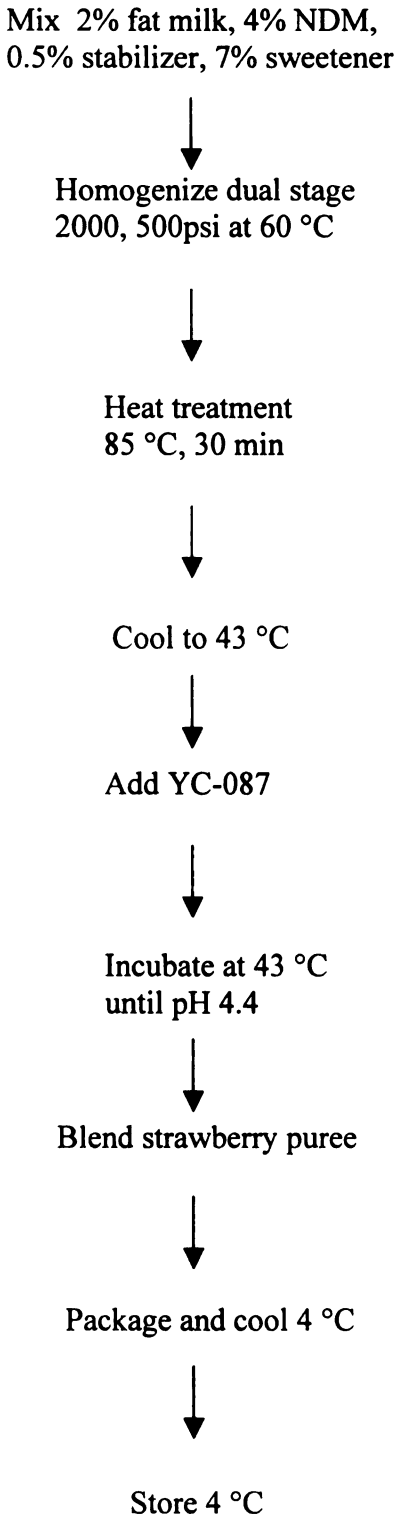

Figure 2.3 Flow diagram for manufacture of low-fat yogurt (Swiss-style)

2.5 SENSORY ANALYSIS OF STRAWBERRY FLAVORED LOW-FAT YOGURT

Yogurts manufactured with different sweeteners were evaluated using a consumer panel. The panelists were recruited by posting flyers around the Michigan State University (MSU) campus, and by sending e-mails containing the flyers to different departments at MSU. One hundred panelists consisting of graduate students, undergraduate students, and faculty participated in the sensory evaluation of the yogurts. Sensory evaluation was conducted in individual illuminated booths in the sensory laboratory in the Department of Food Science and Human Nutrition at MSU. Upon arrival at the sensory laboratory, each subject read an explanation of the study and gave their informed consent. The University Committee on Research Involving Human Subjects (UCRIHS) approved the study (Appendix B). Yogurt samples were stored at refrigerated temperature until evaluation, and spooned into 2 oz plastic cups labeled with randomly selected three-digit numbers. The order of presentation of the samples was randomized across subjects to ascertain that the order of the runs does not introduce bias into the results. Subjects were asked to taste all six samples and indicated their degree of liking on a nine-point hedonic scale from 1 = dislike extremely to 9 = like extremely; 5 = neither like nor dislike. The panelists evaluated each sample for flavor, aroma, sweetness, and overall preference. The panelists were provided with water for rinsing and crackers for palate cleaning.

2.6 VIABILITY DURING REFRIGERATED STORAGE

Viability of lactic acid bacteria and bifidobacteria in the yogurts prepared as described in section 2.4 was monitored at 7-day intervals during 42 days of refrigerated storage (4 °C). For this purpose, a new batch of yogurt was manufactured, using the same formulation as in Table 2.4, and the commercial strains of *S. salivarius* subsp. *thermophilus* (St-133), *L. delbrueckii* subsp. *bulgaricus* (Lr-78), along with the probiotic organisms *L. acidophilus* (La-7) and *B. bifidum* (Bf-1) were used as the starter cultures. Each strain underwent 2 successive 24h at 37 °C transfers in 12% (w/v) NDM pasteurized at 70 °C, 15 min.

Seven aliquots (one for each treatment) containing 500ml of milk base (milk, NDM, sweeteners: sucrose, HFCS, sage honey, alfalfa honey, sourwood honey or inulin, and stabilizer in the concentrations presented in Table 2.4) were inoculated with 1.5% (v/v) level of culture and shaken manually for 5 minutes to ensure even distribution of the organism in the product. Controls were devoid of sweeteners. The culture of bifidobacteria and lactic acid bacteria was added in a 1:1 ratio to each aliquot. The inoculated mixtures were incubated at 43 °C until pH 4.4 was reached. Next, the yogurts were cooled on ice, and strawberry puree (10% w/v) was added to each of the treatments. Each yogurt preparation was aseptically divided into forty two (7 treatments x 6 weeks) 50-mL conical polyethylene centrifuge (Corning) tubes. Next, and then at 7day intervals for 42days, one g of each yogurt sample was diluted with 99 mL of sterile 0.1% (w/v) peptone and subsequent serial dilutions were made to quantify the viability of lactic acid bacteria and bifidobacteria at 0 and 7day intervals. MRS medium containing 1.5% Bacto agar was used to enumerate lactic acid bacteria (*S. salivarius* subsp. *thermophilus*, *L.*

delbrueckii subsp. *bulgaricus*, and *L. acidophilus*). MRSL medium containing 1.5% Bacto agar and 2.5% (v/v) filter sterilized (0.22μm) NPNL antibiotic solution was used for *B. bifidum* enumeration. Shin and others (2000) used 5% (v/v) NPNL antibiotic solution added to MRSL agar for *B. bifidum* enumeration. The antibiotic solution was prepared following the method of Shin and others (2000), but was slightly modified. The antibiotics used and their concentrations in this study were: 1 g/L neomycin sulfate, 4g/L paramomycine, 0.3g/L nalidixic acid and 60g/L lithium chloride. The plates were incubated aerobically at 37 °C for 72 h, with the exception of *B. bifidum*, which was incubated anaerobically using Gas Packs®. The colonies were counted using 920A colony counter (American Bantex Corp., Burlingame, CA). The pH was also monitored at each 7d interval for 42d. Percent viability of lactic acid bacteria and bifidobacteria was calculated as follows:

$$\% \text{ Viability} = (\text{CFU each 7 days storage} / \text{initial CFU}) \times 100$$

2.7 STATISTICAL ANALYSIS

Experiments were replicated three times in a completely randomized design. Fixed effects for growth and activity studies included three factors (sweetener type, time and sweetener level) by four different strains (three lactic acid bacteria and one bifidobacteria) and their interaction terms. For growth with ingredients used in the manufacture of yogurt, fixed effects included two factors (treatment and time) by four different strains (three lactic acid bacteria and one bifidobacteria) and their interaction terms. For viability, fixed effects included two factors (sweetener type, time) by two different groups of strains (lactic acid bacteria and bifidobacteria) and their interaction

terms. Random effects included the replicates.

The data were analyzed using "Proc Mixed" in the SAS system version 9.1 (SAS Institute Inc., 2003, Carry, NC). After running the model by SAS, the assumption of mixed model to check the normality of residuals was tested. The Tukey-Kramer method was used to adjust *P*-values for multiple comparisons. PROC MIXED carries out the estimation and testing of linear combinations of fixed and random effects. The following model statement was used in the present study:

$$y_{ijklm} = t_i + s_j + h_k + w_l + (ts)_{ij} + (th)_{ik} + (tw)_{il} + (sh)_{jk} + (sw)_{jl} + (hw)_{il} + (tsh)_{ijk} + (tsw)_{ijl} + (shw)_{jkl} + (tshw)_{ijkl} + \varepsilon_{ijklm}$$

where:

t_i = main effect of treatment (or sweetener type in the case of growth and acid production

s_j = main effect of strain (culture)

h_k = main effect of time (hours or days)

w_l = main effect of sweeter level

$\varepsilon_{ijklm \sim N(0,\ \sigma^2)}$ = measures between subject variability; assumes that the effect of the subject has a normal distribution with mean 0 and variance sigma S squared

y_{ijklm} = dependent variable (log CFU/mL, or g/L, or % viability)

The rest of the equation is represented by interactions between main effects: two, three or four way interactions. Eventhough PROC MIXED allows only for one dependent variable in the model statement, is possible to use to model multivariate repeated measures.

The sensory analysis data were analyzed using one-way ANOVA on Sigma Stat 1.0 (Jandel Corp., San Rafael, CA). Student-Newman-Keuls method was used as test for multiple comparisons. Differences were considered significant at the $P < 0.05$ level.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 INFLUENCE OF SWEETENER TYPE ON GROWTH AND ACTIVITY OF LACTIC ACID BACTERIA AND BIFIDOBACTERIA

Table 3.1 shows Analysis of Variance (ANOVA) for the independent variables (main effects): sweetener type (sucrose, HFCS, sage, alfalfa, sourwood honeys or inulin), sweetener level (5, 10%), incubation time (0, 24 h), and culture (*Streptococcus salivarius* subsp. *thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus acidophilus*, and *Bifidobacterium bifidum*), their two-way, three-way and four-way interactions on the dependent variable growth of lactic acid bacteria and bifidobacteria. All main effects and their interactions had a significant effect on growth of lactic acid bacteria (LAB) and bifidobacteria. The only exception was the two-way interaction between incubation time and sweetener level.

Table 3.2 reports (in log CFU/mL) the growth of LAB and bifidobacteria as influenced by sweetener type and level over a 24 h incubation period. After 24 h of incubation, there was a one log increase in growth of the microorganisms investigated in the unsweetened (control) as well as the sweetened treatments. Although not statistically significant, overall, higher numbers were reached when cultures were grown in the presence of the three honeys (sage, alfalfa, sourwood) compared to the control, sucrose, and inulin at 5 % sweetener level. Sucrose sweetened yogurt (10%) was lower ($P < 0.05$) than that of alfalfa honey sweetened yogurt (8.31 log CFU/mL vs. 8.83 log CFU/mL). In case of HFCS, at 5% level growth of LAB and bifidobacteria was similar to control, sucrose and inulin treatments, but at 10% HFCS growth of microorganisms was similar to

Table 3.1 Analysis of variance for dependent variable growth of lactic acid bacteria and bifidobacteria

Effect	**Num [1] DF**	**Den[2] DF**	**F value**	**Pr > F**
Sweetener type	5	96	10.31	<0.0001
Culture	3	96	13.85	<0.0001
Time	1	96	4263.09	<0.0001
Sweetener level	1	96	9.90	0.0022
Sweetener type * Culture	15	96	2.30	0.0079
Sweetener type * Time	5	96	9.54	<0.0001
Culture * Time	3	96	445.18	<0.0001
Sweetener type * Sweetener level	5	96	4.26	0.0015
Culture * Sweetener level	3	96	4.86	0.0034
Time * Sweetener level	1	96	0.19	0.6675
Sweetener type * Culture * Time	15	96	4.26	<0.0001
Sweetener type * Culture * Sweetener level	15	96	1.90	0.0326
Sweetener type * Time * Sweetener level	5	96	3.66	0.0045
Culture * Time* Sweetener level	3	96	18.19	<0.0001
Sweet. type * Culture * Time * Sweet. level	15	96	2.13	0.0142

[1]Num DF = numerator degrees of freedom
[2]Den DF = denominator degrees of freedom

Table 3.2 Effect of sweetener type and level on growth of lactic acid bacteria and bifidobacteria over 24 h incubation

Treatment	5%		10%	
	0 h (log CFU/mL)	**24 h** (log CFU/mL)	**0 h** (log CFU/mL)	**24 h** (log CFU/mL)
Control[1]	7.44±0.48[a]	8.37±0.38[a]	7.44±0.48[a]	8.37±0.39[ab]
Sucrose	7.46±0.47[a]	8.50±0.57[a]	7.43±0.49[a]	8.31±0.41[b]
HFCS[2]	7.46±0.49[a]	8.54±0.32[a]	7.56±0.47[a]	8.76±0.35[ab]
Sage	7.55±0.45[a]	8.60±0.34[a]	7.43±0.65[a]	8.66±0.43[ab]
Alfalfa	7.44±0.54[a]	8.71±0.27[a]	7.53±0.55[a]	8.83±0.38[a]
Sourwood	7.39±0.57[a]	8.64±0.33[a]	7.54±0.44[a]	8.77±0.40[ab]
Inulin	7.33±0.53[a]	8.44±0.28[a]	7.58±0.46[a]	8.45±0.51[ab]

[a-b] Means with different superscripts are significantly different ($P < 0.05$). Comparisons are made only within the same column; n = 3 for all treatments.

[1]Control devoid of sweetener

[2]HFCS = high fructose corn syrup.

the three honeys (8.76 log CFU/mL for HFCS and 8.66, 8.83 and 8.77 log CFU/mL for sage, alfalfa and sourwood honey, respectively). Corn sweeteners are produced by hydrolysis of starch catalyzed by α-amylase, which produce glucose and various oligosaccharide chain lengths of 10-13 glucose residues. The enzyme glucoamylase produces glucose from the nonreducing end of the oligosaccharides that vary in their degree of polymerization. High fructose corn syrup is produced by treating corn syrup with the enzymes isomerase to convert a portion of the glucose into fructose. The HFCS in this study was of 42DE (dextrose equivalent) (MinTose™ 3400-42 HFCS Minnesota Corn Processors; Marshall, MI) indicating medium level hydrolysis with significant amount of medium to longer chain oligosaccharides, which perhaps contributed in promoting the growth of LAB and bifidobacteria similar to honey.

Among the sweeteners investigated in this study, inulin and sucrose were the least effective in promoting the growth of the organisms studied after 24 h of incubation (8.45 CFU/mL and 8.31CFU/mL respectively). With respect to inulin, these results are contradictory to those obtained by Kajiwara and others (2002) for bifidobacteria species. They reported 5% inulin being as effective as honey, FOS and GOS ($P < 0.05$) in sustaining the growth of *Bifidobacterium* spp. after 24 h incubation in reinforced clostridial medium supplemented with 5% sweetener as compared with the control reinforced clostridial medium. However, the study was focused on human intestinal bifidobacteria growth and acid production as influenced by honey in comparison with commercial oligosaccharides and inulin. In the current study commercial bifidobacteria typically used in dairy products were investigated in reconstituted non-fat dry milk (12%).

Shin and others (2000) reported that 5% FOS and GOS were more effective than 5% inulin on the growth of commercial *Bifidobacterium* spp. Consistent with their findings in the current study 5 or 10% honey and HFCS were more effective than 5 or 10% inulin for growth of commercial bifidobacteria.

Overall, the effect of sage, alfalfa and sourwood honeys on growth of LAB and bifidobacteria were similar. Chick and others (2001) reported an enhanced growth of bifidobacteria in the presence of 5% clover honey. The researchers suggested that the enhanced growth is due to the various oligosaccharides present in honey, since bifidobacteria tend to prefer more complex carbohydrates for their growth (Shin and others 2000). Most of the oligosaccharides in honey such as isomaltose and melezitose have a low DP (Weston and Brocklebank 1998). It has been reported (Hopkins and

others 1998) that GOS and FOS having lower DP were best in supporting growth of bifidobacteria. Because not very substantial research has been conducted on the mechanism of carbohydrate uptake by bifidobacteria there is the assumption that the substrate transport system is more efficient for dimeric and oligomeric carbohydrate sources (Bruno and others 2002) in bifidobacteria. In the present study honey with different oligosaccharide contents (low, medium and high) were selected and investigated: 3.8% in sage (low), 5.5% in alfalfa (medium) and 10.9% in sourwood (high). However, based on the results of this study the effect of honey on the overall growth of LAB and bifidobacteria was not influenced by oligosaccharide content of the honey and their floral source.

The effects of sweetener type on growth and activity of each microorganism investigated will be discussed individually in the next four sections.

3.1.1 Influence of sweetener type on growth and activity of *Streptococcus salivarius* subsp. *thermophilus* (St-133)

Table 3.3 shows the ANOVA for the independent variable and their various interactions on growth of *S. salivarius* subsp. *thermophilus* (St-133). Table 3.4 reports (in log CFU/mL) the growth of *S. salivarius* subsp. *thermophilus* (St-133) as influenced by sweetener type and level over a 24 h incubation period. Although not statistically significant, after 24 h of incubation at 5% sweetener level only alfalfa honey (8.75 log CFU/mL) appeared to enhance the growth of *S. salivarius* subsp. *thermophilus*. The least effective in promoting the growth of this strain at 5% level was sourwood honey followed by inulin (8.39 log CFU/mL and 8.42 log CFU/mL, respectively). No previous research

has been conducted to determine the growth of this particular strain with 5% sourwood honey. However, 5% clover honey in supporting growth of *S. salivarius* subsp. *thermophilus* (St-133) was investigated, and compared with sucrose and fructose (Chick and others 2001), it was concluded that all sweeteners supported the growth of this organism in a similar manner during 24 h incubation. In the present study at 10% sweetener level sourwood honey significantly enhanced growth of *S. salivarius* subsp. *thermophilus* (St-133) compared to sucrose. Although it is not clear which components in honey may have contributed to the growth of *S. salivarius* subsp. *thermophilus* (St-133), the presence of oligosaccharides in honey may have some influence.

Production of organic acids, particularly lactic acid during fermentation is important in fermented dairy products because acid production determines many of the characteristics of the product as well as their sensory properties. Lactic acid production is also a valuable indicator of bacterial activity (Bouzas and others 1991). In this study in addition to growth determination, lactic acid production by each organism in the presence of each sweetener was also determined. Contrary to the growth studies lactic acid production by *S. salivarius* subsp. *thermophilus* (St-133) (Table 3.5) was enhanced in the presence of inulin (21.08 g/L) and HFCS (20.40 g/L) after 24 h fermentation. In the presence of 5% alfalfa honey lactic acid production was lower (18.56 g/L) than all the treatments, which ranged between 16.63 g/L and 21.08 g/L after 24h incubation. Limitation on growth of cultures but enhancement in lactic acid production was previously reported (Desjardin and others 1990). The researchers called this uncoupling of growth and acid production for *Bifidobacterium* species. The limitation on growth of bifidobacteria in their study was due to lactate and acetate accumulation that resulted in

an uncoupling of biomass and product formation. On the current study the presence of inulin at 5% level produced high levels of lactic acid for *S. salivarius* subsp. *thermophilus* that may have limited the growth of the same strain in 5% inulin. Lactic acid bacteria may change their metabolism in response to various conditions, resulting in different end products pattern. In most of the cases, the changes can be attributed to an altered pyruvate metabolism, the use of external electron acceptors, or both, as these may be connected to each other. Homolatic fermentation by *S. salivarius* subsp. *thermophilus* follows the Embden-Meyerhof-Parnas (EMP) pathway for glucose catabolism through pyruvate and lactic acid. When lactose is metabolized glucose is catabolized to pyruvate and galactose is excreted from the cell. Once all the glucose is utilized *S. salivarius* subsp. *thermophilus* utilizes galactose via Leloir pathway. Vachon (1998) reported increased growth and acid production for the same strain after 24 h incubation at 37 °C. However, the study was done with Grade A Clover honey in comparison with sucrose and fructose and no others complex carbohydrates, except honey were investigated.

Table 3.3 Analysis of variance for dependent variable growth of *Streptococcus salivarius* subsp. *thermophilus* (St-133) in 12% nonfat dry milk

Effect	**Num [1] DF**	**Den[2] DF**	**F value**	**Probability > F**
Sweetener type	5	24	3.53	0.0157
Time	1	24	213.56	<0.0001
Sweetener level	1	24	0.02	0.9030
Sweetener type * Time	5	24	1.74	0.1642
Sweetener type * Sweetener level	5	24	3.31	0.0204
Time * Sweetener level	1	24	4.92	0.0363
Sweetener type* Time* Sweetener level	5	24	1.59	0.2018

[1]Num DF = numerator degrees of freedom
[2]Den DF = denominator degrees of freedom

Table 3.4 Effect of sweetener type and level on growth of *Streptococcus salivarius* subsp. *thermophilus* (St-133) over 24 h incubation

Treatment	5%		10%	
	0 h (log CFU/mL)	**24 h** (log CFU/mL)	**0 h** (log CFU/mL)	**24 h** (log CFU/mL)
Control[1]	8.03 ± 0.01[a]	8.53 ± 0.16[ab]	8.03 ± 0.01[a]	8.53 ± 0.16[ab]
Sucrose	8.09 ± 0.09[a]	8.54 ± 0.04[ab]	7.81 ± 0.02[a]	8.31 ± 0.19[b]
HFCS[2]	8.10 ± 0.10[a]	8.51 ± 0.11[ab]	8.07 ± 0.06[a]	8.47 ± 0.03[ab]
Sage	8.14 ± 0.08[a]	8.49 ± 0.04[ab]	7.97 ± 0.18[a]	8.60 ± 0.51[ab]
Alfalfa	8.09 ± 0.07[a]	8.75 ± 0.30[a]	8.03 ± 0.02[a]	8.84 ± 0.11[ab]
Sourwood	8.07 ± 0.06[a]	8.39 ± 0.03[b]	8.01 ± 0.06[a]	8.87 ± 0.11[a]
Inulin	7.92 ± 0.12[a]	8.42 ± 0.08[ab]	8.08 ± 0.04[a]	8.52 ± 0.18[ab]

[a-b] Means with different superscripts are significantly different ($P < 0.05$). Comparisons are made only within the same column; n = 3 for all treatments.

[1]Control devoid of sweetener

[2]HFCS = high fructose corn syrup.

Table 3.5 Effect of sweetener type on lactic acid production by *Streptococcus salivarius* subsp. *thermophilus* (St-133)

Treatment[1]	Lactic acid (g/L) 0h	Lactic acid (g/L) 24h
Control[2]	2.54 ± 0.36[a]	19.94 ± 0.50[ab]
Sucrose	2.34 ± 0.23[a]	16.63 ± 0.60[b]
HFCS[3]	2.25 ± 0.03[a]	20.40 ± 0.76[ab]
Sage	2.52 ± 0.35[a]	19.49 ± 0.77[ab]
Alfalfa	2.14 ± 0.51[a]	18.56 ± 0.81[b]
Sourwood	1.78 ± 0.37[a]	19.23 ± 0.38[ab]
Inulin	2.28 ± 0.19[a]	21.08 ± 1.00[a]

[a-b] Means with different superscripts are significantly different ($P < 0.05$). Comparisons are made only within the same column; n = 3 for all treatments.

[1]5% sweetener level

[2]Control devoid of sweetener

[3]HFCS = high fructose corn syrup.

3.1.2 Influence of sweetener type on growth and activity of *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lr-78)

Table 3.6 shows the ANOVA for the independent variable and their various interactions on growth of *L. delbrueckii* subsp. *bulgaricus* (Lr-78). Table 3.7 reports (in log CFU/mL) the growth of *L. delbrueckii* subsp. *bulgaricus* (Lr-78) as influenced by sweetener type and level over a 24 h incubation period. After 24 h of incubation, there was nearly a two log increase in the growth of the microorganism investigated in the unsweetened (control) as well as the sweetened treatments. Higher numbers were reached when cultures were grown in the presence of honeys (sourwood, alfalfa and sage) compared to the control and sucrose both at 5 and 10% sweetener levels. Inulin was as effective as the rest of the treatments in enhancing the growth of this strain after 24h incubation in comparison with control (8.72 log CFU/mL and 8.24 log CFU/mL respectively). HFCS at 10% concentration was also effective in stimulating the growth of *L. delbrueckii* subsp. *bulgaricus* (Lr-78) in the same manner as the three honeys used at the same concentrations. At 0h incubation for this strain, significant differences between treatments at 5 and 10% were noticed (Table 3.7). For the enumeration of cell counts, it is commonplace for samples to be diluted sufficiently to obtain between 20-200 colonies on the plate. These results demonstrate the possibility of inaccurate starter counts due to the dilution factor.

Consistent with the data on growth, the activity of *L. delbrueckii* subsp. *bulgaricus* (Lr-78) (Table 3.8) was also enhanced in the presence of HFCS and the three honey varieties. At 5% concentration alfalfa and sourwood honey were the most effective in stimulating acid production by *L. delbrueckii* subsp. *bulgaricus* (Lr-78): 27.60 g/L and 26.52 g/L respectively. Chick and others (2001) also reported 5% clover

honey in supporting growth and lactic acid production by *L. delbrueckii* subsp. *bulgaricus* (Lr-78).

Table 3.6 Analysis of variance for dependent variable growth of *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lr-78) in 12% nonfat dry milk

Effect	Num [1] DF	Den[2] DF	F value	Probability > F
Sweetener type	5	24	7.60	0.0002
Time	1	24	3476.43	<0.0001
Sweetener level	1	24	20.49	0.0001
Sweetener type * Time	5	24	8.26	0.0001
Sweetener type * Sweetener level	5	24	8.37	0.0001
Time * Sweetener level	1	24	18.82	0.0002
Sweetener type* Time * Sweetener level	5	24	5.64	0.0014

[1]Num DF = numerator degrees of freedom
[2]Den DF = denominator degrees of freedom

Table 3.7 Effect of sweetener type on growth of *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lr-78) over 24 h incubation

Treatment	5%		10%	
	0 h (log CFU/mL)	24 h (log CFU/mL)	0 h (log CFU/mL)	24 h (log CFU/mL)
Control[1]	6.91 ± 0.04^{abc}	8.24 ± 0.12^{c}	6.91 ± 0.04^{a}	8.24 ± 0.12^{b}
Sucrose	6.96 ± 0.05^{ab}	8.51 ± 0.15^{b}	6.74 ± 0.22^{ab}	8.33 ± 0.04^{b}
HFCS[2]	6.89 ± 0.08^{abc}	8.53 ± 0.14^{b}	7.05 ± 0.05^{a}	9.17 ± 0.35^{a}
Sage	7.03 ± 0.05^{a}	8.57 ± 0.04^{b}	6.51 ± 0.03^{b}	8.91 ± 0.18^{a}
Alfalfa	6.76 ± 0.13^{abc}	8.64 ± 0.07^{ab}	6.81 ± 0.22^{ab}	9.19 ± 0.21^{a}
Sourwood	6.67 ± 0.17^{bc}	8.95 ± 0.14^{a}	6.97 ± 0.02^{a}	9.23 ± 0.18^{a}
Inulin	6.64 ± 0.03^{c}	8.72 ± 0.07^{ab}	6.97 ± 0.12^{a}	8.89 ± 0.14^{a}

[a-c] Means with different superscripts are significantly different ($P < 0.05$). Comparisons are made only within the same column; n = 3 for all treatments.

[1]Control devoid of sweetener

[2]HFCS = high fructose corn syrup.

Table 3.8 Effect of sweetener type on lactic acid production by *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lr-78)

Treatment[1]	Lactic acid (g/L) 0 h	Lactic acid (g/L) 24 h
Control[2]	3.15 ± 0.26^{a}	22.58 ± 0.84^{d}
Sucrose	3.02 ± 0.25^{a}	21.87 ± 0.20^{d}
HFCS[3]	2.24 ± 0.79^{a}	25.35 ± 0.35^{bc}
Sage	3.04 ± 0.13^{a}	24.41 ± 0.24^{c}
Alfalfa	2.67 ± 0.51^{a}	27.60 ± 0.76^{a}
Sourwood	2.17 ± 0.36^{a}	26.52 ± 0.99^{ab}
Inulin	2.28 ± 0.20^{a}	24.45 ± 0.46^{c}

[a-d] Means with different superscripts are significantly different ($P < 0.05$). Comparisons are made only within the same column; n = 3 for all treatments.

[1]5% sweetener level

[2]Control devoid of sweetener

[3]HFCS = high fructose corn syrup.

3.1.3 Influence of sweetener type on growth and activity of *Lactobacillus acidophilus* (La-7)

Table 3.9 shows the ANOVA for the independent variable and their various interactions on growth of *L. acidophilus* (La-7). Table 3.10 reports (in log CFU/mL) the growth of *L. acidophilus* (La-7) as influenced by sweetener type and level over a 24 h incubation period. After 24 h of incubation, there was more than one log increase in the growth of the microorganism investigated in the unsweetened (control) as well as the sweetened treatments. Higher numbers were reached when cultures were grown in the presence of sucrose HFCS and honeys (alfalfa, sage and sourwood) compared to inulin treatment at 5 and 10% sweetener levels. Vachon (1998) obtained similar results for *L. acidophilus* (La-7) in 5% sucrose (3.87×10^{9} CFU/mL) and clover honey (2.73×10^{9}) in skim milk. Shamala and others (2000) also reported enhanced growth of *L. acidophilus*

(La-7) with honey in *vitro* and in *vivo* conditions. The study was conducted in comparison with sucrose and they concluded that sucrose was not effective in supporting good growth of *L. acidophilus* with same conditions, which contradicts our findings as well as Vachon's (1998) study. However, *L. acidophilus* strain used in their study was grown in a standard medium containing as major components yeast extract, beef extract and sodium acetate. To this medium sweeteners were added: sucrose (1%) or glucose (0.5%) and lactose (0.5%) or honey (1%). In the present study and also the study by Vachon (1998) the organism was grown in skim milk and much higher levels of sweetener were used. Sucrose and HFCS in the present study, at 5 and 10% concentration were efficient in stimulating the growth of *L. acidophilus* (La-7) in the same manner as honey sweeteners at the same concentration. Inulin was the least effective in stimulating the growth of *L. acidophilus* (La-7) among the carbohydrates sources studied. This could be explained by the effect of pH on the function of different enzymes during the fermentation of inulin.

Consistent with the growth data presented in Table 3.10, production of lactic acid (Table 3.11) by *L. acidophilus* (La-7) was enhanced by honeys (sourwood, alfalfa and sage). These results are in accordance with those obtained by Chick and others (2001) with the same strain in the presence of clover honey. Sourwood honey with the highest oligosaccharide content (10.9%) was the most efficient in enhancing lactic acid production of *L. acidophilus* (La-7) (32.00 g/L) and significantly different ($P < 0.05$) from sage honey (30.39 g/L). In terms of oligosaccharide content, sage honey contains lower level (3.8%) than sourwood and alfalfa honey (5.5%). However, the composition of these oligosacharides (same or different monosaccharides), their anomeric

configuration and/or linkage position is not known and a definite conclusion is hard to be made regarding how these complex carbohydrates may be metabolized by this organism. Further research is required before any specific conclusion could be made.

Table 3.9 Analysis of variance for dependent variable growth of *Lactobacillus acidophilus* (La-7) in 12% nonfat dry milk

Effect	**Num [1] DF**	**Den[2] DF**	**F value**	**Probability > F**
Sweetener type	5	24	1.24	0.3236
Time	1	24	2842.28	<0.0001
Sweetener level	1	24	0.29	0.5974
Sweetener type * Time	5	24	9.15	<0.0001
Sweetener type * Sweetener level	5	24	0.36	0.8704
Time * Sweetener level	1	24	5.01	0.0347
Sweetener type* Time * Sweetener level	5	24	2.32	0.0750

[1]Num DF = numerator degrees of freedom
[2]Den DF = denominator degrees of freedom

Table 3.10 Effect of sweetener type and level on growth of *Lactobacillus acidophilus* (La-7) over 24 h incubation

Treatment	**5%**		**10%**	
	0 h (log CFU/mL)	**24 h** (log CFU/mL)	**0 h** (log CFU/mL)	**24 h** (log CFU/mL)
Control[1]	7.23 ± 0.33[a]	8.84 ± 0.22[ab]	7.23 ± 0.33[a]	8.84 ± 0.22[a]
Sucrose	7.28 ± 0.13[a]	9.16 ± 0.12[a]	7.40 ± 0.14[a]	8.82 ± 0.21[a]
HFCS[2]	7.41 ± 0.10[a]	8.95 ± 0.07[ab]	7.29 ± 0.13[a]	8.94 ± 0.09[a]
Sage	7.47 ± 0.10[a]	9.06 ± 0.21[a]	7.44 ± 0.11[a]	9.06 ± 0.19[a]
Alfalfa	7.40 ± 0.10[a]	9.07 ± 0.20[a]	7.40 ± 0.07[a]	9.00 ± 0.15[a]
Sourwood	7.37 ± 0.12[a]	8.91 ± 0.31[ab]	7.45 ± 0.34[a]	8.70 ± 0.27[a]
Inulin	7.40 ± 0.11[a]	8.56 ± 0.13[b]	7.56 ± 0.36[a]	8.65 ± 0.30[a]

[a-b] Means with different superscripts are significantly different ($P < 0.05$). Comparisons are made only within the same column; n = 3 for all treatments.
[1]Control devoid of sweetener
[2]HFCS = high fructose corn syrup.

Table 3.11 Effect of sweetener type on lactic acid production by *Lactobacillus acidophilus* (La–7)

Treatment[1]	Lactic acid (g/L) 0 h	Lactic acid (g/L) 24 h
Control[2]	3.59 ± 0.39^{b}	28.28 ± 0.35^{c}
Sucrose	3.48 ± 0.19^{b}	30.41 ± 0.66^{b}
HFCS[3]	4.77 ± 0.19^{a}	29.85 ± 0.23^{b}
Sage	4.70 ± 0.59^{a}	30.39 ± 0.24^{b}
Alfalfa	4.26 ± 0.28^{ab}	30.99 ± 0.21^{ab}
Sourwood	4.89 ± 0.13^{a}	32.00 ± 0.55^{a}
Inulin	3.82 ± 0.14^{b}	30.85 ± 0.61^{ab}

[a-c] Means with different superscripts are significantly different ($P < 0.05$). Comparisons are made only within the same column; n = 3 for all treatments.

[1]5% sweetener level

[2]Control devoid of sweetener

[3]HFCS = high fructose corn syrup.

3.1.4 Influence of sweetener type on growth and activity of *Bifidobacterium bifidum* (Bf-1)

Table 3.12 shows the ANOVA for the independent variable and their various interactions on growth of *B. bifidum* (Bf-1). Table 3.13 reports (in log CFU/mL) the growth of *B. bifidum* (Bf-1) as influenced by sweetener type and level over a 24 h incubation period. After 24 h of incubation, there was one log increase in the growth of the microorganism investigated in the unsweetened (control) as well as the sweetened treatments. Higher numbers were reached when cultures were grown in the presence of 5% alfalfa (8.41 log CFU/mL), sourwood (8.33 log CFU/mL) and sage (8.26 log CFU/mL) honey compared to control (7.94 log CFU/mL) and sucrose (7.76 log CFU/mL) treatments. These results are in accordance with previous studies on growth of commercial (Chick and others 2001; Ustunol and Gandhi 2001) and intestinal (Kajiwara

Table 3.12 Analysis of variance for dependent variable growth of *Bifidobacterium bifidum* (Bf-1) in 12% nonfat dry milk

Effect	Num [1] DF	Den[2] DF	F value	Probability > F
Sweetener type	5	24	7.96	0.0002
Time	1	24	189.48	<0.0001
Sweetener level	1	24	11.12	0.0028
Sweetener type * Time	5	24	5.74	0.0013
Sweetener type * Sweetener level	5	24	1.42	0.2512
Time * Sweetener level	1	24	26.89	<0.0001
Sweetener type* Time * Sweetener level	5	24	1.31	0.2930

[1]Num DF = numerator degrees of freedom
[2]Den DF = denominator degrees of freedom

Table 3.13 Effect of sweetener type and level on growth of *Bifidobacterium bifidum* (Bf-1) over 24 h incubation

Treatment	5%		10%	
	0 h (log CFU/mL)	24 h (log CFU/mL)	0 h (log CFU/mL)	24 h (log CFU/mL)
Control[1]	7.58 ± 0.44^{a}	7.94 ± 0.04^{bc}	7.58 ± 0.44^{a}	7.94 ± 0.04^{abc}
Sucrose	7.48 ± 0.05^{a}	7.76 ± 0.07^{c}	7.76 ± 0.20^{a}	7.80 ± 0.19^{bc}
HFCS[2]	7.45 ± 0.05^{a}	8.17 ± 0.27^{ab}	7.84 ± 0.09^{a}	8.47 ± 0.09^{a}
Sage	7.55 ± 0.07^{a}	8.26 ± 0.09^{ab}	7.81 ± 0.12^{a}	8.08 ± 0.35^{abc}
Alfalfa	7.51 ± 0.14^{a}	8.41 ± 0.09^{a}	7.90 ± 0.07^{a}	8.31 ± 0.09^{ab}
Sourwood	7.44 ± 0.11^{a}	8.33 ± 0.19^{a}	7.73 ± 0.13^{a}	8.26 ± 0.33^{ab}
Inulin	7.36 ± 0.17^{a}	8.06 ± 0.11^{abc}	7.70 ± 0.15^{a}	7.72 ± 0.23^{c}

[a-c] Means with different superscripts are significantly different ($P < 0.05$). Comparisons are made only within the same column; n = 3 for all treatments.
[1]Control devoid of sweetener
[2]HFCS = high fructose corn syrup.

and others 2002; Shin and Ustunol 2005) *B. bifidum* spp. in honey. It has been hypothesized (Ustunol and Gandhi 2001) that honey selectively supports growth of

beneficial intestinal microflora such as bifidobacteria. Based on the assumption that mono and disaccharides are absorbed on the distal gastrointestinal (GI) tract and the non-digestible oligosaccharides reach the proximal GI tract and influence the colonic microflora on a selective basis, Shin and Ustunol (2005) studied other predominant gut bacteria and their ability to utilize honey for their growth. The researchers concluded that at 5% concentration sourwood, alfalfa and sage honeys were particularly effective in enhancing ($P < 0.05$) the growth of bifidobacteria after 24h incubation. Growth of *Clostridium perfringens* and *Enterococcus faecalis* was inhibited ($P < 0.05$) in the presence of honey and further inhibited when these microorganisms were co-cultured with *Bifidobacterium* spp in the same study. This inhibition was not attributed to the honey type, but to bifidobacterial strains under the influence of honey, which enhances the growth, and acid production of bifidobacteria. Although the study was focused on intestinal bifidobacteria the same type of honeys varying in their oligosaccharides content was used and enhanced the growth of microorganisms studied as in the current study.

The influence of honey in supporting the growth of bifidobacteria could be due to a synergistic effect among the different sugar components of honey (Ustunol 1998). Also, effective in stimulating the growth of bifidobacteria in the present study was HFCS at 10% concentration. Oligosaccharides content of HFCS may have influenced the growth of *B. bifidum* (Bf-1) in a similar manner as honey.

An important characteristic of bifidobacteria is the production of both lactic and acetic acid as end products of sugar fermentation. In an ideal synthetic medium, the bifidobacteria fermentation pathway results in 3 mol of acetic acid and 2 mol of lactic acid per 2 mol of glucose (Scardovi and Trovatelli 1965). Production of lactic acid by *B.*

bifidum (Bf-1) (Table 3.14) was statistically significant in the presence alfalfa honey (29.08 g/L) and control (28.32g/L) in comparison with the rest of the treatments. Although, sourwood honey enhanced the growth of *B. bifidum* (Bf-1) the same pattern was not observed for acid production in the present study. Desjardins and others (1990) found that, the accumulation of lactic and acetic acid caused limitation on growth of *Bifidobacterium* ssp., some being more susceptible (*Bifidobacterium breve*) than others (*B. bifidum*, *B. longum* and *B. infantis*) to the inhibitory effect of organic acids. However, in Desjardin and others (1990) study, the different bifidobacteria were grown in MRS containing different concentration of L-cysteine-HCl, Bacto-agar, Na_2CO_3, and $CaCl_2 \cdot 2H_2O$ with no sweeteners added, and then transferred into 10% non fat dry milk. In the present study growth of bifidobacteria after 24 h was uncoupled from lactic acid production mainly for control treatment: 7.94 CFU/mL for growth versus 28.32 g/L for acid production.

Inulin among the carbohydrate sources tested for lactic acid production of *B. bifidum* (Bf-1) in this study was significantly different from control (26.16 g/L and 28.32 g/L, respectively. Inulin is a complex carbohydrate with a degree of polymerization (DP) that ranges from 3 to 60. Roberfroid and others (1998) reported better *in vitro* fermentation of inulin by human fecal bacteria when molecules had DP > 10. Hopkins and others (1998) reported GOS and FOS with low DP being best in supporting growth of bifidobacteria. Bruno and others (2002) suggested that substrate transport system for commercial bifidobacteria is more efficient for dimeric and oligomeric carbohydrates.

Table 3.14 Effect of sweetener type on lactic acid production by *Bifidobacterium bifidum* (Bf-1)

Treatment[1]	Lactic acid (g/L) 0 h	Lactic acid (g/L) 24 h
Control[2]	3.08 ± 0.29^{a}	28.32 ± 0.99^{ab}
Sucrose	2.75 ± 0.08^{a}	24.78 ± 0.72^{dc}
HFCS[3]	3.07 ± 0.20^{a}	25.47 ± 0.32^{dc}
Sage	3.27 ± 0.60^{a}	26.71 ± 0.73^{bc}
Alfalfa	2.97 ± 0.44^{a}	29.08 ± 0.80^{a}
Sourwood	2.81 ± 0.36^{a}	24.07 ± 0.97^{d}
Inulin	2.40 ± 0.37^{a}	26.16 ± 0.31^{c}

[a-d] Means with different superscripts are significantly different ($P < 0.05$). Comparisons are made only within the same column; n = 3 for all treatments.

[1]5% sweetener level

[2]Control devoid of sweetener;

[3]HFCS = high fructose corn syrup.

All of these reports are to some extent confusing in that they do not take into account the strains specificity of carbohydrate preferences of these organisms. In this study, the theoretical molar ratio (mentioned above) of 3: 2 for acetic: lactic of *B. bifidum* (Bf-1) was not obtained. This ratio is applicable for an ideal synthetic medium. However, in the present study 12% NDM medium containing 5% sucrose, HFCS, honeys and inulin was investigated. The results (Table 3.15) show a significant increase in acetic acid production after 24 h incubation in the presence of inulin (4.11 g/L) and sourwood honey (3.59 g/L) in comparison with the rest of the treatments that have values ranging from 0.77 to 1.49 g/L. These results are not consistent with those reported by Shin and others (2000) with respect to acetic acid production by commercial bifidobacteria Bf-1 and Bf-6 in skim milk containing oligosaccharide and inulin. However, the culture

activity in their study was determined by measuring end products of fermentation (lactic and acetic acid) using High Performance Liquid Chromatography (HPLC). Chick and others (2001) using same HPLC method obtained high acetic acid levels in skim milk fermented with *B. bifidum* (Bf-1) in the presence of honey. The method used in this study in determining acetic acid production was an enzymatic determination based on the formation of reduced nicotinamide-adenine dinucleotide (NADH) measured by the increase in light absorbance at 340 nm and not at 220nm as used in HPLC method. Also, the sample preparation follows completely different steps in both methods. Although HPLC method may be more accurate, the enzymatic method is cost advantageous and less time consuming. More lactic acid was produced after 24h incubation in comparison with acetic acid production in the present study. This change in the acetate to lactate ratio for this particularly strain would be beneficial and desirable from the technological point of view, as the organoleptic characteristics of the product would improve with a higher lactate proportion and the lower acetate production since high acetate levels tend to make products taste "vinegary".

3.2 EFFECT OF YOGURT INGREDIENTS ON GROWTH OF LACTIC ACID BACTERIA AND BIFIDOBACTERIA

In the process of developing a dairy product the desired sensory properties must be taken into account together with the tolerance of the specific dairy microorganisms to the ingredients used to attain those properties (Vinderola and others 2002b). The effect of ingredients on the growth of starter cultures used in the manufacture of yogurt has not been extensively studied and further information is still needed. Table 3.16 shows ANOVA for the independent variables (main effects): treatments (unsweetened control,

Table 3.15 Effect of sweetener type on acetic acid production by *Bifidobacterium bifidum* (Bf-1)

Treatment[1]	Acetic acid (g/L) 0 h	Acetic acid (g/L) 24 h
Control[2]	1.16 ± 0.39[a]	1.34 ± 0.72[b]
Sucrose	0.60 ± 0.39[a]	1.49 ± 0.57[b]
HFCS[3]	0.05 ± 0.03[a]	0.94 ± 0.45[b]
Sage	0.96 ± 0.12[a]	0.97 ± 0.24[b]
Alfalfa	0.24 ± 0.22[a]	0.77 ±0.62[b]
Sourwood	0.55 ± 0.44[a]	3.59 ± 1.11[a]
Inulin	0.53 ± 0.23[a]	4.11 ± 0.69[a]

[a-b] Means with different superscripts are significantly different ($P < 0.05$). Comparisons are made only within the same column; n = 3 for all treatments.

[1]5% sweetener level

[2]Control devoid of sweetener

[3]HFCS = high fructose corn syrup.

Table 3.16 Analysis of variance for dependent variable growth of lactic acid bacteria and bifidobacteria in 12% nonfat dry milk in the presence of yogurt ingredients

Effect	Num[1] DF	Den[2] DF	F value	Probability > F
Treatment	8	72	8.23	<0.0001
Culture	3	72	97.82	<0.0001
Time	1	72	1567.00	<0.0001
Treatment * Culture	24	72	0.78	0.7466
Treatment * Time	8	72	0.27	0.9732
Culture * Time	3	72	135.52	<0.0001
Treatment * Culture * Time	24	72	1.84	0.0255

[1]Num DF = numerator degrees of freedom

[2]Den DF = denominator degrees of freedom

stabilizer, unsweetened strawberry puree, and a mixture of unsweetened strawberry puree with sucrose, HFCS, sage, alfalfa, sourwood honeys or inulin), incubation time (0, 24 h), and cultures (*S. salivarius* subsp. *thermophilus* (St-133), *L. delbrueckii* subsp. *bulgaricus* (Lr-78), *L. acidophilus* (La-7) and *B. bifidum* (Bf-1), their two-way and three-way interactions on the dependent variable growth (with ingredients) of lactic acid bacteria and bifidobacteria. Almost all main effects and their interactions had a significant effect on growth with ingredients of LAB and bifidobacteria. The two-way interaction between treatments and culture and treatments and time did not have a significant effect on growth with ingredients of LAB and bifidobacteria.

Table 3.17 shows that the ingredients, their different combinations and concentrations (commonly used in the manufacture of yogurt) did not inhibit the growth of *S. salivarius* subsp. *thermophilus* (St-133) after 24 h incubation at 37 °C. Growth of *S. salivarius* subsp. *thermophilus* (St-133) in the presence of stabilizer (8.68 log CFU/mL) and strawberry puree (8.57 log CFU/mL) was slightly enhanced in comparison with sage and sourwood honey in combination with strawberry puree: 8.02 and 8.08 log CFU/mL respectively. However, no significant differences were observed after 24 h incubation between treatments. Unsweetened strawberry puree was used in the present study as a flavoring agent in proportion of 10% (w/w), level recommended in the manufacture of strawberry-flavored yogurt. Commercial flavorings at levels between 0.16 and 0.2% (w/w) of strawberry as well as vanilla or banana have been reported to affect strains of *S. thermophilus* as well as *L. delbrueckii* subsp. *bulgaricus* (Vinderola and others 2000b). However, the strains investigated were grown in liquid media: MRS or Elliker broth, not in 12% NDM as in the present study.

Table 3.17 Effect of yogurt ingredients on growth of *Streptococcus salivarius subsp. thermophilus* (St-133)

Treatment	**Ingredients (%)**	**0 h** (log CFU/mL)	**24 h** (log CFU/mL)
Control[1]	0	7.61 ± 0.03[a]	8.30 ± 0.39[a]
Stabilizer	0.5	7.65 ± 0.07[a]	8.68 ± 0.31[a]
Strawberry puree	10	7.62 ± 0.09[a]	8.57 ± 0.40[a]
Sucrose + Strawberry puree	7+10	7.61 ± 0.13[a]	8.16 ± 0.43[a]
HFCS[2] +Strawberry puree	7+10	7.61 ± 0.07[a]	8.35 ± 0.47[a]
Sage + Strawberry puree	7+10	7.56 ± 0.12[a]	8.02 ± 0.45[a]
Alfalfa+ Strawberry puree	7+10	7.56 ± 0.20[a]	8.20 ± 0.23[a]
Sourwood+ Strawberry puree	7+10	7.61 ± 0.03[a]	8.08 ± 0.36[a]
Inulin + Strawberry puree	7+10	7.66 ± 0.05[a]	8.33 ± 0.45[a]

[a-a] Means with different superscripts are significantly different ($P < 0.05$). Comparisons are made only within the same column; n = 3 for all treatments.

[1]Control contains 12% nonfat dry milk and is devoid of other ingredients;

[2]HFCS = high fructose corn syrup.

Table 3.18 reports the effect of ingredients on growth of *L. delbrueckii* subsp. *bulgaricus* (Lr-78). After 24 h of incubation, there was more than one log increase in growth of *L. delbrueckii* subsp. *bulgaricus* (Lr-78) in the presence of the ingredients investigated. Although, slightly high numbers were reached in the presence of stabilizer no significant differences were observed between treatments after 24 h incubation at 37 °C. Stabilizers are used in the manufacture of yogurt to improve the texture, increase the firmness and prevent syneresis. In the current study the stabilizer used was pectin based. Previous reports show that the type of stabilizers had no effect on count of lactic acid bacteria or development of acidity (El-Sayed and others 2002). The researchers studied the effect of xanthan gum either singly or in combination with other gums and concluded no marked effect on the count of LAB, particularly right after manufacturing. Same

results were reported during storage (El-Sayed and others 2002).

Table 3.18 Effect of yogurt ingredients on growth of *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lr-78)

Treatments	**Ingredients (%)**	**0 h** (log CFU/mL)	**24 h** (log CFU/mL)
Control[1]	0	6.98 ± 0.06^{a}	8.11 ± 0.19^{a}
Stabilizer	0.5	7.06 ± 0.06^{a}	8.26 ± 0.13^{a}
Strawberry puree	10	7.07 ± 0.02^{a}	8.15 ± 0.04^{a}
Sucrose + Strawberry puree	7+10	6.40 ± 0.09^{b}	8.14 ± 0.08^{a}
HFCS[2] +Strawberry puree	7+10	6.65 ± 0.10^{b}	8.06 ± 0.16^{a}
Sage + Strawberry puree	7+10	6.65 ± 0.18^{b}	8.11 ± 0.03^{a}
Alfalfa+ Strawberry puree	7+10	6.64 ± 0.18^{b}	8.15 ± 0.03^{a}
Sourwood+ Strawberry puree	7+10	6.42 ± 0.12^{b}	8.23 ± 0.01^{a}
Inulin + Strawberry puree	7+10	6.52 ± 0.06^{b}	8.10 ± 0.18^{a}

[a-b] Means with different superscripts are significantly different ($p<0.05$). Comparisons are made only within the same column; n = 3 for all treatments.

[1]Control contains 12% non fat dry milk and is devoid of other ingredients;

[2]HFCS = high fructose corn syrup.

Table 3.19 shows growth of *L. acidophilus* (La-7) in the presence of the ingredients used in the manufacture of strawberry flavored low fat yogurt. Overall, increase of approximately 2 log were noticed for *L. acidophilus* (La-7) after 24 h incubation. The results are not significant different after 24 h. However, it can be observed less counts for *L. acidophilus* (La-7) obtained with inulin in combination with strawberry puree (8.36 CFU/mL). The same trend was observed for growth of *L. acidophilus* (La-7) in the presence of inulin at 5 or 10%, and it can be concluded that inulin is not the best in promoting the growth for this specific strain.

Table 3.19 Effect of yogurt ingredients on growth of *Lactobacillus acidophilus* (La-7)

Treatments	**Ingredients (%)**	**0 h (log CFU/mL)**	**24 h (log CFU/mL)**
Control[1]	0	6.90 ± 0.32[a]	8.92 ± 0.08[a]
Stabilizer	0.5	7.06 ± 0.04[a]	8.96 ± 0.10[a]
Strawberry puree	10	6.74 ± 0.31[a]	8.82 ± 0.08[a]
Sucrose + Strawberry puree	7+10	6.64 ± 0.13[a]	8.70 ± 0.22[a]
HFCS[2] +Strawberry puree	7+10	6.87 ± 0.05[a]	8.69 ± 0.33[a]
Sage + Strawberry puree	7+10	6.55 ± 0.08[a]	8.63 ± 0.33[a]
Alfalfa+ Strawberry puree	7+10	6.59 ± 0.13[a]	8.65 ± 0.36[a]
Sourwood+ Strawberry puree	7+10	6.58 ± 0.05[a]	8.55 ± 0.22[a]
Inulin + Strawberry puree	7+10	6.84 ± 0.23[a]	8.36 ± 0.46[a]

[a-a] Means with different superscripts are significantly different ($p<0.05$). Comparisons are made only within the same column; n = 3 for all treatments.

[1]Control contains 12% non fat dry milk and is devoid of other ingredients

[2]HFCS = high fructose corn syrup.

Bifidobacteria in the present study (Table 3.20) did not increase significantly after 24 h. Even though a significant difference was noted between control and sourwood honey in combination with strawberry puree, overall ingredients used in this study did not affect the growth of the lactic acid bacteria and bifidobacteria. Reports showed that fruit juices, strawberry flavorings, vanilla flavors and nisin at concentration used in dairy products, can inhibit growth of probiotics such as bifidobacteria (Vinderola and others 2002b). Sensitivity vary between probiotics and *B.bifidum* (Bf-1) used in the present study was more sensitive that the other probiotic used *L. acidophilus* (La-7) to the ingredients used since after 24 incubation there was more increase in *L. acidophilus* (La-7) counts numbers than for *B.bifidum* (Bf-1).

These results are in accordance with Con and others (1996) who showed that the

addition of fruit flavors (cherries, oranges, strawberries and bananas) or sweeteners to yogurt did not significantly affect the growth of starter cultures. In a recent study regarding the influence of compounds associated with fermented dairy products on the growth of lactic acid bacteria and probiotics (Vinderolla and others 2002a) it was concluded that probiotic bacteria were more resistant to dairy ingredients than lactic acid bacteria, which contradicts the results with bifidobacteria, but supports the results obtained with *L. acidophilus* in this study. However, Vinderola and others (2002a) reported some of the compounds used at the concentration used for industrial manufacturing not inhibitory while for others strain dependent effects. In the present study, the strains used in conjunction with typical yogurt manufacturing ingredients did not experience any inhibitory effect after 24 h incubation, which enables their use in the manufacture of low-fat yogurt.

Table 3.20 Effect of yogurt ingredients on growth of *Bifidobacterium bifidum* (Bf-1)

Treatments	**Ingredients (%)**	**0 h (log CFU/mL)**	**24 h (log CFU/mL)**
Control[1]	0	7.11 ± 0.06^{a}	7.73 ± 0.15^{ab}
Stabilizer	0.5	7.18 ± 0.11^{a}	7.97 ± 0.04^{a}
Strawberry puree	10	7.09 ± 0.14^{a}	7.51 ± 0.05^{abc}
Sucrose + Strawberry puree	7+10	7.24 ± 0.14^{a}	7.49 ± 0.05^{bc}
HFCS[2] +Strawberry puree	7+10	7.11 ± 0.12^{a}	7.64 ± 0.22^{abc}
Sage + Strawberry puree	7+10	6.93 ± 0.28^{a}	7.63 ± 0.33^{abc}
Alfalfa+ Strawberry puree	7+10	7.03 ± 0.25^{a}	7.34 ± 0.08^{bc}
Sourwood+ Strawberry puree	7+10	6.88 ± 0.28^{a}	7.24 ± 0.09^{c}
Inulin + Strawberry puree	7+10	7.15 ± 0.22^{a}	7.64 ± 0.17^{abc}

[a-c] Means with different superscripts are significantly different ($p<0.05$). Comparisons are made only within the same column; n = 3 for all treatments.

[1]Control contains 12% non fat dry milk and is devoid of other ingredients

[2]HFCS = high fructose corn syrup.

3.3 ACCEPTABILITY OF STRAWBERRY FLAVORED LOW-FAT YOGURT AS DETERMINED BY A CONSUMER PANEL

The ratings for the sensory parameters: appearance, aroma, sweetness and flavor of the prepared low-fat strawberry yogurt are presented in Table 3.21. Each treatment was individually evaluated.

Table 3.21 Overall acceptability of strawberry flavored low-fat yogurt by an untrained consumer panel (n = 99)

Sweetener	Appearance	Aroma	Sweetness	Flavor
Sucrose	6.41 ± 1.55^{a}	6.09 ± 1.53^{a}	6.64 ± 1.76^{a}	7.12 ± 1.62^{a}
HFCS[1]	6.73 ± 1.43^{b}	5.96 ± 1.69^{a}	5.51 ± 1.99^{b}	5.86 ± 2.13^{b}
Sage	6.24 ± 1.67^{a}	5.53 ± 1.72^{a}	5.88 ± 1.96^{b}	6.04 ± 1.92^{b}
Alfalfa	6.09 ± 1.65^{a}	5.46 ± 1.67^{a}	5.77 ± 1.96^{b}	5.64 ± 1.83^{c}
Sourwood	5.67 ± 1.83^{c}	5.52 ± 1.87^{a}	5.44 ± 2.11^{b}	5.27 ± 1.94^{d}
Inulin	6.25 ± 1.77^{a}	5.81 ± 1.68^{a}	4.01 ± 1.87^{c}	4.07 ± 1.87^{e}

$^{a-c}$ Means with different superscripts are significantly different ($P < 0.05$). Comparisons are made only within the same column. Scale: 9 – like extremely; 5 - neither like/nor dislike; 1 – dislike extremely;
[1]HFCS = high fructose corn syrup.

For appearance, the average rating ranged from 5.67 for the yogurt sweetened with sourwood honey to 6.73 for the yogurt sweetened with HFCS. In other words, the participants "liked slightly" to "moderately" the HFCS sweetened yogurt (average rating above 6) and "neither like nor dislike" the sourwood honey sweetened yogurt. The results for appearance might be due to the slightly low viscosity of some yogurt samples, especially of the yogurt sweetened with honey samples. Another reason for thes low results for this attribute might be the intense pink color of the product, which for some panelists was unexpected, resulting in comments like: "too pink for my taste". In the

manufacture of the yogurt samples we did not use additional food coloring. The 10% unsweetened strawberry puree already had a pronounced red color. The use of a lower concentration of strawberry puree may resolve this problem in the future.

Although, there was no significant difference for aroma between the six yogurt samples, the sucrose-sweetened yogurt was rated higher (average rating 6.09) than the rest of the yogurts. Yogurt aroma is a combination of both volatiles initially present in milk and compounds produced during fermentation (Ott and others 1997). Kneifel and others (1992) found acetaldehyde to be the most significant compound participating in the typical yogurt aroma. Unifloral honeys have highly characteristic aromas, presumably derived from their nectar (Bonvehi and Coll 2003). Since half of the yogurt samples in the present study were manufactured with honey as the sweetness agent, not a very common practice at the industrial level, a mixture of aromatic compounds from yogurt and honey could have driven the lower panelist rating.

Consumer preference for food is motivated by many criteria and particularly for flavor. The most preferred in terms of flavor was the sucrose-sweetened yogurt (average rating 7.12). However, the flavor attribute was rated significantly different for inulin, indicating that this treatment was perceived to have flavor characteristics that were not adequately fitted with the consumer preference. Results obtained in the present study contradict the conclusion that inulin has the ability to act as a sugar replacer without adversely affecting flavor (Tungland 2000).

In terms of sweetener preference consumers preferred the sucrose-sweetened yogurt samples followed by sage honey sweetened yogurt samples. The average ratings for sweetness ranged from 4.01 ("dislike slightly") for inulin to 6.64 ("like slightly" to

"like moderately") for sucrose. Sucrose is widely used in the manufacture of dairy products as well as HFCS. Although no significant differences were between honey and HFCS sweetened yogurts in terms of sweeteners acceptance, the consumers rated sage (5.88) and alfalfa (5.77) honey higher than HFCS (5.51). In summary, the use of honey as a sweetener in low-fat yogurt would meet the consumer acceptance for sweetness in the same manner as for HFCS, however less acceptable than sucrose.

3.4 EFFECT OF SWEETENER TYPE ON VIABILITY OF LACTIC ACID BACTERIA AND BIFIDOBACTERIA DURING REFRIGERATED STORAGE

Table 3.22 shows ANOVA for the independent variables (main effects): sweetener type (sucrose, HFCS, sage, alfalfa, sourwood honeys or inulin), time (0, 7,14, 21, 28, 35, 42), and culture: lactic acid bacteria (*Streptococcus salivarius* subsp. *thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus acidophilus*), and their two-way interactions on the dependent variable viability of lactic acid bacteria. The main effect time had a significant effect on viability of lactic acid bacteria. The sweetener type as well as interaction between sweetener type and time was not significant for viability of lactic acid bacteria.

Table 3.22 Analysis of variance for dependent variable viability of lactic acid bacteria

Effect	**Num [1] DF**	**Den[2] DF**	**F value**	**Probability > F**
Sweetener type	6	14	1.94	0.1432
Time	6	84	15.92	<0.0001
Sweetener type * time	36	84	1.01	0.4736

[1]Num DF = numerator degrees of freedom
[2]Den DF = denominator degrees of freedom

Table 3.23 reports the percent viability of total lactic acid bacteria as influenced by sweetener type over a 42 d of refrigerated storage. The viability of all 3 strains of lactic acid bacteria from day 0 to day 42 was significantly different ($p<0.05$) for unsweetened yogurt treatment (100 vs. 86.73%), yogurt sweetened with sourwood honey (100 vs. 84.35%) and yogurt sweetened with inulin (100 vs. 85.29%). However, the retention of viability during 42 d of storage was higher when LAB were grown in yogurt sweetened with sucrose or sage honey (94.74 and 93.42% respectively). For yogurt sweetened with sucrose, HFCS, sage and alfalfa honey at the end of the storage the retention of viability was not significantly different ($P > 0.05$) from the initial ones. Vinderola and others (2000b) reported similar results for lactic acid bacteria from commercial cultures at the end of the storage (5°C for 4 weeks) of full and reduced fat yogurt where the initial counts (approximately 10^8 to 10^9 CFU/mL) were not significantly different from the counts at the end of storage.

Shin and others (2000) reported population maintained above 10^7 CFU/mL for lactic acid bacteria from two brands of commercial yogurt during 6 wks at refrigerated storage. However, on the expiration day of the product a significant decline ($P < 0.05$) was observed for lactic acid bacteria. The least effective in retaining the viability of LAB in the present study was yogurt sweetened with sourwood honey (84.35%) followed by inulin (85.29%) at the end of the storage period. These values were lower than those obtained with control yogurts (86.73% viability).

Table 3.23 Effect of sweetener type on viability of lactic acid bacteria[1] (St-133, Lr-78, La-7, Bf-1) during refrigerated storage (4°C, 42 d)

Treatment	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
	% Viability	% Viability	% Viability	% Viability	% Viability	% Viability	% Viability
Control[1]	$100\pm2.34^{a;A}$	$94.27\pm4.67^{a;AB}$	$94.49\pm1.54^{b;AB}$	$92.11\pm1.15^{a;AB}$	$94.34\pm4.26^{a;AB}$	$92.95\pm3.80^{a;AB}$	$86.73\pm1.41^{a;B}$
Sucrose	$100\pm2.15^{a;A}$	$101.18\pm5.85^{a;A}$	$104.10\pm6.05^{a;A}$	$97.81\pm4.72^{a;A}$	$100.38\pm8.11^{a;A}$	$96.04\pm1.53^{a;A}$	$94.74\pm4.33^{a;A}$
HFCS[2]	$100\pm2.71^{a;A}$	$99.41\pm0.62^{a;A}$	$95.77\pm3.26^{ab;A}$	$88.75\pm11.52^{a;A}$	$90.14\pm7.43^{a;A}$	$88.71\pm5.92^{a;A}$	$89.23\pm10.35^{a;A}$
Sage	$100\pm2.38^{a;A}$	$97.77\pm0.36^{a;A}$	$97.14\pm2.34^{ab;A}$	$97.81\pm1.75^{a;A}$	$98.20\pm6.69^{a;A}$	$96.55\pm3.92^{a;A}$	$93.42\pm2.75^{a;A}$
Alfalfa	$100\pm5.16^{a;A}$	$93.91\pm1.28^{a;A}$	$97.59\pm2.03^{ab;A}$	$92.93\pm7.66^{a;A}$	$91.43\pm9.13^{a;A}$	$92.97\pm7.39^{a;A}$	$89.36\pm2.52^{a;A}$
Sourwood	$100\pm1.46^{a;A}$	$94.16\pm4.77^{a;AB}$	$96.04\pm1.49^{ab;AB}$	$94.15\pm4.39^{a;AB}$	$90.51\pm5.66^{a;AB}$	$86.74\pm2.82^{a;B}$	$84.35\pm8.14^{a;B}$
Inulin	$100\pm0.47^{a;A}$	$98.79\pm4.35^{a;A}$	$97.51\pm2.55^{ab;AB}$	$99.92\pm6.45^{a;A}$	$92.23\pm3.76^{a;AB}$	$89.78\pm4.79^{a;AB}$	$85.29\pm6.35^{a;B}$

$^{a\text{-}b;A\text{-}B}$ Means with different superscripts are significantly different ($p < 0.05$); Comparisons are made within the same column$^{(a\text{-}b)}$ and within the same row$^{(A\text{-}B)}$; n = 3 for all treatments;

[1]Lactic acid bacteria: *S. salivarius* subsp. *thermophilus* (St-133), *L. delbrueckii* subsp. *bulgaricus* (Lr-78), *L. acidophilus* (La-7);

[2]Control devoid of sweetener;

[3]HFCS = high fructose corn syrup.

The comparison between treatments within each week showed a significant difference ($P < 0.05$) only on day 14 between unsweetened yogurt and yogurt sweetened with sucrose, HFCS, honeys (sage, alfalfa and sourwood) and inulin. There was an increase in cell numbers in yogurt sweetened with sucrose on day 14 of storage, but this could be related to the splitting of the bacteria from the chains into single cells (Saxelin and others 1999). The majority of LAB grown in chains, and not enough attention is given to chain length in relationship to CFU counts (Champagne and others 2005).

Overall, high retention of viability was noticed for all treatments during 42 d refrigerated storage. Some factors that could have been contributed to greater viability of LAB in this study are: freshly autoclaved media for all samples, aseptical inoculation of culture and storage in sterile plastic tubes. Interactions between strains could be another factor to take into account; therefore selection of cultures in the manufacture of a dairy product is critical. Yogurt cultures that have proteolytic or oxygen-scavenging properties have been shown to be beneficial for bifidobacteria and could be considered in the selection of cultures compatible to probiotic strains (Kneifel and others 1992).

Table 3.24 shows the ANOVA for the independent variable and their various interactions on viability of *B. bifidum* (Bf-1). Table 3.25 reports the percent viability of bifidobacteria as influenced by sweetener type over a 42 d of refrigerated storage. Best retention of viability after 42 d of refrigerated storage was observed when bifidobacteria were grown in yogurt sweetened with sucrose (102.29%), sage honey (97.87%) and inulin (97.22%). The lowest viability of 86.13% after 42 d was recorded by yogurt sweetened with sourwood honey. High bifidobacteria viability suggest that strain *B. bifidum* (Bf-1) used in the current study may be more acid tolerant, taking into account

that pH value is a critical factor in the stability of probiotic strains during storage.

Table 3.24 Analysis of variance for dependent variable viability of bifidobacteria

Effect	**Num [1] DF**	**Den[2] DF**	**F value**	**Probability > F**
Sweetener type	6	14	3.15	0.0359
Time	6	84	2.22	0.0488
Sweetener type * time	36	84	0.64	0.9311

[1]Num DF = numerator degrees of freedom
[2]Den DF = denominator degrees of freedom

Increased viability of bifidobacteria (Bf-1 and Bf-6) was obtained in the presence of 5% fructooligosaccharides or galactooligosaccharides (Shin and others 2000) in comparison with control. Minimal decreases in cell numbers of probiotic strain of *L. acidophilus* (NCFM) were found in strawberry and plain yogurt (Iturriria and others 1999) after 52 d of storage at 4° C. The counts of *L. acidophilus* (NCFM) in strawberry yogurt fell only from 1.2 x 10^7 CFU/g to 8.7 x 10^6 CFU/g and in plain yogurt from 2.4 x 10^7 CFU/g to 1.5 x 10^7 CFU/g. However, Roy and others (1997) observed that the viable cells of bifidobacteria in yogurt could not be maintained in sufficient amounts (>10^6 CFU/g) for more than 1 wk during storage at 4 °C. Also, Lamoureux and others (2002) have reported levels of bifidobacteria < 10^6 CFU/g after 7 d of storage in yogurts made with *B. bifidum*, *B. breve*, *B. infantis*, and *B. longum* as first or second inoculum. Different strains of bifidobacteria were used in these studies and differences in environmental conditions during the preparation of the inocula have also been used.

Table 3.25 Effect of sweetener type on viability of bifidobacteria during refrigerated storage (4°C, 42 d)

Treatment	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
	% Viability	% Viability	% Viability	% Viability	% Viability	% Viability	% Viability
Control[1]	100±7.37^{a,A}	95.80±5.09^{a,A}	97.46±5.76^{ab,A}	95.84±4.57^{a,A}	99.21±5.25^{a,A}	94.93±5.59^{a,A}	94.56±4.43^{a,A}
Sucrose	100±5.44^{a,A}	100.79±3.02^{a,A}	108.81±3.89^{a,A}	104.11±7.06^{a,A}	107.07±11.57^{a,A}	101.29±3.43^{a,A}	102.29±3.90^{a,A}
HFCS[2]	100±4.65^{a,A}	101.40±4.15^{a,A}	98.81±5.93^{ab,A}	96.99±8.08^{a,A}	95.47±9.06^{a,A}	93.99±9.83^{a,A}	96.32±10.91^{a,A}
Sage	100±6.35^{a,A}	98.21±5.21^{a,A}	101.29±3.03^{ab,A}	102.21±1.92^{a,A}	104.87±4.68^{a,A}	96.63±4.95^{a,A}	97.87±2.98^{a,A}
Alfalfa	100±3.69^{a,A}	97.85±4.01^{a,A}	96.15±8.54^{ab,A}	96.84±5.77^{a,A}	97.36±10.33^{a,A}	96.07±8.39^{a,A}	94.69±4.41^{a,A}
Sourwood	100±6.32^{a,A}	96.36±5.06^{a,A}	87.72±14.46^{b,A}	89.39±7.61^{a,A}	93.65±0.95^{a,A}	87.72±5.90^{a,A}	86.13±8.84^{a,A}
Inulin	100±6.30^{a,A}	100.25±4.77^{a,A}	103.23±3.48^{ab,A}	103.40±4.73^{a,A}	100.17±5.88^{a,A}	97.39±7.75^{a,A}	97.22±3.37^{a,A}

$^{a\text{-}b;A\text{-}B}$ Means with different superscripts are significantly different ($p < 0.05$); Comparisons are made within the same column$^{(a\text{-}b)}$ and within the same row$^{(A\text{-}B)}$; n = 3 for all treatments;

[1]Control devoid of sweetener;

[2]HFCS = high fructose corn syrup.

Numerous authors have reported viability losses between 0 and 3 log units during refrigerated storage of fermented milks (Medina and Jordano 1994; Dave and Shah 1997a; Shin and others 2000; Gilliland and others 2002). Shah and others (1995) found considerably higher population decreases for *B. bifidum* (3.5-7 log units) and *L. acidophilus* (1.5-6 log units). Variation in probiotic bacteria viability data among different authors may be attributed to differences among strains tested and the influence of factors such as acidity, pH, other starter microorganisms and oxygen dissolved in the milk (Dave and Shah 1997b; Shah 2000).

Increased viability of bifidobacteria in the present study may be attributed to higher inocula of *B. bifidum* (Bf-1) in comparison with LAB, and addition of this probiotic strain at the same time with the traditional starters and *L. acidophilus* at the beginning of fermentation. Losses in *L. acidophilus* during storage of yogurt at 5 °C have been previously reported (Hull and others 1984) when probiotic strain was added to the yogurt prior to storage rather then with the starter at the beginning of fermentation. Table 3.26 shows the ANOVA for the independent variable and their various interactions on pH of yogurt during viability study of lactic acid bacteria and bifidobacteria.

Table 3.26 Analysis of variance for dependent variable pH viability of lactic acid bacteria and bifidobacteria

Effect	**Num [1] DF**	**Den[2] DF**	**F value**	**Probability > F**
Sweetener type	6	15	0.68	0.6672
Time	6	82	29.90	<0.0001
Sweetener type * time	36	82	0.56	0.9733

[1]Num DF = numerator degrees of freedom

[2]Den DF = denominator degrees of freedom

As previously mentioned, a critical factor in the in the stability of bifidobacteria during storage is the pH. The acidity in yogurt during storage may increase ("over-acidification") and the pH may drop to 3.6 (Kailasapathy and Rybka 1997). In the present study the pH of all yogurt samples slightly decreased during storage. The initial pH (d 0) of the prepared low-fat strawberry flavored yogurt was 4.4 to 4.5 and did not change significantly during the 42 days of storage at 4°C. This pattern was observed for unsweetened yogurt as well as for yogurt sweetened with sucrose, HFCS, honeys (sage, alfalfa, and sourwood) and inulin (Table 3.27). A gradual decrease of the pH was observed throughout the storage period of 28 days for yogurts prepared using a mixed culture of bifidobacteria (Lamoureux and others 2002) with values lower (pH 4.0) than the higher pH values recommended (pH 4.6) for survival of bifidobacteria (Shah 1997). Lankaputhra and others (1996) reported the survival of only three out of nine bifidobacterial strains in the pH range of 3.7 to 4.3. In the present study, the values of pH 4.3 corresponded to a viability of bifidobacteria above 90% after 42 days of refrigerated storage. The viability of bifidobacteria may thus be strain-dependent. In addition, the preparation of freshly autoclaved media for all samples, followed by aseptically inoculation of culture and storage in sterile plastic tubes, may have contributed to greater viability in this study. Many of the technological operations used in the processing of foods have an effect of how the probiotics grow and survive in the food product such as: type of substrate, competing lactic cultures, changes in incubation temperature, addition of enzymes, adding compounds that affect the redox conditions of the medium are the most notable (Champagne and others 2005).

Table 3.27 Effect of sweetener type on pH for viability study of lactic acid bacteria and bifidobacteria during refrigerated storage (4°C, 42 d)

Treatment	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Control[1]	$4.47\pm0.06^{a;A}$	$4.47\pm0.10^{a;A}$	$4.47\pm0.10^{a;A}$	$4.48\pm0.06^{a;A}$	$4.39\pm0.10^{a;A}$	$4.38\pm0.10^{a;A}$	$4.31\pm0.06^{a;A}$
Sucrose	$4.47\pm0.06^{a;A}$	$4.45\pm0.15^{a;AB}$	$4.49\pm0.06^{a;A}$	$4.44\pm0.06^{a;AB}$	$4.38\pm0.00^{a;AB}$	$4.35\pm0.06^{a;AB}$	$4.32\pm0.06^{a;B}$
HFCS[2]	$4.45\pm0.12^{a;A}$	$4.41\pm0.17^{a;A}$	$4.45\pm0.10^{a;A}$	$4.44\pm0.12^{a;A}$	$4.40\pm0.12^{a;A}$	$4.36\pm0.06^{a;A}$	$4.31\pm0.10^{a;A}$
Sage	$4.44\pm0.06^{a;A}$	$4.44\pm0.15^{a;A}$	$4.48\pm0.12^{a;A}$	$4.43\pm0.06^{a;A}$	$4.39\pm0.10^{a;A}$	$4.36\pm0.12^{a;A}$	$4.29\pm0.10^{a;A}$
Alfalfa	$4.42\pm0.06^{a;A}$	$4.45\pm0.15^{a;A}$	$4.46\pm0.12^{a;A}$	$4.43\pm0.06^{a;A}$	$4.38\pm0.12^{a;A}$	$4.36\pm0.12^{a;A}$	$4.28\pm0.10^{a;A}$
Sourwood	$4.42\pm0.06^{a;AB}$	$4.38\pm0.10^{a;AB}$	$4.43\pm0.12^{a;A}$	$4.39\pm0.06^{a;AB}$	$4.35\pm0.06^{a;AB}$	$4.31\pm0.00^{a;AB}$	$4.26\pm0.06^{a;B}$
Inulin	$4.45\pm0.06^{a;A}$	$4.32\pm0.17^{a;A}$	$4.46\pm0.10^{a;A}$	$4.44\pm0.06^{a;A}$	$4.40\pm0.10^{a;A}$	$4.34\pm0.06^{a;A}$	$4.28\pm0.06^{a;A}$

$^{a\text{-}b;A\text{-}B}$ Means with different superscripts are significantly different ($p < 0.05$); Comparisons are made within the same column$^{(a\text{-}b)}$ and within the same row$^{(A\text{-}B)}$; n = 3 for all treatments; [1]Control devoid of sweetener; [2]HFCS = high fru

The effect of heating as a technological step may also be considered a factor able to support the viability of LAB and bifidobacteria. Misra and Kuila (1992) suggested that sterilized milk (121°C-15 min) enables more extensive growth of bifidobacteria that does steamed milk. Since milk formulation intended for yogurt manufacture is typically heated between 85 °C and 95 °C this heating temperature is appropriate for the subsequent growth and stability of probiotic cultures. The milk base consisting of milk and dry ingredients was heated at 85 °C for 30 min in the heating step of the manufacture of the strawberry low-fat yogurt in this study. This could be another factor that may have contributed to greater viability of lactic acid bacteria and bifidobacteria in this study.

CHAPTER 4

CONCLUSIONS

- Overall honeys (sage, alfalfa and inulin) from different floral sources and varying in carbohydrate composition used as a sweetener supported growth and activity of yogurt cultures *S. salivarius* subsp. *thermophilus* St-133, *L.delbrueckii* subsp. *bulgaricus* Lr-78, and *L. acidophilus* La-7, and bifidobacteria *B. bifidum* Bf-1 similar to high fructose corn syrup (HFCS).
- Overall and particularly at 10% sweetener level there were no differences between the three honeys. The differences in carbohydrate composition did not appear to make a difference.
- Overall lactic acid production by LAB was supported by the three honey varieties similar to HFCS. The effect of these sweeteners on lactic and acetic acid production by bifidobacteria was inconclusive.
- Although inulin was not very effective in supporting the growth of the microorganisms investigated it enhanced acid production in a similar manner to other sweeteners.
- The ingredients used in the yogurt manufacture showed no inhibitory effect on growth of aforementioned microorganisms in the presence of different sweeteners.
- Sensory analysis of low-fat strawberry flavored yogurt using the untrained consumer panel indicated that the panel preferred the yogurt sweetened with the traditional sweeteners followed by yogurt sweetened with honey (sage, alfalfa and

sourwood) to yogurt sweetened with inulin.

- Overall, high retention of viability of lactic acid bacteria and bifidobacteria was noticed for all treatments during 42 d of refrigerated storage. Yogurt sweetened with honey was as effective as yogurt sweetened with sucrose and HFCS in maintaining the viability of the microorganisms studied.

Overall honey did not have a negative influence on growth, activity and viability of starter cultures and probiotics used in the present study. The final product met the National Yogurt Association live and active culture seal criteria indicating that the manufacture of a low-fat yogurt incorporating probiotics and prebiotics is feasible. Further research is also necessary to better understand the mechanism by which honey components support growth, activity and viability of lactic acid bacteria and bifidobacteria.

CHAPTER 5

FUTURE RESEARCH

Honey and its prebiotic activity are based on the oligosaccharides content that varies within floral sources. Many carbohydrates have been reported to exert prebiotic activity; however the mechanism by which prebiotics selectively stimulate the growth and/or activity of probiotics lacks basic understanding. Hence, further research needs to be done in order to obtain structure-function information of a range of carbohydrates in honey.

Research is also required to precisely determine the degree of polymerization (DP) of oligosaccharide constituents of honey since these complex carbohydrates differ in their DP and the content of mono or disaccharides.

APPENDIX A

Table A 1 Analysis of variance for dependent variable pH of lactic acid bacteria and bifidobacteria

Effect	**Num [1] DF**	**Den [2] DF**	**F value**	**Pr[3] > F**
Sweetener type	5	96	162.63	<0.0001
Culture	3	96	2365.87	<0.0001
Time	1	96	772088	<0.0001
Sweetener level	1	96	1183.89	<0.0001
Sweetener type * Culture	15	96	66.77	<0.0001
Sweetener type * Time	5	96	29.04	<0.0001
Culture * Time	3	96	1250.57	<0.0001
Sweetener type * Sweetener level	5	96	12.44	<0.0001
Culture * Sweetener level	3	96	1420.29	<0.0001
Time * Sweetener level	1	96	0.57	0.4760
Sweetener type * Culture * Time	15	96	63.77	<0.0001
Sweetener type * Culture * Sweetener level	15	96	33.88	<0.0001
Sweetener type * Time * Sweetener level	5	96	59.08	<0.0001
Culture * Time* Sweetener level	3	96	634.03	<0.0001
Sweetener type * Culture * Time * Sweetener level	15	96	44.26	<0.0001

[1]Num DF = numerator degrees of freedom
[2]Den DF = denominator degrees of freedom
[3]Pr = probability

Table A 2 Effect of sweetener type and level on pH of lactic acid bacteria and bifidobacteria over 24 h incubation

Treatment	5%		10%	
	0 h	24 h	0 h	24 h
Control[1]	6.07±0.09[a]	4.07±0.09[ab]	6.10±0.08[a]	4.05±0.13[a]
Sucrose	6.07±0.09[a]	4.12±0.33[b]	6.03±0.17[ab]	3.97±0.15[ab]
HFCS[2]	6.10±0.08[a]	4.10±0.32[ab]	6.03±0.17[ab]	3.98±0.30[ab]
Sage	6.05±0.06[a]	3.95±0.24[cd]	6.00±0.14[bc]	3.95±0.23[b]
Alfalfa	6.05±0.06[a]	3.92±0.21[c]	5.93±0.17[b]	3.93±0.34[b]
Sourwood	6.07±0.09[a]	4.02±0.27[ad]	5.97±0.13[bc]	3.93±0.27[b]
Inulin	6.10±0.08[a]	4.10±0.26[ab]	6.02±0.17[ac]	3.92±0.15[b]

[a-c] Means with different superscripts are significantly different ($p<0.05$). Comparisons are made only within the same column; n = 3 for all treatments.
[1]Control devoid of sweetener
[2]HFCS = high fructose corn syrup.

Table A 3 Analysis of variance for dependent variable pH of *Streptococcus salivarius* subsp. *thermo*philus (St-133) in 12% nonfat dry milk

Effect	Num[1] DF	Den[2] DF	F value	Pr[3] > F
Sweetener type	5	24	70.36	<0.0001
Time	1	24	169857	<0.0001
Sweetener level	1	24	1191.68	<0.0001
Sweetener type * Time	5	24	24.87	<0.0001
Sweetener type * Sweetener level	5	24	5.84	0.0011
Time * sweetener level	1	24	327.05	<0.0001
Sweetener type* Time* sweetener level	5	24	1.73	0.1676

[1]Num DF = numerator degrees of freedom
[2]Den DF = denominator degrees of freedom
[3]Pr = probability

Table A 4 Effect of sweetener type and level on pH of *Streptococcus salivarius* subsp. *thermophilus* (St-133) over 24 h incubation

Treatment	5%		10%	
	0 h	24 h	0 h	24 h
Control[1]	6.18±0.01^{ab}	4.22±0.11^{b}	6.18±0.01^{a}	4.22±0.11^{a}
Sucrose	6.18±0.01^{ab}	4.34±0.06^{a}	6.09±0.02^{b}	4.09±0.01^{b}
HFCS[2]	6.16±0.01^{b}	4.21±0.03^{b}	6.08±0.01^{bc}	3.94±0.02^{c}
Sage	6.15±0.02^{bc}	4.14±0.03^{b}	6.05±0.01^{cd}	3.89±0.01^{d}
Alfalfa	6.11±0.02^{c}	4.13±0.02^{b}	5.97±0.01^{e}	3.83±0.01^{e}
Sourwood	6.16±0.01^{ab}	4.19±0.03^{ad}	6.03±0.01^{d}	3.83±0.01^{e}
Inulin	6.20±0.01^{a}	4.21±0.06^{ab}	6.11±0.02^{b}	3.96±0.03^{c}

$^{a-e}$ Means with different superscripts are significantly different ($p<0.05$). Comparisons are made only within the same column; n = 3 for all treatments.
[1]Control devoid of sweetener
[2]HFCS = high fructose corn syrup.

Table A 5 Analysis of variance for dependent variable pH of *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lr-78) in 12% nonfat dry milk

Effect	Num[1] DF	Den[2] DF	F value	Pr[3] > F
Sweetener type	5	24	25.17	<0.0001
Time	1	24	108802	<0.0001
Sweetener level	1	24	667.85	<0.0001
Sweetener type * Time	5	24	15.04	<0.0001
Sweetener type * Sweetener level	5	24	32.28	<0.0001
Time * sweetener level	1	24	601.39	<0.0001
Sweetener type* Time* sweetener level	5	24	62.00	<0.0001

[1]Num DF = numerator degrees of freedom
[2]Den DF = denominator degrees of freedom
[3]Pr = probability

Table A 6 Effect of sweetener type and level on pH of *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lr-78) over 24 h incubation

Treatment	5%		10%	
	0 h	24 h	0 h	24 h
Control[1]	6.14±0.04[a]	3.93±0.09[a]	6.14±0.04[a]	3.93±0.09[d]
Sucrose	6.09±0.02[a]	3.76±0.02[b]	6.16±0.01[a]	3.86±0.03[d]
HFCS[2]	6.13±0.01[a]	3.75±0.01[b]	6.15±0.02[a]	4.29±0.13[ab]
Sage	6.07±0.01[a]	3.74±0.01[b]	6.11±0.02[a]	4.12±0.02[c]
Alfalfa	6.08±0.01[a]	3.72±0.01[b]	6.07±0.01[a]	4.34±0.02[a]
Sourwood	6.07±0.01[a]	3.70±0.01[b]	6.10±0.02[a]	4.16±0.03[bc]
Inulin	6.11±0.01[a]	3.76±0.01[b]	6.16±0.01[a]	3.82±0.02[d]

[a-d] Means with different superscripts are significantly different ($p<0.05$). Comparisons are made only within the same column; n = 3 for all treatments.
[1]Control devoid of sweetener
[2]HFCS = high fructose corn syrup.

Table A 7 Analysis of variance for dependent variable pH of *Lactobacillus acidophilus* (La-7) in 12% nonfat dry milk

Effect	Num[1] DF	Den[2] DF	F value	Pr[3] > F
Sweetener type	5	24	181.20	<0.0001
Time	1	24	394241	<0.0001
Sweetener level	1	24	4139.63	<0.0001
Sweetener type * Time	5	24	50.46	<0.0001
Sweetener type * Sweetener level	5	24	30.03	<0.0001
Time * sweetener level	1	24	18.03	0.0003
Sweetener type* Time* sweetener level	5	24	10.86	<0.0001

[1]Num DF = numerator degrees of freedom
[2]Den DF = denominator degrees of freedom
[3]Pr = probability

Table A 8 Effect of sweetener type and level on pH of *Lactobacillus acidophilus* (La-7) over 24 h incubation

Treatment	5%		10%	
	0 h	24 h	0 h	24 h
Control[1]	5.98±0.09^{b}	4.01±0.05^{a}	5.98±0.09^{a}	4.01±0.05^{a}
Sucrose	6.03±0.01^{a}	3.91±0.01^{c}	5,83±0.02^{b}	3.83±0.03^{b}
HFCS[2]	6.02±0.02^{a}	3.85±0.02^{d}	5.79±0.01^{bc}	3.63±0.02^{d}
Sage	6.01±0.01^{ab}	3.77±0.01^{f}	5.76±0.02^{cd}	3.60±0.01^{de}
Alfalfa	5.99±0.01^{b}	3.81±0.02^{e}	5.72±0.02^{d}	3.55±0.02^{f}
Sourwood	6.01±0.01^{ab}	3.86±0.01^{d}	5.76±0.02^{cd}	3.59±0.01^{ef}
Inulin	6.03±0.01^{a}	3.97±0.01^{b}	5.83±0.01^{b}	3.76±0.02^{c}

[a-f] Means with different superscripts are significantly different ($p<0.05$). Comparisons are made only within the same column; n = 3 for all treatments.
[1]Control devoid of sweetener
[2]HFCS = high fructose corn syrup.

Table A 9 Analysis of variance for dependent variable pH of *Bifidobacterium bifidum* (Bf-1) in 12% nonfat dry milk

Effect	Num[1] DF	Den[2] DF	F value	Pr[3] > F
Sweetener type	5	24	679.11	<0.0001
Time	1	24	668102	<0.0001
Sweetener level	1	24	3305.16	<0.0001
Sweetener type * Time	5	24	270.65	<0.0001
Sweetener type * Sweetener level	5	24	107.50	<0.0001
Time * sweetener level	1	24	1323.14	<0.0001
Sweetener type* Time* sweetener level	5	24	256.00	<0.0001

[1]Num DF = numerator degrees of freedom
[2]Den DF = denominator degrees of freedom
[3]Pr = probability

Table A 10 Effect of sweetener type and level on pH of *Bifidobacterium bifidum* (Bf-1) over 24 h incubation

Treatment	5%		10%	
	0 h	**24 h**	**0 h**	**24 h**
Control[1]	6.12±0.04^{a}	4.11±0.07^{e}	6.12±0.04^{a}	4.11±0.07^{ab}
Sucrose	6.03±0.01^{cd}	4.47±0.01^{a}	5.03±0.01^{b}	4.13±0.01^{a}
HFCS[2]	6.05±0.02^{bc}	4.48±0.02^{a}	6.01±0.01^{bc}	4.08±0.01^{bc}
Sage	6.03±0.01^{cd}	4.16±0.01^{d}	5.97±0.01^{cd}	4.06±0.01^{c}
Alfalfa	6.00±0.01^{d}	4.01±0.01^{f}	5.90±0.01^{e}	3.99±0.01^{d}
Sourwood	6.03±0.01^{cd}	4.29±0.02^{c}	5.95±0.01^{d}	4.11±0.03^{ab}
Inulin	6.07±0.01^{b}	4.36±0.03^{b}	6.02±0.01^{b}	4.13±0.01^{a}

$^{a-f}$ Means with different superscripts are significantly different ($p<0.05$). Comparisons are made only within the same column; n = 3 for all treatments.

[1]Control devoid of sweetener

[2]HFCS = high fructose corn syrup.

Titratable acidity

Levels of lactic acid produced by lactic acid bacteria and bifidobacteria as well as acetic acid produced by bifidobacteria only when grown in non-fat dry milk, were determined using titrateble acidity according to Standard Methods for the Examination of Dairy Products (Marshall 1992). Nine ml of sample for lactic acid determination and 6 ml of sample for acetic acid determination were transferred into an Erlenmeyer flask. Each sample was titrated with standardized 0.1 N NaOH alkali solution to the phenolphthalein endpoint. Percent titratable acidity was calculated as follows:

% Titratable acidity = [base normality (mEq/mL) x mL base x Eq. Wt. Of acid mg/Eq) / sample weight (g) x 10

Table A 11 Analysis of variance for dependent variable lactic acid production (TA method) of lactic acid bacteria and bifidobacteria

Effect	**Num[1] DF**	**Den[2] DF**	**F value**	**Pr[3] > F**
Sweetener type	5	96	135.38	<0.0001
Culture	3	96	166.40	<0.0001
Time	1	96	718671	<0.0001
Sweetener level	1	96	926.16	<0.0001
Sweetener type * Culture	15	96	10.76	<0.0001
Sweetener type * Time	5	96	69.70	<0.0001
Culture * Time	3	96	164.92	<0.0001
Sweetener type * Sweetener level	5	96	10.34	<0.0001
Culture * Sweetener level	3	96	45.93	<0.0001
Time * Sweetener level	1	96	769.64	<0.0001
Sweetener type * Culture * Time	15	96	13.77	<0.0001
Sweetener type * Culture * Sweetener level	15	96	15.93	<0.0001
Sweetener type * Time * Sweetener level	5	96	17.34	<0.0001
Culture * Time* Sweetener level	3	96	48.23	<0.0001
Sweetener type * Culture * Time * Sweetener level	15	96	17.63	<0.0001

[1]Num DF = numerator degrees of freedom
[2]Den DF = denominator degrees of freedom
[3]Pr = probability

Table A 12 Effect of sweetener type and level on lactic acid production (TA method) of lactic acid bacteria and bifidobacteria over 24 h incubation

Treatment	5%		10%	
	0 h	24 h	0 h	24 h
Control[1]	0.27±0.04[a]	1.06±0.19[a]	0.28±0.04[a]	1.06±0.18[ac]
Sucrose	0.28±0.04[a]	1.12±0.26[bd]	0.26±0.03[a]	1.01±0.19[cb]
HFCS[2]	0.28±0.05[a]	1.17±0.25[b]	0.27±0.05[a]	0.96±0.21[b]
Sage	0.30±0.04[a]	1.29±0.29[c]	0.30±0.05[a]	1.04±0.17[ac]
Alfalfa	0.31±0.04[a]	1.26±0.21[c]	0.31±0.04[a]	1.10±0.20[a]
Sourwood	0.30±0.04[a]	1.31±0.31[c]	0.31±0.05[a]	1.09±0.23[a]
Inulin	0.29±0.04[a]	1.11±0.19[ad]	0.26±0.04[a]	0.96±0.21[b]

[a-d] Means with different superscripts are significantly different ($p<0.05$). Comparisons are made only within the same column; n = 3 for all treatments.
[1]Control devoid of sweetener
[2]HFCS = high fructose corn syrup.

Table A 13 Analysis of variance for dependent variable titratable acidity (TA) of *Streptococcus salivarius* subsp. *thermophilus* (St-133) in 12% nonfat dry milk

Effect	Num[1] DF	Den[2] DF	F value	Pr[3] > F
Sweetener type	5	24	52.51	<0.0001
Time	1	24	7940.88	<0.0001
Sweetener level	1	24	36.17	<0.0001
Sweetener type * Time	5	24	39.84	<0.0001
Sweetener type * Sweetener level	5	24	7.15	0.0003
Time * sweetener level	1	24	20.14	0.0002
Sweetener type* Time* sweetener level	5	24	5.75	0.0013

[1]Num DF = numerator degrees of freedom
[2]Den DF = denominator degrees of freedom
[3]Pr = probability

Table A 14 Effect of sweetener type and level on lactic acid production (TA method) of *Streptococcus salivarius* subsp. *thermophilus* (St-133) over 24 h incubation

Treatment	5%		10%	
	0 h	24 h	0 h	24 h
Control[1]	0.30 ± 0.01^{ab}	1.03 ± 0.14^{dc}	0.30 ± 0.01^{ab}	1.03 ± 0.14^{bc}
Sucrose	0.29 ± 0.01^{b}	0.97 ± 0.02^{d}	0.28 ± 0.02^{ab}	0.91 ± 0.01^{dc}
HFCS[2]	0.29 ± 0.01^{b}	1.07 ± 0.03^{c}	0.30 ± 0.03^{ab}	1.10 ± 0.02^{ab}
Sage	0.31 ± 0.02^{ab}	1.32 ± 0.06^{a}	0.30 ± 0.01^{ab}	1.14 ± 0.03^{ab}
Alfalfa	0.33 ± 0.02^{a}	1.17 ± 0.02^{b}	0.30 ± 0.01^{ab}	1.20 ± 0.01^{a}
Sourwood	0.32 ± 0.01^{ab}	1.37 ± 0.05^{a}	0.31 ± 0.01^{a}	1.22 ± 0.02^{a}
Inulin	0.31 ± 0.01^{ab}	1.06 ± 0.02^{c}	0.27 ± 0.01^{b}	0.82 ± 0.16^{d}

[a-d] Means with different superscripts are significantly different ($p<0.05$). Comparisons are made only within the same column; n = 3 for all treatments.
[1]Control devoid of sweetener
[2]HFCS = high fructose corn syrup.

Table A 15 Analysis of variance for dependent variable titratable acidity (TA) of *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lr-78) in 12% nonfat dry milk

Effect	Num[1] DF	Den[2] DF	F value	Pr[3] > F
Sweetener type	5	24	31.87	<0.0001
Time	1	24	21901.3	<0.0001
Sweetener level	1	24	598.96	<0.0001
Sweetener type * Time	5	24	17.55	<0.0001
Sweetener type * Sweetener level	5	24	36.81	<0.0001
Time * sweetener level	1	24	530.22	<0.0001
Sweetener type* Time* sweetener level	5	24	46.08	<0.0001

[1]Num DF = numerator degrees of freedom
[2]Den DF = denominator degrees of freedom
[3]Pr = probability

Table A 16 Effect of sweetener type and level on lactic acid production (TA method) of *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lr-78) over 24 h incubation

Treatment	5%		10%	
	0 h	24 h	0 h	24 h
Control[1]	0.28±0.01[b]	1.15±0.02[d]	0.28±0.01[bc]	1.15±0.02[ab]
Sucrose	0.29±0.01[ab]	1.30±0.04[c]	0.27±0.02[c]	1.20±0.02[a]
HFCS[2]	0.30±0.01[ab]	1.43±0.05[b]	0.26±0.01[c]	1.00±0.04[c]
Sage	0.32±0.02[a]	1.53±0.03[a]	0.30±0.01[b]	1.01±0.07[bc]
Alfalfa	0.31±0.01[ab]	1.54±0.04[a]	0.34±0.01[a]	1.01±0.06[c]
Sourwood	0.31±0.01[ab]	1.54±0.02[a]	0.30±0.01[b]	1.23±0.04[a]
Inulin	0.29±0.01[ab]	1.17±0.02[a]	0.27±0.01[c]	1.14±0.01[ab]

[a-d] Means with different superscripts are significantly different ($p<0.05$). Comparisons are made only within the same column; n = 3 for all treatments.
[1]Control devoid of sweetener
[2]HFCS = high fructose corn syrup.

Table A 17 Analysis of variance for dependent variable titratable acidity (TA) of *Lactobacillus acidophilus* (La-7) in 12% nonfat dry milk

Effect	Num[1] DF	Den[2] DF	F value	Pr[3] > F
Sweetener type	5	24	24.55	<0.0001
Time	1	24	46680.5	<0.0001
Sweetener level	1	24	463.79	<0.0001
Sweetener type * Time	5	24	14.10	<0.0001
Sweetener type * Sweetener level	5	24	4.76	0.0037
Time * sweetener level	1	24	553.08	<0.0001
Sweetener type* Time* sweetener level	5	24	10.36	<0.0001

[1]Num DF = numerator degrees of freedom
[2]Den DF = denominator degrees of freedom
[3]Pr = probability

Table A 18 Effect of sweetener type and level on lactic acid production (TA method) of *Lactobacillus acidophilus* (La-7) over 24 h incubation

Treatment	5%		10%	
	0 h	24 h	0 h	24 h
Control[1]	0.30±0.01[a]	1.26±0.15[d]	0.30±0.01[bc]	1.26±0.15[a]
Sucrose	0.32±0.02[a]	1.39±0.03[b]	0.30±0.01[bc]	1.11±0.02[c]
HFCS[2]	0.31±0.01[a]	1.39±0.04[b]	0.28±0.02[c]	1.14±0.04[bc]
Sage	0.32±0.01[a]	1.50±0.03[a]	0.32±0.01[abc]	1.20±0.02[ab]
Alfalfa	0.34±0.01[a]	1.39±0.01[bc]	0.32±0.01[ab]	1.25±0.02[a]
Sourwood	0.34±0.03[a]	1.49±0.04[a]	0.34±0.01[a]	1.25±0.04[a]
Inulin	0.32±0.02[a]	1.32±0.03[cd]	0.29±0.01[bc]	1.15±0.02[bc]

[a-d] Means with different superscripts are significantly different ($p<0.05$). Comparisons are made only within the same column; n = 3 for all treatments.
[1]Control devoid of sweetener
[2]HFCS = high fructose corn syrup.

Table A 19 Analysis of variance for dependent variable lactic acid (TA method) of *Bifidobacterium bifidum* (Bf1) in 12% nonfat dry milk

Effect	Num[1] DF	Den[2] DF	F value	Pr[3] > F
Sweetener type	5	24	33.91	<0.0001
Time	1	24	28695.4	<0.0001
Sweetener level	1	24	163.76	<0.0001
Sweetener type * Time	5	24	27.17	<0.0001
Sweetener type * Sweetener level	5	24	10.14	<0.0001
Time * sweetener level	1	24	225.42	<0.0001
Sweetener type* Time* sweetener level	5	24	20.87	<0.0001

[1]Num DF = numerator degrees of freedom
[2]Den DF = denominator degrees of freedom
[3]Pr = probability

Table A 20 Effect of sweetener type and level on lactic acid production (TA method) of *Bifidobacterium bifidum* (Bf1) over 24 h incubation

Treatment	5%		10%	
	0 h	24 h	0 h	24 h
Control[1]	0.30±0.01[a]	1.10±0.01[d]	0.30±0.01[c]	1.10±0.01[bc]
Sucrose	0.31±0.02[a]	1.19±0.05[c]	0.28±0.01[c]	1.08±0.02[bc]
HFCS[2]	0.32±0.02[a]	1.16±0.05[dc]	0.32±0.02[abc]	0.94±0.04[d]
Sage	0.33±0.01[a]	1.30±0.01[ab]	0.34±0.02[ab]	1.11±0.02[b]
Alfalfa	0.34±0.02[a]	1.24±0.04[bc]	0.34±0.01[ab]	1.26±0.03[a]
Sourwood	0.32±0.02[a]	1.36±0.02[a]	0.35±0.02[a]	1.09±0.01[bc]
Inulin	0.33±0.01[a]	1.20±0.02[c]	0.28±0.01[c]	1.04±0.01[c]

[a-d] Means with different superscripts are significantly different ($p<0.05$). Comparisons are made only within the same column; n = 3 for all treatments.
[1]Control devoid of sweetener
[2]HFCS = high fructose corn syrup.

Table A 21 Analysis of variance for dependent variable acetic acid (TA method) of *Bifidobacterium bifidum* (Bf1) in 12% nonfat dry milk

Effect	Num[1] DF	Den[2] DF	F value	Pr[3] > F
Sweetener type	5	24	33.67	<0.0001
Time	1	24	11067.9	<0.0001
Sweetener level	1	24	130.24	<0.0001
Sweetener type * Time	5	24	16.80	<0.0001
Sweetener type * Sweetener level	5	24	7.23	0.0003
Time * sweetener level	1	24	93.34	<0.0001
Sweetener type* Time* sweetener level	5	24	7.46	0.0002

[1]Num DF = numerator degrees of freedom
[2]Den DF = denominator degrees of freedom
[3]Pr = probability

Table A 20 Effect of sweetener type and level on acetic acid production (TA method) of *Bifidobacterium bifidum* (Bf1) over 24 h incubation

Treatment	5%		10%	
	0 h	24 h	0 h	24 h
Control[1]	0.21±0.01[a]	0.76±0.01[d]	0.21±0.01[bc]	0.76±0.01[a]
Sucrose	0.21±0.01[a]	0.76±0.02[b]	0.20±0.01[bc]	0.73±0.02[c]
HFCS[2]	0.20±0.02[a]	0.80±0.02[b]	0.19±0.01[c]	0.61±0.03[bc]
Sage	0.23±0.01[a]	0.81±0.04[a]	0.22±0.01[abc]	0.75±0.01[ab]
Alfalfa	0.24±0.02[a]	0.98±0.06[bc]	0.23±0.01[ab]	0.79±0.01[a]
Sourwood	0.23±0.02[a]	0.77±0.05[a]	0.23±0.01[a]	0.70±0.02[a]
Inulin	0.22±0.01[a]	0.81±0.01[cd]	0.19±0.01[bc]	0.67±0.03[bc]

[a-d] Means with different superscripts are significantly different ($p<0.05$). Comparisons are made only within the same column; n = 3 for all treatments.

[1]Control devoid of sweetener

[2]HFCS = high fructose corn syrup.

Table A 21 Analysis of variance for dependent variable pH of lactic acid bacteria and bifidobacteria in the presence of yogurt ingredients

Effect	**Num [1] DF**	**Den[2] DF**	**F value**	**Pr [3]> F**
Sweetener type	8	72	35.24	<0.0001
Culture	3	72	61.32	<0.0001
Time	1	72	20183.5	0.0001
Sweetener type * Culture	24	72	3.10	<0.0001
Sweetener type * Time	8	72	9.73	<0.0001
Culture * Time	3	72	43.38	<0.0001
Sweetener type* Culture * Time	24	72	2.19	0.0507

[1]Num DF = numerator degrees of freedom
[2]Den DF = denominator degrees of freedom
[3]Pr = probability

Table A 22 Analysis of variance for dependent variable pH of *Streptococcus salivarius* subsp. *thermophilus* (St-133) in the presence of yogurt ingredients

Effect	**Num [1] DF**	**Den[2] DF**	**F value**	**Pr [3] > F**
Sweetener type	8	18	21.12	<0.0001
Time	1	18	8696.95	<0.0001
Sweetener type * time	8	18	1.18	0.3644

[1]Num DF = numerator degrees of freedom
[2]Den DF = denominator degrees of freedom
[3]Pr = probability

Table A 23 Effect of yogurt ingredients on pH of *Streptococcus salivarius* subsp. *thermophilus* (St-133)

Treatments	Ingredients (%)	0 h	24 h
Control[1]	0	6.35 ± 0.04^{a}	4.53 ± 0.18^{a}
Stabilizer	0.5	6.35 ± 0.02^{a}	4.49 ± 0.04^{a}
Strawberry puree	10	6.04 ± 0.03^{b}	4.30 ± 0.14^{ab}
Sucrose + Strawberry puree	7+10	5.93 ± 0.03^{c}	4.22 ± 0.07^{ab}
HFCS[2] +Strawberry puree	7+10	5.91 ± 0.06^{c}	4.13 ± 0.02^{b}
Sage + Strawberry puree	7+10	5.90 ± 0.03^{c}	4.09 ± 0.03^{b}
Alfalfa+ Strawberry puree	7+10	5.91 ± 0.02^{c}	4.15 ± 0.05^{b}
Sourwood+ Strawberry puree	7+10	5.93 ± 0.03^{c}	4.17 ± 0.06^{b}
Inulin + Strawberry puree	7+10	5.96 ± 0.04^{bc}	4.31 ± 0.07^{ab}

[a-c] Means with different superscripts are significantly different ($p<0.05$). Comparisons are made only within the same column; n = 3 for all treatments.
[1]Control contains 12% non fat dry milk and is devoid of other ingredients
[2]HFCS = high fructose corn syrup.

Table A 24 Analysis of variance for dependent variable pH of *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lr-78) in the presence of yogurt ingredients

Effect	Num[1] DF	Den[2] DF	F value	Pr[3]> F
Sweetener type	8	18	1.88	0.1266
Time	1	18	2484.07	<0.0001
Sweetener type * time	8	18	5.74	0.0010

[1]Num DF = numerator degrees of freedom
[2]Den DF = denominator degrees of freedom
[3]Pr = probability

Table A 25 Effect of yogurt ingredients on pH of *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lr-78)

Treatments	Ingredients (%)	0 h	24 h
Control[1]	0	6.20 ± 0.03^{a}	4.12 ± 0.10^{a}
Stabilizer	0.5	6.19 ± 0.03^{a}	4.10 ± 0.01^{a}
Strawberry puree	10	5.93 ± 0.07^{a}	4.11 ± 0.01^{a}
Sucrose + Strawberry puree	7+10	5.84 ± 0.04^{b}	4.14 ± 0.04^{a}
HFCS[2] +Strawberry puree	7+10	5.84 ± 0.04^{b}	4.32 ± 0.24^{a}
Sage + Strawberry puree	7+10	5.86 ± 0.06^{b}	4.34 ± 0.20^{a}
Alfalfa+ Strawberry puree	7+10	5.83 ± 0.06^{b}	4.47 ± 0.36^{a}
Sourwood+ Strawberry puree	7+10	5.86 ± 0.02^{b}	4.18 ± 0.15^{a}
Inulin + Strawberry puree	7+10	5.89 ± 0.04^{b}	4.09 ± 0.03^{a}

[a-c] Means with different superscripts are significantly different ($p<0.05$). Comparisons are made only within the same column; n = 3 for all treatments.
[1]Control contains 12% non fat dry milk and is devoid of other ingredients
[2]HFCS = high fructose corn syrup.

Table A 26 Analysis of variance for dependent variable pH of *Lactobacillus acidophilus* (La-7) in the presence of yogurt ingredients

Effect	Num[1] DF	Den[2] DF	F value	Pr[3] > F
Sweetener type	8	18	32.90	<0.0001
Time	1	18	20992.9	<0.0001
Sweetener type * time	8	18	5.98	0.0008

[1]Num DF = numerator degrees of freedom
[2]Den DF = denominator degrees of freedom
[3]Pr = probability

Table A 27 Effect of yogurt ingredients on pH of *Lactobacillus acidophilus* (La-7)

Treatments	**Ingredients (%)**	**0 h**	**24 h**
Control[1]	0	6.22 ± 0.03^{a}	4.01 ± 0.07^{a}
Stabilizer	0.5	6.22 ± 0.03^{a}	3.99 ± 0.08^{a}
Strawberry puree	10	5.94 ± 0.04^{b}	3.93 ± 0.08^{ab}
Sucrose + Strawberry puree	7+10	5.85 ± 0.02^{bc}	3.91 ± 0.06^{ab}
HFCS[2] +Strawberry puree	7+10	5.84 ± 0.04^{c}	3.80 ± 0.05^{b}
Sage + Strawberry puree	7+10	5.81 ± 0.04^{c}	3.79 ± 0.06^{b}
Alfalfa+ Strawberry puree	7+10	5.83 ± 0.02^{c}	3.79 ± 0.06^{b}
Sourwood+ Strawberry puree	7+10	5.84 ± 0.01^{bc}	3.80 ± 0.04^{b}
Inulin + Strawberry puree	7+10	5.86 ± 0.07^{bc}	3.93 ± 0.04^{ab}

[a-c] Means with different superscripts are significantly different ($p<0.05$). Comparisons are made only within the same column; n = 3 for all treatments.
[1]Control contains 12% non fat dry milk and is devoid of other ingredients
[2]HFCS = high fructose corn syrup.

Table A 28 Analysis of variance for dependent variable pH of *Bifidobacterium bifidum* (Bf1) in the presence of yogurt ingredients

Effect	**Num[1] DF**	**Den[2] DF**	**F value**	**Pr[3] > F**
Sweetener type	8	18	9.25	<0.0001
Time	1	18	4550.06	<0.0001
Sweetener type * time	8	18	3.26	0.0177

[1]Num DF = numerator degrees of freedom
[2]Den DF = denominator degrees of freedom
[3]Pr = probability

Table A 29 Effect of yogurt ingredients on pH of *Bifidobacterium bifidum* (Bf1)

Treatments	**Ingredients (%)**	**0 h**	**24 h**
Control[1]	0	6.21 ± 0.05^{a}	4.35 ± 0.19^{a}
Stabilizer	0.5	6.19 ± 0.02^{a}	4.27 ± 0.14^{a}
Strawberry puree	10	5.91 ± 0.06^{b}	4.21 ± 0.14^{a}
Sucrose + Strawberry puree	7+10	5.74 ± 0.04^{c}	4.25 ± 0.17^{a}
HFCS[2] +Strawberry puree	7+10	5.76 ± 0.07^{c}	4.17 ± 0.11^{a}
Sage + Strawberry puree	7+10	5.76 ± 0.05^{c}	4.10 ± 0.85^{a}
Alfalfa+ Strawberry puree	7+10	5.77 ± 0.03^{bc}	4.08 ± 0.04^{a}
Sourwood+ Strawberry puree	7+10	5.80 ± 0.04^{bc}	4.18 ± 0.08^{a}
Inulin + Strawberry puree	7+10	5.81 ± 0.04^{bc}	4.22 ± 0.12^{a}

[a-c] Means with different superscripts are significantly different ($p<0.05$). Comparisons are made only within the same column; n = 3 for all treatments.
[1]Control contains 12% non fat dry milk and is devoid of other ingredients
[2]HFCS = high fructose corn syrup.

Table A 30 Effect of sweetener type on viability of lactic acid bacteria during refrigerated storage (4°C, 42 d)

Treatment	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g
Control[1]	$8.66\pm0.20^{a;A}$	$8.17\pm0.40^{a;A}_{B}$	$8.19\pm0.13^{b;AB}$	$7.98\pm0.10^{a;AB}$	$8.17\pm0.37^{a;AB}$	$8.05\pm0.33^{a;AB}$	$7.51\pm0.12^{a;B}$
Sucrose	$8.69\pm0.19^{a;A}$	$8.79\pm0.51^{a;A}$	$9.04\pm0.53^{a;A}$	$8.50\pm0.41^{a;A}$	$8.72\pm0.70^{a;A}$	$8.34\pm0.13^{a;A}$	$8.23\pm0.38^{a;A}$
HFCS[2]	$8.45\pm0.23^{a;A}$	$8.40\pm0.05^{a;A}$	$8.09\pm0.28^{b;A}$	$7.50\pm0.97^{a;A}$	$7.62\pm0.63^{a;A}$	$7.50\pm0.50^{a;A}$	$7.54\pm0.88^{a;A}$
Sage	$8.52\pm0.20^{a;A}$	$8.33\pm0.03^{a;A}$	$8.28\pm0.20^{b;A}$	$8.33\pm0.15^{a;A}$	$8.37\pm0.57^{a;A}$	$8.23\pm0.33^{a;A}$	$7.96\pm0.23^{a;A}$
Alfalfa	$8.87\pm0.46^{a;A}$	$8.33\pm0.11^{a;A}$	$8.66\pm0.18^{ab;A}$	$8.24\pm0.68^{a;A}$	$8.11\pm0.81^{a;A}$	$8.24\pm0.66^{a;A}$	$7.92\pm0.22^{a;A}$
Sourwood	$8.67\pm0.13^{a;A}$	$8.16\pm0.41^{a;A}_{B}$	$8.33\pm0.13^{ab;AB}$	$8.16\pm0.38^{a;AB}$	$7.85\pm0.49^{a;AB}$	$7.52\pm0.25^{a;B}$	$7.31\pm0.71^{a;B}$
Inulin	$8.84\pm0.04^{a;A}$	$8.74\pm0.38^{a;A}$	$8.62\pm0.23^{ab;AB}$	$8.83\pm0.57^{a;A}$	$8.16\pm0.33^{a;AB}$	$7.94\pm0.42^{a;AB}$	$7.54\pm0.56^{a;B}$

[a-b;A-B] Means with different superscripts are significantly different ($p < 0.05$); Comparisons are made within the same column[(a-b)] and within the same row[(A-B)] ; n = 3 for all treatments; [1]Control devoid of sweetener; [2]HFCS = high fructose corn syrup.

Table A 31 Effect of sweetener type on viability of bifidobacteria during refrigerated storage (4°C, 42 d)

Treatment	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g	log CFU/g
Control[1]	8.03±0.59$^{a;A}$	7.69±0.41$^{a;A}$	7.82±0.46$^{ab;A}$	7.69±0.37$^{a;A}$	7.96±0.42$^{a;A}$	7.62±0.45$^{a;A}$	7.59±0.36$^{a;A}$
Sucrose	8.01±0.44$^{a;A}$	8.08±0.24$^{a;A}$	8.72±0.31$^{a;A}$	8.34±0.57$^{a;A}$	8.58±0.93$^{a;A}$	8.12±0.27$^{a;A}$	8.20±0.31$^{a;A}$
HFCS[2]	7.88±0.37$^{a;A}$	7.99±0.33$^{a;A}$	7.79±0.47$^{ab;A}$	7.65±0.64$^{a;A}$	7.53±0.72$^{a;A}$	7.41±0.77$^{a;A}$	7.59±0.86$^{a;A}$
Sage	8.01±0.50$^{a;A}$	7.86±0.42$^{a;A}$	8.11±0.24$^{ab;A}$	8.18±0.15$^{a;A}$	7.39±0.38$^{a;A}$	7.74±0.39$^{a;A}$	7.84±0.24$^{a;A}$
Alfalfa	8.24±0.30$^{a;A}$	8.06±0.3$^{a;A}$	7.92±0.70$^{ab;A}$	7.98±0.47$^{a;A}$	8.02±0.85$^{a;A}$	7.91±0.69$^{a;A}$	7.80±0.36$^{a;A}$
Sourwood	7.98±0.50$^{a;A}$	7.69±0.40$^{a;A}$	7.00±1.15$^{b;A}$	7.14±0.61$^{a;A}$	7.48±0.07$^{a;A}$	7.00±0.47$^{a;A}$	6.88±0.71$^{a;A}$
Inulin	8.03±0.59$^{a;A}$	7.69±0.41$^{a;A}$	7.82±0.46$^{ab;A}$	7.69±0.37$^{a;A}$	7.96±0.42$^{a;A}$	7.62±0.45$^{a;A}$	7.59±0.36$^{a;A}$

$^{a\text{-}b;A\text{-}B}$ Means with different superscripts are significantly different ($p < 0.05$); Comparisons are made within the same column$^{(a\text{-}b)}$ and within the same row$^{(A\text{-}B)}$; n = 3 for all treatments; [1]Control devoid of sweetener; [2]HFCS = high fructose corn syrup.

APPENDIX B

MICHIGAN STATE
UNIVERSITY

September 25, 2003

TO: Zeynep USTUNOL
2105 S. Anthony Hall
MSU

RE: **IRB# 03-672** CATEGORY: EXEMPT 1-6

APPROVAL DATE: September 19, 2003
EXPIRATION DATE: August 19, 2004

TITLE: DEVELOPMENT AND PROPERTY OF A HONEY SWEETENED LOW-FAT YOGURT WITH ENHANCED PROBIOTIC ACTIVITY

The University Committee on Research Involving Human Subjects' (UCRIHS) review of this project is complete and I am pleased to advise that the rights and welfare of the human subjects appear to be adequately protected and methods to obtain informed consent are appropriate. Therefore, the **UCRIHS approved this project.**

RENEWALS: UCRIHS approval is valid until the expiration date listed above. Projects continuing beyond this date must be renewed with the renewal form. A maximum of four such expedited renewals are possible. Investigators wishing to continue a project beyond that time need to submit a 5-year application for a complete review.

REVISIONS: UCRIHS must review any changes in procedures involving human subjects, prior to initiation of the change. If this is done at the time of renewal, please include a revision form with the renewal. To revise an approved protocol at any other time during the year, send your written request with an attached revision cover sheet to the UCRIHS Chair, requesting revised approval and referencing the project's IRB# and title. Include in your request a description of the change and any revised instruments, consent forms or advertisements that are applicable.

PROBLEMS/CHANGES: Should either of the following arise during the course of the work, notify UCRIHS promptly: 1) problems (unexpected side effects, complaints, etc.) involving human subjects or 2) changes in the research environment or new information indicating greater risk to the human subjects than existed when the protocol was previously reviewed and approved.

If we can be of further assistance, please contact us at (517) 355-2180 or via email: UCRIHS@msu.edu. Please note that all UCRIHS forms are located on the web: http://www.humanresearch.msu.edu

Sincerely,

Peter Vasilenko III, Ph.D.
UCRIHS Chair

PV: rt

cc: Darcelee S. Popa
2125 South Anthony
East Lansing, MI 48824

OFFICE OF
RESEARCH ETHICS AND STANDARDS
University Committee on Research Involving Human Subjects
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517/355-2180
FAX: 517/432-4503
Web: www.msu.edu/user/ucrihs
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Questionnaire

Product: Strawberry flavored low-fat yogurt

You will be provided with 6 yogurt samples and a questionnaire. Please evaluate each sample in the order they are presented from left to right. Rinse mouth between each sample and indicate how much you like or dislike the sample using the scale provided (1 to 9).

1. Appearance
How do you like the appearance of the sample?

719	195	588	416	121	242
___	___	___	___	___	___

9 – like extremely
8 – like very much
7 – like moderatelly
6 – like slightly
5 – neither like/nor dislike
4 – dislike slightly
3 – dislike moderatelly
2 – dislike very much
1 – dislike extremely

Comments:

2. Aroma
Lift the sample, hold it approximately one inch from your nose, smell the sample and evaluate how you like its aroma.

719	195	588	416	121	242
___	___	___	___	___	___

9 – like extremely
8 – like very much
7 – like moderatelly
6 – like slightly
5 – neither like/nor dislike
4 – dislike slightly
3 – dislike moderatelly
2 – dislike very much
1 – dislike extremely

Comments:

3. Sweeteners

Place a spoon of yogurt sample in your mouth, roll it five times, and evaluate how you like the sweeteness.

719	195	588	416	121	242
___	___	___	___	___	___

9 – like extremely
8 – like very much
7 – like moderatelly
6 – like slightly
5 – neither like/nor dislike
4 – dislike slightly
3 – dislike moderatelly
2 – dislike very much
1 – dislike extremely

Comments:

4. Flavor

How do you like the overall flavor of the sample?

719	195	588	416	121	242
___	___	___	___	___	___

9 – like extremely
8 – like very much
7 – like moderatelly
6 – like slightly
5 – neither like/nor dislike
4 – dislike slightly
3 – dislike moderatelly
2 – dislike very much
1 – dislike extremely

Comments:

5. Mouth residue
After swallowing, how much residue do you have in your mouth? Circle the answer.

4 – A lot of residue
3 – Some residue
2 – Very little residue
1 – No residue

Comments:

General Questionnaire:

1. Do you eat yogurt regularly?
    a. Yes
    b. No

2. How often do you eat yogurt?

    a. 3-4 times a week
    b. Once a week
    c. 2-3 times a month

d. Once a month or less

3. How often do you eat low-fat yogurt?

a. 3-4 times a week
b. Once a week
c. 2-3 times a month
d. Once a month or less

Comments:

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