EFFECT OF STARTER CULTURES ON *LACTOBACILLUS ACIDOPHILUS*
SURVIVAL AND GENE EXPRESSION IN YOGURT

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By
Elizabeth Wei-Yin Ng
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COMMITTEE MEMBERSHIP

TITLE: EFEECT OF STARTER CULTURE ON
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                                          GENE EXPRESSION IN YOGURT

AUTHOR: ELIZABETH WEI-YIN NG

DATE SUBMITTED: May, 2009

COMMITTEE CHAIR: Phillip S. Tong, Ph. D., Professor

COMMITTEE MEMBER: Po Sai Marie Yeung, Ph. D., Assistant Professor

COMMITTEE MEMBER: Anya Goodman, Ph. D., Assistant Professor

ABSTRACT

The Effect of Starter Culture on *Lactobacillus acidophilus* Survival and Gene Expression in Yogurt

Elizabeth Wei-Yin Ng

Probiotics, especially *Lactobacillus acidophilus*, strains have increasingly been incorporated into food products as dietary adjuncts due to their purported health benefits. Dairy products, especially yogurt, are frequently used as probiotic delivery systems. To realize therapeutic effects, it is important to consume a high amount of probiotics. However, maintaining a sufficient amount of viable probiotics in yogurt throughout the shelf life has been a challenge as starter cultures have shown to possess some deleterious effects on probiotic survival. The main objective of this thesis project was to determine the extent of antagonistic effects imposed by yogurt starter cultures (*Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*) and their metabolic products on the survival of five different *L. acidophilus* strains designated as ATCC700396, LA-5, NCFM, PIM703 and SBT2062. These probiotic strains were incorporated as adjuncts in yogurts, which were made with and without starter cultures. To simulate pH reduction in regular yogurt, yogurt without starter cultures was manufactured by adding an acidulant (glucono-delta-lactone). The pH of both yogurts was approximately 4.6 throughout the 28-day storage period at 4°C. Viable cell counts of the probiotic strains were enumerated weekly on a selective medium (MRS-maltose agar). While cell count of *L. acidophilus* PIM703 and SBT2062 remained steady ($\sim 6 \times 10^7$ CFU/g) in yogurt with starter cultures during storage period, the survival of ATCC700396 and NCFM was reduced significantly ($p < 0.05$) by 3 log and 4.6 log, respectively. In contrast, in yogurt without starter cultures, all *L. acidophilus* strains survived well with a

maximum of 1-log reduction. Our results indicated that while the presence of starter cultures (especially *L. delbrueckii* ssp. *bulgaricus*) had a negative effect on the survival of some probiotic strains, low pH was not an important factor impairing their viable cell counts.

In addition to survival studies, gene expression of selected stress genes (*dnaK* and *groEL*) was determined using semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) method. This preliminary study demonstrated the important of *dnaK* in *L. acidophilus* NCFM to maintain its viability under various stress in yogurt during refrigerated storage period. These studies underscore the selection criteria of probiotic strains in fermented food should include their abilities to coexist with the starter cultures. Our future work is to provide a better understanding of strain differences in survival of probiotics in yogurt.

Keywords: Survival, probiotic, *Lactobacillus acidophilus*, yogurt, starter cultures

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1.0

INTRODUCTION

In recent years foods that can be shown to provide health benefits have attracted consumer interest. These foods should also fit into current lifestyles, such as good flavor and convenience with an acceptable price. Such products are commonly referred to probiotic-containing products, especially yogurt. The worldwide consumption of yogurt has been increasing, and this is predominantly due to the unique flavor, great nutritional sources, and health benefits (from probiotics) (Chandan, 1999; Sanders, 1998). Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2001). The health benefits of consuming probiotics have been demonstrated in the number of scientific publications, including antimicrobial production (Olivares et al., 2005; Shah, 1999), immune system modulation (Schlee, et al., 2008), inhibition of *Helicobacter pylori* (Aiba et al., 1998; Gotteland and Cruchet, 2003), improvement of lactose intolerance (de Vrese et al., 2001; Montes et al., 1995), cholesterol assimilation (Liong and Shah, 2005; Walker and Gilliland, 1993), prevention of autoimmunity (Bolton et al., 2008), and antimutagenic properties (Choi et al., 2005; Lankaputhra and Shah, 1998). However, these therapeutic effects are believed to be strain specific. Thus, clinical experiments studied from one strain should not be expected in other strains even from the same genus or species.

One of the most important characteristics of a probiotic strain is that it must be non-pathogenic. Also, they should exhibit some desirable properties, such as maintaining its viability during the manufacture and storage, and resistance to the physico-chemical processing of the food product. Because efficacy of probiotics is directly related to the

number of live and active culture cells ingested, it is important to perpetuate high numbers of the microorganisms during processing, storage and host ingestion. A minimum dose of greater than 10^8 viable probiotic cells has often been suggested for daily ingestion to realize the desired health benefits (Parvez et al., 2006). To date, maintaining a sufficient amount of probiotics during processing and shelf-life of the product is a primary challenge for industries and scientists. Regardless of the viability of probiotics, molecular analysis, such as gene expression, has also become a key tool in probiotic research by identifying the beneficial microorganisms in the gastrointestinal tract and unraveling their specific characteristics. Taken all together, we will propose shelf-life studies and gene expression studies based upon survival of *L. acidophilus* in yogurt. This may provide a better understanding of the relationship between survival of *L. acidophilus* and any possible negative impacts from yogurt starter cultures.

2.0

LITERATURE REVIEW

Consumption of fermented dairy products, especially yogurt, is rapidly growing. In addition to the nutritional value of these products, health benefits associated from the probiotics that are commonly used in these products, drive consumers' demand. Typical yogurt in the US is mainly fermented by two different species of bacteria (*Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*), and sometimes probiotics (*L. acidophilus* in particular) are added as adjuncts due to their potential therapeutic effects on human. This chapter will provide background information regarding probiotics, their purported health benefits and their physiological characteristics (such as survivability) in dairy foods. Some perspectives on the molecular responses to stress will also be discussed near the end of this chapter.

2.1 History and Definition of Probiotics

The concept of “probiotics” was first realized in the early 1900s by a Nobel Prize winning scientist, Elie Metchnikoff, who observed that Bulgarian peasants' average life expectancy greatly exceeded that of the general population. He associated their longevity to the frequent consumption of fermented milks, and he hypothesized that fermented milks contained beneficial bacteria, which could potentially replace harmful microorganisms in human bodies (Heller, 2001; Del Piano et al., 2006). Thereafter, Metchnikoff developed Bulgarian yogurt, which was fermented by the bacterium that he called “Bulgarian bacillus” or “*Lactobacillus bulgaricus*” (Vasiljevic and Shah, 2008). In fact, this microorganism was previously isolated from “podkvassa” (a starter for the

production of Bulgarian sour milk) by a Bulgarian physician named Stamen Grigoroff. During this time, Grigoroff also identified *Streptococcus thermophilus*. However, this microorganism did not receive much attention because it was considered a pathogen. Therefore, Metchnikoff's work was based on *L. bulgaricus*, and many of his experiments convinced his belief that this microorganism improved immune system and digestion. (Anukam and Reid, 2007). *L. bulgaricus* is now commonly used as one of the starter cultures for commercial yogurt in the US. After conducting many experiments, Metchnikoff proposed that proliferation of enteric pathogens and their toxins had deleterious effects on human health. However, these impacts could be prevented or reduced by lactic acid-producing bacteria, mainly lactobacilli, commonly found in fermented milk. Since then, his discovery and assertion have stimulated scientific discussion on the association between human health and microorganisms. More recently, the food and dairy industries have shown great interest in adding probiotics to a variety of products and marketing them as functional foods. High consumer demand has also driven the increased production of these foods.

The word “probiotics” comes from two Greek words (προ and βιοτοσ) meaning “for life” (Hamilton-Miller et al., 2003), and was initially used as an antonym of the word “antibiotic”. The first description of probiotics was proposed by Kollath in 1953. He defined “probiotics are common in vegetable food as vitamins, aromatic substances, enzymes and possibly other substances connected with vital process”. Later, many other definitions had been proposed over time (Table 2.1), and the most recent one was proposed by the Food and Agriculture Organization of the United Nations/the World Health Organization (FAO/WHO) working group (2001), which defined “probiotics” as

“live microorganisms which when administered in adequate amounts confer a health benefit on the host”. This definition implies that sufficient amounts of viable probiotic cells should be ingested at the time of consumption to exert the desirable effects.

Table 2.1. Cited definitions and descriptions of probiotics over the past 50 years

Definition and Description	Reference
Probiotics are common in vegetable food as vitamins, aromatic substances, enzymes and possibly other substances connected with vital process	Kollath, 1953
Probiotics are opposite of antibiotics	Vergin, 1954
Deleterious effects of antibiotics can be prevented by probiotic therapy	Kolb, 1955
Substances produced by microorganisms (this is interesting...the “substances” refer to metabolites instead of actual cells) which promote the growth of other microorganisms	Lilly and Stillwell 1965
Compounds that build resistance to infection in the host but do not inhibit the growth of microorganisms in vitro	Fujii and Cook, 1973
Organisms and substances which contribute to intestinal microbial balance	Parker, 1974
A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance	Fuller, 1989
A viable mono- or mixed-culture of microorganisms which applied to animal or man, beneficially affects the host by improving the properties of the indigenous microflora	Havenaar and Huis in't Veld, 1992
Live microbial culture or cultured dairy product which beneficially influences the health and nutrition of the host	Salminen, 1996
Living microorganisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition	Schaafsma, 1996
A microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract	Naidu et al. 1999
Microbial cell preparations or compounds of microbial cells that have a beneficial effect of the health and well-being of the host	Salminen et al., 1999
A preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host	Schrezenmeir and de Vrese, 2001
Live microorganisms which when administered in adequate amounts confer a health benefit on the host	FAO/WHO report, 2001

Source: Adapted from Sanders, 2008; Vasiljevic and Shah, 2008

2.2 Probiotic Selection Criteria and Their Potential Health Benefits

In order to capitalize most desired health benefits, which require their activities, probiotics should survive and grow in sufficient numbers when reached the gastrointestinal tract. Numerous selection criteria have been recommended (Reid, 1999); among them several key criteria (Table 2.2), including safety, origin of isolation, survival and stability of probiotics in the product during storage, resistance to gastric juice and bile toxicity, adhesion to gut epithelial cells, specifically pertain probiotic food applications (Charteris et al. 1998; Chandan, 2006; Vasiljevic and Shah, 2008). One of the most important characteristics of a probiotic strains is that it must be non-pathogenic to the host. Members of the genera *Lactobacillus* and *Bifidobacterium* are mainly used as probiotics. This is due to their long and safe history, and their ubiquitous nature in many mammalian gastrointestinal environments (Adams and Marteau, 1995; Kailasapathy and Chin, 2000; Percival, 1997; Salminen et al., 1998). On very rare occasions, there have been reports of infections linked to the consumption of commercial probiotics fermented products by individuals who are immunocompromised or have underlying medical conditions such as patients with HIV infection, bacteremia or endocarditis (Borriello et al., 2003). These immunocompromised patients generally are more vulnerable to infection with pathogens and have higher incidence of opportunistic infections. Moreover, there is no evidence that consumption of probiotics alone increases the risk of opportunistic infections in these patients. Therefore, the probiotic used are not necessarily related (Cannon et al., 2005; Mackey et al., 1999; Rautio et al., 1999).

It is suggested that probiotics should be of human origin if humans are the host because some health benefits might be host-specific (Lee and Salminen, 1995; Chandan,

1999). Thus, taxonomic classification of probiotic is thought to be an important criterion. The taxonomic analyses should provide information on the origin and normal habitat of the probiotic strain. However, the idea of human origin has recently been challenged by two arguments: 1) Human infants are born with virtually sterile gastrointestinal tract (FAO/WHO, 2001). The development of microflora varies among individuals and therefore there is no clear consensus on the origin of human microflora; 2) Increasing number of studies support the notion that the beneficial effects are strain-dependent. Therefore, species thought to be originated from human origin do not necessary confer the same effect to the host (FAO/WHO, 2001; Morelli, 2007; Playne, et al., 2003; Sanders et al., 1996).

Some probiotic characteristics such as the ability to attach and interact with the intestinal epithelial cells are considered essential for probiotic selection. It is generally believed that adhesion of probiotic strains to mucosal surfaces contributes to their efficacies, since adherent strains likely have a competitive advantage against normal microflora community in the gastrointestinal tract (Parvez et al., 2006). When probiotics block the microbial attachment sites in human guts, they may prevent infections caused by intestinal pathogens from daily food consumption (Tamime, 2005). Probiotics may render the intestinal milieu unfavorable to pathogens through the production of lactic acid and volatile short-chain fatty acids (acetates, propionates and butyrates) upon fermentation, which subsequently leads to a decrease in colonic pH. Probiotics may also produce other substances (such as hydrogen peroxide and bacteriocins) that are inhibitory to potential enteric pathogens.

Table 2.2. Selection criteria for potentially probiotic microorganism	
Group	Criterion ¹
Technology	Inexpensive cultivation Ease of concentration to high cell density Freeze drying stability (with cryoprotection) Process application robustness Product application robustness Genetically stable strains Phage resistance Good sensory properties (food application) Large scale production
Medical – scientific (<i>in vitro</i> selectable)	Genera of human origin Nontoxic and nonpathogenic Viability at high populations, preferably at least 10 ⁶ - 10 ⁸ cfu/g or mL Demonstrable efficacy Ability to withstand technological processes Ability to remain viable throughout the shelf life Antagonistic toward pathogenic bacteria Gastric transit tolerance Small intestinal transit tolerance Bile salt tolerance Epithelial adhesion and growth Antimicrobial production and susceptibility
Medical – scientific (<i>in vivo</i> selectable)	Amelioration of lactose maldigestion Stimulation of the immune system Diminution of cardiovascular disease risk

¹Adapted from Charteris et al. 1998; Chandan, 2006; Vasiljevic and Shah, 2008

When the epithelial cells or normal microflora of the intestines become damaged or disturbed as a results of infection or irritation, altered gut permeability and inflammation may occur (Vaughan and Mollet, 1999). As mentioned above, probiotics have the potential to prevent and/or reduce infection associated with enteric pathogens. Nevertheless, in order to attach to and colonize on epithelial cells, probiotics must survive through gastrointestinal tract after ingestion. Two main challenging factors for probiotics during transit are an extremely low pH in the stomach and high concentration

of bile salt in the intestine. Therefore, acid tolerance and bile salt resistance are desirable probiotic characteristics (Sanders et al., 1996; Chandan, 1999).

Rational selection and validation of promising probiotic candidates should be based on experimental evidence in well-designed experiments. A comprehensive evaluation should include *in vitro* data (Reid, 2005) and *in vivo* determination using animal models such as mice or pigs, and eventually in clinical studies (Holzapfel et al., 1998). As mentioned previously, a desirable probiotic strain that survive acid and bile must possess a variety of other properties, including the ability to adhere to intestinal epithelial cells, colonize the gastrointestinal tract, produce antimicrobial factors, and inhibit the growth and attachment of enteric pathogens. Additional desirable traits include stimulating and modulating immunity, inactivating procarcinogens, preventing or reducing cancer, and lowering cholesterol (Agerholm-Larsen et al., 2000; Gibson and Fuller, 2000; Holzapfel et al., 1998; Reid, 1999). Numerous studies have documented probiotics effectively mitigate a variety of gastrointestinal and extra-intestinal disorders including inflammatory bowel disease (IBD), irritable syndrome (IBS), vaginal infections, and boost immune response. Some probiotics have also been investigated in relation to atopic eczema, rheumatoid arthritis, and liver cirrhosis. Scarpellini et al. (2008) reviewed that ingestion of yogurt containing *Lactobacillus acidophilus* and *Bifidobacterium bifidum* induce the production of cytokines by enhancing the non-specific phagocytic activity of circulating granulocytes (a category of white blood cells). Besides the immunomodulation, some clinical studies demonstrated the role of some probiotics (such as *L. acidophilus* and *L. reuteri*) in lowering cholesterol (Sanders and Klaenhammer, 2001; Taranto et al., 1998). However, the results of some proposed health

benefits are conflicting. In general, the strongest clinical evidence for probiotics is related to their use in improving gut health and stimulating immune function.

Many commercially available probiotics may provide one or several proposed health benefits according to clinical studies (Parvez et al., 2006). Table 2.3 summarizes the therapeutic effects of some probiotic strains based on clinical trials (Shah, 2006). It was reported that probiotic effects do not tie to specific genus or species, but instead are strain-specific (Gorbach, 2000). Therefore, not all the strains of the same species possess beneficial activities. More importantly, each health benefit is often associated with a few groups of probiotic bacteria, implying that there is no single strain that would provide all the potential health benefits. Due to this specificity, probiotics must be identified at the strain level, and experiments should be carried out with multiple strains (Shortt, 1999).

Table2.3. Probiotic strains with peer review published evidence from clinical studies

Strain	Health Benefits in Clinical Studies	Scientifically Established Effects
<i>L. johnsonii</i> LA1	Adherence to human intestinal cells, balances intestinal flora	Mucosal adherence, immune enhancement
<i>L. acidophilus</i> NCFM	Lowering of fecal enzyme activity, improvement in lactose absorption, production of bacteriocin	Alleviation of lactose malabsorption
<i>L. rhamnosus</i> GG	Prevention of antibiotic associated, diarrhea and rotavirus diarrhea	Management of <i>Clostridium difficile</i> diarrhea, prevention of acute diarrhea, prevention of antibiotic diarrhea, reduction in fecal enzyme associated diarrhea
<i>L. casei</i> Shirota	Prevention of intestinal disturbance, balancing intestinal flora, lowering of fecal enzyme activity	
<i>L. reuteri</i>	Colonizing the intestinal tract in animal studies, shortening of duration rotavirus diarrhea, immune enhancement	Shortening of duration of rotavirus
<i>B. animalis</i> BB12	Treatment of rotavirus diarrhea, balancing intestinal flora	Shortening of duration of rotavirus
<i>S. cerevisiae</i> Boulardii	Prevention of antibiotic associated diarrhea, treatment of <i>C. difficile</i> colitis	Prevention of antibiotic associated diarrhea
<i>E. faecium</i> (Gaio)	Reduction in cholesterol	Reduction in cholesterol

Adapted from Shah (2006)

2.3 Probiotics and Dairy Products

Probiotic cultures have been exploited extensively by the dairy industry as a tool for the development of novel functional products. Today, more than 100 probiotic-containing products, including pasteurized milk, ice-cream, fermented milk, cheese, infant formula, fruit juices, single shots and oat-based products, are available worldwide (Lourens-Hattingh and Viljoen, 2001; Stanton et al., 2002; Shah, 2004). Probiotics are also exclusively available in the form of powders, tablets or capsules as dietary supplements, which are frequently produced by freeze drying method. The exposure of probiotics to freezing and dehydration may lead to cell injury and decreased viability during processing and storage. During processing, the temperature/phase changes and dehydration can lead to the damage of cell membranes, cell walls, ribosomes and DNA (Stanton et al., 2005). Moreover, membrane lipid oxidation may occur during storage. Therefore, by the time the product is consumed, viability of probiotics may be depleted to a level where the desirable health benefits cannot be provided.

As capsules or tablets frequently contain low number of viable probiotic cells, incorporation of probiotics in foods, especially in dairy products, is more common. In fact, commercial dairy products fermented by probiotic cultures have had a history of safety. Among dairy products normally sold in the US, the most familiar is yogurt. According to the standards of identity listed in the United States Code of Federal Regulations (CFR), yogurt (in section 21 CFR part 131.200) is produced by culturing with the lactic-acid producing bacteria *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* (Chandan, 2006). Additional cultures known as “probiotics” may be added for the purpose of conferring their presumptive health benefits. Whether *L.*

delbrueckii ssp. *bulgaricus* and *S. thermophilus* are considered probiotics, this is still debatable to their use because they have no promising demonstrable probiotic effects (Kailasapathy and Chin, 2000). Regardless of their non-human origins, they (especially *S. thermophilus*) have low resistance to gastric acidic conditions (Conway et al., 1987). Accordingly, they may not survive and reach the attachment sites of the intestines in great numbers. Moreover, they lack capability of adhesion in the gut (Chandan, 1999). Thus, *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* are used as the main yogurt starters, and probiotics are added as supplements. Probiotics used in commercial products today are mainly members of the genera *Lactobacillus* and *Bifidobacterium*.

As mentioned previously, probiotics should survive during transit through the gastrointestinal tract. This implies they should withstand the extreme low pH of gastric juice (pH 1-3) in the stomach, and tolerate bile salts in the small intestine. Yogurt is an effective delivery system for probiotics due to the buffering action of the milk proteins that protect probiotics from those harsh environments.

2.4 Factors Affecting the Survival of Probiotics

Some probiotics (especially bifidobacteria) are fastidious microorganisms, and are susceptible to environmental conditions, such as organic acid accumulation, high temperature, hyperosmotic pressure, low water activity, high redox potential (presence of oxygen) and the presence of inhibitory substances (Lourens-Hattingh and Viljoen, 2002). Consequently, survivability and stability of probiotics has become a marketing and technological challenge for the industry. The viability and activity of probiotics in the products have been frequently cited as a prerequisite for achieving numerous beneficial

health benefits (Galdeano and Perdigón, 2004). Therefore, in order to obtain their desirable functional properties, probiotics need to be delivered in sufficient numbers with high viable rate (Godward et al., 2000; Talwalker and Kailasapathy, 2004). A general agreement is that high dose is required, which range from 1×10^6 cfu/mL (Kurmann and Rasic, 1991; Tamime et al., 2005) to over 1×10^8 cfu/mL (Laurens-Hattingh and Viljoen, 2001). Kailasapathy and Chin (2000) suggested that, in general, the minimum therapeutic dose should be 1×10^8 to 1×10^{10} cfu/day. High dosage is likely required to compensate for the possible decline on the amount of the viable probiotic cells during processing and storage of probiotic product as well as passage through the upper and lower parts of the gastrointestinal tract.

Numerous studies were conducted in Asia, Australia, Europe and the US to determine the presence or viability of *Lactobacillus* and *Bifidobacterium* species (Vinderola et al., 2001; Lourens-Hattingh and Viljoen, 2002; Tamime, 2002; Weese, 2002; La Torre et al., 2003; Tharmaraj and Shah, 2003; Varga et al., 2003; Huys et al., 2006; Lin et al., 2006), and most showed the probiotic species were either misidentified or had low viability.

Many factors affect the viability of probiotics in a delivery system (Table 2.4), and most are dependent on particular strains. These factors include their interactions with other bacterial species, the final acidity of the product, the availability of nutrients, the presence of growth promoters (such as small peptides or free amino acids) and inhibitors (chemical or biological), osmotic pressure (concentration of sugar), oxygen content and concentration of inoculums (Shah, 2000). All microorganisms interact with their environment by utilizing components in the medium and secrete metabolic products.

Thus, the composition of the dairy product is a significance factor influencing the metabolic activities of probiotics (Heller, 2001). Addition of casein or whey protein isolates and yeast extract can enhance the growth of probiotics in milk due to their lack of the capability to synthesize most amino acids, cofactors and vitamins (Claesson et al., 2007; Desai et al., 2004; Lucas et al., 2004). Furthermore, the addition of milk protein also increases the buffering capacity of fermented milks and allows better survival of probiotics.

Table 2.4. Summary of different factors affecting the viability of probiotics

Step	Stress Factor
Preparation of probiotic cultures	Presence of organic acids during cultivation Concentration - high osmotic pressure, low water activity, higher concentration of particular ions Temperature - freezing, vacuum and spray drying Drying Prolong storage - oxygen exposure, temperature fluctuation
Production , storage and transportation of probiotic-containing products	Nutrient depletion Strain antagonism Increased acidity Positive redox potential (presence of oxygen) Presence of antimicrobial compounds (hydrogen peroxide and bacteriocins) Storage temperature
Gastrointestinal transit	Gastric acid and juices Bile salt Microbial antagonism

Source: Adapted from Vasiljevic and Shah, 2008

Since lactobacilli and bifidobacteria in general are microaerophilic and obligate anaerobic microorganisms, respectively, oxygen toxicity is an important factor affecting

their viability. Several studies have focused on the prevention of detrimental effects of oxygen on probiotics strains, including the use of anti-oxidants or oxygen scavengers (Dave and Shah, 1997b; Talwalkar and Kailasapathy, 2003; Talwalkar et al., 2004). Unlike aerobic microorganisms, which can completely reduce oxygen to water, the oxygen-scavenging system in probiotics is either reduced or completely absent due to the lack of a functional electron-transport system. This results in the incomplete reduction of oxygen to hydrogen peroxide (H_2O_2). Probiotics do not possess catalase, an enzyme needed for the decomposition of hydrogen peroxide. The intracellular accumulation of toxic oxygenic metabolites, such as superoxide anion (O_2^-), hydroxyl radical ($OH^\cdot$), and hydrogen H_2O_2 can eventually lead to cell death (Talwalkar and Kailasapathy, 2003; Villegas and Gilliland, 1998). In the production of yogurt, *L. delbrueckii* ssp. *bulgaricus* is known to produce a high amount of H_2O_2 , and this appears to decreased viability of probiotics in the product (Tamime, 2005).

In addition to the oxygen toxicity, another well-studied factor affecting the viability of probiotics is organic acid (primarily lactic acid) production during fermentation. When lactic acid content increases, pH level correspondingly decreases, and this may inhibit the survival of probiotics. Below pH 4.4, most probiotics encounter cellular damage and substantial decrease in the number of viable probiotic bacteria is usually observed. Extended refrigeration storage and transportation is also critical in maintaining the viability of probiotics. Yogurt starter cultures, particularly *L. delbrueckii* ssp. *bulgaricus*, possess β -galactosidase that is active at low pH and low temperature, which eventually increases the accumulation of lactic acids during refrigerated storage (Donkora et al., 2006). Some studies reported that the low pH is the most important factor

affecting the viability of *L. acidophilus* (most common *Lactobacillus* species incorporated in commercial yogurts in the US) and *Bifidobacterium* species (Laroia and Martin, 1991; Shah and Ravula, 2000) post-fermentation. On the other hand, Ruis et al. (1994) pointed that *L. acidophilus* has a high cytoplasmic buffering capacity (pH 3.72-7.74), which allows it to resist changes in intracellular pH and therefore gain stability under acidic conditions. However, this effect has not been documented universally for all strains.

On the contrary, yogurt starter cultures may enhance the growth and survival of probiotics by producing growth-promoting substrates or by reducing the oxygen content in milk (Kailasapathy and Rybka, 1997; Dave and Shah, 1997a; Saarela et al., 2000; Vinderola et al., 2002). *S. thermophilus* is a facultative anaerobic microorganism, which reduces the redox potential in milk by the consumption of oxygen by NADH oxidase (an enzyme catalyzed $\text{NADH} + \text{H}^+$ with the presence of O_2 to NAD and hydrogen peroxide) and pyruvate oxidase (an enzyme catalyzed pyruvate with the presence of O_2 to acetate and CO_2) (Teraguchi et al., 1987). Accordingly, this can create an anaerobic condition stimulating the growth of *L. delbrueckii* ssp. *bulgaricus* and probiotics in yogurt (Lankaputhra and Shah, 1996). In addition, *L. delbrueckii* ssp. *bulgaricus* possess proteolytic enzymes that liberate amino acids such as valine, glycine, and histidine that are essential to some probiotics (*Bifidobacteria* species) (Tamime, 2005).

Since the efficacy of probiotics is related to the viable number at the time of consumption, they must survive during the processing, storage and transit through the gastrointestinal tract. Although yogurt starters possess some benefits during fermentation, some inhibitory substances may affect the loss of viability of probiotics. However, these synergistic and antagonistic effects are still the subject of further research before a

consensus can be reached as many previous studies used different probiotic strains or yogurt cultures

2.5 *Lactobacillus* species

Lactobacilli are ubiquitous in nature, and are found in carbohydrate rich environments. They are gram-positive, non-spore forming rods or coccobacilli, catalase negative (Charteris, et al., 1997). They are fermentative, microaerophilic and chemoorganotrophic. The G+C content of their DNA is usually between 32 and 53 mol%. They occur naturally in the gastrointestinal tract (primarily small intestine) of humans and animals, in the human mouth and vagina (Felis and Dellaglio, 2007). *L. acidophilus*, *L. salivarius*, *L. casei*, *L. plantarum*, *L. fermentum*, *L. reuteri* and *L. brevis* have been the most common *Lactobacillus* species isolated from the human intestine (Mitsuoka, 1992). A few particular strains of *L. acidophilus*, *L. casei*, *L. paracasei*, *L. johnsonii*, *L. reuteri*, *L. salivarius* and *L. rhamnosus* have been extensively studied as candidates of probiotics and their functional properties and safety have been well-documented. Examples of lactobacilli used as commercial probiotic cultures are shown in Table 2.5.

Table 2.5.Examples of some *Lactobacillus* strains used in commercial applications

Species and strain	Source
<i>L. acidophilus</i> NCFB 1748	Arla (Stockholm, Sweden)
<i>L. paracasei</i> F19	
<i>L. reuteri</i> SD2112 (MM2)	Biogaia (North Carolina, USA)
<i>L. acidophilus</i> LA-1/LA-5	Chr. Hansen, Inc. (Horsholm, Denmark)
<i>L. paracasei</i> CRL 431	
<i>L. acidophilus</i> La-14	Danisco (Brabrand, Denmark)
<i>L. paracasei</i> Immunitas (DN014001)	Danone (Paris, France)
<i>L. acidophilus</i> LAFTI ® L10	DSM Food Specialties (New South Wales, Australia)
<i>L. paracasei</i> LAFTI ® L26	
<i>L. rhamnosus</i> LB21	Essum AB (Umeå, Sweden)
<i>L. rhamnosus</i> DR20 (HN001)	Fonterra Cooperative Group Ltd (Auckland, New Zealand)
<i>L. acidophilus</i> R0011	Institute Rosell (Quebec, Canada)
<i>L. rhamnosus</i> R0052	
<i>L. acidophilus</i> LB	Lacteol Laboratory (Houdan, France)
<i>L. paracasei</i> F19	Medipharm AB (Kågeröd, Sweden)
<i>L. acidophilus</i> DDS-1	Nebraska Cultures, Inc. (Lincoln, NE)
<i>L. johnsonii</i> La-1	Nestlé (Lausanne, Switzerland)
<i>L. plantarum</i> 299V	Probi AB (Lund, Sweden)
<i>L. rhamnosus</i> 271	
<i>L. acidophilu</i> NCFM ®	Rhodia (Wisconsin, USA)
<i>L. acidophilus</i> SBT-2062	Snow Brand Milk Products Co., Ltd. (Tokyo, Japan)
<i>L. salivarius</i> UCC118	University College (Cork, Ireland)
<i>L. rhamnosus</i> GR-1	Urex Biotech, Inc. (Ontario, Canada)
<i>L. reuteri</i> RC-14	
<i>L. rhamnosus</i> GG(ATCC53103)	Valio Dairy (Helsinki, Finland)
<i>L. casei</i> Shirota (YIT 9018)	Yakult (Tokyo, Japan)

Adapted from Holm, 2003; Playne, et al., 2003; Sanders, 2003; Shah, 2004.

Lactobacillus acidophilus

Among many *Lactobacillus* species, *L. acidophilus* is likely the most common probiotics for dietary use. This microorganism is classified by the Food and Drug Administration (FDA) as Generally Regarded As Safe (GRAS) (Parvez, 2006).

Approximately 80% of the yogurt manufactured in the US contains *L. acidophilus* (Sanders, 2003). In addition to its gram positive rod shape with rounded ends, the typical size of *L. acidophilus* is 0.6-0.9 µm in width and 1.5-6.0 µm in length. Its growth condition is generally enhanced under a microaerophilic environment of 5% O₂, 85% N₂, and 10% CO₂ (Shah, 2006). The optimum growth temperature is between 35°C and 40°C; it can grow at as high as 45°C.

The name “*L. acidophilus*” was derived from “acidus” meaning “acid” and “philus” meaning “loving” (Shah, 2006). This microorganism possesses the characteristics necessary to survive the harsh environmental conditions in the gut, namely, high acid content and the surface-reducing effect caused by bile salts. Although *L. acidophilus* grows slowly in milk, which is partly related to low concentration of small peptides and free amino acids, it produces high amounts of lactic acid (Vedamuthu, 2006). This may be based upon its homofermentative nature. Due to its wide range of acid tolerance (0.3-1.9% lactic acid), *L. acidophilus* can grow in slightly acidic media at pH of 4.5-6.4 (optimum growth at pH 5.5-6.0), but its growth ceases at pH 3.6-4.0. Another desirable characteristic for them to be effective probiotics is tolerance to bile salts. Though Walker and Gilliland (1993) reported that *L. acidophilus* ATCC4356 could withstand bile toxicity, it is perceived that not all strains have this ability. Hence, *in vitro* determination of bile salt tolerance should be conducted (Sanders et al., 1996).

In order to study its viability, an appropriate medium is necessary to selectively enumerate *L. acidophilus* from the products amongst other closely related species. deMan-Rogosa-Sharpe (MRS) agar is usually used as a weak selective medium for the isolation of lactic acid bacteria from most fermented food products. Many selective media for *L.*

acidophilus were therefore based on MRS (Table 2.6) (Coeuret et al., 2003; Van de Castele et al., 2006). The list of these selective media is extensive, and selection often reflects the preference of the user.

Table 2.6. Selective media for the enumeration of *L. acidophilus*, *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* from yogurts

Bacteria	Agar	Incubation conditions	Reference
<i>L. acidophilus</i>	NA-salicin agar	Anaerobic, 37°C, 48-72h	Dave and Shah, 1996
	MRS-clindamycin agar		Van de Castele et al., 2006
	MRS-sorbitol agar		Tharmaraj and Shah, 2003
	MRS-ribose agar		Lankaphuthra and Shah, 1996
	MRS-gluconate agar		Lankaphuthra et al., 1996 IDF, 1995
	MRS-arabinose agar		
	MRS-maltose agar		
	MRS-trahalose agar		
	MRS-bile agar		
	TMVC agar		
	X-Glu agar		
	RCABC agar		Darukaradhya et al., 2006
	CLBS agar		Nighswonger et al., 1996
<i>L. delbrueckii</i> ssp. <i>bulgaricus</i>	Acidified MRS agar (pH 4.58)	Anaerobic, 45°C, 48-72h	Tharmaraj and Shah, 2003
	RCA agar (pH 5.3)		Lankaphuthra and Shah, 1996
	RCPB agar (pH 5)		Rybka and Kaisalapathy, 1996
<i>S. thermophilus</i>	<i>S. thermophilus</i> agar M17 agar	Aerobic, 37°C, 48-72h	Tharmaraj and Shah, 2003 Ravula and Shah, 1998

Adapted from Coeuret et al., 2003; Van de Castele et al., 2006

2.6 Molecular Aspects of Probiotics

2.6.1 Genome of *Lactobacillus acidophilus*

The complete genome sequence of *L. acidophilus* NCFM was determined (Altermann et al. 2005). Based on sequence analysis, this microorganism encodes 20 putative peptidases, homologs of PrtP and PrtM (cell-envelope protease and membrane-

bound lipoprotein, respectively), and two complete oligopeptide transport systems (Altermann et al., 2005). Nine two-component regulatory systems were predicted; some associated with determinants implicated in bacteriocins production. *L. acidophilus* NCFM contains genes (and therefore expresses the predicted proteins) including those: involved in the digestion of fructo-oligosaccharides (Barrangou et al., 2003); tolerate acidic conditions (Azcarate-Peril et al., 2004); and affect the cell surface, cell division, and adhesion to epithelial cells (Altermann et al., 2005). It is also predicted to produce a number of stress-related proteins, including most of the highly conserved SOS regulon genes (Altermann, et al., 2005). The F₁F₀-ATPase system, which is encoded by the *atp* operon, has been well characterized in *L. acidophilus* (Kullen and Klaenhammer, 1999). Additionally, a gene *relA* is identified to encode an enzyme putatively involved in osmotolerance by synthesis and hydrolysis of (p)ppGpp (Altermann et al., 2005). All this information help understand the physiology of *L. acidophilus*, elucidate potential mechanisms on their survival during transit through gastrointestinal tract and their interaction with the intestinal mucosa and microbiota.

2.6.2 Stress Responses

For probiotic bacteria destined to be delivered to the human host, they are subjected to a variety of stresses beginning from the production of the culture, incorporation into milk products or dietary supplements, transport and storage, transit along the human GI tract, to finally at the location such as colon where they are transiently colonized (Delcour et al., 2000). When the probiotic cells are exposed to environmental stress, these stress factors (acid, hyperosmotic pressure and oxidation)

may cause protein denaturation, which may affect their survival and eventually lead to cell death (Vorob'eva, 2004). To overcome these challenges, several survival mechanisms exhibited by bacteria are normally referred to as the stress response (Kim et al., 2001), and this response is usually associated with genes encoding a group of protein called heat shock proteins (HSPs) (De Angelis and Gobbetti, 2004). These proteins are called "heat shock" because the concentration of these proteins were found to be increased shortly after the microorganism was exposed to a higher temperature; a process called heat shock response (Ang et al., 1991). In fact, the expression of HSPs can also be induced by other stress conditions, such as high salinity, alcohol and organic acids (Sugimoto et al., 2008). The HSPs are capable of re-folding nascent polypeptides or heat-denatured proteins from denaturation, assembling protein complexes and translocating proteins during cellular stress (Bukau and Horwich, 1998). During normal growth of the cell, HSPs are expressed at low concentration. When the cell is exposed to a heat shock or other stresses, the synthesis rate of HSPs markedly increases and then slowly decreases to normal levels. Among different types of HSPs, DnaK, DnaJ, GrpE, GroEL and GroES are molecular chaperones expressed in significant amounts (Craig et al., 1994). The two major subgroups of molecular chaperones consist of the complex DnaK/DnaJ/GrpE and the complex GroEL/GroES (Zink et al., 2000). Denatured proteins are first bound to the complex DnaK/DnaJ/GrpE. The re-folding mechanism by this complex is widely reviewed (Foster and Hall, 1990; de Angelis and Gobbetti, 2004). It is known that binding and release of substrate proteins are regulated by the ATPase activity of DnaK, which is under tight control by DnaJ and GrpE (Szabo et al., 1994; McCarty et al., 1995). DnaJ accelerates the rate of ATP hydrolysis of DnaK, whereas GrpE promotes the

nucleotide exchange of ATP and ADP (Liberek et al., 1991). DnaJ and GrpE have the utmost importance for the chaperone activity of DnaK *in vivo* and *in vitro* (Bukau and Horwich, 1998; Rosen and Ron, 2002). The partially folded polypeptide (protein) released from the complex DnaK/DnaJ/GrpE is passed to the complex GroEL/GroES for further folding within its cage structure (Vorob'eva, 2004).

It has been generally assumed that any stress, which triggers protein denaturation, will greatly induce the synthesis of the DnaK, DnaJ, GrpE, GroEL and GroES (Broadbent et al., 1998). In several lactic acid bacteria, such as *L. johnsonii*, *L. delbrückii* ssp. *bulgaricus*, *L. plantarum* and *L. acidophilus*, heat-shock chaperones were found to be consistently upregulated presumably due to the need to repair acid-induced damaged proteins and/or to facilitate the folding of nascent proteins (Vaughan and Mollet, 1999; Somkuti and Steinberg, 1999; Van de Guchte et al., 2002). Although Lorca et al. (2002) hypothesized that DnaK, DnaJ, GrpE, GroES and GroEL are important heat shock proteins found in *L. acidophilus*, little experimental evidence of the HSPs mechanism in this microorganism is established. Most reviews on heat shock response use *Escherichia coli*, *Bacillus subtilis*, *Lactococcus lactis* ssp. *lactis* and *Lactobacillus plantarum* as model organisms (Henderson et al., 2006; Sugimoto et al., 2008; Vorob'eva, 2004). Because of the difference in HSPs possession among lactic acid bacteria, a subset of acid-induced heat-shock proteins may vary between species (Champomier-Verges et al., 2002). Accordingly, molecular analyses are required to understand the expression of these genes/proteins in *L. acidophilus*. These analyses may include northern blots, cDNA microarrays reverse-transcription polymerase chain reaction (RT-PCR), proteomics, etc.

2.6.3 Gene Expression Analysis with RT-PCR

Precise regulation of gene expression allows an organism to respond suitably to environmental conditions. For bacteria, gene expression is regulated by specific non-coding DNA sequences called promoters, which are genetic sequences that determines when and to what extent the associated genes are transcribed. An understanding of gene regulation in probiotics may lead to better improvement of probiotic cells for food applications.

Several methods are commonly used for the quantification of transcription level; these methods include northern blotting (Boot et al., 1996), *in situ* hybridization (Parker and Barnes, 1999), RNase protection assays (Hod, 1992), reverse-transcription polymerase reaction (RT-PCR) (Weis et al., 1992) and cDNA microarray (Pieterse et al., 2005; Barrangou et al., 2006). Microarray technology is a powerful tool for analyzing the transcriptional response of microorganisms, but its application is still somewhat limited due to relatively high cost and difficulty in instrument accessibility (Bucher, 1999). Northern blotting provides the information related to mRNA size, alternative splicing and the integrity of RNA samples (Bustin, 2000). The RNase protection assay is a useful method for mapping transcript initiation and termination sites and for discrimination between related mRNAs of similar size, which would migrate at similar positions on a northern blot. Both northern blotting and RNase protection assay methods have been the major techniques used in the past to determine mRNA levels to estimate gene expression (Kelleher, 2001). *In situ* hybridization is a complex method and is the only one that allows localization of transcripts to specific cells within a sample. The limitation of these three methods is their comparatively low sensitivity. Moreover, they require large

amounts of total RNA to estimate the concentration of a specific transcript (Zamorano et al., 1996; Bustin and Nolan, 2004).

RT-PCR has become a standard technique for assessing differential expression of genes (Pfaffl, 2001; Walker, 2002), and it is the most sensitive and specific method for the detection of low-abundance mRNA (Carding et al., 1992; Bustin, 2000). It can be used to compare the levels of mRNAs in different sample populations, to characterize patterns of mRNA expression, to discriminate between closely related mRNAs, and to analyze RNA structure (Wang and Brown, 1999). Numerous studies used RT-PCR for analyzing gene expression of *Lactobacillus* species (Fitzsimons et al., 2003; Piuri et al., 2003; Spano et al., 2004; Marco and Kleerebezem; 2007; Fiocco et al., 2008). Fitzsimons et al. (2003) also suggested that RT-PCR should be applicable in assessing the *in vivo* gene expression producing both long and short half-life mRNAs from enteric probiotic bacteria. Although cDNA microarrays are becoming more sought-after among scientists, RT-PCR is still required to validate the gene expression profile (Bustin and Nolan, 2004).

The use of gene technology in dairy or food microorganisms is controversial in many parts of the world (Dellaglio, 2005). Molecular methods will be useful not only for studying the effects of probiotics on the distribution of normal intestinal flora but also the activity of the probiotic in the gastrointestinal tract. Molecular techniques will certainly play a main role in understanding the mechanism the probiotics. This understanding will help design and develop probiotics strains, and improve industrial production of probiotic food ingredients.

2.7 Objectives and Hypotheses

Probiotics are increasingly popular due to their purported health benefits. Since many health benefits require consumption of sufficient viable cells, many studies have been conducted regarding the survival of probiotics in fermented products, especially in yogurt. However, probiotic activities are strain-dependent, rendering many studies not useful due to the lack of strain identity. Additionally, in the absence of a standard selective medium for probiotic enumeration from commercial fermented milks, it is difficult to compare current published reports on probiotic survival in the products. In this thesis project, we will focus on the survival of multiple *L. acidophilus* strains in yogurt manufactured with commercial starter cultures. Furthermore, we will study the gene expression of stress genes (*dnaK* and *groEL*) in selected *L. acidophilus* strains to better understand the survival capability during refrigerated storage.

The hypotheses of this study were:

1. Some *Lactobacillus acidophilus* strains exhibit poor survival in yogurt due to the presence of yogurt starter cultures, while some strains remain stable throughout the refrigerated storage period.
2. Loss of viable cell counts of some *L. acidophilus* strains in yogurt is not due to low pH.
3. Expression of stress genes in *L. acidophilus* in yogurt is associated with survival capability.

The objectives of this study were:

1. To determine the effect of starter cultures and their metabolic products on survival of multiple *L. acidophilus* strains (ATCC700396, LA-5, NCFM, PIM703 and SBT2062)
2. To determine the survival of *L. acidophilus* in yogurt independent of starter culture fermentation by using glucono-delta-lactone as an acidulant
3. To determine gene expression of stress genes of selected *L. acidophilus* strains in yogurt during refrigerated storage period using reverse-transcription polymerase chain reaction

3.0

JUSTIFICATION

Probiotics have been widely incorporated in fermented foods for years due to their potential therapeutic effects. To realize their desirable health benefits, many publications have suggested that a high amount of viable probiotics ($>10^6$ cfu/g or mL) must be consumed to compensate for any possible loss during transit through the gastrointestinal tract of the host. Although numerous commercial products containing probiotics are available worldwide, many publications have revealed disappointing results with regards to misidentification and low viability of probiotics (less than 10^6 cfu/g or mL by the end of expiration date). However, results among these studies are inconsistent, which may be due to the difference in selective media used for probiotic enumeration.

Probiotics are fastidious microorganisms, and are highly susceptible to a variety of environmental stress, such as organic acid accumulation, high temperature and oxygen toxicity such as production of hydrogen peroxide (refer to Chapter 2.4). Consequently, maintaining a sufficient amount of viable probiotics in fermented foods throughout the shelf life has always been a great challenge to dairy industries and scientists. In the US, yogurt is perhaps the most familiar fermented milk product that is used as a probiotic delivery vehicle. Many studies demonstrated that several factors, such as lactic acid accumulation and production of hydrogen peroxide from yogurt starter cultures (especially *L. delbrueckii* ssp. *bulgaricus*), may affect probiotic survival during fermentation and storage. However, these studies normally used different strains of starter cultures or probiotics. As activity of microorganisms is strain dependent, results may vary, and it is therefore difficult to rely on few publications with different probiotic

strain use. In this project, multiple probiotic strains will be used to study their survival in various types of yogurt during refrigerated storage period. This may show strain-to-strain variation in survivability. Among these probiotic strains, *L. acidophilus* NCFM and LA-5 will be studied due to their well-documented health claims.

When probiotics are subjected to environmental stress, the expression of a group of proteins called “heat shock proteins” is upregulated. Among these proteins, DnaK and GroEL (encoded by *dnaK* and *groEL*) function as intracellular chaperones for denatured proteins by re-folding and assembling the proteins. Many studies have been conducted with the purpose of understanding the relationship between probiotic survival and the regulation of heat shock proteins (or gene). While many investigated pathogens, few studies used *L. acidophilus* as a model. As with survival capabilities of probiotics, mechanisms of gene regulation are likely strain dependent. Therefore, we will study mRNA expression of *dnaK* and *groEL* of *L. acidophilus* while they coferment with yogurt starter cultures by using reverse-transcription polymerase chain reaction. The expression of these genes will help elucidate their roles in aiding microbial survival under stressful environment. Beyond the scope of this project, genome-wide gene expression analysis will provide us a more comprehensive understanding with regards to molecular mechanisms in stress conditions. Once the mechanism of probiotic survival is better understood, later it may be more likely to optimize their viability. This will ultimately lead to more efficacious delivery of probiotic and increased health benefits from yogurt. Moreover, the identification of the heat shock genes will provide targets 1) for specific manipulation (to promote or limit growth), 2) to develop tools to screen for tolerant or sensitive strains and 3) to evaluate the level of adaptation of a strain.

Overall, this thesis project will focus on the survival and gene expression of multiple *L. acidophilus* strains in yogurt during refrigerated storage period. By understanding their physiological properties, we can formulate and/or improve a probiotic strain used in fermented dairy products with better survivability and desired therapeutic effects.

4.0

MATERIALS AND METHODS

4.1 Bacterial Strains, Propagation and Storage

Commercial yogurt starter cultures (YoFlex–L702), which contained *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, were provided from Chr. Hansen (Milwaukee, WI) in Direct Vat Set (DVS) form. To prepare frozen stocks from a commercial DVS containing *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*, approximately 25 g of DVS were transferred into a sterile whirlpak® bag (Fisher Scientific, Tustin, CA), and let thaw at room temperature. Then, a loopful of thawed concentrated suspension was streaked on MRS agar (BD Diagnostics, Sparks, MD) and was incubated at 37°C in a CO₂ incubator (Isotemp Incubator, Fisher Scientific). The aeromicrophilic condition and the presence of CO₂ in this incubator could stimulate the growth of *L. delbrueckii* ssp. *bulgaricus*. After 48 hours of incubation, two different colony morphologies were observed. Each type was tested with a Gram stain kit (BD Diagnostics) to determine their species identity –*L. delbrueckii* ssp. *bulgaricus* is gram positive bacillus whereas *S. thermophilus* is gram positive coccus. The procedure of Gram stain was as follows: on a clean microscopic slide, a single colony was mixed with a drop of water. After the suspension was air-dried and heat-fixed, the slide was stained with crystal violet for 1 min, iodine solution for 1 min, 95% ethanol for 10 s and safranin for 30 s. After each reagent added, the slide was gently rinsed with tap water. Microscopic cell morphologies were observed under a light microscope with a total magnification of 1,000X, and identification of each starter culture was further confirmed by API 50 CHL test (BioMérieux Vitek, Hazelwood, MO) according to the

manufacturer's recommended procedure (refer to section 4.2). Each starter culture was streaked for single colony isolation on M17 (*S. thermophilus*) or MRS media (*L. delbrueckii* ssp. *bulgaricus*) (Ravula and Shah, 1998). After incubated at 37°C for 24-36 h, broth media supplemented with 20% glycerol (Fisher Scientific) were used to make frozen stocks of these cultures in 1.2 mL Nalgene cryogenic vials (Fisher Scientific), which were stored at -50°C.

Five strains of *L. acidophilus* were used as probiotics in this study. These strains include NCFM, ATCC700396, PIM703, SBT2062 and LA-5. The first four strains were obtained from the Dairy Products Technology Center (DPTC) culture collection (Yeung et al., 2002) at California Polytechnic State University, San Luis Obispo, and *L. acidophilus* LA-5 was kindly provided by Chr. Hansen (Milwaukee, WI). The identity of all *L. acidophilus* strains was also confirmed by API 50 CHL and Gram stain tests.

To prepare working stocks, frozen stocks of all strains were scraped with a sterile pipette tip and transferred into 10 mL of M17 (*S. thermophilus*) or MRS (lactobacilli) broth and were incubated overnight at 37°C. On the following day, a loopful of overnight culture was streaked onto an appropriate agar medium. After incubation at 37°C for 24-36 h, all plates were stored at 4°C. Prior to each experiment, inocula of all *L. acidophilus* strains, *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* were individually prepared by sub-culturing twice in 10 mL of the appropriate medium (MRS or 10% [w/w] sterile reconstituted nonfat dry milk [NFDm]) at the rate of 1% and incubated at 37°C for 16-18 h.

4.2 Bacterial Species Identification

API 50 CHL or carbohydrate fermentation test from BioMérieux was performed for bacterial species identification. For each strain, three colonies of working stocks were heavily streaked on MRS (lactobacilli) or M17 (*S. thermophilus*) agar. After incubation at 37°C for 24-48 h, a loopful of cultures were suspended into 2 mL of sterile dH₂O. Increments of 25 µL of this suspension were added to 5 mL sterile dH₂O until the turbidity matched a McFarland Standard #2 (BioMérieux). Then, double amounts of 25 µL were added to the CHL medium (BioMérieux). After mixing by pipette up and down several times, 110 µL of this suspension were added to each cupule of the API 50 CH strips followed by overlaying with few drops of sterile mineral oil. Then, the API strips were incubated at 37°C for 24, 48 and 72 h. A positive reaction was given when the media turned from purple to yellow, except for the esculin test, which turned from purple to black. A negative reaction was interpreted when the media color remained purple. The results were input to the web-based apiweb™ System (<http://apiweb.biomerieux.com>) (BioMérieux) for speciation.

4.3 Selective Media for *Lactobacillus acidophilus*

A selective media was required to enumerate *L. acidophilus* amidst the closely related yogurt starter cultures. To this end, maltose, mannitol and sorbitol (BD Diagnostics, Franklin Lakes, NJ) were tested as dextrose replacement in MRS agar. Modified MRS preparation was made according to the ingredients shown in Table 4.1 and sterilized by autoclave at 121°C for 15 min. Disaccharide or sugar alcohol solution was prepared separately by filter sterilization through a membrane with a pore size of

0.22 μm (Corning, Fisher Scientific). The filter-sterilized solution was then added to the molten media to achieve a final concentration of 2% maltose, mannitol or sorbitol. Preliminary testing was done on all *L. acidophilus* strains, *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* by streaking them heavily on each selective media followed by incubation at 37°C for 48-72 h. Then, results were recorded as growth or no growth. The experiment was done in duplicate. M17 and MRS agar media were used as positive control for *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*, respectively. The best selective media from this experiment, which was MRS-maltose agar, was chosen to be used in subsequent experiments requiring *L. acidophilus* enumeration from samples containing *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*.

Table 4.1. Ingredients of MRS agar without dextrose per 1 liter

Ingredient	Amount (g)
Proteose peptone No.3 ¹	10
Beef extract ¹	10
Yeast extract ¹	5
Polysorbate 80 (Tween 80) ²	1
Ammonium Citrate ²	2
Magnesium Sulfate ²	0.1
Manganese Sulfate ²	0.05
Dipotassium phosphate ²	2
Sodium Acetate ²	5
Granulated agar ¹	15
Deionized water	950

¹Ingredients supplied by BD Diagnostics

²Ingredients supplied by Fisher Scientific

4.4 Enumeration of *L. acidophilus*

Standard plate count (SPC) was used to quantify viable *L. acidophilus* cells. The procedure was as follows: 1 g or mL of sample was diluted with 99 mL of sterile phosphate buffer saline (PBS), pH 7.2 from Hardy Diagnostics (Santa Maria, CA).

Subsequent 10-fold serial dilutions were then made with PBS, and 0.1 mL of the diluted samples was spread on MRS-maltose agar (section 4.3). After incubation at 37°C for 48-72 h, colony-forming units (CFU) per g or mL were calculated.

4.5 Agar Diffusion Assay

Negative impacts of starter cultures on the survival of *L. acidophilus* strains were directly assessed with the agar diffusion assay method. On MRS agar, 10 µL of overnight cultures of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* were spotted as shown in Figure 4.1. All plates were incubated at 37°C for 24 h. For each *L. acidophilus* strain (NCFM, ATCC700396, PIM703, SBT2062 or LA-5), 0.1 mL of overnight culture was inoculated into 10 mL of 0.7% molten MRS agar, and was overlaid on pre-grown starter cultures. After incubation at 37°C for 48 h, the presence of inhibition zones around the starter culture colonies was observed.

Figure 4.1. Agar diffusion assay using MRS agar spotted with overnight starter cultures (10 µL *S. thermophilus* [ST]; 10 µL *L. delbrueckii* ssp. *bulgaricus* [LB]; 10 µL *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* [M])

4.6 Survival Assays of *Lactobacillus acidophilus*

4.6.1 Effect of Yogurt Starter Cultures

To study the effect of the presence and absence of starter cultures on the survival of probiotics strains, two types of yogurt were manufactured – one made with starter cultures and one made with glucono-delta-lactone (GDL) (PMP Fermentation Products, Inc, Peoria, IL) as shown in Figure 4.2. GDL is an acidulant that gradually lowers the pH of milk. Therefore, the purpose of adding GDL was to study the effect of pH independent of any metabolic activity of starter cultures. Table 4.2 summarizes the factor and response variables for this experiment.

Yogurt mixture was made from 14% reconstituted low-heat NFDM (Dairy America, Inc., Fresno, CA) in the laboratory. For yogurt made with starter cultures, a package of 250-g starter cultures (YF-L702) was thawed in a water bath at room temperature. A 10-fold dilution of the starter cultures was made with sterile reconstituted NFDM (10% total solids), and 6 mL of this diluted culture were added into 3 L of pasteurized (heat treatment at 85°C for 30 min) reconstituted NFDM being held at 43°C. For yogurt made without starter cultures, GDL was added to the pasteurized reconstituted NFDM at a concentration of 1.6% (w/v) instead of starter cultures.

To study the survival of *L. acidophilus* strains in these two types of yogurt, they were inoculated (as separate samples) immediately after the addition of starter cultures or GDL at a concentration of 5% (v/v) which was equivalent to $\sim 10^6$ cfu/mL of milk. The pH of the mixtures was monitored periodically during the incubation at 43°C. Incubation was ceased when pH reached 4.6; upon which the mixtures became semisolid and were considered finished products. Samples were stored at 4°C for 28 days, the typical shelf

life of commercial yogurts. Aliquots of each sample were removed from the refrigerator on day 0, 7, 14, 21 and 28, and were tested for pH and viable cell counts with standard plate count procedure. Relative survival for each strain was determined by dividing the number of viable cells on day 7, 14, 21 or 28 by the initial cell count on day 0 and multiplied by 100. The experiment was carried out in triplicate; the strains demonstrating the best (SBT2062) and the poorest (NCFM) survival from this experiment were used in subsequent experiments.

Figure 4.2. Outline of yogurt production with and without starter culture fermentation

Table 4.2. Experimental design (effect of starter culture) factor and response variables

Effect of Starter Cultures in Yogurt		
Factor	Treatment Levels	Response
Acid producer	Starter Cultures	%Survival of <i>L. acidophilus</i>
	GDL	
<i>L. acidophilus</i> strain	ATCC700396	
	LA-5	
	NCFM	
	PIM703	
	SBT2062	

4.6.2 Effect of Yogurt Filtrate

To study the effect of metabolic products produced from starter cultures on probiotics survival, yogurt filtrate was collected and was added into MRS broth containing *L. acidophilus* NCFM. Yogurt filtrate was prepared by adding 100 mL of sterile phosphate buffer, pH 7.2 (Hardy Diagnostics), into 100 g of yogurt made with starter cultures or with GDL. These yogurts did not contain probiotics. The diluted yogurt mixture was prefiltered through a Whatman #42 filter (Fisher Scientific) and then a cellulose acetate membrane with a pore size of 0.22 μm (Fisher Scientific). Various amounts of sterile filtrate collected from two types of yogurt (Table 4.3) was added into MRS broth containing overnight-grown cultures of *L. acidophilus* NCFM. Enumeration of *L. acidophilus* NCFM was performed for 15 days at 3-day intervals. The experiment was carried out in duplicate.

Table 4.3. Amount of yogurt filtrate added into *L. acidophilus* NCFM

Sample	10G	9G1S	8G2S	5G5S	2G8S	1G9S	10S
YF-STLB ^a (mL)	0	1	2	5	8	9	10
YF-GDL ^b (mL)	10	9	8	5	2	1	0

^a YF-STLB is the yogurt filtrate originated from yogurt with starter cultures

^b YF-GDL is the yogurt filtrate originated from yogurt with GDL

4.6.3 Effect of Killed Starter Cultures

This experiment was conducted to investigate the survival of *L. acidophilus* strains in yogurt post-fermentation and in the presence of non-viable starter cultures. Yogurt was made as previously described in section 4.6.1 except that *L. acidophilus* strains were not added. When the pH reached 4.6, the fermentation was ceased by immediately cooling down to 4°C in an ice bath. The finished product was re-pasteurized by heating at 75°C for 15 min to kill all starter cultures. After cooling down to 4°C, 5% (v/v) of *L. acidophilus* NCFM or SBT2062 was mixed thoroughly. Enumeration of *L. acidophilus* was conducted for 15 days at 3-day intervals.

4.6.4 Effect of Individual Starter Culture Species

To further study the competition between individual species of the yogurt starter cultures and *L. acidophilus*, yogurts were made with *S. thermophilus* or *L. delbrueckii* ssp. *bulgaricus* alone using a similar procedure described in section 4.6.1. After pasteurizing 14% reconstituted NFDM, the heated milk was divided into 4 portions. To each portion, 5% (v/v) of the best survival probiotics strain (*L. acidophilus* SBT2062) or the poorest (NCFM) survival of *L. acidophilus* and 1% (v/v) of starter cultures (*S. thermophilus*, ST; *L. delbrueckii* ssp. *bulgaricus*, LB) were separately inoculated as outlined in Figure 4.3. All samples were incubated at 43°C until the pH reached 4.6. After cooling the samples to 4°C in an ice bath, enumeration of *L. acidophilus* was performed on MRS-maltose agar, and the remaining samples were stored at 4°C for 28 days. The experiment was carried out in triplicate. Table 4.4 describes the factor and response variables for this study.

Table 4.4. Experimental design (effect of individual starter culture species) factor and response variables

Effect of Individual Starter Culture Species Fermentation		
Factor	Treatment Levels	Response
Starter Culture Species	<i>S. thermophilus</i>	% Survival of <i>L. acidophilus</i>
	<i>L. delbrueckii</i> ssp. <i>bulgaricus</i>	
<i>L. acidophilus</i> strain	NCFM	
	SBT2062	

Figure 4.3. Outline of yogurt production with individual starter culture species (ST: *S. thermophilus*; LB: *L. delbrueckii* ssp. *bulgaricus*) and *L. acidophilus* strains (SBT2062 and NCFM)

4.6.5 Survival of Probiotics in Soy Milk and Reconstituted Milk

Due to the popularity of soy-based products along with probiotics, we conducted a supplemental experiment regarding the survival of *L. acidophilus* in soy milk and in reconstituted milk. Commercial plain soymilk was purchased from a local supermarket, and milk was prepared from 9% reconstituted low-heat NFDm. Prior to inoculation of *L. acidophilus* strains, soymilk and milk (9% total solids) were sterilized by autoclave (121°C for 15 min). After cooling down to 37°C, 2% (v/v) of overnight cultures (refer to section 4.1 for inoculum preparation) was added to each medium, and was incubated in a water bath at 37°C for 6 h. All samples were immediately cooled down to 4°C in an ice water bath, then stored for enumeration of *L. acidophilus* weekly for 28 days.

4.6.6 pH Measurement

Prior to pH measurement, the pH meter (Orion model 410 A plus, USA) was calibrated with standard buffer pH 4 and 7 (Fisher Scientific). Then, the pH of each sample was measured at room temperature.

4.6.7 Statistical Analysis

Log transformation was used to evaluate the relative survival of *L. acidophilus* strains, and a two-way analysis of variance (ANOVA) was performed using the general linear model in Minitab software version 15.1 to analyze the differences between samples and within *L. acidophilus* strains. Data reported in all tables were the means $\pm$ SD of two or three replicates.

4.7 Gene Expression Study of *Lactobacillus acidophilus*

To further understand the physiology of survival of *L. acidophilus* NCFM and SBT2062 in yogurt, gene expression analysis using semi-quantitative RT-PCR was performed. Table 4.5 describes the factor and response variables for this study.

Table 4.5. Experimental design (gene expression) factor and response variables

Effect of Starter Cultures on Gene Expression		
Factor	Treatment Levels	Response
Stress genes	<i>dnaK</i>	Intensity of amplification products (Intensity*mm ²)
	<i>groEL</i>	
<i>L. acidophilus</i> strain	NCFM	
	SBT2062	

4.7.1 Stress Genes and Primer Design

Seven genes related to stress response (*dnaJ*, *dnaK*, *gadC*, *groEL*, *groES*, *grpE* and *hrcA*) and one housekeeping gene (16S rDNA) were chosen to determine the gene expression profiles of *L. acidophilus* in yogurt. Species-specific primers (see Table 4.6) were designed by using Amplicon (Jarman, 2004). For each gene, DNA sequences from *L. acidophilus* NCFM (Altermann et al., 2005), *L. delbrueckii* ssp. *bulgaricus* ATCC11847 (Van de Guchte et al., 2006) and *S. thermophilus* CNRZ1066 (Bolotin et al., 2004) were aligned using CLUSTAL W version 1.4 (Higgins, 1994) in the BioEdit program (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>), and then the most unique regions to *L. acidophilus* were selected for designing the forward and reverse primers. Primers were purchased from Integrated DNA Technologies (San Diego, CA).

Table 4.6. Sequences of primer sets for *L. acidophilus*

Gene	Primer	Sequence (5' to 3')
<i>dnaJ</i>	Forward	AAGCCACCTTGACCATTGAC
	Reverse	CATGAACCTGGTGCAGAAGA
<i>dnaK</i>	Forward	GCACCACCATTGTTACCATC
	Reverse	GCTTTATCAAAAGCTGCTCAAG
<i>gadC</i>	Forward	TGGCAAGACTGGTGCTACTG
	Reverse	TCGCCCAGTGAACCTTTTAC
<i>groEL</i>	Forward	GAAATTGGTTACAATGCTGCAAC
	Reverse	CTTGAGGAGCTTCTGGCTTATC
<i>groES</i>	Forward	TTGGCGGTATTGTTTTAGCA
	Reverse	TCGTTAAAGTTAAAGAAGAAGAAG
<i>grpE</i>	Forward	TAAGTTGCGCACGTTCTTTG
	Reverse	GGCTAAAGGCGAAGAAACAA
<i>hrcA</i>	Forward	TCCTTCACATTATGCTCAGATG
	Reverse	AGTGAGCTAGGCGGAGAACA
16S rDNA	Forward	AGCAGATCGCATGATCAGCT
	Reverse	TTGCCTTCGCAGGCTTG

The specificity of the designed primers was evaluated by polymerase chain reaction (PCR) with template DNA of *L. acidophilus* NCFM and SBT2062, *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* in a GeneAmp PCR System 2400 (Perkin-Elmer, Foster City, CA). The amplification condition is described in section 4.7.3.

4.7.2 Total RNA Extraction

Extracting RNA directly from yogurt was not successfully achieved due to the high viscosity of the yogurt matrix. Therefore, yogurt samples must be diluted prior to RNA extraction: 2 g of yogurt were diluted with 6 mL of warm (~40°C) PBS. The diluted

yogurt was mixed by vortexing at room temperature for 30 sec. Then, 2 mL of the sample were added into 4 mL of RNAProtect Bacteria Reagent (Qiagen, Valencia, CA). After incubation at room temperature for 5 min, the sample was centrifuged at 3,000 x *g* at room temperature for 25 min. The supernatant was carefully removed, and the pellet was re-suspended with 1 mL of PBS. To the suspension, 0.5 mL of clearing solution [0.15M *N*-[2-Acetamido]iminodiacetic acid (ADA) (Sigma-Aldrich, Atlanta, GA); 0.01% w/v uniform polystyrene particles (Bangs Laboratories, Inc., Fisher, IN, size 0.80 µm); 0.5% w/v Triton-X100, Promega, Madison, WI] was added. After mixing by inverting 10 times, the sample was centrifuged at 14,000 x *g* for 10 min. Then, the supernatant was carefully discarded.

Total RNA extraction was performed by using RNeasy Mini kit from Qiagen (Valencia, CA). In a separate microfuge tube, 20 µL of Proteinase K (Qiagen) were added to 100 µL of TE buffer (30 mM Tris-Cl; 1 mM EDTA, pH 8.0) (Promega) containing 15 mg/mL lysozyme (Sigma-Aldrich), and the entire mixture was added to the pellet. The suspension was mixed at room temperature by vortexing every 2 min for 10 min.

To the suspension, 700 µL of Buffer RTL from the RNeasy Mini kit was added, and the entire volume was transferred to a new 2 mL safe-lock tube containing 50 mg of acid-washed glass beads (Sigma). The cells were disrupted in a Tissue Lyser at the maximum speed for 5 min. After the cell disruption step, the tube was briefly centrifuged at 14,000 x *g* for 10 sec. Then, 760 µL of the supernatant was carefully transferred to a new 2-mL microcentrifuge tube, and 590 µL of 80% ethanol (Fisher Scientific, Tustin, CA) was added. After mixing by pipetting up and down several times, the lysate was

transferred into an RNeasy Mini spin column and was centrifuged at 10,000 x g for 15 sec. Three hundred and fifty μ L of Buffer RW1 were added to the RNeasy spin column, and the column was centrifuged at 10,000 x g for 15 sec.

DNase Digestion was applied at this point to eliminate any possible DNA contamination during the RNA extraction by using RNase-Free DNase set from Qiagen (Valencia, CA). In a separate tube, 10 μ L of DNase I stock solution was added to 70 μ L Buffer RDD. Then 80 μ L of the diluted DNase I was added directly to the column membrane and was incubated at room temperature for 15 min. This step was repeated after centrifugation (at 10,000 x g for 15 sec) for efficient DNA elimination.

Three hundred and fifty μ L Buffer of RW1 was added to the column and was incubated for additional 5 min. The column was centrifuged at 10,000 x g at room temperature for 15 s. Then, the column was placed in a new 2-mL collection tube, and 500 μ L Buffer RPE was added followed by centrifugation at 10,000 x g for 15 s. After the flow-through was discarded, addition of 500 μ L Buffer RPE was repeated, and the column was centrifuged at 10,000 x g at room temperature for 2 min. The column was once again placed to a new 1.5-mL RNase-free microfuge tube, and 30 μ L of RNase-free water were added directly to the column membrane followed by centrifugation at 10,000 x g at room temperature for 1 min. This step was repeated with the RNA elute for higher RNA concentration.

The integrity of RNA was determined by gel electrophoresis using FlashGel® RNA system (Lonzo, Cottonwood, AZ). Five μ L of each sample were loaded to each well. The electrophoresis was conducted at room temperature at 225V for 5 min. Gels were stained with ethidium bromide and were visualized on a UV transilluminator

(ChemiDoc XRS imaging device, Bio-Rad, Hercules, CA). RNA concentration and purity were determined by a UV spectrophotometer at 260 and 280 nm, respectively. All samples were stored at -20°C.

4.7.3 Reverse Transcription – Polymerase Chain Reaction (RT-PCR)

Reverse transcription (mRNAs converted to cDNAs) was carried out by using Superscript III (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. All reagents and supplies used in this step were nuclease-free and were purchased from Invitrogen (Carlsbad, CA). One hundred ng of total RNA, 2.5 µL of random hexamers (100 ng/µL) and 1 µL of 10 mM dNTPs were mixed in a 0.2-mL microcentrifuge tube. Then, nuclease-free water was added to a total volume of 13 µL. The mixture was heated to 65°C for 5 min and was incubated on ice for at least 1 min. After a brief centrifugation, 4 µL of 5X First-Strand buffer, 1 µL of 0.1 M DTT, 1 µL of RNaseOUT™ Recombinant RNase Inhibitor and 1 µL of SuperScript™III RT were added to the contents. The sample was mixed carefully by pipette up and down followed by incubating at 25°C for 5 min, 50°C for 60 and 70°C for 15 min. After cooling down to 37°C, 2 units of *E. coli* RNase H were added to remove DNA:RNA hybrids as the sample was incubated at 37°C for 20 min. All cDNA samples were stored at -20°C until use.

PCR was then conducted using the above cDNA as templates and the primer sets specified in Table 4.6 in a GeneAmp PCR System 2400 (Perkin-Elmer). For each gene, a reaction mixture of 50 µL consisted of 5 µL of 10X buffer without MgCl₂ (Roche Applied Science), 3 µL of 25 mM MgCl₂ (Roche), 0.5 µL of 10 mM dNTP mix (Invitrogen), 1.5 µL of 10 µM forward and reverse primers, 0.25 µL of 5U/µL *Taq*

Polymerase (Roche), 2 μL of template DNA and 36.25 μL of nuclease-free water (Invitrogen). The thermocycling condition was programmed as follows: 94°C for 5 min; 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min; and 72°C for 10 min. Ten μL of the PCR products were electrophoresed in 1.5% (w/v) agarose gel (Fisher Scientific) in 0.5X TBE buffer (44.5 mM Tris-borate and 1 mM EDTA, pH 8.3) (Sigma) containing ethidium bromide (Fisher Scientific) (final concentration 0.5 $\mu\text{g/mL}$) at 90 V for 45 min. Gels were visualized with a UV transilluminator (ChemiDoc XRS imaging device, Bio-Rad), and the density (units of signal intensity) of each band was quantified using Quantity One® Software program version 4.6.4 (Bio-Rad).

5.0

RESULTS

5.1 Identification of Bacterial Species

Carbohydrate fermentation (API 50 CH) was performed on seven strains (five *Lactobacillus acidophilus*, one *Lactobacillus delbrueckii* ssp. *bulgaricus*, and one *Streptococcus thermophilus*) to confirm the bacterial species from the Dairy Products Technology Center (DPTC) culture collection and from the manufacturer. After 24, 48 and 72 hours of incubation, a numeric value of 0 to 5 was given according to the color of the media. Values of 0-2 (negative results) represent media color change from purple to blue, and values of 3-5 (positive results) denote the color change from purple to yellow. After inputting the data to the manufacturer's web-based program (<http://apiweb.biomerieux.com>), speciation of each bacterial strain was returned with relative identification and profile status as seen in Table 5.1. The profile status describes the reliability of the speciation. The carbohydrate fermentation raw data are shown in Appendix A.

Table 5.1. Speciation results obtained from carbohydrate fermentation tests

Bacteria ID provided by the supplier	Speciation Result	Relative Identification	Profile Status	Microscopic Cell Morphology
<i>L. acidophilus</i> ATCC700396	<i>L. acidophilus</i>	99.0%	Good	Gram positive rods
<i>L. acidophilus</i> LA-5	<i>L. acidophilus</i>	99.0%	Good	Gram positive rods
<i>L. acidophilus</i> NCFM	<i>L. acidophilus</i>	99.0%	Good	Gram positive rods
<i>L. acidophilus</i> PIM703	<i>L. acidophilus</i>	99.0%	Good	Gram positive rods
<i>L. acidophilus</i> SBT2062	<i>L. acidophilus</i>	97.9%	Good	Gram positive rods
<i>L. delbrueckii</i> ssp. <i>delbrueckii</i>	<i>L. delbrueckii</i> ssp. <i>delbrueckii</i>	92.8%	Doubtful	Gram positive rods
<i>S. thermophilus</i>	<i>S. thermophilus</i>	64.7%	Low discrimination	Gram Positive cocci

5.2 Selective Media for *Lactobacillus acidophilus*

Three selective media, MRS-maltose, MRS-mannitol and MRS-sorbitol agars, were preliminarily tested as selective media for *L. acidophilus* but not the yogurt starter cultures. After 48 hours of incubation at 37°C, all five *L. acidophilus* strains grew on these media, while no growth of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* was observed. The colony morphology of *L. acidophilus* on MRS-maltose and MRS-mannitol agars was slightly whitish (PIM703 was white and creamy) with colony size of 1-2 mm. However, a technical disadvantage of mannitol was that its solubility was fairly low, and re-crystallization of this sugar alcohol occurred quickly. For MRS-sorbitol agar, the colony size of all *L. acidophilus* strains was pin-point and translucent, rendering colony counting rather difficult. Therefore, MRS-maltose agar was selected for the enumeration of *L. acidophilus* from yogurt samples containing *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*.

5.3 Determination of Survival of *Lactobacillus acidophilus*

5.3.1 Effect of Yogurt Starter Cultures

Enumeration of *L. acidophilus* at various time points during the refrigerated storage period was determined by standard plate count on MRS-maltose. To evaluate the difference in the strains' survival in two types of yogurts (made with GDL or starter cultures), the mean relative survival of three replicates was plotted as shown in Figures 5.1 and 5.2. The initial viable cell counts post-fermentation of *L. acidophilus* strains (day 0) ranged from 1×10^7 to 1×10^8 cfu/g. During the refrigerated storage period, the pH of both types of yogurts stayed at ~pH 4.6 (ranged from 4.59 to 4.62). Statistical analysis

was performed using a two-way analysis of variance (ANOVA) with the log relative survival as the response variable, the yogurt type (GDL or starter cultures) and *L. acidophilus* strain (ATCC700396, LA-5, NCFM, PIM703 and SBT2062) as factors. All relative survival data were transformed using a based-10 logarithm (log relative survival), and a value of 0.00001 was added to all data values, since some values were zero. The transformation for statistical analysis was performed due to the correction of normality and constant variance tests. Although, among *L. acidophilus* strains in yogurt in the absence of starter cultures (i.e., yogurt made with GDL), the p-values also demonstrated a significant difference ($p < 0.05$), survival of all strains remained stable throughout the storage period with a maximum of 1 log reduction (NCFM and PIM703) (see Table 5.2 and Figure 5.1), and *L. acidophilus* SBT2062 exhibited the best relative survival.

On the other hand, throughout the 28-day storage period of the standard yogurt (made with starter cultures), survival of five probiotic strains showed strain-to-strain variation as shown in Figure 5.2 suggesting a distinction in probiotic survival behavior within the same species. Among these five strains, the best survival was *L. acidophilus* SBT2062, and the poorest survival was *L. acidophilus* NCFM exhibiting a 4.6-log reduction, followed by ATCC700396 and LA-5 (3.0-log and 1.6-log reduction, respectively)(see Table 5.3 and Figure 5.2). The drastic drops of *L. acidophilus* NCFM and ATCC700396 started on day 14, and the final viable cell counts on day 28 were 1.3×10^4 and 1.1×10^5 , respectively. Statistical analysis on the average relative survival of *L. acidophilus* in regular yogurt showed that the survival of NCFM was significantly lowered than that of LA-5, PIM703 and SBT2062 ($p < 0.05$).

By analyzing the survival of *L. acidophilus* based on the presence and absence of starter cultures in yogurt, we expected to see difference in the survival behavior of some strains due to some inhibitory effects from starter cultures (refer to Chapter 2.4). After 28-day storage period, *L. acidophilus* ATCC700396 and NCFM showed that there is a significant difference in survival in two types of yogurts ($p < 0.05$), which indicates that the survival of these two strains was adversely affected by the presence of starter cultures. Since *L. acidophilus* SBT2062 and NCFM exhibited the best and the poorest survival in both types of yogurts, respectively, these strains were subsequently used in other experiments.

Table 5.2. Viable cell count of *L. acidophilus* strains in yogurt made with GDL during 28 days of storage period at 4°C. Means and standard deviations were calculated from three replicates.

<i>L. acidophilus</i> Strain	Colony forming unit (CFU)/g ($\times 10^6$)				
	Day 0	Day 7	Day 14	Day 21	Day 28
ATCC700396	39.0 $\pm$ 21.0	21.8 $\pm$ 7.6	23.0 $\pm$ 13.5	21.2 $\pm$ 19.9	15.1 $\pm$ 14.7
LA-5	84.0 $\pm$ 20.0	75.7 $\pm$ 16.0	73.7 $\pm$ 14.5	52.2 $\pm$ 28.7	29.2 $\pm$ 15.6
NCFM	48.3 $\pm$ 10.5	33.9 $\pm$ 10.6	23.3 $\pm$ 14.9	10.7 $\pm$ 9.8	7.4 $\pm$ 8.0
PIM703	33.3 $\pm$ 7.1	27.5 $\pm$ 6.1	24.9 $\pm$ 10.3	13.7 $\pm$ 5.1	6.0 $\pm$ 1.9
SBT2062	11.4 $\pm$ 6.0	13.2 $\pm$ 9.0	13.2 $\pm$ 7.53	11.7 $\pm$ 6.1	11.0 $\pm$ 5.8

Figure 5.1. Relative survival of five different *L. acidophilus* strains in yogurt made with GDL during 28 days of storage period at 4°C.

Table 5.3. Viable cell count of *L. acidophilus* strains in yogurt made with starter cultures during 28 days of storage period at 4°C. Means and standard deviations were calculated from three replicates.

<i>L. acidophilus</i> Strain	Colony forming unit (CFU)/g ($\times 10^6$)				
	Day 0	Day 7	Day 14	Day 21	Day 28
ATCC700396	99.7 $\pm$ 34.2	53.8 $\pm$ 25.3	6.6 $\pm$ 7.1	1.0 $\pm$ 0.8	0.1 $\pm$ 0.1
LA-5	136.0 $\pm$ 33.9	73.0 $\pm$ 35.2	35.7 $\pm$ 52.2	21.6 $\pm$ 33.7	3.3 $\pm$ 5.1
NCFM	61.2 $\pm$ 18.1	10.4 $\pm$ 10.0	0.9 $\pm$ 1.2	0.1 $\pm$ 0.1	0.01 $\pm$ 0.02
PIM703	68.3 $\pm$ 21.5	51.2 $\pm$ 21.3	31.5 $\pm$ 23.5	14.3 $\pm$ 9.6	19.5 $\pm$ 30.8
SBT2062	105.0 $\pm$ 10.0	97.7 $\pm$ 45.6	93.7 $\pm$ 53.2	69.6 $\pm$ 69.6	94.0 $\pm$ 67.2

Figure 5.2. Relative survival of five different *L. acidophilus* strains in yogurt made with starter cultures during 28 days of storage period at 4°C.

5.3.2 Effect of Yogurt Filtrate

To determine if yogurt starter cultures produced secretory metabolic inhibitory to the *L. acidophilus* strains, yogurt filtrate was collected and was added directly to overnight-grown cultures of *L. acidophilus* NCFM (the poorest survival from section 5.3.1). The final volume of each sample was brought to equal amount with yogurt filtrate originated from yogurt made with GDL instead of dI H₂O or PBS to control for possible variations due to nutrient loss. Figure 5.3 shows the average relative survival of two replicates of *L. acidophilus* NCFM in MRS with varying amounts of filtrate from

different types of yogurt. Survival of *L. acidophilus* NCFM in all samples remained stable for 15 days, except it exhibited 1.7-log reduction (2.2×10^9 to 4.1×10^7 cfu/mL) in the treatment where the 2 mL of “yogurt filtrate” and 8 mL of “GDL filtrate” was added. which (Table 5.4). Statistical analysis was performed using one-way ANOVA using the log relative survival as the response variable, yogurt filtrate amount as the factor. The transformation of relative survival values was similarly performed as mentioned in section 5.3.1. Yogurt filtrate was found to pose no negative effect on *L. acidophilus* survival.

Table 5.4. Viable cell count of *L. acidophilus* NCFM in MRS broth containing various amounts of yogurt filtrate during 15 days of storage period at 4°C. Means and standard deviations were calculated from two replicates.

Treatment	Colony forming unit (CFU)/g ($\times 10^7$)					
	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15
10G	34.5 ± 7.8	28.7 ± 6.9	13.4 ± 5.6	9.6 ± 3.4	7.2 ± 3.1	5.9 ± 3.2
9G1S	33.0 ± 3.5	27.4 ± 0.2	13.0 ± 1.7	10.8 ± 4.7	8.4 ± 4.0	6.3 ± 3.9
8G2S	60.0 ± 33.2	29.3 ± 1.1	13.0 ± 1.9	7.8 ± 0.3	7.5 ± 3.7	6.8 ± 1.3
5G5S	32.5 ± 2.8	34.1 ± 4.9	8.4 ± 4.2	5.1 ± 2.4	3.8 ± 2.9	3.7 ± 2.9
2G8S	216.0 ± 247.0	31.7 ± 4.7	9.98 ± 6.3	7.2 ± 5.2	5.8 ± 3.9	4.1 ± 3.0
1G9S	41.3 ± 15.2	30.0 ± 9.9	8.4 ± 1.2 ⁷	7.4 ± 2.3	6.1 ± 1.8	4.5 ± 0.7
10S	34.8 ± 7.4	30.7 ± 3.9	7.5 ± 0.6	6.0 ± 0.7	4.6 ± 1.5	4.1 ± 0.6

Figure 5.3. Relative survival of *L. acidophilus* NCFM in MRS broth containing various amounts of yogurt filtrate (GDL [G] and starter cultures [S]) during 15 days of storage period at 4°C.

5.3.3 Effect of Killed Starter Cultures

Another approach was taken to further study if starter cultures produced metabolites that are inhibitory to *L. acidophilus*. Yogurt was pasteurized to eliminate the effect of viable starter cultures still present in yogurt. Yogurt made with GDL was also heated with the same pasteurization condition for comparison. During the 15-day storage period, *L. acidophilus* NCFM and SBT2062 showed steady survival with a maximum of 0.9-log reduction (1.4×10^7 to 9.9×10^6 cfu/g) in both types of yogurt (Table 5.5 and Figure 5.4). Since the experiment was done only once, statistical analysis was not performed. However, the results from this preliminary study indicate that killed starter cultures do not impair the survival of *L. acidophilus* NCFM and SBT2062.

Table 5.5. Viable cell count (CFU/g) of *L. acidophilus* strains (NCFM and SBT2062) in pasteurized yogurt during 15 days of storage period at 4°C.

Sample	Colony forming unit (CFU)/g				
	Day 0	Day 3	Day 9	Day 12	Day 15
GNCFM^a	1.4×10^7	1.4×10^7	8.1×10^6	8.4×10^6	1.2×10^7
SNCFM^b	1.1×10^7	1.3×10^7	9.5×10^6	1.1×10^7	9.8×10^6
GSBT2062^c	1.4×10^7	1.1×10^7	1.3×10^7	8.2×10^6	9.9×10^6
SSBT2062^d	1.5×10^7	1.1×10^7	1.1×10^7	1.2×10^7	1.2×10^7

^a GNCFM = *L. acidophilus* NCFM in pasteurized yogurt made with GDL

^b SNCFM = *L. acidophilus* NCFM in pasteurized yogurt made with starter cultures

^c GSBT2062 = *L. acidophilus* SBT2062 in pasteurized yogurt made with GDL

^d SSBT2062 = *L. acidophilus* SBT2062 in pasteurized yogurt made with starter cultures

Figure 5.4. Relative survival of *L. acidophilus* NCFM and SBT2062 in pasteurized yogurts made with GDL (G) or starter cultures (S) during 15 days of storage period at 4°C.

5.3.4 Effect of Individual Starter Culture Species

To study the antagonistic effects imposed by each starter culture species on the survival of *L. acidophilus* NCFM and SBT2062, two types of yogurts were made: one with *S. thermophilus* alone and another with *L. delbrueckii* ssp. *bulgaricus* alone. The fermentation duration (to acidify milk to pH 4.6) for both types of yogurt was approximately 6 hours. Viable cell counts of *L. acidophilus* were monitored weekly for 28 days. Figure 5.5 shows the mean $\pm$ SD relative survival of *L. acidophilus* NCFM and SBT2062 in both types of yogurts from three replicates. *L. acidophilus* NCFM exhibited the poorest survival in yogurt made with *L. delbrueckii* ssp. *bulgaricus* alone, showing a 2.5-log reduction (4.0×10^7 to 1.3×10^5 cfu/g). *L. acidophilus* SBT2062 showed a 0.6-log reduction in yogurt made with *L. delbrueckii* ssp. *bulgaricus* alone. On the other hand, these two strains survived well in yogurt made with *S. thermophilus* alone (Table 5.6 and Figure 5.5). Statistical analyses on transformed data were performed as described in section 5.3.1. The type of starter cultures (*S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*) and *L. acidophilus* strain (NCFM and SBT2062) were analyzed as factors. The p-values from Minitab demonstrated the difference was not significant, except that a marginal significant difference was observed in the survival of *L. acidophilus* NCFM in yogurt made with *L. delbrueckii* ssp. *bulgaricus* with that of SBT2062 in yogurt made with *S. thermophilus* ($p=0.0569$).

Table 5.6. Viable cell count (CFU/g) of *L. acidophilus* strains (NCFM and SBT2062) in yogurt with *L. delbrueckii* ssp. *bulgaricus* or *S. thermophilus* during 28 days of storage period at 4°C. Means and standard deviations were calculated from three replicates.

Sample	Colony forming unit (CFU)/g (x10 ⁶)				
	Day 0	Day 7	Day 14	Day 21	Day 28
NCFM-ST^a	68.7 ± 20.2	46.0 ± 43.2	29.2 ± 46.6	21.3 ± 36.5	21.8 ± 25.1
NCFM-LB^b	39.6 ± 30.8	3.6 ± 3.3	0.4 ± 0.5	0.1 ± 0.2	0.1 ± 0.2
SBT2062-ST^c	119.0 ± 15.2	129.0 ± 14.8	125.0 ± 25.7	127.0 ± 35.8	127.0 ± 33.9
SBT2062-LB^d	71.2 ± 6.3	47.7 ± 14.0	29.2 ± 10.8	14.4 ± 2.2	18.3 ± 6.3

^a NCFM-ST = *L. acidophilus* NCFM in yogurt made with *S. thermophilus*

^b NCFM-LB = *L. acidophilus* NCFM in yogurt made with *L. delbrueckii* ssp. *bulgaricus*

^c SBT2062-ST = *L. acidophilus* SBT2062 in yogurt made with *S. thermophilus*

^d SBT2062-LB = *L. acidophilus* SBT2062 in yogurt made with *L. delbrueckii* ssp. *bulgaricus*

Figure 5.5. Relative survival of *L. acidophilus* NCFM and SBT2062 in yogurt made with individual starter culture species (*S. thermophilus* or *L. delbrueckii* ssp. *bulgaricus*) during 28 days of storage period at 4°C (NCFM in yogurt made with *S. thermophilus* (NCFM-ST); NCFM in yogurt made with *L. delbrueckii* ssp. *bulgaricus* (NCFM-LB); SBT2062 in yogurt made with *S. thermophilus* (SBT2062-ST); SBT2062 in yogurt made with *L. delbrueckii* ssp. *bulgaricus* (SBT2062-LB)).

5.3.5 Survival of *L. acidophilus* in Soy Milk and Milk

Soy milk has always been an alternative product for lactose intolerant or non-dairy consumers due to the absence of lactose. To deliver probiotics to this consumer group, soy milk should be an effective vehicle that does not affect the viability of the probiotic strains. Therefore, survival of four *L. acidophilus* strains in soy milk were investigated as a preliminary study; and the results were compared with data using (cow) milk. The relative survival in milk and in soy milk was plotted in Figure 5.6 and 5.7, respectively. Table 5.7 shows that the viable cell counts (CFU/mL) of all four *L. acidophilus* strains in milk remained stable throughout the 28-day storage period with a maximum of 0.6-log reduction for ATCC700395 (2.6×10^7 to 6.5×10^6 cfu/mL). In contrast, survival of *L. acidophilus* in soy milk exhibited strain-to-strain variation as observed in Figure 5.7; the poorest survival strain was *L. acidophilus* NCFM with 2.8-log reduction (6.5×10^6 to 9.6×10^3 cfu/g; Table 6.7) followed by *L. acidophilus* ATCC700396 and SBT2062 with 2.47-log and 1.60-log reduction (1.7×10^7 to 5.8×10^4 cfu/mL and 8.0×10^7 to 2.0×10^6 cfu/mL; Table 5.8), respectively. A drastic drop of the relative survival of all four strains in soy milk was noted on day 14.

Table 5.7. Viable cell count (CFU/mL) of *L. acidophilus* strains in milk during 28 days of storage period at 4°C.

<i>L. acidophilus</i> Strain	Colony forming unit (CFU)/mL				
	Day 0	Day 7	Day 14	Day 21	Day 28
NCFM	2.1×10^7	3.1×10^7	1.5×10^7	3.6×10^7	5.0×10^7
ATCC700396	2.6×10^7	3.9×10^7	2.7×10^7	4.1×10^7	6.5×10^7
PIM703	4.0×10^7	4.9×10^7	6.5×10^7	2.5×10^7	1.7×10^7
SBT2062	9.0×10^7	1.1×10^8	1.0×10^8	9.6×10^7	9.3×10^7

Figure 5.6. Relative survival of *L. acidophilus* strains in milk during 28 days of storage period at 4°C.

Table 5.8. Viable cell count (CFU/mL) of *L. acidophilus* strains in soymilk during 28 days of storage period at 4°C.

<i>L. acidophilus</i> Strain	Colony forming unit (CFU)/mL				
	Day 0	Day 7	Day 14	Day 21	Day 28
NCFM	6.5×10^6	7.4×10^6	2.4×10^6	1.2×10^5	9.6×10^3
ATCC700396	1.7×10^7	5.9×10^6	1.8×10^6	2.9×10^5	5.8×10^4
PIM703	1.2×10^6	1.7×10^6	1.5×10^6	3.8×10^5	1.8×10^5
SBT2062	8.0×10^7	5.6×10^7	3.1×10^7	7.0×10^6	2.0×10^6

Figure 5.7. Relative survival of *L. acidophilus* strains in soymilk during 28 days of storage period at 4°C.

5.4 Agar Diffusion Assay

The diffusion assay used in this experiment aimed to determine if inhibitors were produced from *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* cells against *L. acidophilus*. In this assay, the presence of inhibitors would be indicated by an inhibition zone, or a clearance ring, around the producers. *L. acidophilus* NCFM was chosen in this experiment because its viability was the most impacted from previous experiments (section 5.3.1, 5.3.3 and 5.3.4). We hypothesized that this strain was more sensitive to the inhibitors produced by the starter cultures. Figure 5.8 shows an experiment with *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* grown on MRS, either alone or as a mixture, followed by overlaying with *L. acidophilus* NCFM. After incubation, a lawn of

culture of *L. acidophilus* NCFM was developed and no distinct inhibition zone was observed around the starter cultures. Fuzzy zones appeared around *L. delbrueckii* ssp. *bulgaricus* (LB) and the mixture of both species (M) colonies.

Figure 5.8. Agar diffusion assay on MRS agar spotted with starter cultures (ST: *S. thermophilus*; LB: *L. delbrueckii* ssp. *bulgaricus*; M: *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*) using *L. acidophilus* NCFM as an indicator.

5.5 Expression of Stress Genes in *Lactobacillus acidophilus*

5.5.1 Designing Species-Specific Primers

Primers specific to *L. acidophilus* were designed on eight genes (seven stress and one housekeeping genes). For each gene, the specificity of the primers was determined by polymerase chain reaction using DNA from *L. acidophilus* NCFM and/or SBT2062, *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* as template. A PCR mix without DNA template was used as negative control. Based on sequence homology to published

sequence, all primers were expected to solely bind to DNA template of *L. acidophilus* NCFM and SBT2062. However, empirical data (Fig. 5.9) showed that only two stress genes (*dnaK* and *groEL*) and the housekeeping gene (16S rDNA) were specific to *L. acidophilus* strains. The rest cross-reacted with yogurt starter cultures to various degrees. Therefore, *dnaK*, *groEL* and 16S rDNA genes were further studied in the subsequent experiments.

Figure 5.9. A 1.5% agarose gel of PCR products using species-specific primers to stress genes (*dnaK*, *dnaJ*, *grpE* and *groES*, *hrcA*, *gadC* and *groEL*). Gels were stained using ethidium bromide, and images were taken using a BioRad ChemiDoc XRS Densitometer. Lanes 1, 5, 9, 13, 17, 21, 25- *L. acidophilus* NCFM; lanes 2, 6, 10, 14, 18, 22- *L. acidophilus* SBT2062; lanes 3, 7, 11, 15, 19, 23, 26- *L. delbrueckii* ssp. *bulgaricus*; and lanes 4, 8, 12, 16, 20, 24, 27- *S. thermophilus*; *- 100bp Ladder (100-1000 bp in 100 bp increments); N- negative control

5.5.2 Semi-Quantitative Reverse-Transcription-Polymerase Chain Reaction (RT-PCR)

The purpose of this experiment was to provide preliminary data on the stress response of *L. acidophilus* at a molecular level. Yogurt was similarly prepared as described in Section 4.6.1. After a 14-day storage period of yogurt, PCR reaction was

conducted, and the amplicons were electrophoresed through 1.5% agarose gel containing ethidium bromide at 90 V for 50 min. The gel was visualized using a BioRad ChemiDoc XRS Densitometer (see Figure 5.10), and the adjusted volume (intensity*mm²), which is the reading with background subtracted, of each band was quantified using Quantity® One software. To normalize the adjusted volume values (intensity*mm²), the ratio of each sample was calculated by dividing the adjusted volume of the stress gene (*dnaK* or *groEL*) by the adjusted volume of the housekeeping gene (16S rDNA). Results for all samples are shown in Table 5.9. A comparison of the average adjusted volume (intensity*mm²) between *dnaK* and *groEL* is plotted in Figure 5.11. This figure shows that the expression of *groEL* of both *L. acidophilus* strains remained steady during the 14-day refrigerated storage period, while that of *dnaK* of *L. acidophilus* NCFM increased as the storage time increased. Statistical analysis was performed using one-way analysis of variance (ANOVA) with the ratio of intensity as the response variable, and showed that the average ratio of intensity of these two genes was significantly different ($p < 0.01$) for both strains. By the end of day 14, expression of *dnaK* of both strains was the strongest, suggesting that this gene was required to counteract the stressful conditions in yogurt.

Figure 5.10. A 1.5% agarose gel of PCR products of *dnaK*, *groEL* and 16S rDNA genes. Gels were stained using ethidium bromide, and images were taken using a BioRad ChemiDoc XRS Densitometer. Lanes 1, 9, 17- negative control; lanes 2, 10, 18- *L. acidophilus* NCFM from day1; lanes 3, 11, 19- *L. acidophilus* SBT2062 from day1; lanes 4, 12, 20- NCFM from day7; lanes 5, 13, 21- SBT2062 from day7; lanes 6, 14, 22- NCFM from day14; lanes 7, 15, 23- SBT2062 from day14; and lanes 8, 16, 24- positive control using DNA template of *L. acidophilus* NCFM; *- 100bp Ladder (100-1000 bp in 100 bp increments)

Table 5.9. Average adjusted volume (intensity*mm²) comparison of *dnaK* gene from *Lactobacillus acidophilus* NCFM and SBT2062 in yogurt during 14 days of storage period at 4°C. Means and standard deviations were calculated from two replicates.

Strain	Refrigerated Storage Period (day)		
	1	7	14
NCFM	0.2079 ± 0.0770	0.2271 ± 0.0469	0.3257 ± 0.1104
SBT2062	0.2817 ± 0.0739	0.1904 ± 0.0080	0.2971 ± 0.1654

Figure 5.11. Average adjusted volume (intensity*mm²) comparison of *dnaK* gene from *Lactobacillus acidophilus* NCFM and SBT2062 in yogurt during 14 days of storage period at 4°C.

6.0

DISCUSSION

6.1 Bacterial identification

The purpose of identifying the microorganisms used in this study was to validate the species identification of the bacterial strains, which were previously isolated (Yeung et al., 2002) or provided by the manufacturer. Identities of all microorganisms used in this study were verified with Gram stain and API 50 CH test. Gram stain is a screening method to differentiating bacteria into two large groups based upon their cell wall composition. Microscopically, *Lactobacillus* species and *Streptococcus thermophilus* are gram positive (purple after Gram stain) rods and cocci, respectively. These results confirmed the morphology as mentioned previously in section 2.4. API 50 CH is a common biochemical test for further bacterial identification based on the ability of the microorganisms to ferment different types of carbohydrates. This test is widely used in many scientific research studies for bacteria identification (Champagne et al., 2008; Hedberg et al., 2008; Klein et al., 1998; Nighswonger et al., 1996). In our study, commercial starter cultures (*L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*) obtained from the manufacturer fermented lactose, and lactose and saccharose, respectively, and lactose was the disaccharide fermented by both microorganisms. For *L. acidophilus*, carbohydrate fermentation profile varied among strains, suggesting that carbohydrate utilization is a strain-dependent trait. Hamilton-Miller and Shah (1998) reported that commercial kits did not always provide accurate species identification results. Therefore, further tests (e.g. pulsed field gel electrophoresis or 16S rDNA sequencing) are desired for bacterial identification. In accordance with Yeung et al. (2002), who previously

isolated and identified *L. acidophilus* ATCC700396, NCFM, PIM703 and SBT2062 by pulsed field gel electrophoresis, our API 50 CH results confirm their findings, although DNA typing methods were not performed in this study.

6.2 Survival of *Lactobacillus acidophilus* in Yogurt

Delivering probiotics via food vehicle has been an interest to food scientists for decades due to their purported health-associated benefits to customers. As mentioned in Chapter 2, maintaining viable probiotic cell count above the recommendation level ($>10^6$ cfu/g or mL) by the end of the expiration date is required for most health benefit. Nevertheless, it has been a challenge since probiotics are subjected to various environmental stresses during food production and process, thereby decreasing their survival. Since most commercial fermented dairy products in worldwide contain viable probiotic cells less than the recommendation level, foundation of factors negatively affecting survival of probiotics can lead to improvement of better probiotic strains as well as formulation of better fermented products. In this project, we studied the survival of *L. acidophilus* in four different conditions to determine 1) the effect of yogurt starter cultures; 2) the effect of yogurt filtrate; 3) the effect of pasteurized yogurt; and 4) the effect of individual starter culture species fermentation.

Many studies have reported that one of the most important contributing factors for the loss of cell viability of probiotics is low pH and/or the accumulation of organic acids, especially lactic acid (produced mainly by *L. delbrueckii* ssp. *bulgaricus*), in the products (Lourens-Hattingh and Viljeon, 2001; Kailasapathy and Chin, 2000; Vinderola et al., 2002). Since there is a correlation between acid accumulation and low pH (the lactic

acid increases, pH level correspondingly decreases), well-designed experiments regarding these two factors on the survival of probiotics are important. Kailasapathy et al. (2007) studied the survival of *L. acidophilus* LAFTI® L10 in stirred fruit yogurt and concluded that the pH from the fruit preparation might influence the viability of the probiotics.

On the other hand, Donkor et al. (2006) showed that the decrease in cell numbers of probiotics in yogurt was, in fact, the result of organic acids (lactic acid and acetic acid) accumulation rather than the actual pH value. Additionally, Azcarate-Peril et al. (2004) found that *L. acidophilus* NCK1398 drastically lost its viability in acidified MRS broth with lactic acid, whereas no loss was detected when exposed to acidified MRS broth with hydrochloric acid to the same pH. In our study, we also found that low pH alone did not explain the loss of viable cell count of all five *L. acidophilus* strains (ATCC700396, NCFM, LA-5, PIM703 and SBT2062) because the survival of all *L. acidophilus* strains in yogurt made with GDL was stable throughout the 28 days of refrigerated storage period. According to Lorca and de Valdez (2001a; 2001b), this may be due to the acid tolerance response in *L. acidophilus*. In the presence of organic acids, the F₁F₀-ATPase played an important role in maintaining the intracellular pH (pH_i). Moreover, the inducible pH homeostasis system, mediated by a proton-translocating ATPase as a mechanism to increase the pH_i, might be an important mechanism in the success of *L. acidophilus* to survive low pH environment. Therefore, we speculate that this may be due their acid tolerance response, since low pH can serve as an important signal for the induction of this mechanism (Lorca et al., 2002). Another explanation is that *L. acidophilus* has high cytoplasmic buffering capacity (pH 3.72-7.73), which may increase its stability and allow the microorganism to resist changes in pH_i under acidic conditions (Ruis et al., 2000).

From our study, we found that the survival of some *L. acidophilus* strains, especially ATCC700396 and NCFM, were negatively affected by the presence of starter cultures, *L. delbrueckii* ssp. *bulgaricus* in particular. In yogurt made with starter cultures, the survival of *L. acidophilus* NCFM decreased markedly after 14 days of refrigerated storage period, whereas *L. acidophilus* SBT2062 remained stable, indicating strain-to-strain variation in survivability. To determine if the negative impact is caused by a particular individual starter culture species, yogurts fermented with *S. thermophilus* alone or *L. delbrueckii* ssp. *bulgaricus* alone were made. Our results show that survival of *L. acidophilus* NCFM was markedly declined in the presence of *L. delbrueckii* ssp. *bulgaricus*, but remained stable in the presence of *S. thermophilus*. According to Tamime (2005), *L. delbrueckii* ssp. *bulgaricus* is known for its post-acidification and high production of hydrogen peroxide (H_2O_2). As mentioned previously that low pH in yogurt was not the main factor impairing the survival of *L. acidophilus*. Therefore, the negative impact on survival of *L. acidophilus* NCFM was presumably based upon the presence of *L. delbrueckii* ssp. *bulgaricus* and/or its metabolites (such as H_2O_2). Due to its lack of catalase (an enzyme for decomposition of H_2O_2) along with the presence of H_2O_2 , *L. acidophilus* is subject to oxidative stress, and this may lead to protein and DNA damage and eventually lead to cell death.

Gilliland and Speck (1997) demonstrated the instability of *L. acidophilus* in yogurt with *L. delbrueckii* ssp. *bulgaricus*. They found that the addition of catalase (an enzyme, which hydrolyze H_2O_2 to water and oxygen, and therefore, reduce the level of H_2O_2 in yogurt) improved the viability of *L. acidophilus*. They also suggested that *L. delbrueckii* ssp. *bulgaricus* was responsible for the loss in viability of *L. acidophilus*

when added in yogurt. Therefore, exclusion of *L. delbrueckii* ssp. *bulgaricus* in yogurt production has been suggested to eliminate the antagonistic effects caused by this starter culture (Lourens-Hattingh and Viljeon, 2001).

In agreement with Dave and Shah (1997a), they demonstrated that H_2O_2 was higher in yogurts manufactured from starter cultures, which contained *L. delbrueckii* ssp. *bulgaricus* along with *S. thermophilus*, *L. acidophilus* and bifidobacteria, than in yogurt with only *S. thermophilus* and probiotics. Moreover, they found that viable cell counts of *L. acidophilus* markedly decreased in yogurt with *L. delbrueckii* ssp. *bulgaricus*, which might be responsible for the production of H_2O_2 . According to Talwalkar and Kailasapathy (2003), another enzyme known as NADH peroxidase in *L. acidophilus* 2400 and 2409 can scavenge the presence of H_2O_2 . However, this enzyme functions optimally at pH 5, and the activity of the enzyme markedly dropped at pH 4.5. Since the pH of yogurt is ~pH 4.6, NADH peroxidase may not be as active. Thus, further studies regarding the activity of NADH peroxidase of *L. acidophilus* NCFM and SBT2062 in milk and yogurt at various pH may be conducted to better understand the mechanism of the enzyme and the survival of *L. acidophilus*.

To study the effect of metabolites (such as H_2O_2) produced from yogurt starter cultures, we conducted a survival study using yogurt filtrate and an agar diffusion assay spotted with starter cultures using *L. acidophilus* NCFM as an indicator. During the incubation of agar diffusion assay, *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* were presumed to produce some metabolites that can inhibit the growth of *L. acidophilus*. However, no inhibition zone was observed around *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*. This also agrees with our survival study of *L. acidophilus* NCFM in MRS

broth with yogurt filtrate that the survival of *L. acidophilus* NCFM remained stable throughout the 15-refrigerated storage period. To further study the effect of metabolites in yogurt, we conducted an additional survival study of *L. acidophilus* in pasteurized yogurt. Similarly, survival of *L. acidophilus* NCFM and SBT2062 was not significantly different. Both strains remained stable during the 15-day refrigerated storage period. With respect to our results, the loss of viable cells of *L. acidophilus* in yogurt may not be due to metabolites from starter cultures.

We also proposed a supplemental experiment regarding the survival of *L. acidophilus* NCFM in soymilk, which is an alternative product for lactose intolerance consumers. Our results show that except for *L. acidophilus* SBT2062, all other strains (ATCC700396, NCFM and PIM703) exhibited poor survival during the 28-day storage. This might be due to the lack of fermentable carbohydrates to the probiotics. Soymilk contains about 1 percent of fermentable carbohydrate (stachyose and raffinose); however, these carbohydrates are not fermentable by most lactic acid bacteria. Wang et al. (2002) reported that soymilk could support the growth of *L. acidophilus* CCRC 14079, but, in our study, three of four *L. acidophilus* strains had $<10^6$ CFU/mL after 28-day storage (the final cfu/ml does not sound very low). In addition, Canganella et al. (2000) demonstrated that the survivability of *L. acidophilus* in cowmilk yogurt and soymilk yogurt and speculated that survival of probiotics might be higher in soymilk yogurt than in cowmilk yogurt. As mentioned previously that survival of microorganisms is strain dependent, *L. acidophilus* strains used in our study may be more susceptible to soymilk environment.

6.3. Expression of stress genes in *L. acidophilus*

From our survival studies, we found that there was a strain-to-strain variation in survival of *L. acidophilus*. Consequently, we questioned that the variation in this survival property might be due to the difference at a molecular level, gene expression in particular. To address this question, total RNA was extracted and was transcribed into cDNA followed by DNA amplification. The amplicons were then electrophoresed. This entire process is known as “RT-PCR”. However, due to the homogeneity of gene sequences among *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus* and *L. acidophilus* strains, selection of specific primers for *L. acidophilus* was strongly challenging, especially highly conserved regions. Therefore, out of our selected seven stress genes, primers specific to *L. acidophilus* were successfully designed for only two genes (*dnaK* and *groEL*).

Gene expression of two stress responsive genes (*dnaK* and *groEL*) of *L. acidophilus* in yogurt during 14 days of refrigerated storage period was conducted as a preliminary study to understand the difference in survival capability between two strains of *L. acidophilus* (NCFM and SBT2062) at a molecular level. In yogurt, microorganisms may be subjected to many environmental stresses, such as high acidity, the presence of inhibitors (lactic acid and H₂O₂) and low temperature. Consequently, we hypothesized that these various types of stress present in yogurt made with starter cultures may strongly affect the survival of *L. acidophilus* NCFM. After conducting the study of gene expression, we found that *dnaK* was highly expressed in both strains (NCFM and SBT2062), whereas the expression of *groEL* was not observed. Moreover, the expression of *dnaK* in *L. acidophilus* NCFM was positively associated with the storage time. There

was a difference in gene expression of *dnaK* in *L. acidophilus* NCFM and SBT2062, indicating that the physiology with respect to gene expression capacity was strain specific. Although the results were not significantly different, we speculated that *dnaK* was important in maintaining the survival of both *L. acidophilus* strains in yogurt during the storage period. In correlation with Lorca et al. (2002), they demonstrated that GroES, GrpE and DnaJ of *L. acidophilus* were highly expressed after exposure to a synthetic medium at pH 5.0. As mentioned in section 2.6.2, DnaJ and GrpE have the utmost importance for the chaperone activity of DnaK *in vivo* and *in vitro* (Bukau and Horwich, 1998; Rosen and Ron, 2002). The partially folded polypeptide (protein) released from the DnaK chaperone is passed to the GroES chaperone for further folding within its cage structure (Vorob'eva, 2004). From our study, expression of *groEL* was not observed; therefore, most denatured proteins may be successfully re-folded by the complex DnaK/DnaJ/GrpE. Sugimoto et al. (2008) also reviewed that a *dnaK* mutant of *Lactococcus lactis* ssp. *lactis* (one of well-studied lactic acid bacteria) showed thermosensitivity, which can create cross-responses (such as acid and alcohol sensitivity). With the limitation of primer design in this study, we could not determine the expression of *grpE*, *dnaJ* and other stress responsive genes. Thus, further experiments regarding these stress-related genes would be interesting to address the importance of the complex DnaK/DnaJ/GrpE and GroEL/GroES. This can also elucidate the mechanisms of survival action of this probiotic species as well as others.

7.0

CONCLUSIONS

Probiotics are widely incorporated into fermented dairy products, especially yogurt, due to their purported health-promoting effects. As viability of probiotics is crucial for delivery most desirable health benefit, the survival capabilities and physiological characteristics of several strains of *Lactobacillus acidophilus* in yogurt were addressed in this thesis project. Two primary studies were performed: 1) survival studies of multiple strains of *L. acidophilus*, and 2) gene expression analyses of *dnaK* and *groEL* of selected *L. acidophilus* strains (NCFM and SBT2062).

The survival of *L. acidophilus* in yogurt during 28 days of storage period showed strain-to-strain variation. Among the strains used in this study, the survival of *L. acidophilus* SBT2062 was the best and that of *L. acidophilus* NCFM the poorest. In addition, the results demonstrated that the presence of starter cultures, predominantly *L. delbrueckii* ssp. *bulgaricus*, had a significant detrimental effect on the survival of *L. acidophilus* NCFM, presumably due to the production of hydrogen peroxide.

To understand the variation of survivability between these two *L. acidophilus* strains, gene expression of two stress response genes (*dnaK* and *groEL*) was conducted. Overall, this experiment demonstrated that *dnaK* is highly expressed in both strains, whereas the expression of *groEL* was not observed. Moreover, the expression of *dnaK* of *L. acidophilus* NCFM is associated with the storage time; the level increases as storage time increases. This indicates that *dnaK* is highly expressed under refrigerated storage conditions in yogurt, suggesting that it is necessary to maintain the viability of *L. acidophilus*. Although results from the experiment exploring the gene expression

between two *L. acidophilus* strains were not significantly different, this thesis project provides preliminary results for future studies. Additional studies on more genes would still be useful to elucidate the molecular mechanisms of survival capability of this probiotic species as well as others. Other gene candidates including *dnaJ*, *grpE*, *groES*, *hrcA* and *gadC* should be investigated to provide additional understanding into the physiology and function of *L. acidophilus*. The new insights will help develop novel strains or optimize existing strains with robust survival and a suite of desirable properties. Accordingly, this would ultimately guarantee the delivery of certain probiotic strains conferring therapeutic effects to the consumers.

8.0

FUTURE RESEARCH

Additional research for further exploration in the relationship between the survivability and stress response of probiotics in fermented dairy products during storage period may provide a better understanding in their physiological characteristics and mechanisms. Following is a list of possible research directions:

1. Perform survival study on other commercial *Lactobacillus* and *Bifidobacterium* species
2. Further investigate the effect of soy- and milk-based media on the survival of probiotics to validate milk-based medium is a better delivery system for probiotics
3. Measure the amount of hydrogen peroxide and lactic acid in yogurt made with commercial starter cultures and GDL for the purpose of comparing the inhibitors produced from *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*
4. Additional experiments on the survival of *L. acidophilus* in acidified milk using lactic acid and GDL to verify the effect of organic acids in yogurt
5. Optimize species-specific primers and further study the gene expression of other stress response genes, such as *dnaJ*, *grpE*, *groES*, *hrcA* and *gadC*, of *L. acidophilus* using reverse-transcription polymerase chain reaction to correlate the survival capability and expression of these genes during refrigerated storage period

6. Develop a method for extracting mRNA from probiotics in the presence of yogurt starter cultures so that gene expression profile can be determine by using cDNA microarray analysis

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APPENDIX A. Carbohydrate fermentation profile using API 50 CH after 24 hours of incubation at 37°C. Positive reaction is indicated by a color change from purple to yellow (purple to black for esculin), and negative reaction is assigned when the color remain purple. A score of 0 to 2 was given to purple to blue color, and 3 to 5 then was given to green to yellow color. Numerical scores were converted to positive or negative before speciation.

Legend:

No.	Carbohydrate
0	Control
1	Glycerol
2	Erythriol
3	D-Arabinose
4	L-Arabinose
5	Ribose
6	D-Xylose
7	L-Xylose
8	Adonitol
9	β -Methyl-xyloside
10	Galactose
11	D-Glucose
12	D-Fructose
13	D-Mannose
14	L-Sorbose
15	Rhamnose
16	Dulcitol
17	Inositol
18	Mannitol
19	Sorbitol
20	α -Methyl-D-manniside
21	α -Methyl-D-glucosamine
22	N-Acetyl-glucosamine
23	Amygdaline
24	Arbutine

No.	Carbohydrate
25	Esculine
26	Salicine
27	Cellobiose
28	Maltose
29	Lactose
30	Melibiose
31	Saccharose
32	Trehalose
33	Inuline
34	Melezitose
35	D-Raffinose
36	Amidone
37	Glycogene
38	Xylitol
39	β -Gentiobiose
40	D-Turanose
41	D-Lyxose
42	D-Tagatose
43	D-Fucose
44	L-Fucose
45	D-Arabitol
46	L-Arabitol
47	Gluconate
48	2-Ceto-gluconate
49	5-Ceto-gluconate

Strain	#	Carbohydrate No.																								
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
ATCC 700396 ¹	1	0	0	0	0	0	0	0	0	0	0	5	5	5	0	0	0	0	0	0	0	0	0	5	4	0
	2	0	0	0	0	0	0	0	0	0	0	5	5	5	0	0	0	0	0	0	0	0	0	5	4	0
	3	0	0	0	0	0	0	0	0	0	0	5	5	5	3	0	0	0	0	0	0	0	0	5	3	0
LA-5 ¹	1	0	0	0	0	0	0	0	0	0	0	5	5	5	3	0	0	0	0	0	0	0	0	5	3	0
	2	0	0	0	0	0	0	0	0	0	0	5	5	5	3	0	0	0	0	0	0	0	0	5	3	0
NCFM ¹	1	0	0	0	0	0	0	0	0	0	0	4	5	5	0	0	0	0	0	0	0	0	0	5	4	0
	2	0	0	0	0	0	0	0	0	0	0	5	5	5	0	0	0	0	0	0	0	0	0	5	4	0
	3	0	0	0	0	0	0	0	0	0	0	5	5	5	0	0	0	0	0	0	0	0	0	5	3	5
PIM 703 ¹	1	0	0	0	0	0	0	0	0	0	0	0	5	5	5	0	0	0	0	0	0	0	0	5	5	0
	2	0	0	0	0	0	0	0	0	0	0	0	5	5	5	0	0	0	0	0	0	0	0	5	4	0
	3	0	0	0	0	0	0	0	0	0	0	0	5	5	5	0	0	0	0	0	0	0	0	5	5	0
SBT 2062 ¹	1	0	0	0	0	0	0	0	0	0	0	5	5	5	5	0	0	0	0	0	0	0	0	5	0	0
	2	0	0	0	0	0	0	0	0	0	0	5	5	3	5	0	0	0	0	0	0	0	0	0	0	0
ST ²	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LB ³	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Strain	#	Carbohydrate No.																									
		25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	
ATCC 700396 ¹	1	5	5	4	5	4	0	5	5	0	0	5	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0
	2	5	5	5	5	5	0	5	5	0	0	5	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0
	3	5	5	5	5	5	0	5	5	0	0	5	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0
LA-5 ¹	1	5	5	5	5	5	0	5	5	0	0	5	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0
	2	5	5	4	5	5	0	5	5	0	0	5	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0
NCFM ¹	1	5	5	5	5	5	0	5	5	0	0	5	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0
	2	5	5	5	5	5	0	5	5	0	0	5	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0
	3	5	5	5	5	5	0	5	5	0	0	5	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0
PIM 703 ¹	1	5	5	5	4	5	0	5	5	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0
	2	5	5	5	4	5	0	5	5	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0
	3	5	5	5	3	5	0	5	5	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0
SBT 2062 ¹	1	5	5	3	5	5	0	5	5	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	5	5	3	5	5	0	5	5	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ST ²	1	0	0	0	0	5	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	5	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LB ³	1	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

¹Lactobacillus acidophilus strains

²Streptococcus thermophilus

³Lactobacillus delbrueckii ssp. bulgaricus

APPENDIX B. Carbohydrate fermentation profile using API 50 CH after 48 hours of incubation at 37°C. Positive reaction is indicated by a color change from purple to yellow (purple to black for esculin), and negative reaction is assigned when the color remain purple. A score of 0 to 2 was given to purple to blue color, and 3 to 5 then was given to green to yellow color. Numerical scores were converted to positive or negative before speciation.

Strain	#	Carbohydrate No.																								
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
ATCC 700396 ¹	1	0	0	0	0	0	0	0	0	0	0	5	5	5	5	0	0	0	0	0	0	0	0	5	4	0
	2	0	0	0	0	0	0	0	0	0	0	5	5	5	5	0	0	0	0	0	0	0	0	5	5	0
	3	0	0	0	0	0	0	0	0	0	0	5	5	5	5	0	0	0	0	0	0	0	0	5	3	0
LA-5 ¹	1	0	0	0	0	0	0	0	0	0	0	5	5	5	5	0	0	0	0	0	0	0	0	5	5	0
	2	0	0	0	0	0	0	0	0	0	0	5	5	5	5	0	0	0	0	0	0	0	0	5	5	0
NCFM ¹	1	0	0	0	0	0	0	0	0	0	0	4	5	5	5	0	0	0	0	0	0	0	0	5	4	0
	2	0	0	0	0	0	0	0	0	0	0	5	5	5	5	0	0	0	0	0	0	0	0	5	3	0
	3	0	0	0	0	0	0	0	0	0	0	5	5	5	5	0	0	0	0	0	0	0	0	5	3	0
PIM 703 ¹	1	0	0	0	0	0	0	0	0	0	0	0	5	5	5	0	0	0	0	0	0	0	0	5	5	0
	2	0	0	0	0	0	0	0	0	0	0	0	5	5	5	0	0	0	0	0	0	0	0	5	5	0
	3	0	0	0	0	0	0	0	0	0	0	0	5	5	5	0	0	0	0	0	0	0	0	5	5	0
SBT 2062 ¹	1	0	0	0	0	0	0	0	0	0	0	5	5	5	5	0	0	0	0	0	0	0	0	5	0	0
	2	0	0	0	0	0	0	0	0	0	0	5	5	5	5	0	0	0	0	0	0	0	0	5	0	0
ST ²	1	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LB ³	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Strain	#	Carbohydrate No.																									
		25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	
ATCC 700396 ¹	1	5	5	5	5	5	0	5	5	0	0	5	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0
	2	5	5	5	5	5	0	5	5	0	0	5	0	0	0	5	0	0	0	0	0	0	0	0	0	0	
	3	5	5	5	5	5	0	5	5	0	0	5	0	0	0	5	0	0	0	0	0	0	0	0	0	0	
LA-5 ¹	1	5	5	5	5	5	0	5	5	0	0	5	0	0	0	5	0	0	0	0	0	0	0	0	0	0	
	2	5	5	5	5	5	0	5	5	0	0	5	0	0	0	5	0	0	0	0	0	0	0	0	0	0	
NCFM ¹	1	5	5	5	5	5	5	5	5	0	0	5	0	0	0	4	0	0	0	0	0	0	0	0	0	0	
	2	5	5	5	5	5	0	5	5	0	0	5	0	0	0	4	0	0	0	0	0	0	0	0	0		
	3	5	5	5	5	5	0	5	5	0	0	5	0	0	0	3	0	0	0	0	0	0	0	0	0		
PIM 703 ¹	1	5	5	5	5	5	0	5	5	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	
	2	5	5	5	5	5	0	5	5	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0		
	3	5	5	5	5	5	0	5	5	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0		
SBT 2062 ¹	1	5	5	5	5	5	0	5	5	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	2	5	5	5	5	5	0	5	5	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0		
ST ²	1	0	0	0	0	5	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	2	0	0	0	0	5	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
LB ³	1	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	2	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

¹*Lactobacillus acidophilus* strains

²*Streptococcus thermophilus*

³*Lactobacillus delbrueckii* ssp. *bulgaricus*

APPENDIX C. Carbohydrate fermentation profile using API 50 CH after 72 hours of incubation at 37°C. Positive reaction is indicated by a color change from purple to yellow (purple to black for esculin), and negative reaction is assigned when the color remain purple. A score of 0 to 2 was given to purple to blue color, and 3 to 5 then was given to green to yellow color. Numerical scores were converted to positive or negative before speciation.

Strain	#	Carbohydrate No.																								
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
ATCC 700396 ¹	1	0	0	0	0	0	0	0	0	0	0	5	5	5	5	0	0	0	0	0	0	0	0	5	4	0
	2	0	0	0	0	0	0	0	0	0	0	5	5	5	5	0	0	0	0	0	0	0	0	5	5	0
	3	0	0	0	0	0	0	0	0	0	0	5	5	5	5	0	0	0	0	0	0	0	0	5	5	0
LA-5 ¹	1	0	0	0	0	0	0	0	0	0	0	5	5	5	5	0	0	0	0	0	0	0	0	5	5	0
	2	0	0	0	0	0	0	0	0	0	0	5	5	5	5	0	0	0	0	0	0	0	0	5	5	0
NCFM ¹	1	0	0	0	0	0	0	0	0	0	0	5	5	5	5	0	0	0	0	0	0	0	0	5	3	0
	2	0	0	0	0	0	0	0	0	0	0	5	5	5	5	0	0	0	0	0	0	0	0	5	5	0
	3	0	0	0	0	0	0	0	0	0	0	5	5	5	5	0	0	0	0	0	0	0	0	5	5	0
PIM 703 ¹	1	0	0	0	0	0	0	0	0	0	0	5	5	5	5	0	0	0	0	0	0	0	0	5	5	0
	2	0	0	0	0	0	0	0	0	0	0	3	5	5	5	0	0	0	0	0	0	0	0	5	5	0
	3	0	0	0	0	0	0	0	0	0	0	0	5	5	5	0	0	0	0	0	0	0	0	5	5	0
SBT 2062 ¹	1	0	0	0	0	0	0	0	0	0	0	5	5	5	5	0	0	0	0	0	0	0	0	5	0	0
	2	0	0	0	0	0	0	0	0	0	0	5	5	5	5	0	0	0	0	0	0	0	0	5	0	0
ST ²	1	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LB ³	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Strain	#	Carbohydrate No.																								
		25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49
ATCC 700396 ¹	1	5	5	5	5	5	0	5	5	0	0	5	0	0	0	5	0	0	0	0	0	0	0	0	0	0
	2	5	5	5	5	5	4	5	5	0	0	5	0	0	0	5	0	0	0	0	0	0	0	0	0	0
	3	5	5	5	5	5	0	5	5	0	0	5	0	0	0	5	0	0	0	0	0	0	0	0	0	0
LA-5 ¹	1	5	5	5	5	5	0	5	5	0	0	5	0	0	0	5	0	0	0	0	0	0	0	0	0	0
	2	5	5	5	5	5	0	5	5	0	0	5	0	0	0	5	0	0	0	0	0	0	0	0	0	0
NCFM ¹	1	5	5	5	5	5	4	5	5	0	0	5	0	0	0	4	0	0	0	0	0	0	0	0	0	0
	2	5	5	5	5	5	0	5	5	0	0	5	0	0	0	5	0	0	0	0	0	0	0	0	0	0
	3	5	5	5	5	5	0	5	5	0	0	5	0	0	0	3	0	0	0	0	0	0	0	0	0	0
PIM 703 ¹	1	5	5	5	5	5	0	5	5	0	0	4	0	0	0	5	0	0	0	0	0	0	0	0	0	0
	2	5	5	5	5	5	0	5	5	0	0	4	0	0	0	5	0	0	0	0	0	0	0	0	0	0
	3	5	5	5	5	5	0	5	5	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0
SBT 2062 ¹	1	5	5	5	5	5	0	5	5	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	5	5	5	5	5	0	5	5	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ST ²	1	0	0	0	0	5	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	5	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LB ³	1	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

¹Lactobacillus acidophilus strains

²Streptococcus thermophilus

³Lactobacillus delbrueckii ssp. bulgaricus

APPENDIX D. Statistical results of survival of *L. acidophilus* using Minitab software version 15.1

1. Effect of Yogurt Starter Cultures on Day 28 (General Linear Model: log prop [log survival] versus Block, Yogurt type, Bacteria)

Factor	Type	Levels	Values
Block	random	3	1, 2, 3
Yogurt type	fixed	2	GDL, STLB
Bacteria	fixed	5	ATCC700396, LA5, NCFM, PIM703, SBT2062

Analysis of Variance for log prop, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Block	2	1.5995	1.5995	0.7998	2.24	0.136
Yogurt type	1	21.0696	21.0696	21.0696	58.93	0.000
Bacteria	4	22.9581	22.9581	5.7395	16.05	0.000
Yogurt type*Bacteria	4	11.8573	11.8573	2.9643	8.29	0.001
Error	18	6.4352	6.4352	0.3575		
Total	29	63.9198				

S = 0.597923 R-Sq = 89.93% R-Sq(adj) = 83.78%

Unusual Observations for log prop

Obs	log prop	Fit	SE Fit	Residual	St Resid
13	0.04140	-1.09194	0.37816	1.13334	2.45 R

R denotes an observation with a large standardized residual.

Tukey 95.0% Simultaneous Confidence Intervals

Response Variable log prop

All Pairwise Comparisons among Levels of Yogurt type*Bacteria

Yogurt type = GDL

Bacteria = ATCC700396 subtracted from:

Yogurt

type	Bacteria	Lower	Center	Upper
GDL	LA5	-1.734	0.017	1.767
GDL	NCFM	-2.264	-0.514	1.237
GDL	PIM703	-1.994	-0.243	1.507
GDL	SBT2062	-1.264	0.486	2.236
STLB	ATCC700396	-4.538	-2.788	-1.038
STLB	LA5	-3.429	-1.679	0.071
STLB	NCFM	-5.594	-3.844	-2.094
STLB	PIM703	-2.437	-0.687	1.063
STLB	SBT2062	-1.387	0.363	2.113

Yogurt

type	Bacteria	-----+-----+-----+-----
GDL	LA5	(---*---)
GDL	NCFM	(---*---)
GDL	PIM703	(---*---)
GDL	SBT2062	(---*---)
STLB	ATCC700396	(---*---)
STLB	LA5	(---*---)
STLB	NCFM	(---*---)
STLB	PIM703	(---*---)
STLB	SBT2062	(---*---)
		-----+-----+-----+-----

-3.5   0.0   3.5

Yogurt type = GDL

Bacteria = LA5 subtracted from:

Yogurt

type	Bacteria	Lower	Center	Upper
GDL	NCFM	-2.280	-0.530	1.220
GDL	PIM703	-2.010	-0.260	1.490
GDL	SBT2062	-1.281	0.469	2.219
STLB	ATCC700396	-4.555	-2.805	-1.054
STLB	LA5	-3.446	-1.696	0.055
STLB	NCFM	-5.611	-3.861	-2.110
STLB	PIM703	-2.454	-0.704	1.046
STLB	SBT2062	-1.404	0.346	2.097

Yogurt

type	Bacteria	
GDL	NCFM	(---*---)
GDL	PIM703	(---*---)
GDL	SBT2062	(---*---)
STLB	ATCC700396	(---*---)
STLB	LA5	(---*---)
STLB	NCFM	(---*---)
STLB	PIM703	(---*---)
STLB	SBT2062	(---*---)

-----+-----+-----+-----
-3.5   0.0   3.5

Yogurt type = GDL

Bacteria = NCFM subtracted from:

Yogurt

type	Bacteria	Lower	Center	Upper
GDL	PIM703	-1.480	0.270	2.020
GDL	SBT2062	-0.751	0.999	2.750
STLB	ATCC700396	-4.025	-2.275	-0.524
STLB	LA5	-2.916	-1.165	0.585
STLB	NCFM	-5.081	-3.330	-1.580
STLB	PIM703	-1.924	-0.174	1.576
STLB	SBT2062	-0.874	0.877	2.627

Yogurt

type	Bacteria	
GDL	PIM703	(---*---)
GDL	SBT2062	(---*---)
STLB	ATCC700396	(---*---)
STLB	LA5	(---*---)
STLB	NCFM	(---*---)
STLB	PIM703	(---*---)
STLB	SBT2062	(---*---)

-----+-----+-----+-----
-3.5   0.0   3.5

Yogurt type = GDL

Bacteria = PIM703 subtracted from:

Yogurt

type	Bacteria	Lower	Center	Upper
GDL	SBT2062	-1.021	0.729	2.479
STLB	ATCC700396	-4.295	-2.545	-0.794
STLB	LA5	-3.186	-1.435	0.315

STLB	NCFM	-5.351	-3.600	-1.850
STLB	PIM703	-2.194	-0.444	1.306
STLB	SBT2062	-1.144	0.607	2.357

Yogurt

type	Bacteria	-----+-----+-----+-----
GDL	SBT2062	(---*---)
STLB	ATCC700396	(---*---)
STLB	LA5	(---*---)
STLB	NCFM	(---*---)
STLB	PIM703	(---*---)
STLB	SBT2062	(---*---)

-----+-----+-----+-----
-3.5   0.0   3.5

Yogurt type = GDL
 Bacteria = SBT2062 subtracted from:

Yogurt

type	Bacteria	Lower	Center	Upper
STLB	ATCC700396	-5.024	-3.274	-1.524
STLB	LA5	-3.915	-2.165	-0.414
STLB	NCFM	-6.080	-4.330	-2.579
STLB	PIM703	-2.923	-1.173	0.577
STLB	SBT2062	-1.873	-0.123	1.628

Yogurt

type	Bacteria	-----+-----+-----+-----
STLB	ATCC700396	(---*---)
STLB	LA5	(---*---)
STLB	NCFM	(---*---)
STLB	PIM703	(---*---)
STLB	SBT2062	(---*---)

-----+-----+-----+-----
-3.5   0.0   3.5

Yogurt type = STLB
 Bacteria = ATCC700396 subtracted from:

Yogurt

type	Bacteria	Lower	Center	Upper	-----+-----+-----+-----
STLB	LA5	-0.641	1.109	2.8594	(---*---)
STLB	NCFM	-2.806	-1.056	0.6943	(---*---)
STLB	PIM703	0.351	2.101	3.8510	(---*---)
STLB	SBT2062	1.401	3.151	4.9013	(---*---)

-----+-----+-----+-----
-3.5   0.0   3.5

Yogurt type = STLB
 Bacteria = LA5 subtracted from:

Yogurt

type	Bacteria	Lower	Center	Upper
STLB	NCFM	-3.915	-2.165	-0.4148
STLB	PIM703	-0.759	0.992	2.7419
STLB	SBT2062	0.292	2.042	3.7922

Yogurt

type	Bacteria	-----+-----+-----+-----
STLB	NCFM	(---*---)
STLB	PIM703	(---*---)
STLB	SBT2062	(---*---)

-----+-----+-----+-----

-3.5   0.0   3.5

Yogurt type = STL

Bacteria = NCFM subtracted from:

Yogurt

type	Bacteria	Lower	Center	Upper	
STLB	PIM703	1.406	3.157	4.907	(---*---)
STLB	SBT2062	2.457	4.207	5.957	(---*---)

-----+-----+-----+-----
-3.5   0.0   3.5

Yogurt type = STL

Bacteria = PIM703 subtracted from:

Yogurt

type	Bacteria	Lower	Center	Upper	
STLB	SBT2062	-0.6999	1.050	2.801	(---*---)

-----+-----+-----+-----
-3.5   0.0   3.5

Tukey Simultaneous Tests

Response Variable log prop

All Pairwise Comparisons among Levels of Yogurt type*Bacteria

Yogurt type = GDL

Bacteria = ATCC700396 subtracted from:

Yogurt type	Bacteria	Difference of Means	SE of Difference	Adjusted T-Value	P-Value
GDL	LA5	0.017	0.4882	0.034	1.0000
GDL	NCFM	-0.514	0.4882	-1.052	0.9838
GDL	PIM703	-0.243	0.4882	-0.499	0.9999
GDL	SBT2062	0.486	0.4882	0.995	0.9888
STLB	ATCC700396	-2.788	0.4882	-5.711	0.0007
STLB	LA5	-1.679	0.4882	-3.439	0.0663
STLB	NCFM	-3.844	0.4882	-7.874	0.0000
STLB	PIM703	-0.687	0.4882	-1.408	0.9095
STLB	SBT2062	0.363	0.4882	0.744	0.9986

Yogurt type = GDL

Bacteria = LA5 subtracted from:

Yogurt type	Bacteria	Difference of Means	SE of Difference	Adjusted T-Value	P-Value
GDL	NCFM	-0.530	0.4882	-1.086	0.9801
GDL	PIM703	-0.260	0.4882	-0.533	0.9999
GDL	SBT2062	0.469	0.4882	0.961	0.9912
STLB	ATCC700396	-2.805	0.4882	-5.745	0.0006
STLB	LA5	-1.696	0.4882	-3.473	0.0622
STLB	NCFM	-3.861	0.4882	-7.908	0.0000
STLB	PIM703	-0.704	0.4882	-1.442	0.8977
STLB	SBT2062	0.346	0.4882	0.710	0.9991

Yogurt type = GDL

Bacteria = NCFM subtracted from:

Yogurt type	Bacteria	Difference of Means	SE of Difference	Adjusted T-Value	P-Value
GDL	PIM703	0.270	0.4882	0.553	0.9999
GDL	SBT2062	0.999	0.4882	2.047	0.5810
STLB	ATCC700396	-2.275	0.4882	-4.659	0.0057

STLB	LA5	-1.165	0.4882	-2.387	0.3863
STLB	NCFM	-3.330	0.4882	-6.822	0.0001
STLB	PIM703	-0.174	0.4882	-0.356	1.0000
STLB	SBT2062	0.877	0.4882	1.796	0.7301

Yogurt type = GDL

Bacteria = PIM703 subtracted from:

Yogurt type	Bacteria	Difference of Means	SE of Difference	Adjusted T-Value	P-Value
GDL	SBT2062	0.729	0.4882	1.494	0.8781
STLB	ATCC700396	-2.545	0.4882	-5.212	0.0018
STLB	LA5	-1.435	0.4882	-2.940	0.1643
STLB	NCFM	-3.600	0.4882	-7.375	0.0001
STLB	PIM703	-0.444	0.4882	-0.909	0.9940
STLB	SBT2062	0.607	0.4882	1.242	0.9546

Yogurt type = GDL

Bacteria = SBT2062 subtracted from:

Yogurt type	Bacteria	Difference of Means	SE of Difference	Adjusted T-Value	P-Value
STLB	ATCC700396	-3.274	0.4882	-6.706	0.0001
STLB	LA5	-2.165	0.4882	-4.434	0.0091
STLB	NCFM	-4.330	0.4882	-8.869	0.0000
STLB	PIM703	-1.173	0.4882	-2.403	0.3782
STLB	SBT2062	-0.123	0.4882	-0.251	1.0000

Yogurt type = STLB

Bacteria = ATCC700396 subtracted from:

Yogurt type	Bacteria	Difference of Means	SE of Difference	Adjusted T-Value	P-Value
STLB	LA5	1.109	0.4882	2.272	0.4488
STLB	NCFM	-1.056	0.4882	-2.163	0.5117
STLB	PIM703	2.101	0.4882	4.303	0.0119
STLB	SBT2062	3.151	0.4882	6.455	0.0002

Yogurt type = STLB

Bacteria = LA5 subtracted from:

Yogurt type	Bacteria	Difference of Means	SE of Difference	Adjusted T-Value	P-Value
STLB	NCFM	-2.165	0.4882	-4.435	0.0091
STLB	PIM703	0.992	0.4882	2.031	0.5905
STLB	SBT2062	2.042	0.4882	4.183	0.0152

Yogurt type = STLB

Bacteria = NCFM subtracted from:

Yogurt type	Bacteria	Difference of Means	SE of Difference	Adjusted T-Value	P-Value
STLB	PIM703	3.157	0.4882	6.466	0.0002
STLB	SBT2062	4.207	0.4882	8.617	0.0000

Yogurt type = STLB

Bacteria = PIM703 subtracted from:

Yogurt type	Bacteria	Difference of Means	SE of Difference	Adjusted T-Value	P-Value
STLB	SBT2062	1.050	0.4882	2.151	0.5184

2. Effect of Individual Starter Culture Species on Day 28 (General Linear Model: log prop [log survival] versus Block, Sample)

Factor Type Levels Values
 Block random 3 1, 2, 3
 Sample fixed 4 NCFM-LB, NCFM-ST, SBT2062-LB, SBT2062-ST

Analysis of Variance for log prop, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Block	2	1.764	1.764	0.882	0.65	0.556
Sample	3	18.627	18.627	6.209	4.57	0.054
Error	6	8.155	8.155	1.359		
Total	11	28.545				

S = 1.16580 R-Sq = 71.43% R-Sq(adj) = 47.63%

Tukey 95.0% Simultaneous Confidence Intervals
 Response Variable log prop
 All Pairwise Comparisons among Levels of Sample
 Sample = NCFM-LB subtracted from:

Sample	Lower	Center	Upper
NCFM-ST	-2.231	1.068	4.366
SBT2062-LB	-0.760	2.538	5.836
SBT2062-ST	-0.109	3.189	6.487

-2.5 0.0 2.5 5.0

Sample = NCFM-ST subtracted from:

Sample	Lower	Center	Upper
SBT2062-LB	-1.827	1.471	4.769
SBT2062-ST	-1.177	2.121	5.419

-2.5 0.0 2.5 5.0

Sample = SBT2062-LB subtracted from:

Sample	Lower	Center	Upper
SBT2062-ST	-2.648	0.6504	3.949

-2.5 0.0 2.5 5.0

Tukey Simultaneous Tests
 Response Variable log prop
 All Pairwise Comparisons among Levels of Sample
 Sample = NCFM-LB subtracted from:

Sample	Difference of Means	SE of Difference	Adjusted T-Value	P-Value
NCFM-ST	1.068	0.9519	1.121	0.6911
SBT2062-LB	2.538	0.9519	2.667	0.1285
SBT2062-ST	3.189	0.9519	3.350	0.0569

Sample = NCFM-ST subtracted from:

Sample	Difference of Means	SE of Difference	Adjusted T-Value	P-Value
SBT2062-LB	1.471	0.9519	1.545	0.4700

SBT2062-ST 2.121 0.9519 2.228 0.2178

Sample = SBT2062-LB subtracted from:

	Difference of Means	SE of Difference	Adjusted T-Value	P-Value
SBT2062-ST	0.6504	0.9519	0.6833	0.8998

3. Effect of Yogurt Filtrate on Day 28 (General Linear Model: log prop [log survival] versus Block, Sample)

Factor	Type	Levels	Values
Block	random	2	1, 2
Sample	fixed	7	10G, 10S, 1S9G, 2S8G, 5S5G, 8S2G, 9S1G

Analysis of Variance for log prop, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Block	1	0.00457	0.00457	0.00457	0.07	0.805
Sample	6	0.84334	0.84334	0.14056	2.06	0.200
Error	6	0.40952	0.40952	0.06825		
Total	13	1.25743				

S = 0.261254 R-Sq = 67.43% R-Sq(adj) = 29.44%

Tukey 95.0% Simultaneous Confidence Intervals
 Response Variable log prop
 All Pairwise Comparisons among Levels of Sample

Sample = 10G subtracted from:

Sample	Lower	Center	Upper	-----+-----+-----+-----
10S	-1.220	-0.1303	0.9596	(-----*-----)
1S9G	-1.059	0.0305	1.1204	(-----*-----)
2S8G	-1.210	-0.1197	0.9703	(-----*-----)
5S5G	-1.315	-0.2251	0.8648	(-----*-----)
8S2G	-1.851	-0.7613	0.3286	(-----*-----)
9S1G	-1.249	-0.1590	0.9309	(-----*-----)
				-----+-----+-----+-----
	-1.0	0.0	1.0	

Sample = 10S subtracted from:

Sample	Lower	Center	Upper	-----+-----+-----+-----
1S9G	-0.929	0.1608	1.2507	(-----*-----)
2S8G	-1.079	0.0106	1.1006	(-----*-----)
5S5G	-1.185	-0.0948	0.9951	(-----*-----)
8S2G	-1.721	-0.6311	0.4589	(-----*-----)
9S1G	-1.119	-0.0288	1.0612	(-----*-----)
				-----+-----+-----+-----
	-1.0	0.0	1.0	

Sample = 1S9G subtracted from:

Sample	Lower	Center	Upper	-----+-----+-----+-----
2S8G	-1.240	-0.1501	0.9398	(-----*-----)
5S5G	-1.346	-0.2556	0.8344	(-----*-----)
8S2G	-1.882	-0.7918	0.2981	(-----*-----)
9S1G	-1.279	-0.1895	0.9004	(-----*-----)
				-----+-----+-----+-----
	-1.0	0.0	1.0	

Sample = 2S8G subtracted from:

Sample	Lower	Center	Upper	-----+-----+-----+-----
5S5G	-1.195	-0.1054	0.9845	(-----*-----)
8S2G	-1.732	-0.6417	0.4483	(-----*-----)
9S1G	-1.129	-0.0394	1.0506	(-----*-----)
				-----+-----+-----+-----
	-1.0	0.0	1.0	

Sample = 5S5G subtracted from:

Sample	Lower	Center	Upper	-----+-----+-----+-----
8S2G	-1.626	-0.5362	0.5537	(-----*-----)
9S1G	-1.024	0.0661	1.1560	(-----*-----)
				-----+-----+-----+-----
	-1.0	0.0	1.0	

Sample = 8S2G subtracted from:

Sample	Lower	Center	Upper	-----+-----+-----+-----
9S1G	-0.4876	0.6023	1.692	(-----*-----)
				-----+-----+-----+-----
	-1.0	0.0	1.0	

Tukey Simultaneous Tests
Response Variable log prop

All Pairwise Comparisons among Levels of Sample
Sample = 10G subtracted from:

	Difference	SE of	Adjusted	
Sample	of Means	Difference	T-Value	P-Value
10S	-0.1303	0.2613	-0.499	0.9979
1S9G	0.0305	0.2613	0.117	1.0000
2S8G	-0.1197	0.2613	-0.458	0.9987
5S5G	-0.2251	0.2613	-0.862	0.9666
8S2G	-0.7613	0.2613	-2.914	0.1915
9S1G	-0.1590	0.2613	-0.609	0.9938

Sample = 10S subtracted from:

	Difference	SE of	Adjusted	
Sample	of Means	Difference	T-Value	P-Value
1S9G	0.1608	0.2613	0.615	0.9935
2S8G	0.0106	0.2613	0.041	1.0000
5S5G	-0.0948	0.2613	-0.363	0.9996
8S2G	-0.6311	0.2613	-2.415	0.3269
9S1G	-0.0288	0.2613	-0.110	1.0000

Sample = 1S9G subtracted from:

	Difference	SE of	Adjusted	
Sample	of Means	Difference	T-Value	P-Value
2S8G	-0.1501	0.2613	-0.575	0.9954
5S5G	-0.2556	0.2613	-0.978	0.9421
8S2G	-0.7918	0.2613	-3.031	0.1686
9S1G	-0.1895	0.2613	-0.725	0.9851

Sample = 2S8G subtracted from:

	Difference	SE of	Adjusted	
Sample	of Means	Difference	T-Value	P-Value
5S5G	-0.1054	0.2613	-0.404	0.9993
8S2G	-0.6417	0.2613	-2.456	0.3133
9S1G	-0.0394	0.2613	-0.151	1.0000

Sample = 5S5G subtracted from:

	Difference	SE of	Adjusted	
Sample	of Means	Difference	T-Value	P-Value
8S2G	-0.5362	0.2613	-2.053	0.4698
9S1G	0.0661	0.2613	0.253	1.0000

Sample = 8S2G subtracted from:

	Difference	SE of	Adjusted	
Sample	of Means	Difference	T-Value	P-Value
9S1G	0.6023	0.2613	2.305	0.3661

Residual Plots for log prop