EVALUATION OF VIABILITY OF *LACTOBACILLUS ACIDOPHILUS* LA-5
DURING SIMULATED DIGESTION PROCESS USING A DYNAMIC *IN VITRO*
MODEL STOMACH

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COMMITTEE MEMBERSHIP

TITLE: EVALUATION OF VIABILITY OF *LACTOBACILLUS*
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ABSTRACT

Evaluation of viability of *Lactobacillus acidophilus* La-5 during simulated digestion process using a dynamic *in vitro* model

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In recent years, there has been an upsurge in medical research assessing the therapeutic benefits of probiotic bacteria and growing commercial interest in food fortification with these bacteria. Probiotic bacteria such as *L. acidophilus* are known to be predominant Lactobacilli species in the intestinal tract of healthy humans and suggested to provide clinical health benefits such as enhancement of immunity against intestinal infections, prevention of diarrhea and hypercholesterolaemia and improvement in lactose utilization. Many studies have demonstrated the possibility of incorporating probiotic bacteria in an ice cream matrix and shown its viability can be maintained throughout the shelf life of the ice cream. However, there is limited information about the protective effect of ice cream on viability of incorporated probiotic bacteria during simulated gastric digestion using an *in vitro* dynamic model stomach.

In phase one of this study, a preliminary study was conducted to determine the effect of air addition on the viability of *L. acidophilus* La-5. This was done by manufacturing low fat (4%) non-fermented ice cream mix supplemented with *L. acidophilus* La-5 to yield an initial population of 10^7 cfu/g. The mix was processed with 60% and 100% overrun (OR) and stored at -10°C for 90 days. The effect of air addition at different levels was tested post freezing and every 30 days throughout its shelf life of 90 days. The results showed less than one log reduction in the viable counts of *L.*

acidophilus La-5 for both samples incorporated with 60% and 100% OR after freezing and the number of viable cells did not differ significantly ($p>0.05$) from day 1 to day 90.

In phase two of this study, a 2^2 full factorial experimental design was used to evaluate whether the viscous nature of ice cream mix plays an important role in improving the survivability of *L. acidophilus* La-5 during simulated digestion against low pH and presence of mechanical shear and to determine whether initial inoculation level has any effect on the viability of *L. acidophilus* La-5 at the end of 2 hr simulated digestion. Non-fermented low fat (5%) ice cream mixes with high and low viscosity were produced by changing the amount of stabilizer/emulsifier blend and each of the two mixes were supplemented with two levels of *L. acidophilus* La-5 to obtain an initial population of 10^8 cfu/g and 10^6 cfu/g before freezing. These mixes were frozen with 60% overrun. The ice cream samples were digested for 2 hr in an *in vitro* model stomach called Human Gastric Simulator (HGS). This model included factors such as gastric secretions, mechanical shearing due to peristaltic contractions and temperature and pH control. No significant effect ($p>0.05$) of different levels of viscosity on the survivability of *L. acidophilus* La-5 was found during and at the end of 2 hr simulated *in vitro* digestion, irrespective of the difference in initial inoculation level. The initial supplementation level of *L. acidophilus* La-5 had a significant impact ($p<0.05$) on its survivability during the simulated digestion of ice cream samples, irrespective of the difference in viscosity. The log survival of *L. acidophilus* La-5 was on an average 3.64 log cfu/g and 4.08 log cfu/g for ice cream samples supplemented with higher and lower amount of *L. acidophilus* La-5, respectively at the end of 2 hr. Nevertheless, this difference in overall survival was not statistically significant ($p>0.05$).

These studies demonstrated the efficacy of low fat non-fermented ice cream in maintaining high viable numbers of *L. acidophilus* La-5 throughout its tested shelf life of 90 days. In addition, protective effect of ice cream on the viability of *L. acidophilus* La-5 against harsh stomach conditions was observed, but this effect was not as a result of viscosity of ice cream. It was also found that an ice cream supplemented with 10^6 cfu/g would result in a similar overall log reduction of *L. acidophilus* La-5 at the end of 2 hr simulated digestion compared to an ice cream supplemented with 10^8 cfu/g.

The aggressive stomach conditions had a negative impact on the survivability of *L. acidophilus* La-5 during digestion of all the ice cream samples, but this detrimental effect can be reduced by incorporating *L. acidophilus* La-5 into an ice cream matrix which would increase the opportunity of bacteria to reach the small intestine and provide the desired health benefit.

Keywords: *L. acidophilus* La-5, ice cream, overrun, viscosity, inoculation, HGS, survival

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1.0 Introduction

Fermented dairy products especially yogurt and cheese have enjoyed consumer's attention because they are made from milk which is rich source of proteins and calcium and they contain live bacteria which provides health benefits. On the contrary, dairy product like ice cream is considered as a rich, indulgent treat because of high fat and sugar content and typically consumed during summer time. Low fat has become a buzz word among consumers and therefore, majority of them prefer low fat frozen desserts. Customers associate words like low fat and low sugar with a reduced risk of obesity, coronary heart diseases and diabetes (El-Nagar et al., 2002). Hence, food producers are concentrating their efforts in making ice cream like products nutritional and functional by supplementing them with probiotic bacteria so that they could be enjoyed every day.

Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2001). *Lactobacillus* and *Bifidobacterium* are the most common genera of bacteria used as probiotics for the production of fermented dairy products (Fuller, 1992). Generally, strains belonging to genus *Lactobacillus* have proven to be highly resistant to detrimental conditions during ice cream manufacturing process as well as more aerotolerant in comparison with *Bifidobacteria* strains (Talwalkar et al., 2001; Homayouni et al., 2008; Tamime et al., 2005). Some common probiotic species of *Lactobacillus* are *L. acidophilus*, *L. reuteri*, *L. helveticus*, and *L. rhamnosus* (Shah, 2007; Senok et al., 2005). Many studies have focused on survival of probiotic bacteria in fermented (Hekmat and McMahon, 1992; Magarinos et al., 2007; Turgut and Cakmakci, 2009) and non-fermented (Alamprese et al., 2002; Abghari et al., 2011; Nousia et al., 2011) ice cream and most of these studies have shown that ice cream can be an excellent environment for maintaining the viability

of probiotic bacteria above the recommended level of 10^6 cfu/g throughout its shelf life of more than 90 days. However, the viability of probiotic bacteria in an ice cream must be maintained not only during processing and shelf life, but also during transit through consumer's gastrointestinal (GI) tract to be able to reach the intestine in high viable cells to provide intended health benefits. Acid and bile tolerance are the two fundamental properties of probiotic microorganisms to be able to survive conditions in stomach and intestine (Prasad et al., 1998; Alamprese et al., 2002; Bhadoria and Mahapatra, 2011). To study the viability of bacteria in GI conditions, both *in vivo* and *in vitro* tests have been carried out.

In vivo studies are complex, labor intensive, and expensive to be used for preliminary and routine evaluation of foods containing probiotic bacteria (Sumeri et al., 2008; Fernández de Palencia et al., 2008). Thus, static (incubation of probiotic bacteria in the presence of acid and bile) and dynamic (TNO, Dynamic Gastric Model) *in vitro* models have been developed and used in numerous studies to determine the efficiency of dairy products such as fermented milk, yogurt, kefir as probiotic bacteria carrying vehicles (Conway et al., 1987; Charteris et al., 1998; Marteau et al., 1997; Mainville et al., 2005; Faye et al., 2012). A non-fermented ice cream supplemented with probiotic bacteria has been rarely studied in an *in vitro* digestion model to determine the contribution of its components (stabilizers, fat, and proteins) and properties (viscosity, buffering capacity and initial pH) on survivability of probiotic bacteria during its simulated digestion in a model stomach. Therefore, in this study, efficacy of a non-fermented low fat ice cream as a probiotic bacteria carrying vehicle was tested by subjecting it to simulated digestion process using a dynamic *in vitro* model stomach called Human Gastric Simulator (HGS).

2.0 Literature Review

2.1 Concept of Probiotics

One thing most people may have experienced more than once in their lifetime is taking antibiotics to cure bacterial infections. Antibiotics have been the “gold standards” in the management of diseases. However, the side effects like hypersensitivity, induction of yeast vaginitis, and sometimes even death associated with antibiotic cannot be ignored. In 21st century, consumers are extremely concerned about living healthier and happier today and beyond. Hence, the food and pharmaceutical industry is spending research dollars to understand the mechanism of probiotic bacteria so that its ingestion can provide health benefits. In recent years, there has been an upsurge in clinical research assessing the therapeutic benefits of probiotic bacteria as well as growing commercial interest in food fortification with them (Czinn and Blanchard, 2009). Scientists are looking at possibilities of substituting antibiotics with probiotics by finding a link between probiotics and prevention of human diseases (Oliveira et al., 2001; Schrezenmeir and de Vrese, 2001; Teitelbaum and Walker, 2002).

The concept of probiotics came into existence around 1900 when the Nobel Prize winner Elie Metchnikoff made a remarkable observation and hypothesized that the Bulgarian peasants lived longer and healthier lives as a result of their consumption of fermented dairy products containing genus *Lactobacillus* (Ross et al, 2005). The word “probiotics” comes from two Greek words 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 (1953) who defined it as “probiotics are common in vegetable food as vitamins, aromatic substances, enzymes and possibly other substances connected with vital process”. Later, various definitions for the term

probiotics were proposed by scientists like Parker (1974), Fuller (1989), Salminen et al. (1998). In 2001, Food and Agriculture Organization of United Nations and World Health Organization (FAO/WHO, 2001) defined probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host”. Many commercially available probiotic bacteria may provide one or several proposed health benefits according to clinical studies (Parvez et al., 2006). A few examples of probiotic strain with clinically proven health benefits are shown in Table 2.1.1. Gorbach (2000) and Figueroa-Gonzalez et al. (2011) reported that beneficial effects of probiotic bacteria do not tie to specific genus or species, but instead are strain-specific which is also demonstrated in Table 2.1.1.

Table 2.1.1: Probiotic strains and their specific clinically proven health benefits (Shah, 2006)

Probiotic Strain	Clinical Benefits
<i>L. acidophilus</i> NCFM	Lowers fecal enzyme activity, improves lactose absorption and produces bacteriocin
<i>L. rhamnosus</i> GG	Plays a role in prevention of antibiotic and rotavirus associated diarrhea
<i>L. casei</i> shirota	Helps in prevention of intestinal disturbance, balancing intestinal flora and lowering of fecal enzyme activity
<i>L. reuteri</i>	Colonizes the intestinal tract, shortens the duration of rotavirus diarrhea, and helps in immune enhancement
<i>B. animalis</i> BB-12	Plays a role in treatment of rotavirus diarrhea and balancing intestinal flora

2.1.1 Probiotic bacteria and current scenario

Currently, there is not an approved standard of identity for probiotics, but an established suitable level of viable cells to be ingested for therapeutic benefits is 10^6 cfu/g or ml, representing a daily dose of 8 log (Cruz et al., 2009; Ding and Shah, 2009; Abghari et al., 2011). Additionally, the concentration of probiotic microorganisms needed for

biological health benefits depends on the strain, the delivery medium and the desired health effect (Sanders et al., 2008; Champagne et al., 2005). High dosage is likely required to compensate for the possible decline of the number of viable probiotic cells during processing and storage of probiotic containing products and passage through the stomach and intestine (Waterman and Small, 1998).

Due to the increasing awareness about the health benefits provided by live bacteria, the number of commercially marketed probiotic products in the USA has tripled in the past 10 years. According to a new market research report, ‘Probiotics Market (2009-2014)’, published by Markets and Markets (2009), the global probiotics market is expected to be worth US\$ 32.6 billion by 2014, where Europe and Asia are expected to account for nearly 42% and 30% of the total revenues, respectively. Probiotic dairy products are expected to hold the highest market share among all the products containing probiotic bacteria reaching a market size of almost \$24 billion by the end of 2014. It was predicted that products supplemented with probiotic bacteria will enjoy the largest market share by the year 2015 (Anonymous, 2009; Figure 2.1.1.1).

Figure 2.1.1.1: Projected shares of global sales of probiotic ingredients, supplements and foods in 2015 (%) compared to 2010 (%) (Anonymous, 2009)

It is important to note that incorporating probiotic bacteria in a food product does not guarantee health benefits. It's essential to ensure that viability of probiotic bacteria during food production and throughout product's shelf life is maintained above recommended level. Many quality control studies of commercialized probiotic products have often revealed deficiencies in the number of viable *Bifidobacteria* (Gueimonde et al., 2004; Jayamanne and Adams, 2006; Masco et al., 2007; Shah et al., 1995; Tamime, 2002). For instance, a recent study tested viable numbers of *Bifidobacteria* in ten bioyoghurts sold in the UK market at the time of purchase and at the end of expiration date. The results from this comparison study found that most products contained optimum level of viable *Bifidobacteria* at the time of purchase but only few maintained high level of viable cells until the end of shelf life (Sanz, 2007). These discrepancies in results emphasize the importance of conducting studies to determine the viability of probiotic bacteria in a food matrix of interest throughout its shelf life and ensure that the viability is maintained at a high level of 10^6 cfu/g at the time of its consumption.

Once the survivability of probiotic bacteria is maintained throughout product's shelf life, another important concern is whether the selected strain is resistant to gastric acidity and shear stresses in the stomach and bile toxicity in the small intestine (Collins and Thornton, 1998; Sanders et al., 1996; Chandan, 1999) and whether it's able to provide intended health benefits in vivo. Although, the number of articles on probiotic bacteria has increased exponentially in recent years, there are very few articles about randomized clinical trials (Figure 2.1.1.2; Hibberd and Davidson, 2008). Therefore, it's essential to conduct well planned experiments using *in vitro* digestion models to screen

for gastric tolerance of probiotic bacteria followed by *in vivo* studies to firmly establish health benefits (Chou and Weimer, 1999).

Figure 2.1.1.2: Research and randomized trials of probiotics published in the Medline database, 1996–2006 (Hibberd and Davidson, 2008)

Various species of genera *Lactobacillus* and *Bifidobacteria* have been incorporated in dairy and non dairy products (Table 2.1.1.1 and 2.1.1.2, respectively) over the years to study the effect of food vehicle on the survivability and functionality of selected probiotic bacteria. The *Lactobacillus* and *Bifidobacteria* genera are most commonly studied genera and have played an extensive role as probiotics because of their association with healthy human intestinal tract and specifically in the case of *Lactobacilli*, due to their association with fermented foods.

Table 2.1.1.1: Dairy products and probiotic bacteria

Products	Probiotic bacteria	References
Crescenza cheese; Cheddar cheese; Cheese	<i>L.paracasei</i> A13 and <i>L. acidophilus</i> H5; <i>L. acidophilus</i> 4962, <i>L. casei</i> 279, <i>B. longum</i> 1941, <i>L. acidophilus</i> LAFTI® L10, <i>L. paracasei</i> LAFTI® L26, <i>B. lactis</i> LAFTI® B94; <i>L. casei</i> ATCC 393	Burns et al., 2008; Ong et al., 2006; Kourkoutas et al., 2006
Frozen yogurt, ice cream	<i>B. longum</i> , <i>L. acidophilus</i> ; <i>B. infantis</i> , <i>B. brevis</i> , <i>B. longum</i> ; <i>L. acidophilus</i> La-5, <i>B. animalis</i> subsp. <i>lactis</i> Bb-12; <i>L. johnsonii</i> La1; <i>B. bifidum</i> Bb-12, <i>L. acidophilus</i> La-5, <i>L. casei</i> 01; <i>L. gasseri</i> B-14168, <i>L. rhamnosus</i> B-445, <i>L. reuteri</i> B-14171, <i>L. acidophilus</i> La-5, <i>B. bifidum</i> Bb-12; <i>L. acidophilus</i> La-5, <i>L. casei</i> Lc-01, <i>B. bifidum</i> BB-12, <i>B. longum</i> BB-46; <i>L. casei</i> Lc-01, <i>B. lactis</i> Bb-12; <i>L. acidophilus</i> DSMZ 20079 <i>B. bifidum</i> DSMZ 200456; <i>L. acidophilus</i> 74-2, <i>L. acidophilus</i> LAC 4; <i>L. acidophilus</i> , <i>L. Rhamnosus</i> ; <i>L. acidophilus</i> LMGP-21381;	Davidson et al., 2000; Modler et al., 1990; Magarinos et al., 2007; Alamprese et al., 2002; Taha et al., 2005; Salem et al., 2005; Homayouni et al., 2008; Turguti and Cakmakci, 2009; Favaro-Trindade et al., 2007; Abghari et al., 2011; Nousia et al., 2011
Fermented milk	<i>L. acidophilus</i> LAC4, <i>B. longum</i> BL; <i>L. acidophilus</i> La-5 and <i>L. rhamnosus</i> LR35	Zacarchenco and Massaguer-Roig, 2006; Sodini et al, 2002
Buttermilk	<i>B. animalis</i> subsp. <i>lactis</i> Bb12	Antunes et al, 2009
Yogurt and yogurt drink	<i>L. acidophilus</i> and <i>B. lactis</i> ; <i>L. acidophilus</i> and <i>Bifidobacteria</i> ; <i>L. acidophilus</i> , <i>B. bifidum</i> ; <i>L. acidophilus</i> La-5, <i>B. animalis</i> ssp. <i>lactis</i> , BB-12, <i>Streptococcus thermophilus</i> , <i>L. rhamnosus</i> LGG	Seelee et al, 2009; Dave and Shah, 1998; Mortazavian et al, 2008; Ramasubramanian et al, 2008

Table 2.1.1.2: Non dairy products and probiotic bacteria

Product	Probiotic bacteria	Reference
Mayonnaise	<i>B. bifidum</i> DI, <i>B. infantis</i> 4038	Khalil and Mansour, 1998
Fermented Pork Sausage	<i>L. acidophilus</i> JCM1132T, <i>L. crispatus</i> JCM1185T, <i>L. amylovorus</i> JCM1126T, <i>L. gallinarum</i> JCM2011T, <i>L. gasseri</i> JCM1131T <i>L. johnsonii</i> JCM2012T	Arihara et al, 1998
Reduced fat (60% w/w) edible table biospread	<i>L. casei</i> ACA-DC 212.3, <i>B. infantis</i> ATCC 25962, Mixed-strain powder preparation of <i>L. acidophilus</i> and <i>B. bifidum</i> AB® Sweet	Charteris et al, 2002
Coleslaw	<i>L. acidophilus</i> , <i>B. lactis</i> , <i>L. casei</i>	Rodgers and Odongo, 2002
French onion cheese based dip	<i>L. acidophilus</i> LAC1, <i>L. paracasei</i> subsp. <i>paracasei</i> LCS1, <i>B. animalis</i> Bb12 <i>P. freudenreichii</i> subsp. <i>shermanii</i> PS1, <i>L. rhamnosus</i> LC 705	Tharmaraj and Shah, 2004
Oat Based Cereal Bar	<i>B. lactis</i> Bb-12	Ouwehand et al., 2004
Table Olives	<i>L. rhamnosus</i> ATCC 53103, <i>B. bifidum</i> ATCC 15696, <i>B. longum</i> ATCC 15708, <i>L. rhamnosus</i> IMPC11 & IMPC19, <i>L. paracasei</i> IMPC2.1	Lavermicocca et al., 2005
Chocolate mousse	<i>L. paracasei</i> subsp. <i>paracasei</i> LBC 82	Cardarelli et al., 2008
Fresh cut apple slices	<i>L. rhamnosus</i> GG	Roble et al., 2010
Nonfermented frozen soy dessert	<i>L. acidophilus</i> MJLA1, <i>L. rhamnosus</i> 100-C <i>B. lactis</i> BDBB2, <i>L. paracasei</i> ssp. <i>paracasei</i> Lp-01, <i>B. lactis</i> Bb-12, <i>Saccharomyces boulardii</i> 74012	Heenan et al., 2004

2.1.1.1 *Lactobacillus*

Lactobacilli are ubiquitous in nature, found in carbohydrate rich environments and are natural inhabitant of human gut. They are gram-positive rods or coccobacilli, non-spore forming and catalase negative organisms (Hammes and Vogel, 1995). They are fermentative (ferment carbohydrates into lactic acid) and facultative anaerobes (produce ATP energy in the presence of oxygen, but switch to fermentation in anaerobic conditions). Their preferred growth temperature depends on the specific species and subtype, but the range is generally 35-38°C, with 37°C optimal for many and the ideal pH values for growth are slightly acidic, for example, 5.5-6.0 for *Lactobacillus acidophilus*. This is the most extensively studied genus comprising of 106 species and a few most commonly isolated species from human intestine are *L. acidophilus*, *L. salivarius*, *L. casei*, *L. plantarum*, *L. fermentum*, *L. reuteri* and *L. brevis* (Mitsuoka, 1992).

2.1.1.1.1 *Lactobacillus acidophilus*

L. acidophilus has been considered to be predominant *Lactobacillus* species in the intestinal tract of healthy humans (Arihara and Luchansky, 1994; Ray, 1996). Thus, it is the most studied species and variety of its strains such as La-5 and La-1 are widely used in commercial dairy and food products as well as dietary supplements (Salminen et al., 1998; Shah, 2001; Tannock, 2002; Holzapfel, 2006). Acidophilus milk is one of the oldest and most commonly found fermented milk products and approximately 80% of the yogurt manufactured in the US contains *L. acidophilus* (Sanders, 2003). Important characteristics of *L. acidophilus* include,

1. Gram- positive, rod shaped non motile, non-flagellated and non-sporing species of genus *Lactobacillus*.

2. It is usually around 0.6 to 0.9 μm in width and 1.5 to 6.0 μm in length with rounded ends.
3. Cells may appear singularly or in pairs as well as in short chains.
4. It belongs to the homofermentative group of *Lactobacilli* and lactic acid is the only end product of the fermentation process.
5. The optimum growth occurs within 35-40°C, but it can tolerate temperatures as high as 45°C.
6. It can utilize lactose as well as sucrose effectively.
7. *L. acidophilus* has a high cytoplasmic buffering capacity in the pH range 3.72-7.74 which allows it to resist changes in cytoplasmic pH and become stable under acidic conditions (Godward et al., 2000; Tamime et al., 2005).

Several studies have been conducted to determine the potential of *L. acidophilus* to provide health benefits to humans. Lin and Chen (2000) tested six strains of *L. acidophilus* for their cholesterol reducing ability and found that they were able to reduce cholesterol *in vivo*. This ability of *L. acidophilus* was attributed to the assimilation of cholesterol by the cells and/or the attachment of cholesterol to the surface of *L. acidophilus* cells. Several other studies such as Gilliland and Walker (1990) and Liong and Shah (2004) have found that *L. acidophilus* strains are capable of reducing cholesterol. Some strains of *L. acidophilus* namely *L. acidophilus* M92 was found to possess proteinaceous components called S-layer proteins which aid in its aggregation and adhesion to epithelial cells in the intestine as well as play a protective role during its transit through gastrointestinal tract (Frece et al., 2003 and 2005). *In vitro* tests conducted

by Bhatia et al. (1989) showed that lactic acid produced by *L. acidophilus* inhibited the growth of *Helicobacter pylori*. Sheu et al. (2002) also conducted a clinical study to determine the effect of consumption of probiotic yogurt containing *L. acidophilus* and *B. bifidum* on suppression of *H. pylori* and found that supplementation of drugs with live cultures reduced the number of side effects and may enhance the action of drugs on inhibition of *H. pylori*. Another study conducted by Medellin-Pena and Griffiths (2008) tested the effect of presence of *L. acidophilus* La-5 on prevention of *E. coli* infection *in vitro* and *in vivo*. They found that in the presence of specific fractions of *L. acidophilus* La-5, adhesion and formation of lesions by *E. coli* was reduced *in vitro*. In addition, the gut colonization of mice by *L. acidophilus* La-5 resulted in decreased amount of *E. coli* counts in feces. *L. acidophilus* La-5 produces certain molecules that prevent adhesion of *E. coli* cells to the intestinal epithelial cells. Rajpal and Kansal (2009) demonstrated that consumption of yogurt containing *L. acidophilus* and *B. bifidum* may play a role in slowing down the process of ageing as a result of improved antioxidant activity in rats. These studies have demonstrated potential clinical health benefits that could be provided by *L. acidophilus* strains when consumed in food products or as supplements.

2.1.2 Probiotic bacteria and dairy products

Our diet is considered as one of the major factors participating in the regulation of intestinal flora. In addition, food also acts as a buffer for bacterial transit through the stomach to the intestine. Food matrix with appropriate pH and buffering capacity could play an important role in maintaining the viability of probiotic bacteria during its transit through the stomach. Other matrix factors influencing probiotic viability include fat content, concentration and type of proteins, sugars, the processing and storage conditions

during product's shelf life (Kailasapathy and Chin, 2000). Some evidence has indicated that adequate colonization may be achieved in the intestine as a result of maintenance of viability of probiotic bacteria through the stomach when administered in food (Alander et al., 1999). In the United States, as mentioned earlier, the major outlets for probiotic bacteria are dairy foods and dietary supplements.

2.1.2.1 Fermented dairy products

Dairy products like yogurt and fermented milk form the largest segment of the market for probiotic products mainly because they are considered as an ideal vehicle for successfully delivering probiotic bacteria to the human intestine because of their suitable pH, buffering capacity (significantly increases gastric pH), presence of fat and possible encapsulation by milk proteins (Ross et al., 2005; Charteris et al., 1998). Fermented dairy foods seem to fit naturally with probiotics because of their long term association with live cultures and positive health image. It's easy for consumers to naturally correlate fermented dairy products with live cultures and perceive a benefit in their presence (Sanders, 2000). However, it is essential to take into account possible synergistic or antagonistic interaction that may occur during production of fermented probiotic dairy product between probiotic bacteria and starter cultures. Studies have reported few antagonistic effects due to the production of bacteriocins (peptides or proteins exhibiting antibiotic properties) and lactic acid (lowering pH of the product) by starter culture and probiotic bacteria during fermentation causing negative effects on each other's survivability. The ability to produce bacteriocins and lactic acid are both desirable characteristic of probiotic bacteria (Salminen et al., 1996), but when supplemented in addition to starter bacteria could result in detrimental effects limiting the possibility of

co-existence of starter cultures and probiotic bacteria in the same product (Joseph et al., 1998). Tabasco et al. (2009) conducted a study to identify the bacteriocin produced by *L. acidophilus* La-5 and the factors causing the production of bacteriocin. They found that presence of live yogurt starter cultures such as *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* induced bacteriocin production by *L. acidophilus* La-5. On the contrary, *L. acidophilus* La-5 did not produce any bacteriocin in the presence of non viable cells of yogurt bacteria.

Other factors influencing the viability of probiotic bacteria in fermented dairy products include hydrogen peroxide produced by starter bacteria, availability of nutrients, osmotic pressure due to presence of sugars, dissolved oxygen, inoculation level, fermentation time and storage temperature (Shah, 2000). Kopeloff et al. (1934), Gilliland and Speck (1977) and Ng (2009) found that some strains of *L. acidophilus* had limited stability in fermented products like traditional acidophilus milk and yogurt because of progressive increase in the acidity of products during storage termed as post acidification since the starter culture bacteria are active at refrigerated temperature.

A few other dairy products which have been explored for supplementation with probiotic bacteria are frozen yogurt, fermented ice cream and cheese. The combination of starter culture and probiotic bacteria could possibly defeat the purpose of addition of probiotic bacteria in the ice cream due to the reasons mentioned above in case of yogurt. Hekmat and McMahon (1992) and Akalin and Erisir (2008) reported significant decrease in *L. acidophilus* counts in fermented ice cream after storage for 17 weeks at -29°C and 13 weeks at -18°C, respectively. The sensory properties of ice cream could be negatively affected due to acidification of the ice cream mix. In addition, there is some evidence that

consumers prefer less acidic products like frozen probiotic ice cream over fermented probiotic yoghurt like products (Hekmat and McMahon, 1992; Mashayekh and Brown, 1992; Christiansen et al., 1996). In a study conducted Salem et al. (2005), all fermented ice cream treatments scored slightly lower values in melting quality and color attributes than control treatment. This indicates that fermentation of ice cream may result in adverse effects on its attributes. They also found that the viable counts of *L. acidophilus* La-5 reduced by 2 logs at the end of 12 weeks of frozen storage at -26°C.

2.1.2.2 Ice cream, a non-fermented dairy product

Ice cream is a popular frozen dessert and enjoyed by all ages. Its production has become a profitable industry because of recent advances and rapidly developing technology (Turgut and Cakmakci, 2009). In 2003, 5333 million liters of ice cream were produced in the US utilizing 10% of the total milk production and 16% of the processed milk for this purpose. In 2006, although an overall decrease in the product sales was registered in the American supermarkets, the sale of light and diet ice creams increased by 15% from January to June (Cruz et al., 2009). This data suggests that consumers are quickly adopting healthier ice cream alternatives when given those choices. What if food producers could give consumers a functional and therapeutic ice cream alternative by supplementing it with probiotic bacteria? Incorporation of probiotic bacteria in ice cream would improve its value as a frozen dessert and provide alternatives to consumers. It may also offer certain advantages over fermented dairy products like frozen yogurt, fermented milk, yogurt in terms of delivery of viable probiotic bacteria, such as neutral pH, low storage temperature and absence of metabolic activity during storage preventing changes in organoleptic properties of ice cream (Taha et al., 2005; Cruz et al., 2009). Ice cream is

a frozen mixture of fat, proteins, sweeteners, stabilizers, emulsifiers and flavoring agents (Marshall et al., 2003) which makes it a good vehicle for probiotic bacteria. Milk fat and air bubbles act as insulators by reducing the transfer of heat through the frozen foam (Magarinos et al, 2007). Stabilizers bind to the water molecules restricting the growth of ice crystals during storage resulting in minimum damage caused to microbial cells due to increasing size of ice crystals (Jay, 1992).

As a general rule, the addition of probiotic strains into a food product requires producers to ensure that the survivability of probiotic bacteria is maintained at above recommended level at the end of shelf life (Stanton et al., 2003). In order to achieve this goal, a number of factors should be strictly controlled such as the selection of oxygen tolerant and low pH resistant probiotic bacteria, the initial supplementation level, the appropriate step for adding cultures and the strict control of the storage temperature (Cruz et al, 2009). Successful incorporation of probiotic bacteria involves overcoming intrinsic hurdles (Table 2.1.2.1.1) during processing of the ice cream. These include mixing and shearing step when air is incorporated and freezing step when ice crystals are formed and storage at subzero temperature (Cruz et al., 2009; Mohammadi et al., 2011).

Table 2.1.2.1.1: Factors affecting viability of probiotic bacteria during ice cream production (Cruz et al, 2009)

Step	Problem	Solutions
Adequate choice of formulation ingredients (fruit pulp/juice)	<ol style="list-style-type: none"> 1. High acidity of final product may lead to decreased probiotic survival 2. Some ingredients may have inhibitory activity against probiotic strains 	Preferably use of fruit pulp/juices with a lower natural acidity
Decision about addition of probiotic bacteria	<ol style="list-style-type: none"> 1. If starter cultures are added along with probiotic bacteria, fermentation due to starter cultures could result in lowering of pH which in turn could result in loss of viability of probiotic strains 2. If probiotic bacteria is added in the ice cream mix and allowed to ferment and produce acid, it can impart acidic flavors. Perception of an acidic taste can have a negative effect on the consumer since ice cream is expected to be a sweet delicacy vs. sour frozen yogurt like. 	<ol style="list-style-type: none"> 1. Start with one probiotic strain of interest and study it from strain level to its efficacy as a therapeutic ingredient 2. Avoid using mixed strains in the ice cream preparation 3. Perform preliminary tests on combination strains of interest to determine any detrimental effects caused by one strain to the other
Beating/air incorporation	Oxygen represents a factor of toxicity for probiotic bacteria	Selection of oxygen tolerant strain
Freezing and storage	Stress induced by freezing results in cell damage. This damaged cell eventually dies during storage	<ol style="list-style-type: none"> 1. Increased inoculum concentration 2. Avoid temperature abuse of the product during storage

Many studies have been conducted to study the effect of ice cream manufacturing process on the viability of added probiotic bacteria after processing and throughout shelf life (Taha et al., 2005; Salem et al., 2005; Magarinos et al., 2007; Abghari et al., 2011; Nousia et al., 2011 and Ferraz et al., 2012). An important aspect to consider while producing ice cream supplemented with probiotic bacteria is the step during ice cream manufacturing process during which probiotic bacteria must be added. Abghari et al. (2011) found that the capacity of *L. acidophilus* to survive the freezing and air incorporation step was higher when it was added before ageing and allowed to interact with ice cream components during ageing at 4°C compared to those reported by other researchers who added probiotic microorganisms after the ageing of ice cream mix (Christiansen et al., 1996; Akin et al., 2005). Taha et al. (2005) compared effect of two treatments on the viability of mixed culture of *L. acidophilus* La-5, *B. bifidum* BB12 and *L. casei* 01 after processing and during storage for three months. They aged the mix and added mixed culture to the ice cream mix an hour before freezing and for another treatment ice cream mix was fermented. They found that freezing and air incorporation step resulted in less than one log reduction for all the three probiotic bacteria in two different treatments. In addition, at the end of storage, the viability of all the three bacteria was maintained above 8 log cfu/g and did not decrease significantly throughout storage for both the treatments.

Once the probiotic bacteria are added to the ice cream mix, a process called freezing is used to convert the unfrozen ice cream mix into a frozen dairy product. It is an important step since it defines the structure of the final product and for palatability and production of ice cream; on the contrary, it can affect the number of viable probiotic cells

incorporated in the mix (Dave and Shah, 1998; Hekmat and McMahon, 1992; Kailasapathy and Sultana, 2003; Ravula and Shah, 1998). In a study conducted by Magarinos et al. (2007), it was found that 8.7% and 9.9% of *L.acidophilus* La-5 and *B. lactis*, respectively was lost after freezing, whereas, 4.3% and 0% for *L. acidophilus* La-5 and *B. lactis*, respectively was lost after storage. These results indicate that the adverse effect of freezing on both the tested probiotic bacteria was higher compared to effect of storage at -25°C for 60 days and survival was species specific. Hagen and Narvhus (1999) found that freezing resulted in ~1 log reduction in viable bacterial cells. The numbers of viable cells were found to be 7 log cfu/g for *B. bifidum* BB-12, 7.4 log cfu/g for *L. rhamnosus* GG and 6.75 log cfu/g for *L. acidophilus* La-5 in the frozen ice cream shortly after freezing. These results indicate that ice crystal formation during freezing results in injury or death of viable cells and the intensity of the detrimental effect is species specific.

Air addition to the ice cream mix during the freezing process results in overrun. It is defined as percent increase in the volume of ice cream mix as a result of air addition. It results in light texture, partial coalescence and destabilization of the fat present in the mixture and influences the physical properties of the ice cream (Bolliger et al., 2000; Sofjan and Hartel, 2004). Oxygen can be introduced into the ice cream at various steps such as during culture addition into the ice cream mix and intended air incorporation during freezing. After production, atmospheric oxygen can enter into the product during filling and packaging as well as it can diffuse through the packaging material during storage (Talwalkar and Kailasapathy, 2004; Talwalkar et al., 2004). Since air incorporation is a valuable step, it is essential to select oxygen tolerant probiotic strains to

maintain their viability during ice cream manufacturing process. Oxygen tolerance is also a strain specific characteristic of probiotic bacteria (Kawasaki et al., 2006). The genus *Bifidobacteria* of probiotic bacteria are more sensitive to oxygen compared to *Lactobacillus* (Vasiljevic and Shah, 2008). Since most of the *Lactobacillus* strains are microaerophilic and *Bifidobacteria* strains are strictly anaerobic, their oxygen scavenging system is reduced or completely absent. This results in an incomplete reduction of oxygen to hydrogen peroxide eventually leading to cell death (Champagne et al., 2005; Talwalkar and Kailasapathy, 2004; Vasiljevic and Shah, 2008). Ferraz et al. (2012) conducted a study to determine the effect of 45%, 60% and 90% overrun on the viability of *L. acidophilus* DOWARUTM. The probiotic bacteria were added right before freezing the ice cream mix. They found that the survival of *L. acidophilus* DOWARUTM was maintained at 8 log cfu/g after freezing with 45%, 60% and 90% overrun. The effect of amount of air addition was observed during the frozen storage during which the number of viable cells dropped to 7 log cfu/g and 6.06 log cfu/g for samples incorporated with 60% and 90% overrun, respectively. The authors suggested that the observed effect of different amounts of air addition was a result of accumulation of toxic metabolites interacting with proteins, lipids and nucleic acids resulting in cell death.

The effect of storage on the viability of probiotic bacteria during ice cream's shelf life is also an important factor that must be studied to ensure viability of tested probiotic bacteria is maintained in excess of recommended level of 10⁶cfu/g in the ice cream at the time of consumption. Nousia et al. (2011) studied the effect of low temperature storage of non-fermented ice cream on the survivability of *L. acidophilus* and found that regardless of the storage temperature (-15°C versus -25°C), the viable cells of *L.acidophilus* LMGP-

21381 remained above 10^7 cfu/g and did not differ significantly ($p>0.01$) throughout the storage study of 45 weeks. These findings were in accordance with those reported by other authors (Godward and Kailasapathy 2003; Magarinos et al. 2007; Turgut and Cakmakci 2009) who indicated that *L. acidophilus* counts did not change significantly during storage of ice cream at temperatures lower than -20°C . Buriti et al. (2010) tested the effect of storage temperature on the viability of *L. acidophilus* La-5 incorporated in guava mousses and found that survival of *L. acidophilus* La-5 reduced by more than 1 log at the end of 28 days storage at 4°C . On the contrary, the viable cells were maintained at greater than 7 log cfu/g at the end of frozen storage at -18°C for 112 days and did not differ significantly throughout storage. These results show that storage temperature may also impact the survival of *L. acidophilus* La-5 at the end of product's shelf life. The results from these studies indicate that the combination of *L. acidophilus* and non-fermented ice cream seem to be a promising one that could be further challenged in an *in vitro* digestion model to determine whether ice cream matrix helps *L. acidophilus* to survive through the stomach in high viable counts.

2.2 Digestion Process

The mouth and the stomach are the major parts of human body where food is broken into small size particles whereas small intestine is the major site of nutrient absorption. Digestion of foods begins with chewing in the mouth which is a rapid but important step in digestion. Saliva is secreted in the mouth which consists of mucus, enzyme α -amylase and electrolytes. The gastric digestion of foods includes numerous influencing factors such as fed/fast state pH, gastric acid, enzymatic reactions, and hydrodynamic and mechanical forces. This indicates the complexity of digestion process

which upon ingestion the probiotic bacteria must battle to survive in high numbers and reach the intestine to provide clinical health benefits.

2.2.1 Mouth

“Mastication is a process during which pieces of food are ground into a fine state, mixed with saliva, and brought to approximately body temperature ready for transfer to the stomach where most of the digestion takes place” (Bourne, 2004). The presence of food in the mouth is a powerful stimulus to salivation. Saliva is a clear, mucoserous exocrine secretion which is composed of water, a variety of electrolytes (including sodium, potassium, calcium, magnesium, bicarbonate and phosphates) and enzymes such as α -amylase and mucin (Guyton and Hall, 1996; Drago et al., 2011).

Water helps to moisten the food particles for easier breakdown. The dominating salivary enzyme, α -amylase begins starch digestion in the mouth resulting in a decreased perceived thickness of foods and therefore plays an important role in mouth feel perception. Saliva is secreted from different glands in the mouth and therefore, the magnitude of activity of its components differs based on the location of secretion (Humphery and Williamson, 2001). The magnitude of α -amylase activity was reported to be 60–70 U/ml in whole saliva and 50–60 U/ml in saliva secreted from parotid gland by Mackie and Pangborn (1990) and Froelich et al. (1987), respectively. The salivary mucin has lubrication properties that helps in formation of a food bolus that can be easily be swallowed (Tabak, 1995). Electrolytes like bicarbonates and phosphates modulate pH and the buffering capacity of saliva. In summary, salivary functions include lubrication and protection, buffering action, maintenance of tooth integrity, antibacterial activity, taste perception and digestion (Moss, 1995). Saliva also plays an important role in

clearing the esophageal acid because of normal reflux activity (Helm et al., 1983 and 1984) which indicates that the probiotic bacteria comes in contact with low pH and acid only in the stomach.

In healthy, non-medicated adults, the saliva flow rates are on an average 0.3 mL/min and 1.5 mL/min for unstimulated and stimulated whole saliva, respectively (Bertram, 1967; Heintze et al., 1983; Sreebny, 2000). There is great variation in the values found in literature for unstimulated and stimulated salivary flow rate. This is because the composition of saliva and its flow rate is influenced by the type and size of gland from which saliva is secreted (Ericson, 1971), state of hydration (Shannon, 1966), nutritional state (Johansson et al., 1992), the time of collection (Dawes, 1975), nature, characteristics and duration of stimulus (Dawes, 1969) and emotional state of the subject (Bolwig and Rafaelsen, 1972). The average pH value of saliva reported in literature is between 6 ± 0.5 (unstimulated) to 7 ± 0.5 (stimulated) (Drago et al., 2011; Davidson et al., 1998; Engelen et al., 2003; Humphrey and Williamson, 2001).

The effect of saliva on the viability and *in vitro* oral colonization potential of probiotic bacteria was tested by Haukioja et al. (2006). They found that the *Lactobacillus* and *Bifidobacterium* strains survived for 24 hr in saliva except for two strains that showed decreased counts after 24 hr incubation. None of the tested strains grew in saliva. The results from this study showed that some strains might be sensitive to the component(s) of saliva and therefore, it's essential to consider testing probiotic survivability in a simulated mastication process in addition to simulated *in vitro* gastric transit.

2.2.2 Stomach

The human stomach is a “J”- shaped, hollow and elastic organ that is divided into four major regions: fundus, body, antrum and pylorus. The three main functions of the stomach include storage of large particles of food for further disintegration, mixing of food with the contents of gastric juice and emptying of small particles into the intestine. The reservoir function of the stomach is achieved through its flexible volume which is capable of expanding to accommodate upto 4 L of food. Digestive juice which consists of hydrochloric acid (HCl) and enzymes is secreted to promote the enzymatic splitting of proteins, carbohydrates, and fats, while peristaltic contractions of the entire gastrointestinal tract (GIT) generate mechanical forces to promote mechanical and chemical breakdown of the food, absorption, and emptying. The intensity of secretory and motor responses of the GIT is affected by the individual, digestion time, and the amount, composition, and physicochemistry of the meal (Coupe et al., 1991; Camilleri and Prather, 1994; Mayer 1994; Parada and Aguilera, 2007).

2.2.2.1 Gastric secretion

Food stimulates Central Nervous System (CNS) activity, which results in the release of acid and gastrin hormone (Debas and Carvajal, 1994). Upon ingestion of food, alterations in stomach pH caused due to the food’s buffering capacity and volume results in hydrochloric acid (HCl) secretion. The mechanism of HCl secretion involves G cells in the antrum to secrete gastrin hormone which signals parietal cells to secrete gastric acid until the fasting state pH is restored (Wollin, 1987). Once the fasting state pH is restored, D cells secrete somatostatin to down regulate acid and gastrin hormone release (Chew, 1983; Magami et al., 2002; Schubert et al., 1987) protecting the stomach from excessive

acidity (Guyton and Hall, 1996). Principal components of gastric secretion (Table 2.2.2.1.1) include hydrochloric acid (HCl), 0.8 to 1mg/ml (=1g/L) pepsin, about 1.5 mg/ml (=1.5g/L) mucin (Vertzoni et al, 2005; Dean and Ma, 2007; Guyton and Hall, 1996b) and cations are sodium (about 70mM) and potassium (about 15mM), whereas the principal anion is chloride (about 100mM) (Dressman et al., 1998; Vertzoni et al., 2005; Kalantzi et al., 2006).

Table 2.2.2.1.1: Principal components of gastric juice and their functions (Glass, 1968)

Components	Concentration	Function
HCl	Variable	<ol style="list-style-type: none"> 1. Helps in breaking down of connective tissue and muscular fibers 2. Modifies gastric osmolarity by bringing the hyperosmotic and hyposmotic chyme to isosmotic level 3. Activates pepsinogen into pepsin by lowering the pH to a range where pepsin is active 4. Prevents bacterial growth and colonization 5. Stimulate secretin and pancreozymin production in the duodenum which in turn results in the secretion of bicarbonates and enzymes by pancreas.
Pepsin	0.8-1g/L	<ol style="list-style-type: none"> 1. The activation of pepsinogen results into pepsin at a pH range 1.0-2.0. 2. Pepsin helps in protein digestion. 3. It clots milk in human stomach similar to rennin in cows stomach due to denaturation of proteins in the milk
Mucin	1.5g/L	Mucin forms a gelatinous coating over the mucosal surface to prevent the digestion of stomach itself by hydrochloric acid

The resting volume of stomach in the fasted state rarely exceeds 50ml (Glass, 1968). Dressman et al. (1990) and Malagelada et al. (1976) reported empty (fasting) state stomach pH to be in the range 1.3-2.5 with a considerable degree of inter subject variation. They also found that the fasting state pH increased to on an average pH of 6.7 following ingestion of 6oz of hamburger, 2 slices of bread, 2oz of hash brown potatoes, 1tbsp each of ketchup and mayonnaise, 1 oz each of tomato and lettuce and 8 oz of milk

(total 1000 kcal) meal and pH 5.0 after consumption of coarsely ground tenderloin steak, cooked and seasoned with 0.1 g of salt; 25 g of white bread with 8 g of butter; 60 g of vanilla ice cream topped with 35 g of chocolate syrup and 240ml of water, respectively. This increase in pH upon consumption of meal clearly reflects the buffering effect of the food consumed. The higher the pH upon consumption of a meal, the more acid will be secreted in order to restore the pH back to the fasting state pH. Besides pH, other important factors that could lead to an increased rate of gastric secretion, acidity and dilution include food composition, its properties (protein and fat content, initial pH and viscosity) and the ingested quantity.

Meal viscosity also plays an important role since increase in meal viscosity causes an increase in the rate of secretion. The stomach responds to a high viscosity meal by increasing the rate of gastric secretion resulting in dilution and decrease in ingested meal viscosity. Marciani et al. (1999) reported that the viscosity of a meal containing 1.5 g locust bean gum per 100 g fell from 11 to 2 Pa.s immediately after ingestion and further decreased to 0.3 Pa.s after 30 min of digestion. Malagelada et al (1979) found that solid-liquid meals induced a stronger gastric secretory response (acid, pepsin, and volume) resulting in higher gastric acidity and volume compared to homogenized meals. This demonstrates the dynamic mechanism of gastric secretion in response to the food composition and its properties. Since probiotic bacteria are incorporated in a food product, it's very important to understand how composition of the food is able to enhance rate of gastric secretion and may consequently affect the survivability of probiotic bacteria during gastric transit.

During passage through the stomach, the viability of probiotic bacteria incorporated in a food matrix is influenced by stress factors like acid, enzymes and mechanical shear due to strong peristaltic contractions (Heatley and Sobala, 1993; Simon and Gorbach, 1987). Many probiotic bacteria are known to be acid sensitive (Shah and Jelen 1990; Berrada et al., 1991; Mituoka 1992; Shah and Lankaputhra 1997; Gardiner et al, 2000). *In vivo* and *in vitro* studies have found that initial pH of the stomach and its decrease from fed state pH (4-6) to fasting state pH (<3.0) during digestion are the principal factors affecting the viability of probiotic bacteria during its gastric transit (Conway et al, 1987; Pitino et al, 2010; Kheadr et al, 2009). Therefore, it's crucial to carefully select the food matrix for incorporating probiotic bacteria. Alternatively, a meal with high buffering capacity and fat content may protect the bacteria against acidic conditions during the digestion process before the bacteria is exposed to the restored fasting state pH.

2.2.2.2 Mechanical forces

The pattern of stomach motility is distinctly different in the fasting and fed states. There is a four phase movement in the fasting state and continuous movement in the fed state. There are two types of contractions namely regular tonic contractions and peristaltic contractions. Tonic contractions move food from the top to the bottom of the stomach and peristaltic contractions are responsible for grinding, kneading and mechanical disintegration of food (Kong and Singh, 2010). Gastric waves pass along both the lesser and the greater curvatures. Usually they do not proceed simultaneously along both the curvatures, so that there is generally some backlog between downward moving waves along both curvatures of the stomach (Glass, 1968). As the peristaltic wave

reaches the pylorus, the contraction width increases and indentations deepen (Bilecen et al., 2000; Schulze, 2006). Meanwhile, the pylorus contracts and the sphincter narrows so that only small particles or liquid can empty into the intestine whereas, the large particles are squirted back into the stomach for further disintegration. This action is called retropulsion (Figure 2.2.2.2.1). Magnitude of peristaltic contractions depend on various biological factors such as age, body mass index, hormonal factor, gender, blood glucose level and whether a person is suffering from any disorders. In addition, stomach increases or decreases the force of these contractions depending on the physical properties of food like solid, liquid, high viscosity, fat and protein content to result in complete digestion and emptying of food (Marciani et al., 2001).

Figure 2.2.2.2.1: Propulsion, grinding, and retropulsion of solids by peristaltic contractions of distal stomach (Kong and Singh, 2008)

2.2.3 *In vitro* digestion models

Methods that simulate the gastrointestinal digestion process in the laboratory are known as gastrointestinal models (GIMs) or *in vitro* digestion models (Parada and Aguilera, 2007). *In vivo* assays using humans or animals may provide the most accurate results, but they are costly, labor intensive, time consuming and causes ethical concern (Boisson and Eggum, 1991). Therefore, GIMs are used as a suitable alternative to *in vivo* assays to perform prescreening studies. Based on the results obtained from GIMs,

accurate experimental planning can be done for validating its results in *in vivo* studies.

GIMs can be categorized at static and dynamic models. Static models mainly include chemical digestion process while the mechanical disintegration is often ignored. Dynamic models typically simulate both chemical and mechanical aspects of the complex digestion process (Parada and Aguilera, 2007). A few examples of dynamic models are TNO (TIM) (Figure 2.2.3.1), Dynamic Gastric Model (DGM) (Figure 2.2.3.2), Human Gastric Simulator (HGS) (Figure 2.2.3.3) and Dynamic Model of Human Upper Gastrointestinal Tract (Figure 2.2.3.4). A comparison between the currently available *in vitro* models is shown in Table 2.2.3.1. Hur et al. (2011) did a survey on various *in vitro* digestion models and found that they differ from one another in their operation:

1. The number and type of steps included in the digestion sequence, e.g., mouth, stomach, small intestine, large intestine.
2. The composition of the digestive fluids used in each step, e.g., enzymes, salts, buffers, biological polymers, and surface active components.
3. The mechanical stresses and fluid flows utilized in each step in the digestion sequence, e.g., magnitude and direction of applied stresses, flow geometries, and flow profiles.

Table 2.2.3.1: *In vitro* digestion models

Model	Model Description	Reference
TNO (TIM) developed at TNO Nutrition and Food Research (Zeist, The Netherlands)	<ol style="list-style-type: none"> 1. Four serial compartments simulating the stomach, duodenum, jejunum, and ileum, are connected with each other by computer-controlled valve pumps. 2. The temperature is kept at 37°C and system is kept anaerobic by flushing with N₂ gas. 3. Change in water pressure simulates peristaltic movements 4. The pH values are controlled via the computer by secreting either water or 1M HCl into the stomach, or by secreting either water or 1M NaHCO₃ into the duodenum, via syringe pumps. 5. Secretions of gastric electrolytes and enzymes, bile and pancreatic juices are regulated by using computer-controlled syringe pumps. 	Minekus et al, 1995; Yoo and Chen, 2006
Dynamic Gastric Model Developed at Model Gut Platform - Institute of Food Research Norwich Research Park	<p>The DGM is built on a modular design of two stages:-</p> <ol style="list-style-type: none"> 1. Stomach: The first part simulates the main body of the stomach (fundus). This stage of the model mimics the mixing dynamics, diffusion profiles of both acid and enzymes and emptying cycles measured within the main body of the human stomach. This is followed by a unique emptying routine into a second module simulating the antrum (the lower part of the stomach). Here the digesta is subjected to high shear (as measured using EPI), forcing mechanical breakdown of the food structure. 2. Small intestine: Material emptied from the DGM can be then processed within a simulation of the small intestine. Here, intestinal mixing dynamics and diffusion are integrated with the addition of 	Wickham et al, 2009

	bicarbonate, phospholipids, bile and digestive enzymes simulating the complex environment of the small intestine.	
Dynamic model of human upper gastrointestinal tract developed at Food Research and Development Centre, Agriculture and Agri-Food Canada	<ol style="list-style-type: none"> 1. The model consists of two 1 L jacketed glass beakers representing the stomach and the duodenum. 2. Stomach Reactor include pH electrode, a temperature probe, and two entry ports for meal and HCl delivery into the stomach. 3. For the duodenum reactor, there are three entry ports for stomach digesta, NaOH, and Ovgall bile. 4. A magnetic stir bar is placed inside each vessel and agitation is controlled via a magnetic stirrer plate. This mimics shear caused in the stomach as a result of contractions 5. The temperature inside the reactors is controlled by circulating water at 37°C through the jacketed beakers. 6. Peristaltic pumps are used to control the delivery of the products to be added, as well as the emptying rate of the stomach reactor into the duodenum reactor. 	Mainville et al, 2004
Human Gastric Simulator developed at Dept. of Biological and Agricultural Engineering, Univ. of California, Davis, U.S.A	<ol style="list-style-type: none"> 1. HGS consists of round cylindrical stomach vessel made of latex rubber. 2. The main latex body has a diameter of 102 mm and a depth of 280 mm, and it has a collective volume of 5.7 L. 3. The bottom end of the latex vessel is tapered with an angle of 75 degrees to reduce the diameter to 25 mm. 4. Plastic tubing with internal diameter 3.2 mm connects the vessel bottom to a peristaltic pump for emptying digesta from the vessel. 5. During digestion trials, a thin polyester mesh bag with net pore size of 1.5 mm is placed inside the latex 	Kong and Singh, 2010

	<p>vessel, covering the inner wall of the latex. This bag allows small particulates of < 1 to 2 mm to pass through the mesh for emptying and retains large particulates for further breakdown, thus simulating a “sieving effect” of pylorus.</p> <ol style="list-style-type: none"> 6. A mechanical driving device consisting of 12 rollers, 4 belts, driving shafts, and pulley system has been installed to create peristaltic contractions on 4 sides of the latex stomach vessel. The driver is set to create 3 contractions per minute on the latex vessel, to simulate the actual stomach contraction frequency of 3 cycles per minute. 7. A variable flow mini peristaltic pump delivers simulated gastric juice into the simulated stomach chamber through a 6.4-mm ID plastic pipe splitting into 5 polyethylene tubing (I.D. 0.86 mm). A control valve is used to adjust the flow rate for the tubing. 8. Two 60W light bulbs are installed to maintain the temperature at 37°C and a thermostat is used to turn on/off the bulbs automatically. 	
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Figure 2.2.3.1: The multi-compartmental dynamic TIM-1 model of the gastrointestinal system. Vessels (A–D) constitute the gastric, duodenal, jejunal and ileal compartments, respectively. Modules (E) are semi-permeable hollow-fiber membrane dialysis units. (F) Peristaltic valves, (G) ileo-caecal valves, (H) pH electrodes, (I) temperature sensor, (J) stomach secretion inlets, (K) duodenal secretions inlet, (L) and (M) bicarbonate secretion inlet, and (N) volume detecting sensors (Khalf et al, 2010)

Figure 2.2.3.2: Dynamic Gastric Model. A- Main Body, B- Antrum, C- Emptying (Model Gut called Dynamic Gastric Model, PBLTechnology, 2006)

Figure 2.2.3.3: HGS. (1) Motor; (2) latex lining; (3) mesh bag; (4) secretion tubing; (5) roller; (6) belt; (7) light bulb for temperature control; (8) plastic foam insulation (Kong and Singh, 2010)

Figure 2.2.3.4: The dynamic *in vitro* human upper GI tract model system (Mainville et al., 2005)

During the past few years, food and animal scientists have utilized a number of these *in vitro* digestion models (both static and dynamic) to test the structural and chemical changes that occur in different foods during digestion process. *In vitro* models have been successfully used in assessing the quantitative release of functional ingredients and nutrients from food and drugs, such as tyrosol in enriched custards (Sanz and Luyten, 2006); carotenoids in carrot matrix (Garrett et al., 1999; Hedren et al., 2002); antioxidants in wholegrain foods (Nagah and Seal, 2005); stability and composition of the major polyphenols in chokeberry juice (Bermudez-Soto et al., 2007); isoflavonoids in soy bread (Walsh et al., 2003); disintegration kinetics of solid foods (Kong and Singh, 2008); and determining probiotic bacteria survivability during GI transit (Marteau et al, 1997; Pitino et al., 2010; Mainville et al., 2005). Marteau et al. (1997) reported no significant difference between the *in vitro* and *in vivo* data indicating that DGM has a predictive value for the survival of probiotic bacteria in humans. Limited foods and beverages have been studied using *in vitro* models.

Both static and dynamic models have been extensively used for assessing probiotic survivability during simulated digestion (gastric juice-low pH, presence of bile and peristaltic contractions) (Marteau et al., 1997; Maathuis et al., 2009; Kheadr et al., 2009; Pitino et al., 2010), to determine the protective effect of food matrix on the viability of probiotic bacteria (Khalf et al., 2010; Possemiers et al., 2010), to validate *in vitro* probiotic survivability with *in vivo* data (Ritter et al, 2009; Martinez et al, 2011), to determine the effect of selected prebiotic on the survivability of probiotic bacteria (Buriti et al., 2010; Martinez et al., 2011) as well as to validate the label claim about the level of live bacteria present in commercial products like yogurt, Gefilus milk, Gefilus, Emmental

cheese, Tutteli baby formula (Sumeri et al, 2008). A list of probiotic bacteria that have been tested in dynamic *in vitro* models is shown in Table 2.2.3.3.

Few studies have been conducted to determine the effect of food matrix and resistance of *L.acidophilus* La-5 to gastrointestinal stress and the effect of prior stress such as cold or low pH on its viability during digestion (Fernandez de Palencia et al., 2008; Sumeri et al., 2008; Buriti et al., 2010). Fernandez de Palencia et al. (2008) tested the effect of incubating *L. acidophilus* La-5 in skim milk acidified to pH 5.0, 4.1, 3.0, 2.1 and 1.8 for 20 min at each pH simulating conditions during digestion. They found more than 90% survival at the end of 20 min incubation each at pH 5.0 and 4.1, ~80% survival after incubation at pH 3.0, ~60% survival after incubation at pH 2.1 and less than 20% survival of *L. acidophilus* La-5 at the end of 20 min incubation at pH 1.8. These results indicate that pH below 2.0 caused highest percent mortality of *L. acidophilus* La-5. Buriti et al. (2010) studied the impact of storage temperature and simulated gastric conditions on the viability of *L. acidophilus* La-5 incorporated in guava mousse. The authors subjected *L. acidophilus* La-5 to simulated gastric conditions by incubating at pH 1.9 or lower for 2 hr and found that as the days of storage at 4°C progressed, the sensitivity of *L. acidophilus* La-5 towards low pH increased and on days 21 and 28 its viability was below 2 log cfu/g at the end of 2 hr simulated digestion. Whereas, the viability of *L. acidophilus* La-5 was improved for samples stored at -18°C and found to be 4 log cfu/g or slightly below when subjected to low pH for 2 hr on days 7, 35, 56, 84 and 112. The authors attributed this difference in viability of *L. acidophilus* La-5 to the initial stress of cold storage resulting in their better response to stress due to acidic conditions and they called this “cross-protective stress response”.

Table 2.2.3.3: *In vitro* models and probiotic bacteria

Model	Delivery Matrix	Strains	Reference
TNO	Olifus, yogurt; Lactose solution (5% w/v in water), fructose solution (5% w/v in water), milk; Fermented milk; Maple sap; experimental meals (with prebiotic, skimmed milk and with other probiotic bacteria)	<i>B. Bifidum</i> and <i>L. acidophilus</i> ; <i>L. delbrueckii</i> ssp. <i>bulgaricus</i> strain LB9; <i>Streptococcus thermophilus</i> strain ST20; <i>Bacillus coagulans</i> BC 30; <i>Pediococcus acidilactici</i> UL5; <i>B. animalis</i> E508, <i>L. paracasei</i> E510, <i>L. rhamnosus</i> E522, <i>L. plantarum</i> E98; <i>B. bifidum</i> BB12 and <i>L. rhamnosus</i> GG; <i>L. amylovorus</i> DSM 16698, <i>B. animalis</i> subsp. <i>lactis</i> Bb-12	Marteau et al, 1997; Maathuis et al, 2009; Kheadr et al, 2009; Miettinen et al, 1998; Khalf et al, 2010; Martinez et al, 2011
SHIME	Chocolate	<i>L. helveticus</i> CNCM I-1722 and <i>B. longum</i> CNCM I-3470	Possemiers et al, 2010
DGM	MRS broth; Sterile water and UHT whole milk	Six <i>L. rhmanosus</i> strains: D44, F17, H12, H25, N24, R61; <i>L. casei</i> subsp. <i>shirota</i> , <i>L. casei</i> subsp. <i>immunitas</i> , <i>L. acidophilus</i> subsp. <i>johnsonii</i>	Pitino et al, 2010; Curto et al, 2011
Dynamic model of the human upper gastrointestinal (GI) tract	Irradiated kefir	<i>B. infantis</i> ATCC 27920G, <i>L. acidophilus</i> ATCC 4356, <i>L. johnsonii</i> NCC533 La-1, <i>L. rhamnosus</i> ATCC 53103 GG	Mainville et al, 2005
Single bioreactor system gastrointestinal tract simulator (GITS)	Lactose containing complex, Gefilus milk, Gefilus daily dose drink, Gefilus Emmental cheese, Gefilus peptide Tutteli baby formula; UHT skim milk; MRS medium frozen in nitrogen beads	<i>L. acidophilus</i> La-5, <i>L. johnsonii</i> NCC 533, <i>L. casei</i> strain shirota, <i>L. rhamnosus</i> GG; <i>L. gasseri</i> K7, <i>B. animalis</i> subsp. <i>lactis</i> 14403, <i>B. longum</i> subsp. <i>infantis</i> 14390, <i>B. longum</i> 14383, <i>B. adolescentis</i> 14377, <i>B. longum</i> 14382, <i>B. breve</i> 14938, <i>B. longum</i> 14406; <i>L. acidophilus</i> La-5, <i>L. rhamnosus</i> GG, and <i>L. fermentum</i> ME-3	Sumeri et al, 2008; Ritter et al, 2009; Sumeri et al, 2010

2.3 Justification of work

The most common outlet for probiotic bacteria is found to be fermented dairy products such as fermented milk and yogurt since consumers are well aware of the existence of live bacteria in these products and the positive health image associated with them. However, several studies have shown that fermented dairy products do not sustain adequate populations of viable probiotic bacteria during their shelf life. The main obstacle for achieving and maintaining the required levels of probiotic bacteria in fermented dairy products is the poor survival due to interactions between species present, increased acidity, culture conditions, chemical composition of the product, availability of nutrients, growth promoters and inhibitors, concentration of sugars, level of inoculation, incubation temperature, fermentation time and storage temperature (Shah 2000; Heller 2001; Antunes et al. 2005; Donkor et al. 2007; Mortazavian et al. 2007). Thus, it's important to study the effect of alternative non-fermented dairy products such as ice cream on the survivability of probiotic bacteria during product manufacture, storage and gastric transit. This will eliminate the adverse effects of fermentation process preserving the benefits of milk components like fat and proteins on the survival of probiotic bacteria.

To encourage consumer acceptance of alternate products, it's extremely important to minimize negative effects of probiotic supplementation on the organoleptic properties of these products. Therefore, it's important to choose a form of probiotic bacteria that would cause least impact on the flavor. In addition, it should be convenient and easy for big factories to supplement products with probiotic bacteria to result in better efficiency and productivity. Abghari et al. (2011) conducted a study to evaluate the efficiency of non-fermented ice cream for delivering *L. acidophilus* and *L. rhamnosus* to consumers

and to determine its effect on organoleptic properties of ice cream. Sensory analysis indicated that panelists were able to distinguish between control and inoculated samples based on flavor difference. The cause of off-flavor in the ice cream could possibly be due to harvested cells from MRS medium that were used to inoculate the ice cream mix (Abu-Taraboush et al, 1998). Therefore, using freeze dried form of bacterial culture could possibly ensure better palatability in addition to least processing requirements for ice cream factories.

Numerous studies have tested survival of *L. acidophilus* La-5 during ice cream manufacture and storage at subzero temperature (Salem et al., 2005; Taha et al., 2005; Magarinos et al., 2007). They found that viability of *L. acidophilus* La-5 after freezing and at the end of shelf life is maintained above the recommended level of 10^6 cfu/g. The consumption of high level of *L. acidophilus* La-5 may not guarantee the intended health benefits because it must reach the intestine in high viable numbers to be able to provide these benefits. Hence, it is extremely important to study its viability in the presence of acid, enzymes and mechanical shear caused due to peristaltic contractions during simulated digestion process. A few studies have investigated the effect of food products on the survivability of *L. acidophilus* La-5 when subjected to gastric stress using an *in vitro* digestion model (Fernandez de Palencia et al., 2008; Sumeri et al., 2008; Buriti et al., 2010). None of these studies have looked at the effect of incorporating *L. acidophilus* La-5 in an ice cream on its viability during simulated digestion process. Hence, in the present study, low fat non-fermented ice cream was supplemented with *L. acidophilus* La-5 and its survival was determined during simulated digestion of 2 hr using an *in vitro* model stomach called Human Gastric Simulator (HGS).

Abghari et al. (2011) conducted a conventional acid resistance test by incubating cells in MRS broth adjusted to pH 1.5, 2.5, 3.5 and 6.5 to determine whether frozen thawed cells (from ice cream) displayed a similar or different acid shock response compared to fresh cells. This study was conducted with a possibility of acid tolerant strains becoming acid sensitive after going through the mixing, churning, freezing and air incorporation process as well as storage at sub zero temperatures. Since probiotic bacteria must survive low pH conditions during their transit through the stomach; there would be no reason to incorporate acid tolerant strains into the product that could become acid sensitive at the end of food production and storage. Their results showed that at pH 2.5, the number of *L. acidophilus* cells decreased significantly ($p < 0.05$) by 3 log units after 1 hr of incubation for fresh cells, whereas, frozen thawed cells were completely inhibited at the end of 1 hr incubation at pH 2.5. On the contrary, Alamprese et al (2002 and 2005) reported no significant reduction in frozen thawed cells at the end of 3 hr incubation at pH 2.5 and 3.5 in comparison to fresh cells for *L. johnsonii* La-1 and *L. rhamnosus* GG. Therefore, it's essential to ensure that the selected strain does not become acid sensitive after production and storage.

Convention acid resistance test has its own limitations since the bacteria are exposed to the same pH throughout the incubation time partially contradictory to conditions *in vivo*, it oversimplifies the mixing patterns and does not reproduce temporal nature of gastric and duodenum processing, mechanical forces that probiotic bacteria will encounter in the stomach resulting from contractions of the stomach wall, dilution of food and particle size reduction which can affect the digestion rate are ignored (Kong and Singh, 2010; Curto et al., 2011). This could lead to rejection of probiotic strains that have

potential to reach the intestine in high numbers and provide health benefits (Mainville et al, 2005). Therefore, it's important to test the acid resistance of probiotic bacteria in an *in vitro* model closely simulating digestion conditions *in vivo*. Therefore, in the present study, conventional acid resistance test was conducted using a static model and the results were compared with those obtained from HGS. This *in vitro* dynamic model is capable of simulating both chemical and mechanical conditions that occur during digestion in *in vivo*. In addition, it is the only *in vitro* dynamic digestion model available in the U.S. Using this model will allow more research to be conducted in the U.S and save time and money that would be spent in its absence by going to other countries to use the *in vitro* models.

2.3.1 Research Objectives

- 1) To determine the effect of air addition on the viability of *L. acidophilus* La-5
- 2) To determine the effect of simulated digestion process on the viability of *L. acidophilus* La-5

2.3.2 Research Tasks

Phase I: Survivability of *L. acidophilus* La-5 in nonfermented low fat ice cream

- a) To manufacture low fat nonfermented probiotic ice cream by inoculating the ice cream mix with freeze dried form of *L. acidophilus* La-5 and determine the effect of addition of La-5 on the physicochemical properties of ice cream.
- b) To determine the effect of two levels of over run and freezing on the viability of *L. acidophilus* La-5 immediately after freezing and during storage at -10°C for 90 days.

Phase II: Bench top experiments

- a) To evaluate *in vitro* buffering capacity of ice cream mix, milk and PBS in the absence of *L. acidophilus* La-5 and compare it with buffering capacities of samples in the presence of La-5.
- b) To assess the effect of simulated gastric shear and dilution on the survivability of *L. acidophilus* La-5 in low viscosity and high viscosity ice cream samples.
- c) To determine the effect of incubation of *L. acidophilus* La-5 and *L. paracasei* 431 at pH 2.0 and pH 5.0 in the absence of ice cream mix for two hours on its survivability during and at the end of 2 hr.
- d) To compare survivability of each of the tested probiotic bacteria (*L. acidophilus* La-5 and *L. paracasei* 431) at pH 2.0, pH 5.0 and during simulated static digestion in the presence of ice cream mix.

Phase III: *In vitro* digestion of ice cream

- a) To determine the acid tolerance of *L. acidophilus* La-5 incorporated in a low fat non-fermented ice cream during a closely replicated gastric digestion after its storage at -10°C for 30 days.
- b) To compare the buffering effect of ice cream observed in the static model stomach experiments with two different *in vitro* model stomachs namely shaking water bath and human gastric simulator (HGS).
- c) To determine whether presence of mechanical shear caused due to peristaltic contractions (similar to the ones found in the stomach during digestion) had an impact on the survivability of *L. acidophilus* La-5 in addition to the impact of pH.

- d) To assess the role of viscosity as an important factor in maintaining the viability of *Lactobacillus acidophilus* La-5 during simulated digestion process.

2.3.2 Research Hypotheses

1. The ice cream sample incorporated with 100% overrun will pose significant detrimental impact on the viability of *L. acidophilus* La-5 post freezing process compared to the ice cream sample incorporated with 60% overrun.
2. The modeled digestion of ice cream in human gastric simulator (HGS) will result in significantly lower survival rate of *L. acidophilus* La-5 at the end of 2 hr digestion compared to the shaking water bath model stomach.
3. The high buffering capacity and viscosity of the ice cream will provide protection and improve viability of *L. acidophilus* La-5 during simulated gastric digestion in Human Gastric Simulator.
4. The high initial inoculation level of *L. acidophilus* La-5 in the ice cream will result in higher percent survival at the end of simulated digestion of 2 hr in HGS compared to the low initial inoculation level.

3.0 Materials and Methods

3.1 Phase I: Survivability of *L. acidophilus* La-5 in non-fermented low fat ice cream

3.1.1 Ice cream production

Ice cream was manufactured to study the effect of two levels of over run (OR) namely 60% and 100% on the survivability of *Lactobacillus acidophilus* La-5 (Figure 3.1.1.1). Raw milk, 3.5% fat (Cal Poly Dairy, San Luis Obispo, CA) and pasteurized cream, 38% fat (Producers Dairy, California, U.S.A) were used in the ice cream mix as fat sources. Nonfat dry milk (Grade A, Dairy America, USA), sugar (Extra fine granulated cane sugar, Classic SYSCO), Corn syrup solids (Cargill, USA) and stabilizer/emulsifier blend (Grindsted[®] Ice Pro 2516 LF, Danisco, USA) were also used in making the ice cream mix. The ice cream formulation was based on 4% fat, 12% nonfat milk solids, 15% sugar, 4% Corn Syrup Solids, 0.65% stabilizer/emulsifier and 35% total solids (Appendix I).

Two batches of ice cream mix were made (100 lbs each), one control mix and one probiotic test mix. The probiotic test mix was inoculated with probiotic bacteria strain *Lactobacillus acidophilus* La-5 (Chr. Hansen, Wisconsin, U.S.A) and mixed thoroughly to yield an initial population of 10^7 cfu/g. After 24 hours of ageing at 4°C, for both mixes, half of the mix was frozen with a 60% OR and the other half was frozen with a 100% OR using batch scrap surface freezer (Technogel, Greensboro, NC) with a draw temperature of -4°C. The overrun was regulated by using the air incorporation screw from the heat exchanger, which was adjusted to the desired amount. Throughout processing, the screw was adjusted two times, resulting in the following overrun levels: 60% (P60) and 100% (P100) (Table 3.1.1.1). All the ice cream samples supplemented with La-5 were the

experimental samples (P60 and P100). Control and experimental ice cream samples were filled in 3.5oz cups, placed in the hardening freezer at -26°C and then moved to -10°C freezer for 90 days for shelf life study.

Table 3.1.1.1: Probiotic and control ice creams – features and codes

Ice cream samples	Features	Code
Probiotic ice cream 60	60% over run level	P60
Probiotic ice cream 100	100% over run level	P100
Control ice cream 60	60% over run level	C60
Control ice cream 100	100% over run level	C100

Figure 3.1.1.1: Probiotic ice cream manufacturing procedure

Table 3.1.1.2: Experimental Design

Effect of two levels of over run (OR) on survivability of <i>Lactobacillus acidophilus</i> La-5			
Factor	Treatment levels	Response	Repetition
Level of overrun	60%	Survival of <i>L.acidophilus</i> La-5	2
	100%		

3.1.2 Enumeration of *L. acidophilus* La-5

Experimental samples were collected prior to and immediately after freezing (soft ice cream at -4°C) and after hardening to determine the effect of air addition on the rate of survival of *L. acidophilus* La-5 in the ice cream. During the three months of storage, P60 and P100 ice cream samples were examined every 30 days for *L. acidophilus* La-5 viability. To determine the counts of viable *L. acidophilus* La-5, 11 g of individual experimental samples were transferred to a sterile bag containing 99 mL of phosphate buffered saline (PBS, pH 7.2, Hardy Diagnostics, Santa Maria, CA). Further 10 fold dilutions were made from this dilution, and the counts were performed in duplicate by using the pour plating technique using MRS (de Man, Rogosa and Sharpe, Difco) agar. L- Cys-HCl was added to MRS agar at 0.05% (wt/vol) to reduce the redox potential of the medium for the growth of *L. acidophilus* La-5 (Taha et al., 2005). The plates were placed in an anaerobic jar and the resulting colonies on the plates were counted after 48 hr incubation at 37°C in a closed incubator containing 5% CO₂ (v/v) (Abghari et al., 2011).

3.1.3 Physiochemical analysis of ice cream mix

Microbial analysis, fat, moisture and total solids analysis was conducted after making the ice cream mix, whereas, pH and viscosity tests were conducted after ageing the ice cream mix.

Microbial Analysis: All mixes produced were analyzed for standard plate count and E.coli/Coliform (EC/CC) before inoculation with probiotic bacteria to validate that the mix was properly pasteurized. Standard plate counts were determined by plating 1 mL of each sample in duplicate on aerobic plate count (APC) Petrifilm (3M, St. Paul, MN)

and incubating at 32°C for 48 hr. Coliform counts were determined by plating 1 mL of each sample in duplicate on E.coli/Coliform Petrifilm and incubating at 35°C for 24 hr.

Fat, moisture and total solids (TS): Percent fat was determined using Mojonnier fat extraction method (Wehr, 2004). Each sample was tested in duplicate. Moisture and TS was measured using CEM Corporation LabWave9000, Microwave Moisture/Solids Analyzer (Modler et al, 1990). All the samples were tested in triplicate by spreading approximately 2.0 grams of ice cream mix sample on a sample pad and heated for 5 min at 60% power. The final moisture content and TS were recorded.

pH and titratable acidity: The pH values were measured in triplicate for aged control and experimental samples at room temperature using pH meter (Orion, Model 410) standardized to pH 4.0 and pH 7.0. The average of three readings was recorded. Acidity of mixes was determined in duplicate by titration with NaOH 0.1N, using phenolphthalein as an indicator (Richardson, 1986)

Mix Viscosity: A Brookfield viscometer, model ¼ RV DV II+ (Brookfield Engineering Laboratories, Stoughton, MA, USA) was used to measure the apparent viscosity of the ice cream mix samples (with and without added probiotic bacteria) after overnight ageing at 4°C. Samples were tested in duplicate at 4°C and 37°C with spindle #18. Temperature was maintained by connecting the water bath (VWR North American Ca# 13271-074, Model 1160S) to the sample holder. Samples were loaded into the sample cup and allowed to equilibrate at the desired temperature. Viscosity was then recorded after samples were sheared for about 20 sec in the shear rate range of 2.64 to 66 sec⁻¹ at 4°C and in the range of 13.2 to 66 sec⁻¹ at 37°C. The power law model was used to describe the apparent viscosity of the mix as a function of shear rate and to calculate the

flow behavior index (n) and consistency index (κ) of the different ice-cream mixes (Goff et al, 1994).

$$\eta = \kappa D^{n-1}$$

Where,

η = the relative viscosity

D= the shear rate

n= the flow behavior index

κ = the consistency index

3.1.4 Statistical Analysis

Data was statistically analyzed using General Linear Model of Minitab 16.0 software (PA, USA) to determine significant effects of freezing process, percentage of overrun and storage at -10°C on the survivability of *L. acidophilus* La-5. Significant differences in the viable cells of La-5 between samples pre and post freezing were determined using one way analysis of variance (ANOVA) with viable counts as response and level of overrun as main effect. Significant differences in the viable cells of La-5 during storage (30, 60 and 120 days) were also evaluated using one way analysis of variance (ANOVA) with viable counts as response and storage time and level of over run as main factors. The significance level used for all the analysis was 5%.

3.2 Phase II: Bench top experiments

3.2.1 Buffering capacity of ice cream mix, milk and PBS

A comparison study was conducted to determine the amount of gastric juice (GS) required to restore fasting state stomach pH (~2.0) once the pH of GS in the simulated empty stomach increases upon addition of ice cream mix (Cal Poly ice cream mix, 10% fat), milk (3.5%, pasteurized milk) and 1x PBS, pH ~7.0 (10x PBS, 0.017 M potassium phosphate monobasic (Fisher Sci. cat #P-0662), 0.1 M potassium phosphate dibasic (EMD Chemicals, cat #PX1570-2), 1.49 M NaCl (Fisher Sci. cat #S640-3)). All the

experiments were conducted at room ($22\pm1^{\circ}\text{C}$) and body temperature ($37\pm1^{\circ}\text{C}$), in the presence and absence of *L. acidophilus* La-5 and in the presence of ice cream mix, milk or PBS. Simulated GS was prepared fresh by dissolving pepsin (1 g/L; Laboratory Grade; Powder; cat# P53 100, Fisher Scientific, USA), gastric mucin (1.5 g/L; mucin from porcine stomach, cat# M2378-100G, Sigma-Aldrich, USA), and NaCl (8.775 g/L) in deionized water. The 500 mL of GS was added to an acidometer and placed on a heat plate adjusted to 39°C so that the temperature of the GS in the flask is maintained at $37\pm1^{\circ}\text{C}$ taking into account any heat loss. Once the temperature of GS was at $\sim 37^{\circ}\text{C}$, its pH was adjusted to 1.5 ± 0.05 . A 4 oz glass cup was filled with 9 mL of ice cream, milk or PBS with or without 1g of *L. acidophilus* La-5. The native pH was recorded before adding GS to the samples. A list of indicators used to indicate a decrease in pH by a change in color as GS was consistently added to the sample cup is shown in Table 3.2.1.2. Three to four drops of bromocresol purple (0.04% (w/v) aqueous solution, CAS # 62625-30-3, Ricca Chemical, TX, USA) was added to the sample cup to detect the pH change of the sample from its native pH to pH 5.0. Once, the indicator changed color from purple to yellow (Table 3.2.1.1), amount of GS added as well as pH was recorded. A similar process was repeated with methyl red (0.02% (w/v) aqueous solution, CAS # 845-10-3, Ricca Chemical, TX, USA), bromocresol green (0.04% (w/v) aqueous solution, CAS# 62625-32-5, Ricca Chemical, TX, USA) and congo red (0.1% (w/v) aqueous solution, CAS# 573-58-0, Ricca Chemical, TX, USA) indicators for pH change from native pH of sample to pH 4.0, 3.0, 2.0, respectively.

Table 3.2.1.1: Experimental design – buffering capacity

Factors	Treatment levels	Response	Repetition
Product	Ice cream mix	Amount of gastric juice required to restore pH of ~2.0	2
	Milk		
	PBS		
Temperature	Room		
	Body		
Probiotic bacteria	Presence		
	Absence		

Table 3.2.1.2: Indicators and color change

Indicator	Color (pH>5.0)	Final color change	Final pH
Congo Red	Red	Blue	2
Bromocresol green	Blue	Yellow	3
Methyl red	Red	Pink	4
Bromocresol Purple	Purple	Yellow	5

3.2.2 Effect of shear and dilution on the survivability of *L. acidophilus* La-5

Bench top experimental set up was used to determine the effect of continuous shear for 2 hr and dilution with GS at regular intervals on the survivability of *L. acidophilus* La-5 incorporated in low and high viscosity ice cream samples. The viscosity of ice cream mixes was changed by varying the amount of added stab/emulsifier. The ice cream formulation was based on a) 5% fat, 11% MSNF, 15% sugar, 4% CSS, 0.65% stab/emulsifier (Appendix II) and b) 5% fat, 11% MSNF, 15% sugar, 4% CSS, 1.3% stab/emulsifier (Appendix III). Each of the two ice cream mixes were inoculated with *L. acidophilus* La-5 to yield an initial count of 10^8 cfu/g before freezing. After ageing, the mixes were frozen with 60% overrun. Experimental samples were collected pre and post freezing and plated in duplicate (section 3.1.2). DV II+ Pro Viscometer (Brookfield Engineering Laboratories, Stoughton, MA, USA), consisting of jacketed cylindrical sample holder and connected to water bath (VWR North American Ca# 13271-074,

Model 1160S) was used for these experiments. Spindle SC4-21 was used for low viscosity samples and spindle SC4-27 was used for high viscosity samples.

3.2.2.1 Effect of simulated gastric shear

In order to determine effect of simulated gastric shear on the survivability of *L. acidophilus* La-5, slightly different procedure was followed for low viscosity samples compared to high viscosity samples due to equipment constraints. Low viscosity ice cream cup (3.5oz) was placed at room temperature for thawing. The water bath was set at 4°C. Once the temperature of the water bath was equilibrated, 10 mL of ice cream was added to the sample cup and the temperature of water bath was set at 37°C to simulate change in temperature that occurs upon consumption of ice cream. The sample was sheared at a rate 22.32 sec^{-1} (Brenelli et al., 1997) for 2 hr. One mL of ice cream samples were collected before starting the experiment at 4°C, once the temperature of sample was adjusted to 37°C, at the end of 60 and 120 min and pour plated on MRS agar (Section 3.2.3.1). Similar process was repeated for high viscosity sample except for the shear rate which was 21.76 sec^{-1} .

3.2.2.2 Effect of simulated gastric dilution of ice cream

Simulated GS was prepared (section 3.2.1) and its pH was adjusted at 7.0 by using 0.1N NaOH. Two mL of GS was added to the sample cup. Usually the pH of the empty stomach is in the range of 1.5 to 2.5, but gastric juice at pH 7.0 was used in this experiment to prevent the effect of low pH in addition to effect of dilution on the survivability of *L. acidophilus* La-5. The primary goal was to determine the effect of dilution of ice cream upon ingestion and during digestion on the viability of La-5. Once the temperature of gastric juice was adjusted to 37°C, 5 mL of thawed ice cream (~4°C)

was added to the sample cup containing GS. The mixture was sheared at a rate of 22.32 sec⁻¹ and 21.76 sec⁻¹ for low and high viscosity ice cream samples, respectively. One mL sample was collected before adding ice cream to GS in the sample cup and after the temperature of ice cream and GS increased and equilibrated at 37°C. Four mL GS was added to the mixture after first 15 and 45 min of the experiment to mimic progressive gastric secretion *in vivo*. One mL sample was collected at the end of 30, 60 and 120 min and all the samples were immediately pour plated on MRS agar (section 3.2.3.1).

3.2.3 Conventional acid resistance test in the presence and absence of ice cream

3.2.3.1 Acid resistance test in the absence of ice cream

Probiotic strains of *L.acidophilus* La-5 and *L. paracasei* 431 were obtained from Chr. Hansen (Wisconsin, U.S.A) in the DVS frozen pellet form. One gram was carefully weighed and added to 10 mL of Phosphate Buffered Saline (PBS; section 3.2.1). The mixture was thoroughly mixed using a bench top vortex to dissolve the frozen pellets and disperse homogeneously in a 10 mL PBS tube. One mL of sample was collected, serially diluted, and pour plated on MRS agar to determine the initial number of bacteria (cfu/g). This was the control. To determine acid tolerance of the two probiotic strains, a 50 mL falcon tube was filled with 16 mL of citrate-HCl buffer (29.40g/L tri sodium citrate. 2H₂O and 1M HCl; Ritter et al., 2009) adjusted to pH 2.0. This tube was tied to a bench top rotator (Labquake, Barnstead International, IA, USA; Model # 415110) and placed on a platform shaker (Innova 2000, New Brunswick Scientific, CT, USA) in an incubator set at body temperature (37°C) to allow the temperature of the buffer to equilibrate with the incubator temperature.

Four mL of PBS plus probiotic bacteria mixture was carefully pipetted into 50 mL falcon tube containing citrate-HCl buffer, pH 2.0 to mimic fed state stomach conditions. The mixture was continuously mixed by shaking at 50 rpm to simulate gastric mixing and shear due to peristaltic contractions during digestion (Oomen et al., 2003). A similar procedure was followed to determine acid tolerance of *L. acidophilus* La-5 and *L. paracasei* 431 at pH 5.0 (Ruiz-Moyano et al., 2008). One mL of experimental sample was collected immediately after administration of PBS and probiotic bacteria into citrate-HCl buffer and at the end of 60 and 120 min. One mL of experimental sample was serially diluted with 9 mL of PBS (pH 7.2, Hardy Diagnostics, Santa Maria, CA). Further 10-fold dilutions were made from this dilution and the counts were performed in duplicate using the pour plating technique and MRS agar (section 3.1.2).

3.2.3.2 Acid resistance test in the presence of ice cream

Cal Poly creamery ice cream mix (10% fat) was used to determine protective effect of ice cream components on the survivability of *L. acidophilus* La-5 and *L. paracasei* 431 during simulated digestion conditions. One gram of frozen pellets was carefully weighed and added to 10 mL of PBS (section 3.2.1). The mixture was thoroughly mixed. Four mL of this mixture was added to nine mL of ice cream mix and thoroughly mixed again. To mimic fasting state stomach conditions, 16 mL of simulated GS (section 3.2.1) was added to a 100 mL empty plastic tube and similar experimental setup was followed as in section 3.2.3.1. Once the temperature of GS was equilibrated to $37\pm1^{\circ}\text{C}$, its pH was adjusted to 2.0 (Mainville et al., 2005) using 6N HCl.

To mimic fed state stomach conditions, 4 mL of ice cream mix and probiotic bacteria mixture was carefully pipetted into 100 mL plastic tube containing 16mL GS

(pH 2.0) and immediately the pH of the mixture was recorded. Preliminary studies showed that the pH of the GS increased due to the addition of ice cream mix and probiotic mixture into the GS due to the buffering effect of the ice cream mix. Therefore, during bench top study, GS (pH 2.0, $37\pm1^{\circ}\text{C}$) was manually added in 10-15 mL increments for 120 min to the mixture in order to restore fasting state stomach pH of 2.5 (Malagelada et al., 1976). Sample collection and bacterial enumeration was done as shown in section 3.2.3.1.

Table 3.2.3.1: Experimental Design – conventional acid resistance test

Factors	Treatments levels	Response	Repetition
pH	2.0	Survival of <i>L. acidophilus</i> La-5 and <i>L. paracasei</i> 431	2
	5.0		
Probiotic bacteria	<i>L. acidophilus</i> La-5		
	<i>L. paracasei</i> 431		

3.2.4 Statistical analysis

Buffering capacity of ice cream mix, milk and PBS: Data was statistically analyzed using GLM to determine the significant effect of sample, temperature and presence of *L. acidophilus* La-5 on the buffering capacities of ice cream, milk and PBS. Tukey's pairwise comparison in one way analysis of variance (ANOVA) was used to determine significant differences among samples in the presence and absence of *L. acidophilus* La-5 each at room and body using BC as a response and samples as the main factor. The effect of pH on the BC of samples was determined using GLM. The significance level used for all the analysis was 5%.

Effect of shear and dilution on the survivability of *L. acidophilus* La-5: GLM was used to determine the effect of shear and dilution on the viability of *L. acidophilus* La-5 added in low and high viscosity ice cream samples. Tukey's pairwise comparison test was done using one way analysis of variance (ANOVA) to determine which of the

treatment pairs were significantly different among low viscosity samples by using survival rate of La-5 as response and treatment effect as main factor. The significance level used for all the analyses was 5%.

Conventional acid resistance test in the presence and absence of ice cream: The impact of incubation pH (2.0 and 5.0) and the sampling time (0, 60 and 120min) on the viable counts of *L. acidophilus* La-5 and *L. paracasei* 431 was determined using GLM. Tukey's pairwise comparison test was done using one way analysis of variance (ANOVA) to determine the significant differences between survival rate at different sampling points and between survival rate of *L. acidophilus* La-5 and *L. paracasei* 431 at each sampling point. Tukey's pairwise comparison test was also used to determine significant differences among the overall log reduction of *L. acidophilus* La-5 using survival rate as response and treatment (pH 2.0, pH 5.0 and presence of ice cream) as main effect. Similar comparison was done for *L. paracasei* 431. The significance level used for all the analyses was 5%.

3.3 Phase III: *In vitro* digestion of ice cream

3.3.1 Shaking water bath

Shaking water bath was used as an *in vitro* model stomach to determine the protective effect of frozen ice cream on the survivability of *L. acidophilus* La-5. This method is often used to simulate gastric conditions (Muir and O'Dea, 1992) except for peristaltic contractions observed in the stomach during digestion. As shown in Figure 3.3.1.1, a shaking water bath (Model YB531, American Scientific Products) was adjusted to body temperature (37°C) and two 500 mL glass beakers were held in a rack placed in the water bath. The beakers will be addressed as beaker # 1 and beaker # 2. Fifty mL of

freshly prepared GS (section 3.2.1) adjusted at ~pH 1.8 using 6N HCl (Vertzoni et al, 2005) was added to beaker #1 to simulate fasting state stomach conditions and 400 mL of gastric juice (pH 1.8) was added to beaker #2 to be used during simulated digestion process. Contents in both the beakers were maintained at body temperature.

A 3.5oz ice cream (Appendix II) cup was randomly selected and thawed for about thirty minutes. A mercury thermometer and pH probe connected to a pH meter (IQ Scientific Instruments, ISFET #12 probe) were placed in beaker #1 to consistently record temperature and pH, respectively throughout the 2 hr simulated digestion process. A sample of thawed ice cream was collected to determine the viable cells of *L. acidophilus* La-5 before the simulated digestion process and frozen to inhibit any activity until plating. The rest of the ice cream was added to beaker#1 to simulate fed state stomach conditions. The rack containing beaker #1 and beaker #2 was immediately shaken at a frequency of 120 strokes/min for 2 hr (Kong and Singh, 2010) and the peristaltic pump (Masterflex Pump Controller 7553-50/7090-42 Pump, Cole-Parmer, Chicago, Ill., U.S.A.) was turned on to secrete 3.5 mL/min (Mainville et al, 2005) of GS from beaker #2 into beaker #1 containing ice cream to simulate shear and secretion from stomach walls, respectively during digestion. Once the pH of the simulated gastric digesta in beaker #1 was restored to pH 2.5, the secretion rate was lowered to 0.9 mL/min (Mainville et al, 2005) to simulate gastrin hormone inhibition which signals the stomach to stop secreting HCl.

Samples were collected after 30, 60 and 120 min to determine the effect of simulated digestion on the viability of La-5 and refrigerated until plating. Enumeration of *L. acidophilus* La-5 was performed immediately after the simulated digestion process was

complete according to the procedure in section 3.2.3.1. The whole simulated digestion process was repeated twice.

Figure 3.3.1.1: Effect of gastric digestion simulated using shaking water bath on the survivability of *L. acidophilus* La-5

Table 3.3.1.1: Experimental Design – shaking water bath

Factor	Treatment level	Response	Repetition
<i>In vitro</i> digestion model	Shaking water bath	Survival of <i>L. acidophilus</i> La-5	2
	Human Gastric Simulator (HGS)		

3.3.2 Human Gastric Simulator (HGS)

Protective effect of ice cream on the survivability of *L. acidophilus* La-5 was validated using dynamic *in vitro* model stomach called Human Gastric Simulator (HGS) developed at UC Davis by Dr. Paul Singh. The main components of the HGS are a latex lining chamber to mimic stomach, mechanical driving system composed of 12 rollers secured on belts pushing the stomach walls driven by a motor assembly, gastric secretion and temperature and pH control (Figure 3.3.2.1). The entire system was placed on a large aluminum base plate and covered by specially designed insulated plastic foam chamber to

prevent heat loss. For detail description of the model stomach, refer to Kong and Singh (2010).

Figure 3.3.2.1: Human Gastric Simulator

Two different ice cream mixes were made as per the flow chart in Figure 3.1.1.1. The level of viscosity was different in the two ice cream mix formulations and this was done by changing the amount of added stabilizer/ emulsifier blend. The formulation was based on a) 5% fat, 11% MSNF, 15% sugar, 4% CSS, and 0.65% stab/emulsifier (Appendix II) b) 5% fat, 11% MSNF, 15% sugar, 4% CSS, 1.3% stab/emulsifier (Appendix III). The ice cream mix with lower level of viscosity was divided into two equal parts and each of the two parts was inoculated with *L. acidophilus* La-5 to obtain an initial population of 10^8 cfu/g and 10^6 cfu/g, respectively before freezing. Based on the results from section 3.1.2, approximately one log reduction was estimated upon freezing and air incorporation. The ice cream mix with higher level of viscosity was inoculated in a similar fashion as the one with lower viscosity to obtain an initial population of

10⁸cfu/g and 10⁶cfu/g respectively before freezing. All the ice cream mix samples (Table 3.3.2.1) were frozen with 60% over run after ageing overnight at 4°C and stored at -10°C until the day of *in vitro* digestion experiments. Experimental samples were collected immediately before and after freezing to determine the counts of *L. acidophilus* La-5 in the mix and in the final ice cream, respectively using the procedure described in section 3.1.2.

Table 3.3.2.1 Probiotic ice cream samples and their codes

Ice cream samples	Amount of added stabilizer/emulsifier blend	Amount of inoculation (<i>L. acidophilus</i> La-5)	Code
Low viscosity, high level of La-5	0.65%	10 ⁸ cfu/g	LVHC
Low viscosity, low level of La-5	0.65%	10 ⁶ cfu/g	LVLC
High viscosity, high level of La-5	1.3%	10 ⁸ cfu/g	HVHC
High viscosity, low level of La-5	1.3%	10 ⁶ cfu/g	HVLC

Table 3.3.2.2: Experimental Design - HGS

Factors	Treatment levels	Response	Repetition
Viscosity	Low	Survival of <i>L. acidophilus</i> La-5	2
	High		
<i>L. acidophilus</i> La-5 inoculation amount	Low		
	High		

The simulated digestion process was repeated two times for each of the four ice cream samples (Table 3.3.2.1). Fresh simulated saliva (Table 3.3.2.2) was prepared by dissolving mucin (1 g/L; Mucin from porcine stomach, cat# M2378-100G, Sigma-Aldrich, MO,USA), 50U/ml α -amylase (from porcine pancreas, 22U/mg solid , Cat# A3176-2.5MU, Sigma Aldrich, MO, USA; Engelen et al, 2003) into electrolyte solution containing NaCl (0.117 g/L), KCl (0.149 g/L) and NaHCO₃ (2.1 g/L). It was used to simulate mastication process. Freshly prepared GS was added to a 1000 mL glass beaker

which was placed on a heat/stir plate (Isotemp, Fisher Scientific, 12000 rpm and 500°C max) set at 37°C to maintain its temperature at body temperature throughout 120 min. One end of a small tube was placed in the beaker containing GS and the other end was connected to the peristaltic pump (Figure 3.3.2.1) to deliver gastric juice into the stomach during digestion process. A disposable sterile blue liner bag (SAVL-101-19/25, Animal Repro Systems, Chino, CA) similar to the shape of the latex stomach was placed inside the stomach before each simulated digestion process to minimize contamination of the ice cream samples. The bottom of the liner bag was sealed using Impulse sealer (Type TISH 300, 430W, Electric Heating Equipment Co. Ltd.). The control valve was used to adjust the rate of gastric secretion into the latex stomach.

A randomly selected ice cream cup (3.5oz, ~60g; Table 3.3.2.1) was thawed for about 30 min while waiting for the system temperature to equilibrate at 37°C. In the meanwhile, 50 mL simulated gastric juice, pH 1.75-1.80 (Masco et al., 2007; Sumeri et al., 2008) was added to the latex stomach to simulate fasting state stomach conditions. A temperature controller was set at 37°C to increase and maintain the temperature of the system and GS at body temperature. One and half mL of simulated saliva, pH 6.86-6.90 (Engelen et al., 2003; Madureira et al., 2011) was added to the ice cream, stirred for 10 sec and incubated for 50 sec to mimic mastication in the mouth. The pH of the ice cream plus saliva was recorded and a sample was collected after one minute incubation.

The temperature and pH probe were inserted into the liner bag placed in the latex stomach to record the pH and temperature of the empty stomach as well as gastric digesta during simulated digestion. Then the ice cream was dropped into the stomach and peristaltic contractions (three cycles/ min) and gastric secretion (3.5 mL/min) was started

to simulate gastric mixing, shear and hydrolysis of ice cream during 2 hr digestion. Experimental setup is shown in Figure 3.3.2.3. Postprandial pH and temperature were recorded consistently every minute for first 30 min and every five minutes for last 90 min. Once the pH dropped to 2.5 (Pinto et al., 2006; Pitino et al., 2010; Faye et al., 2012), the rate of gastric juice secretion was lowered to 0.9 mL/min until the end of the 120 min of simulated digestion. Twenty-five mL sample was also collected at each time point to enumerate live cells of *L. acidophilus* La-5 (section 3.2.3.1) and to measure the change in viscosity of ice cream due to shear and dilution in the stomach (section 3.2.2). LV spindle # SC4 21 was used to measure viscosity at a range of shear rate 0.93 sec^{-1} to 186 sec^{-1} at body temperature (37°C). The apparent viscosity was measured at shear rate 49.92 sec^{-1} (Soukoulis et al., 2009). The power law model was used as described earlier.

3.3.3 Statistical analysis

Shaking water bath: The impact of pH and sampling point within each model (shaking water bath and HGS) on the survival of *L. acidophilus* La-5 was evaluated using GLM. Tukey's pairwise comparison test was done using one way analysis of variance (ANOVA) to determine significant differences in survivability of La-5 at each sampling point between the two models stomachs. Tukey's pairwise comparison test was also used to determine significant difference in the overall log reduction of *L. acidophilus* La-5 as a result of digestion in static bench top model, shaking water bath or HGS using survival rate as response and model type as main effect.

Human Gastric Simulator: GLM was used to evaluate the impact of viscosity level and initial inoculation level on the survivability of *L. acidophilus* La-5 when ice cream was digested in HGS. Tukey's pairwise comparison test was done using one way

analysis of variance (ANOVA) to determine the significant differences in the apparent viscosity of gastric digesta at time points 30, 60 and 120 min of digestion between LVHC and HVHC samples (Table 3.3.2.1). Tukey's pairwise comparison test was also used to determine significant differences in survivability of *L. acidophilus* La-5 between sampling points (saliva, 30, 60, 120 min) within each variable and at each sampling point across four variables. The significance level used for all the analyses was 5%.

4.0 Results and Discussion

4.1 Phase I: Survivability of *L. acidophilus* La-5 in nonfermented low fat ice cream

4.1.1 Effect of manufacturing process and storage on the viability of *L. acidophilus* La-5

During freezing of the mix, the counts of viable *L. acidophilus* La-5 decreased by 0.35 and 1.39 log units as a result of 60% and 100% overrun respectively. This log reduction as a result of addition of 60% and 100% overrun was found to be statistically non significant ($p>0.05$).

Table 4.1.1.1: Effect of different amount of air incorporation on the viable cells of *L. acidophilus* La-5

Treatments	Counts of <i>L. acidophilus</i> La-5 ($\log_{10}\text{cfu/g}$)	
	Before freezing (4°C)	After freezing (-4°C)
60% OR	$7.15 \pm 0.00^{\text{aA}}$	$6.80 \pm 0.28^{\text{aA}}$
100% OR	$7.15 \pm 0.00^{\text{aA}}$	$5.76 \pm 0.98^{\text{aA}}$

Data were means $\pm$ standard deviations of two sample cups.

^aMeans within the same column with different letters are significantly different ($p<0.05$).

^AMeans within the same row with different letters are significantly different ($p<0.05$).

The viable counts of *L. acidophilus* La-5 remained in excess of recommended level of 10^6cfu/g throughout the storage of 90 days (Table 4.1.1.2) and did not differ significantly ($p>0.05$) from day 1 to day 90. This shows that storage at -10°C did not have a significant effect on the survival of *L. acidophilus* La-5. The viable counts in the ice cream samples incorporated with 60% overrun were statistically significantly different ($p<0.05$) from those in the ice cream samples incorporated with 100% overrun which shows that level of overrun played a significant role in determining the survival rate of La-5 at the end of shelf life of 90 days (Figure 4.1.1.1). Fluctuations in the viable counts of La-5 were observed throughout storage period (Table 4.1.1.2).

Table 4.1.1.2: Effect of storage of ice cream samples incorporated with 60% and 100% overrun at -10°C for 90 days on the survivability of *L. acidophilus* La-5

Treatments	Counts of <i>L. acidophilus</i> La-5 (log ₁₀ cfu/g)				
	Ice cream mix (4°C)	Day 1	Day 30	Day 60	Day 90
60% OR	7.15±0.00 ^{aA}	6.60±0.44 ^{aA}	6.71±0.11 ^{aA}	6.68±0.13 ^{aA}	6.82±0.10 ^{aA}
100% OR	7.15±0.00 ^{aA}	6.32±0.07 ^{aB}	6.43±0.07 ^{aB}	6.29±0.08 ^{aB}	6.19±0.11 ^{aB}

Data were means ± standard deviations of two sample cups.

^{AB}Means within the same column with different letters are significantly different (p<0.05).

^aMeans within the same row with different letters are significantly different (p<0.05).

Figure 4.1.1.1: Effect of overrun and storage on the survivability of *L. acidophilus* La-5

The results obtained for the viable counts after inclusion of 100% overrun were in contradiction to the results obtained by Magarinos et al. (2007) who found that freezing and inclusion of 108% overrun and the subsequent hardening of the ice cream, permitted the survival rate of 91.3% of the *L. acidophilus* La-5 i.e reduction in viable counts by 0.6 log units immediately after freezing. Abghari et al. (2011) also found that in a non-fermented ice cream, inclusion of 90% over run resulted in a loss of 0.28 log units in the viable counts of *L. acidophilus* immediately after freezing. These differences in survival rate of *L. acidophilus* are possibly due to the additional stress caused by the technological

hurdles during processing of ice cream mix with 100% overrun in the present study resulting in an increased loss of viable counts of *L. acidophilus* La-5 immediately after freezing. Nevertheless, it can be seen in Table 4.1.1.2, that the day 1 counts were higher i.e 6.32 log cfu/g compared to immediately after freezing which was 5.76 log cfu/g since all the samples tested throughout storage for 100% overrun were from new batch of ice cream that was processed after overcoming the hurdle.

The loss of less than one log unit in La-5 counts as a result of inclusion of 60% overrun immediately after freezing was in agreement with studies conducted by Salem et al. (2005) and Turgut and Cakmakci (2009). Although the fat content of ice cream sample in the present study was 4% which was lower than that of ice cream samples (8%) tested in the study conducted by Salem et al. (2005), similar reduction of viable counts of *L. acidophilus* La-5 immediately after freezing was observed in both the studies. This shows that fat content may not influence or provide an additional protection against freezing. Favaro-Trindade et al. (2007) conducted a study on various *L. acidophilus* strains to determine the effect of pH (4.5 and 5) and cream level (5% and 10%) on the survivability of probiotic bacteria during ice cream manufacture process and during storage at -18°C for 105 days. The results indicated that higher fat content did not provide better protection to the tested probiotic microorganisms (*L. acidophilus* 74-2 and *L. acidophilus* LAC 4). Haynes and Playne (2002) also found that the full fat ice cream mixes made with ~10% cream did not result in better survivability of the probiotic microorganisms during storage, when compared to the survival rate of those incorporated in ice cream mix made with 3.8% fat.

The ability of *L. acidophilus* La-5 to survive the freezing and air incorporation process at 60% and 100% overrun in this study was higher than those reported by Ferraz et al. (2012) who chose to add probiotic bacteria after the ageing of ice cream mix and used different *L. acidophilus* strain in a 10% fat ice cream mix . They reported reduction of 1 and 2 log units in the viable counts of *L. acidophilus* DOWARUTM as a result of 60% and 90% air incorporation, respectively during storage. These differences in the results may be attributed to the differences in the strains of *L. acidophilus* that were tested. In addition, exposure of *L. acidophilus* La-5 to the cryoprotective components of ice cream mix such as proteins and sugar during overnight ageing at 4°C may play a role in protecting the bacteria during freezing process (Abghari et al., 2011). In the present study, if more repetitions were conducted, it may be possible to find significant effect of different levels of air addition on the viability of *L. acidophilus* La-5 after freezing.

During frozen storage, the results indicated that the survival rate of *L. acidophilus* La-5 at each of the tested days (1, 30, 60, 90) was higher in samples incorporated with 60% overrun compared to 100% overrun. Ferraz et al. (2012) also found a significant influence of the overrun level on the survival of the probiotic strain throughout frozen storage ($p < 0.05$) beginning on day 15 of storage. The observed effect of different amounts of air incorporation during storage may be due to initial damage caused due to air toxicity since *L. acidophilus* species are microaerophilic in nature and response to stress is found to be strain dependent (Talwalkar and Kailasapathy, 2003). In the present study, although statistical analysis found significant difference between viable cells in samples with 60% overrun compared to 100% overrun, biologically these differences are considered non significant . In both the cases, the log reduction at the end of 90 days was

less than 1 log which is considered biologically non significant reduction. According to Talwalkar and Kailasapathy (2003), an enzyme known as NADH peroxidase in *L. acidophilus* 2400 and 2409 was able to scavenge the presence of H₂O₂ produced as a result of presence of oxygen. Further investigation may be conducted to determine whether an enzyme such as NADH peroxidase is present and active in *L. acidophilus* La-5 to correlate insignificant reduction in cell numbers as a result of air addition during freezing and storage.

4.1.2 Physicochemical analysis of ice cream containing *L. acidophilus* La-5

The results (Table 4.1.2.1) showed that values for % TS and % fat in the ice cream mix did not differ significantly ($p>0.05$) from the calculated values (Appendix I) and thereby, not affected by *L. acidophilus* La-5 addition. The E.coli/Coliform counts were found to be <10 cfu/g which was an indication of successful pasteurization of ice cream mixes. The pH and titratable acidity values were not significantly affected ($p>0.05$) by the addition of *L. acidophilus* La-5 in the ice cream mix and did not change significantly ($p>0.05$) throughout storage at -10°C throughout tested shelf life of 90 days. This confirms that there was no metabolic activity in the ice cream mix inoculated with La-5 during ageing at 4°C and during frozen storage.

Table 4.1.2.1: Physical and microbial analysis of ice cream mix after ageing

Variables	TS (%)	Fat (%)	pH	% TA	EC/CC (cfu/ml)	APC (cfu/ml)
Control (no La-5)	35.17	3.95	6.55	0.20	<10	<10
Experimental (La-5)	35.29	3.84	6.50	0.23	<10	<10

Table 4.1.2.2: pH and titratable acidity (%) during storage study

	30 days		60 days		90 days	
	pH	% acidity	pH	% acidity	pH	% acidity
Control	6.57	0.20	6.54	0.21	6.56	0.21
Experimental	6.52	0.24	6.50	0.23	6.51	0.23

In the present study, viscosity of ice cream mix with added *L. acidophilus* La-5 was compared with that of control mix with no added probiotic in order to determine any significant contribution of the presence of La-5 on the viscosity of aged ice cream mix. The presence of *L. acidophilus* La-5 in the experimental ice cream mix did not have a significant effect ($p>0.05$) on the final viscosity of aged ice cream mix. The results also indicated that the viscosity of the mix decreased with an increase in shear rate and temperature (Figure 4.1.2.1). The testing temperature had a significant effect ($p<0.05$) on the viscosity ($p<0.05$). The consistency coefficient (κ) indicates the relative thickness of a solution. The values of consistency coefficient (κ) were found to decrease significantly ($p<0.05$) with an increase in temperature (Table 4.1.2.3) and were positively correlated with the apparent viscosity values. For all the samples tested in our study at 4°C and 37°C, the values of flow behavior index (n) were found to be less than unity (Table 4.1.2.3). Therefore, it can be said that the samples exhibited pseudoplastic behavior i.e. overtime shear thinning.

Figure 4.1.2.1: Effect of shear rate (sec^{-1}) and temperature ($^{\circ}\text{C}$) on viscosity (Pa.s) of aged ice cream mix with and without added *L. acidophilus* La-5

Table 4.1.2.3: Consistency index (κ) and flow behavior index (n)

Variables	Consistency index (κ) Pas ⁿ	flow behavior index (n)	R ²
Control (4°C)	0.68	0.64	0.9969
Control (37°C)	0.14	0.65	0.9949
Experimental (4°C)	0.63	0.65	0.9966
Experimental (37°C)	0.14	0.68	0.9988

The findings for pH was in accordance with those reported by Nousia et al. (2011) who indicated that pH was influenced neither by addition of freeze dried culture or activated cells of *L. acidophilus* LMPG-2138 during ageing or during frozen storage at -15°C or -25°C for up to 45 weeks. Abghari et al. (2011) conducted a similar study by manufacturing non fermented ice cream and found that pH did not differ significantly before and after ageing and during storage in the samples inoculated with *L. acidophilus* (isolated from probiotic commercial capsules). This indicates that ageing of the inoculated ice cream mix at 4°C does not provide suitable conditions for bacteria to metabolize and produce acid validating that the mix is non-fermented.

Authors who fermented the ice cream mix (Akalin and Erisir, 2008; Hekmat and McMahon, 1992) reported the pH of the final product to be below 5.0 or 6.0 and consequently found that there was a significant reduction in the viable counts of tested probiotic strain, even in the presence of prebiotic (Akalin and Erisir, 2008). This shows that the pH of the final product could be detrimental to the viability of added probiotic bacteria. The pH of the ice cream samples in the present study may provide an advantage over fermented ice cream eliminating one of many stress factors resulting in reduction of bacterial cells during ice cream manufacture and storage.

The viscosity of an ice cream mix is principally influenced by the fat content and the stabilizer blend (Marshall and Arbuckle, 1996; Alamprese et al., 2002; El-Nagar et

al., 2002). Besides fat and stabilizers, some strains of probiotic bacteria are capable of producing exopolysaccharides, which in turn affects the viscosity of the ice cream mix (Goh et al, 2008). Therefore, in the present study, viscosity of ice cream mix with added *L. acidophilus* La-5 was compared with that of control mix without probiotic in order to determine any significant contribution of La-5 on the viscosity of aged ice cream mix. The addition of *L. acidophilus* La-5 did not affect the viscosity in the present study. The unaffected viscosity in the present study could be explained by low acidity of the mix (Table 4.1.2.1). Some studies (Salem et al., 2005; Turgut and Cakmakci, 2009) tested the effect of supplementation of ice cream with *L. acidophilus* on the viscosity of ice cream mix compared to the effect of other probiotic bacteria and often found lowest viscosity values of the mix supplemented with *L. acidophilus* cells compared to the mix inoculated with other probiotic bacteria.

In both the studies, either ice cream mix was fermented or fermented milk was added to the ice cream mix and the low pH as a result of acid production during fermentation could be attributed to the increase in viscosity of the ice cream mix. *L. acidophilus* species is known to grow slowly in milk which may result in less acid production and lowering of pH in turn resulting in least effect on the viscosity compared to other probiotic bacteria such as *L. reuteri* (Salem et al., 2005) or *B. bifidum* (Turgut and Cakmakci, 2009). Investigators like Xu et al. (2006) and Mostafa et al. (2001) attributed high viscosity values to the fermentation process and the increase in acidity which consequently causes slight protein precipitation and low pH value. In a study conducted by Abghari et al. (2011) in which non-fermented ice cream matrix was tested,

the authors found that addition of *L. acidophilus* and *L. rhamnosus* had no significant effect on the viscosity of the ice cream mix similar to the present study.

The results indicated that the mix viscosity decreased with an increase in shear rate and temperature. Similar results have been reported by other authors, who showed that viscosity is a temperature dependent property of the ice cream mix (Marshall and Arbuckle, 1996; Innocente et al, 2002; Metwally, 2007). The consistency coefficient (κ) values in the present study were found to decrease for the samples tested at higher temperature suggesting the temperature dependence nature of ice cream viscosity. These findings were similar to those of Aime et al. (2001).

4.2 Phase II: Bench top experiments

4.2.1 Buffering capacity (BC)

The addition of *L. acidophilus* La-5 in all the three samples had a significant ($p<0.05$) effect on the amount of gastric juice that was required to lower the initial pH of samples to the fasting state stomach pH 2.0. The BC of ice cream mix, milk and PBS increased significantly ($p<0.05$) in the presence of *L. acidophilus* La-5. In the absence of La-5, the BC was highest for the ice cream mix followed by milk and PBS. Whereas, in its presence, BC of ice cream mix did not differ significantly ($p>0.05$) from milk. The BC values were observed to have slightly increased at body temperature versus room temperature for PBS (in the presence and absence of La-5) and for milk and ice cream only in the absence of La-5. Temperature (room or body) had a significant ($p<0.05$) effect on BC of all the samples with added *L. acidophilus* La-5 compared with those without La-5, but it did not have significant ($p>0.05$) effect when samples were compared with each other within each group. Figures 4.2.1.2 and 4.2.1.3 show a graph which compares

BC values between ice cream mix, milk and PBS in the presence and absence of La-5 at body and room temperature, respectively.

Table 4.2.1.1: Comparison of amount of gastric juice required to lower the native pH of PBS, milk and ice cream mix with no added *L. acidophilus* La-5. Data were means $\pm$ standard deviations of two replicates

Product	Temperature	Native pH (w/o La-5)	Amount of GS added (ml) (w/o La-5)	Native pH (with La-5)	Amount of GS added (ml) (with La-5)
PBS	Room	7.01	3.33 $\pm$ 0.11	6.17	12.95 $\pm$ 0.64
Milk	Room	6.67	16.25 $\pm$ 0.35	6.29	25.50 $\pm$ 0.71
Ice cream mix	Room	6.55	20.75 $\pm$ 1.06	6.50	26.75 $\pm$ 0.35
PBS	Body	7.03	4.25 $\pm$ 0.07	6.36	18.25 $\pm$ 1.77
Milk	Body	6.70	17.70 $\pm$ 0.14	6.45	26.00 $\pm$ 1.41
Ice cream mix	Body	6.50	21.50 $\pm$ 2.12	6.32	26.00 $\pm$ 0.71

Figure 4.2.1.1: Buffering capacity (BC) of ice cream mix, milk and PBS at body and room temperature in the presence and absence of *L. acidophilus* La-5

This difference in the BC of samples in the presence and absence of *L. acidophilus* La-5 could be attributed to the addition of freeze dried form of bacteria whose major growth medium was skimmed milk powder/milk permeate in addition to other ingredients such as sodium caseinate, yeast extract and carbohydrates. Dairy products like ice cream mix and milk display high BC due to the presence of proteins and

salts. It may be possible that addition of freeze dried form of La-5 may have added more proteins resulting in resistance to pH change and in turn higher BC. These results indicate that incorporating freeze dried milk based form of probiotic bacteria in dairy products like milk and ice cream mix further enhanced their resistance to change of pH from 3.0 to 2.0. Since, pH below 3.0 is found to be most detrimental to the viability of probiotic bacteria, this increased resistance to the change in pH from 3.0 to 2.0 may allow probiotic bacteria to reach the intestine unaffected in large numbers during gastric emptying.

Al Dabbas et al. (2010) compared the buffering capacity of cow's milk with non dairy products and found that cow's milk had highest BC. They found BC value for cow's milk to be 1.96 ± 0.17 which was slightly lower than the value found in present study (2.52 ± 0.02). This difference could be due the difference between the chemical composition of milk samples used in their study compared to our study. For example, milk from Jersey cows, which has higher protein and phosphate contents, has a higher buffering capacity than milk from Holstein cows (Park, 1992). Parameters affecting the BC and causing differences in BC value of different dairy products depend on several compositional factors including small constituents (inorganic phosphate, citrate, and organic acids), milk proteins (caseins and whey proteins) and solubilization of colloidal calcium phosphate (Lucey et al., 1996; Salaun et al., 2005).

Figure 4.2.1.2: Buffering capacity (BC) of ice cream mix, milk and PBS for different pH values at body temperature in the presence and absence of *L. acidophilus* La-5.

Figure 4.2.1.3: Buffering capacity (BC) of ice cream mix, milk and PBS for different pH values at room temperature in the presence and absence of *L. acidophilus* La-5.

4.2.2 Effect of shear and dilution

As seen in Table 5.2.1.1, there was no significant effect of shear ($p > 0.05$) and dilution ($p > 0.05$) on the survivability of La-5 whether in low viscosity or high viscosity sample. Effect of shear was found to be significantly ($p < 0.05$) different from the effect of dilution on relative log survival of *L. acidophilus* La-5. Therefore, Tukey's pairwise

comparison test was done using one way analysis of variance (ANOVA) to determine which of the treatment pairs were significantly different. As shown in Table 4.2.2.1, the relative log survival at the end of 2hr of shear treatment on low viscosity ice cream sample was significantly ($p<0.05$) higher compared to the relative log survival after dilution treatment on the low viscosity ice cream sample with gastric juice for 2hr. This could be due to an increase in bacterial count as a result of shear treatment which was not found as a result of dilution treatment. Since ice cream samples were thawed and plated when they were about -4°C to -5°C before treatment, this could have resulted in clumping of cells and an underestimation of actual number of colonies present in the control sample. Shearing the same sample for two hours at body temperature of 37°C would have resulted in disruption of chains and higher number of colonies after treatment was observed after the treatment.

Table 4.2.2.1: Effect of shear and dilution on survivability of *L. acidophilus* La-5 incorporated in low and high viscosity samples.

Variable	Effect	Before treatment (4°C)	After treatment (120 min at 37°C)
		$\text{Log}_{10}\text{cfu/ml}$	
Low viscosity	Shear	$7.72 \pm 0.12^{\text{Aa}}$	$7.96 \pm 0.02^{\text{Aaa}}$
High viscosity	Shear	$7.49 \pm 0.06^{\text{Aa}}$	$7.73 \pm 0.23^{\text{Aa}}$
Low viscosity	Dilution	$7.66 \pm 0.18^{\text{Aa}}$	$7.28 \pm 0.06^{\text{Aab}}$
High viscosity	Dilution	$7.16 \pm 0.17^{\text{Aa}}$	$7.02 \pm 0.13^{\text{Aa}}$

Data were means $\pm$ standard deviations of two replicates.

^{ab}Means within the same column with different letters are significantly different ($p<0.05$).

^AMeans within the same row with different letters are significantly different ($p<0.05$).

4.2.3 Conventional acid resistance test

4.2.3.1 Survivability of *L. acidophilus* La-5 and *L. paracasei* 431 at pH 2.0 and pH 5.0 in the absence of ice cream

Incubation of probiotic bacteria in citrate-HCl buffer in the absence of ice cream mix for first 60 min at pH 2.0 (fasting state stomach pH) resulted in a reduction of cell

count from 7.12 log cfu/g to <10 cfu/g for *L. acidophilus* La-5 and from 10.37 log cfu/g to <10cfu/g for *L. paracasei* 431 (Table 4.2.3.1). Whereas, incubation for 120 min at pH 5.0 (pH of the stomach after ingestion of food) resulted in a log reduction from 6.37 log cfu/g to 5.08 log cfu/g for *L. acidophilus* La-5 and from 9.48 log cfu/g to 6.35 log cfu/g for *L. paracasei* 431 (Table 4.2.3.1 and Figure 4.2.3.1). The impact of incubation pH and the sampling time (0, 60 and 120 min) on the viable counts of *L. acidophilus* La-5 and *L. paracasei* 431 was found to be significant ($p<0.05$).

Table 4.2.3.1: Effect of pH 2.0 and 5.0 on viability of selected probiotic strains during 2 hr incubation using conventional acid resistance test

Sampling Time	pH 2	pH 2	pH 5	pH 5
	<i>L. acidophilus</i> La-5	<i>L. paracasei</i> 431	<i>L. acidophilus</i> La-5	<i>L. paracasei</i> 431
	Log ₁₀ cfu/g		Log ₁₀ cfu/g	
Control	7.12±1.29 ^{Aa}	9.69±0.09 ^{Ba}	6.37±0.67 ^{ABa}	9.48±0.07 ^{ABb}
60min	0.00±0.00 ^{Ba}	0.00±0.00 ^{Ca}	5.76±0.13 ^{Ba}	7.64±0.34 ^{BCb}
120mins	0.00±0.00 ^{Ba}	0.00±0.00 ^{Ca}	5.08±0.44 ^{Ba}	6.35±0.89 ^{Ca}

Data were means ± standard deviations of two replicates.

^{ABC} Means within the same column that do not share a letter are significantly different ($p<0.05$).

^{ab} Means within the same row (pH =2) that do not share a letter are significantly different ($p<0.05$) for pH 2.0 and pH 5.0.

Figure 4.2.3.1: Relative log survival of *L. acidophilus* La-5 and *L. paracasei* 431 at pH 2.0 and pH 5.0 during 2 hr incubation in citrate-HCl buffer

Similar effect of pH on the viability of *L. acidophilus* BG2F04 was observed by Hood and Zottola (1988). Their results demonstrated that no cells of *L. acidophilus* BG2F04 were recovered following 45 min exposure to pH 2.0. While at pH 4.0, viability was maintained close to 7 log cfu/ml after 2 hr incubation. Abghari et al. (2011) and Alamprese et al. (2002) also reported significant impact of incubation pH (1.5, 2.5, 3.5 and 6.5) and sampling time on the survivability of tested probiotic bacteria. Abghari et al. (2011) demonstrated a significant reduction in viable counts of *L. acidophilus* (isolated from probiotic capsules) at pH 1.5 and 2.5 overtime during 3 hr incubation in MRS broth. At pH 2.5, approximately 3.5 log cfu/g survived at the end of 3 hr incubation whereas, at pH 1.5, almost no survival was observed similar to present study where the viability was 0% at the end of first hour incubation at pH 2.0 for *L. acidophilus* La-5 as well as *L. paracasei* 431 (Table 4.2.3.1). On the other hand, Alamprese et al. (2002) reported significant reduction in viable counts of *L. johnsonii* La-1 at pH 1.5 at the end of 3 hr incubation, but for pH 2.5 and 3.5, the number of viable cells remained close to 8 log cfu/ml during 3 hr incubation which was not significantly different from the counts at pH 6.5. These differences in the level of impact of pH on the survivability of probiotic bacteria could be attributed to the difference in suspending medium, variability in tested probiotic species and strains and their response to acid shock and growth conditions (Chou and Weimer, 1999). In addition, when the cells are present in an environment of low external pH, the energy consumption, which is required for maintenance of the intracellular pH, is increased. As a result, other crucial cellular functions are depressed of ATP and the cells cannot survive (Corcoran et al., 2005; Shabala et al., 2006). Differences in the level of sensitivity towards stress factors have been reported for

Bifidobacterium species isolated from human gastrointestinal tract in relation to those of animal origin (Sanz, 2007). Sanz (2007) suggested that response to acid stress and strain origin have a significant relationship and this could have an impact on species composition under extreme acidic conditions.

Table 4.2.3.2: Overall Log Reduction at pH 2, pH 5 and in the presence of ice cream for *L. acidophilus* La-5 and *L. paracasei* 431

	<i>L. acidophilus</i> La-5	<i>L. paracasei</i> 431
	overall log reduction (log ₁₀ cfu/g)	
pH 2	7.12±0.92 ^{Aa}	9.69±0.06 ^{Aa}
pH 5	1.30±0.79 ^{Ba}	3.13±0.58 ^{Ba}
Ice cream	2.62±0.63 ^{ABa}	4.87±0.65 ^{Ba}

Data were means ± standard deviations of two replicates.

^{AB}Means within the same column that do not share a letter are significantly different (p<0.05).

^aMeans within the same row that do not share a letter are significantly different (p<0.05).

Even though, *L. acidophilus* La-5 and *L. paracasei* 431 lacked the ability to survive harsh conditions of traditional screening at pH 2 in citrate-HCl buffer, it's not an environment that is usually encountered *in vivo* during and after a meal because of buffering capacity of foods and variations in the response of various gastric juice components (HCl and enzymes) based on food composition in which the bacteria is embedded (section 2.2.2.1). It has also been suggested that buffering of stomach pH by certain foods containing probiotic microbes improves their survival during gastric transit (Saxelin et al., 2010). Therefore, This difference in counts at sampling time points 60 min and 120 min could be attributed to the difference in acid shock response of each of the two bacteria at a specific pH as well as difference in the initial inoculation level of ice cream mix as shown in Table 4.2.3.3. Curto et al. (2011) also reported differences in the survival rate among the three probiotic bacteria that were tested (*L. casei* subsp. *shirota*, *L. casei* subsp. *immunitas*, *L. acidophilus* subsp. *johnsonii*) in the presence of milk. They

also found that the survival rate was higher for all the bacteria in the presence of milk compared to water since water has a low buffering effect compared to milk (Holzapfel et al., 2001). Majority of the studies have conducted acid resistance test in the pH range of 1.0-5.0 and have found that pH 2.0 or below has been most detrimental compared to pH 3.0, 4.0, and 5.0 to the viability of tested probiotic bacteria. At pH 1.0 and 2.0, maximum mortality has been reported (Conway et al., 1987; Hood and Zottola., 1988; Abghari et al., 2011; Kawther et al., 2010), whereas, at pH 3.0 or above, survivability has been more or less maintained steady (Chou and Weimer, 1999; Alamprese et al., 2002; Basyigit et al., 2006) or has shown a slower reduction overtime maintaining viability of up to 4 log units or more (Collado et al., 2005; Faye et al., 2012) depending on the species and strains, initial inoculation level, incubation pH and suspension medium. Similarly, in this study, pH 2.0 was found to be extremely detrimental and progressive reduction in viable counts were observed at pH 5.0.

4.2.3.2. Survivability of *L. acidophilus* La-5 and *L. paracasei* 431 in the presence of ice cream

The effect of ice cream mix was tested by mimicking gastric digestion on bench top in the presence of simulated gastric juice. The results showed that there was a reduction in the viable counts of *L. acidophilus* La-5 from 7.56 log cfu/g to 4.94 log cfu/g which is an overall log reduction of 2.62 log cfu/g at the end of 2 hr simulated digestion in the presence of ice cream. Similar reduction in viable counts from 8.55 log cfu/g to 3.68 log cfu/g, an overall reduction of 4.87 log cfu/g was found for *L. paracasei* 431 in the presence of ice cream mix (Table 4.2.3.3). The results demonstrated a loss in viable counts of both La-5 and 431 at the end of 2 hr simulated digestion, but 65.34% of La-5

and 43.04% of 431 were still alive even though the pH was maintained at ~2.6 for the last hour of digestion in the presence of ice cream compared to 0% for both the bacteria when they were incubated at pH 2.0 for 2 hr in the absence of ice cream. The probiotic strain had a significant ($p<0.05$) impact on the resulting overall log reduction at pH 5.0 in the absence of ice cream and during simulated digestion in the presence of ice cream. This means that even though the presence of ice cream improved survivability of both the probiotic bacteria at low pH, their response was strain dependent. In addition, at each time points (60 and 120 min), there was a significant ($p<0.05$) difference between the counts of La-5 and 431 in the presence of ice cream (Table 4.2.3.3), but the overall reduction as a result of simulated digestion process for each of the two strains was not significantly ($p>0.05$) different.

Table 4.2.3.3: Effect of presence of ice cream mix on the viable counts of selected probiotic strains during 2hr of simulated digestion process.

Sampling time	In the presence of ice cream mix (simulated digestion process)	
	<i>L. acidophilus</i> La-5	<i>L. paracasei</i> 431
	Log ₁₀ cfu/g	
Control	7.56±0.70 ^{Aa}	8.55±0.67 ^{Aa}
60min	6.51±0.16 ^{ABa}	8.09±0.38 ^{Ab}
120mins	4.94±0.19 ^{Ba}	3.68±0.24 ^{Bb}

Data were means ± standard deviations of two replicates.

^{AB} Means within the same column that do not share a letter are significantly different ($p<0.05$).

^{ab} Means within the same row that do not share a letter are significantly different ($p<0.05$).

Figure 4.2.3.2 Relative log survival of *L. acidophilus* La-5 and *L. paracasei* 431 in the presence of ice cream mix during 2 hr simulated digestion

In this study, incubation at pH 2.0 in the absence of ice cream mix was found to be extremely detrimental and progressive reduction in viable counts were observed at pH 5.0. Whereas, the presence of ice cream mix was found to improve survivability of both *L. acidophilus* La-5 and *L. paracasei* 431 at the end of simulated digestion. The observed protective effect of ice cream in this study could be attributed to the high buffering capacity of ice cream mix (section 4.2.1).

Mainville et al. (2005) tested kefir as a suspension medium for selected *Bifidobacterium* and *Lactobacillus* strains and isolates from kefir and reported that kefir improved the survivability of the tested strains during simulated digestion that were not able to survive the conventional acid resistance test i.e incubation at pH 2.0 in the absence of kefir for more than 15 min (*L. rhamnosus* GG, *B. longum* RW002, and *B. infantis* ATCC 27920G). The authors also found that the simulated digestion time below pH 3.0 in the presence of kefir resulted in most cell death. Similar pH dependent survival of probiotic bacteria (*L. johnsonii* B-2178, *L. gasseri* B-14168 and *L. salivarius* B-1950)

and improvement in their survivability in the presence of milk proteins during gastric transit was also demonstrated by Kawther et al. (2010). They found that all the strains showed progressive reduction in viable cell numbers at pH 2.0 and pH 4.0 in the absence of protective matrix such as sodium caseinate, whey protein or starch. The presence of milk proteins and starch played a role in enhancing the viability of tested probiotic bacteria during simulated digestion at pH 2.0 and 3.0.

The results from these studies and the present study demonstrated the importance of pH and the protective effect of food matrix on the survivability of probiotic bacteria against the harsh stomach conditions during digestion. The presence of milk, milk proteins and milk based products have been shown to play a significant role in reducing the detrimental effects of lower pH values and enhancing the viability of strains that were completely inhibited by conventional acid resistance test or showed low acid tolerance (Conway et al., 1987; Miettinen et al., 1998; Mainville et al., 2005 and Fernández de Palencia et al., 2008; Kawther et al., 2010).

Milk and milk products generally have high initial pH and buffering capacity due to the presence of proteins and salts. This leads to an initial increase in the fasted state pH of the stomach and reduces the time of exposure of probiotic bacteria to hostile stomach pH protecting them from dying (Charteris et al., 1998; Conway et al., 1987; Huang and Adams, 2004; Tompkins et al., 2011; Faye et al., 2012). In case of cheese and ice cream, the fat content could be as high as 10-12% or more. The presence of fat (as low as 1%) has been found to have improved the survivability of bacteria during gastric transit as compared to absence of fat as in skim milk (Varcoe et al., 2002) suggesting the importance of fat in ensuring the survival of tested probiotic bacteria through stomach.

Some studies have also been conducted that demonstrated binding between probiotic bacteria and certain components of the milk fat globule membrane (Bachiero et al. 2007; Brisson et al., 2010). Therefore, it may be speculated that the 10% fat content and high buffering capacity of the in ice cream mix tested in the present study could be responsible for protecting *L. acidophilus* La-5 and *L. paracasei* 431 against low pH and presence of enzymes during simulated digestion.

4.3 Phase III: *In vitro* digestion of ice cream

4.3.1 Shaking water bath

Ice cream sample termed as LVHC (Table 3.3.2.1) was digested using shaking water bath model and HGS and the survivability of *L. acidophilus* La-5 during simulated gastric conditions in both the model stomachs was compared. No significant difference in the viable cell count ($p>0.05$) was found between the two models at each sampling time point except at 30 min. It was found that within each model, sampling point and pH had a significant effect ($p<0.05$) on the relative log survival of *L. acidophilus* La-5 (Table 4.3.1.1). The log reduction at the end of first hour of gastric digestion was observed to be 1.74 log cfu/g for shaking water bath model and 2.18 log cfu/g for HGS, whereas, at the end of second hour, the observed reduction was slightly lower i.e. 1.31 log cfu/g and 1.95 log cfu/g for shaking water bath and HGS, respectively. One thing that was common in both the models was higher mortality during the first hour of digestion compared to the second hour of digestion. This indicates that *L. acidophilus* La-5 cells experienced slightly more stress during the first hour compared to the second hour, even though the pH was maintained above 3 for the first 45 minutes and then dropped to ~2.5 in the next 15 min (Figure 4.3.1.2). As it can be seen in Figure 4.3.1.1, the percent survivability of

L. acidophilus La-5 at each sampling points (30, 60 and 120min) during 2 hr digestion was higher when the ice cream was digested using shaking water bath compared to HGS. The overall log reduction in the viable counts of La-5 was higher when ice cream was digested in HGS (4.13 ± 0.65 log cfu/g) compared to shaking water bath (3.05 ± 0.57 log cfu/g), but this difference was not significant ($p > 0.05$) possibly due to the small sample size.

Table 4.3.1.1: Survivability of *L. acidophilus* La-5 during 2 hr simulated gastric digestion of ice cream in shaking water bath model and HGS.

	Shaking Water Bath	HGS
	Log ₁₀ cfu/g	
Initial (ice cream)	7.84 ± 0.10^{Aa}	7.89 ± 0.10^{Aa}
30min	6.77 ± 0.01^{Aab}	6.57 ± 0.01^{Bab}
60min	6.10 ± 0.22^{Abc}	5.71 ± 0.11^{Ab}
120min	4.79 ± 0.69^{Ac}	3.76 ± 0.82^{Ac}

Data were Means $\pm$ Standard deviations of two replicates.

^A Means with different letters within same rows are significantly different ($p < 0.05$).

^{abc} Means with different letters within same column are significantly different ($p < 0.05$).

Figure 4.3.1.1: Relative log survival of *L. acidophilus* La-5 during 120 min simulated digestion in shaking water bath and Human Gastric Simulator (HGS)

Figure 4.3.1.2: Postprandial pH decrease during simulated digestion of ice cream in shaking water bath and HGS

The decrease in viability of *L. acidophilus* La-5 was found to be more during first hour of simulated digestion compared to the second hour in the present study. Similarly, Masco et al. (2007) also reported that the relative decrease in viability of all the tested *Bifidobacterium* stains was higher after 1 min compared to that after 180 min and attributed this to possible adaptation to acidic conditions. Although, this mechanism remains to be investigated for *L. acidophilus* La-5 in the present study, it can be hypothesized that upregulation of genes involved in stress responses could be responsible for enhancing acid tolerance of probiotic bacteria (Klaenhammer and Kullen, 1999).

A more legitimate reason for the initial shock during first hour could be the continuous flood of hydrogen ions during the first 60 min as a result of gastric secretion at a rate of 3.5 ml/min until pH was lowered to 2.5 after which it was reduced to a much lower rate of 0.9 ml/min. Another possible explanation could be chemical stresses caused during melting (freeze-thaw) of the frozen ice cream before it was subjected to gastric digestion. In the present study, ice cream was thawed before simulated digestion to

approximately -5°C and saliva was added and mixed for 10 sec as a result of which cells could be exposed to osmotic effects and melting of ice cream may result in hydrogen ions and oxygen poisonous to probiotic cells (Jay et al., 2005). The reduction in viable cells during second hour was possibly a result of acid shock due to maintenance of low pH (~2.5) throughout that hour in accordance with the findings of Pinto et al. (2006), Zhu et al. (2006) and Ruiz-Moyano et al. (2008). In addition, it could also be a result of decrease in viscosity and increase in dilution exposing the probiotic cells to acid and enzymes. In both model stomachs, gastric emptying was not stimulated. Therefore, viable cells were exposed to pH 2.5 for the whole second hour of digestion, whereas *in vivo*, the viable bacteria would have left the stomach with digested food as a result of emptying and reached the intestine unaffected.

The slightly lower survivability of *L. acidophilus* La-5 during simulated digestion in HGS compared to shaking water bath could be due to stomach contractions that generated fluid flow of the gastric content resulting in a shearing effect on the food causing damage to the cells eventually leading to their death. Therefore, presence of real peristaltic contractions and shear in addition to dilution in HGS could have enhanced the contact between probiotic cells and gastric juice contents resulting in additional detrimental effects compared to shaking water bath model. The morphology of the cell may also contribute to the extent of detrimental effect caused due to shear and grinding in HGS. Since *L. acidophilus* La-5 is rod shaped bacteria, it is probably more prone to adverse effect of mechanical shear compared to a bacteria such as streptococcus.

Even though the survivability of *L. acidophilus* La-5 was slightly, but not significantly affected by HGS, it was able to successfully demonstrate the importance of

incorporating probiotic bacteria in a food product like ice cream since approximately 4 log units of La-5 were still viable at the end of simulated digestion which could reach their site of action to provide health benefits. It was also able to effectively simulate major conditions in the stomach, an increase in pH upon ingestion of food followed by restoration of fasted state stomach pH as well as peristaltic contractions. More studies and repetitions must be conducted with different conditions, strains and food products to further validate the preliminary results obtained in the present study.

It was speculated that the acid tolerance capacity of *L. acidophilus* La-5 that was observed in the presence of ice cream mix (section 4.2.3) would be lower in the frozen ice cream when exposed to low pH during simulated digestion because of the stress due to freezing process and storage at sub zero temperature possibly causing the cells to become acid sensitive. No significant ($p>0.05$) difference was found in the overall log reduction of *L. acidophilus* La-5 numbers in either of the three experiments (cells in ice cream mix, static model; cells in frozen ice cream, shaking water bath and HGS). This suggests that the freezing process during ice cream manufacture and the storage at subzero temperature did not have a detrimental effect on the bacterial acid tolerance capacity. These results indicate the importance of mimicking the actual digestion process, the rise and fall in the pH due to the presence of food matrix, its buffering effect and gastric secretion in response to the ingestion of a meal. In a study conducted by Abghari et al. (2011) authors did not include the effect of presence of food matrix when testing the low pH sensitivity of frozen thawed cells. The frozen thawed cells were washed with PBS and resuspended in saline followed by incubation at pH 2.5 for 2 hr. They found that the frozen thawed cells had become acid sensitive which was in contradiction to the

present study. The variability in the acid stress response by different strains tested in each of these studies could have also lead to differences in results.

4.3.2 Human Gastric Simulator

The results indicated that there was no significant difference ($p>0.05$) in the apparent viscosity of gastric digesta measured at shear rate of 49.92 sec^{-1} (Soukoulis et al., 2009) collected at 30, 60 and 120 min for both LVHC and HVHC samples (Figure 4.3.2.1 and 4.3.2.2). Analysis also showed that there was no significant difference ($p>0.05$) in viscosities tested at a range of shear rates at time points 30, 60 and 120 min once both the samples (LVHC and HVHC) were diluted for first 30 min, irrespective of their initial viscosity level.

Figure 4.3.2.1: Effect of gastric dilution on low viscosity ice cream sample during simulated digestion in HGS

Figure 4.3.2.2: Effect of gastric dilution on high viscosity ice cream sample during simulated digestion in HGS

Power law model of non Newtonian fluid was used to describe the behavior of gastric digesta (Takahashi et al., 2004). The value of n indicates the flow behavior of the product. When $n=1$, the fluid displays Newtonian flow behavior. The value of n was highest (closest to 1) for HVHC, 120 min sample and lowest for LVHC, 30 min sample (Table 4.3.2.1). Hence, the HVHC, 120 min sample displayed least shear thinning behavior and more Newtonian like fluid. The consistency coefficient (κ) indicates the relative thickness of a solution. The values of consistency coefficient (κ) in Table 4.3.2.1 were found to decrease as the digestion process progressed towards 120 min for both LVHC and HVHC samples. This shows that the relative thickness was highest for samples collected at 30 min and it kept decreasing as samples were further diluted at 60 min until at 120 min during simulated digestion.

Table 4.3.2.1: Consistency index (κ) and flow behavior index (n)

Variables	30min	60min	120min	30min	60min	120min
	Consistency index (κ)			flow behavior index (n)		
Low viscosity high conc.	1.7567	1.6787	1.6065	0.0381	0.0607	0.0765
High viscosity, high conc.	1.7165	1.5433	1.4864	0.053	0.0817	0.0949

Statistical analysis was also conducted to determine the effect of saliva addition, viscosity level and initial inoculation amount on the survivability of *L. acidophilus* La-5 during 2 hr simulated digestion. There was no significant detrimental effect ($p>0.05$) of addition of saliva to each of the four ice cream samples on the viability of *L. acidophilus* La-5 (Table 4.3.2.2) indicating no cumulative inhibitory action caused due to presence of α -amylase and mucin on *L. acidophilus* La-5. It was also found that the high level of viscosity did not have a significantly different effect ($p>0.05$) on the survivability of La-5 at the end of digestion compared to that of low level of viscosity, irrespective of the initial inoculation level of *L. acidophilus* La-5. In addition, there was no significant difference ($p>0.05$) in the relative log survival of *L. acidophilus* La-5 between high and low viscosity ice cream samples, irrespective of the initial inoculation level at time points 30, 60 and 120 min (Table 4.3.2.2). The overall log survival of *L. acidophilus* La-5 at the end of 120 min for LVHC and HVHC ice cream samples was 3.76 ± 0.82 and 3.52 ± 0.74 , respectively and did not differ significantly ($p>0.05$). Similarly, the overall log survival of La-5 for LVLC and HVLC ice cream samples was 4.13 ± 0.75 and 4.04 ± 0.31 , respectively and did not differ significantly ($p>0.05$) (Figure 4.3.2.3).

Table 4.3.2.2: Effect of difference in viscosity and initial inoculation amount of *L. acidophilus* La-5 on its survivability during 120 min of simulated digestion

Sampling points	LVHC	HVHC	LVLC	HVLC
	Log ₁₀ cfu/ml			
Initial (ice cream)	7.89±0.10 ^{Aa}	7.54±0.06 ^{Aa}	6.40±0.14 ^{Ba}	6.26±0.25 ^{Ba}
Ice cream + Saliva	7.84±0.00 ^{Aa}	7.61±0.06 ^{Ba}	6.38±0.03 ^{Ca}	6.08±0.00 ^{Da}
30min	6.57±0.01 ^{Aab}	6.65±0.19 ^{Aab}	5.71±0.57 ^{Aab}	5.37±0.27 ^{Aab}
60min	5.71±0.11 ^{Ab}	5.57±0.27 ^{Ab}	5.17±0.80 ^{Aab}	4.97±0.21 ^{Abc}
120min	3.76±0.82 ^{Ac}	3.52±0.74 ^{Ac}	4.13±0.75 ^{Ab}	4.04±0.31 ^{Ac}
Overall Log Reduction	4.14±0.91 ^A	4.02±0.67 ^A	2.28±0.60 ^A	2.22±0.06 ^A

Data were Means ± standard deviations of two replicates.

^{ABCD} Means with different letters within same row are significantly different (p<0.05).

^{abc} Means with different letters within same column are significantly different (p<0.05).

The initial inoculation level of *L. acidophilus* La-5 had a significant impact (p<0.05) on its survivability during the simulated digestion of ice cream samples, irrespective of the difference in viscosity. Minimum differences were observed in the log survival for both LVLC and HVLC samples compared to LVHC and HVHC samples, these differences were found to be statistically non significant (p>0.05). The overall reduction on an average for LVLC and HVLC samples was found to be 4.08 log cfu/g and for LVHC and HVHC samples was 2.25 log cfu/g. These differences were also found to be statistically non significant (p>0.05) (Table 4.3.2.2.). Even though, most of the bacterial death occurred after first 30 min and decreased progressively until 120 min, the survivability of La-5 was normalized at about 4 log cfu/g for all the samples (LVHC, HVHC, LVLC and HVLC) at the end of digestion, irrespective of the difference in initial supplementation amount of *L. acidophilus* La-5 and in the viscosity (Figure 4.3.2.2). The pH of gastric digesta was also found to have a significant effect (p<0.05) on the viable counts of *L. acidophilus* La-5 during 120 min modeled digestion. As the pH decreased progressively throughout 120 min, the survivability of *L. acidophilus* La-5 also

decreased and resulted in ~4 log cfu/g survival for all the samples (LVHC, HVHC, LVLC and HVLC), irrespective of difference in initial level of La-5 and initial viscosity.

Figure 4.3.2.3: Relative survival ($\log_{10}\text{cfu/g}$) of *L. acidophilus* La-5 during simulated digestion of ice cream in the HGS

Few studies conducted by Marciani et al. (1999 and 2001) tested the effect of digestion process on the viscosity of samples *in vivo*. They found that the viscosity of the ingested food, irrespective of initial viscosity reduced significantly *in vivo* in the first 40 min of digestion as a result of progressive dilution, acidification by gastric juice and shear due to peristaltic contractions. Cameron-Smith et al. (1994) and Cherbut et al. (1990) also reported in line marked reductions in meal viscosity after ingestion of meal in rats and pigs largely due to dilution. Marciani et al. (1999) reported that the dilution of each ingested meal was similar, but viscosity of most viscous meal was found to decrease more compared to the less viscous meal. This was suggested to be due to the exponential relationship between viscosity and concentration of added gums and increase in gastric secretion because of increased distending of the stomach walls in response to high

viscosity of the meal. Edwards et al. (1987) found that the acidification changed the viscosity of the X/LBG (1:1 combination of xanthan and locust bean gum) and X/Mey (1:1 combination of xanthan and Meyprodyn®) gum mixtures. These gum mixtures had the highest initial viscosity, but upon acidification resulted in lowest viscosity compared to other gums.

In the present study, similar decrease in viscosity was observed because as the time of digestion increased, dilution of the ice cream also increased in the presence of constant mechanical shear. Even though, viscosity of both LVHC and HVHC ice cream samples reduced significantly ($p < 0.05$) at the end of first 30min itself and remained low throughout gastric digestion, the protective effect of ice cream still persisted which was demonstrated by survival of 4 log units of *L. acidophilus* La-5 at the end of 120 min simulated digestion. Hence, it could be suggested that viscosity is not the factor that was mainly responsible for the high viability of *L. acidophilus* La-5 at the end of digestion. One thing to consider is that in this study, gastric secretion rate (3.5 ml/min) and peristaltic contractions (3 cycles/min) were kept constant for consistency purposes, irrespective of viscosity levels of ice cream contradictory to the situation *in vivo*. This may be a reason why an effect of initial viscosity was not observed on the viability of *L. acidophilus* La-5 during simulated digestion.

Another factor tested in this study was the initial inoculation level. It was found that there was no statistically significant difference in the overall log reduction in viable cells of *L. acidophilus* La-5 at the end of 2 hr digestion. It is important to note that absence of statistical significant difference could be a result of small sample size because biologically difference of 2 log units in the overall log reduction between low

concentration and high concentration samples is significant. Nevertheless, an important finding from the present study was the survival of ~4 log cfu/g for all the four tested samples (LVLC, HVLC, LVHC and HVHC) irrespective of the initial amount of inoculation of *L. acidophilus* La-5 in the ice cream.

The survivability results obtained in the present study indicated that supplementation with lower amount of *L. acidophilus* La-5 in ice cream could be as effective as higher amount and this may be cost effective for ice cream producers and may eliminate possible adverse effects of adding high amount of *L. acidophilus* La-5 on the organoleptic properties and texture of the ice cream. Gomes et al. (2011) and Olson and Aryana (2008) reported changes in properties such as appearance, aroma, taste and texture of cheese and yogurt, respectively due to supplementation with high concentration of *L. acidophilus*. In addition, sensory defects and consumer rejection of cheeses supplemented with high levels of *L. acidophilus* in comparison to control cheese were also reported (Martin-Diana et al., 2003 and Gomes et al., 2011). Daily consumption of probiotic bacteria containing products is important for the probiotic bacteria to provide health benefits. Therefore, it's important for producers to consider making healthy products which have pleasant taste and texture so that consumers like the products and look forward to consuming them everyday (Saxelin et al., 1999; Champagne et al., 2005).

Results for survival of *L. acidophilus* La-5 found in the present study were in contradiction with those reported by Fernández de Palencia et al. (2008). They reported close to 20% survivability of *L. acidophilus* La-5 incorporated in skim milk at the end of simulated *in vitro* digestion. Whereas in this study, minimum survivability observed for same strain at the end of 2 hr simulated digestion was close to 50% or more depending on

the ice cream sample (LVLC, HVLC, LVHC or HVHC) that was digested. This difference in viability of same *L. acidophilus* La-5 strain in two different studies may be attributed to the difference in matrix that was tested (skim milk versus ice cream), components incorporated in the *in vitro* digestion model such as pH and time of digestion and fat content (<0.3% versus 5%). In a study conducted by Sumeri et al. (2008), they tested survivability of *L. rhamnosus* GG in different commercial products in addition to MRS as a model food and found that it behaved differently in different products during its transit through an *in vitro* gastrointestinal model. It survived very well in GEFILUS cheese and milk compared to its low survival <0.1% when digested with MRS matrix and Tütty baby formula. In addition to food properties such as buffering capacity as observed in the present study, fat may also play an important role in protecting the probiotic bacteria against harsh stomach conditions during digestion. Buriti et al. (2010) found that addition of milk fat to frozen guava mousse improved survival of tested probiotic bacteria during simulated digestion conditions when incubated at pH 1.4-1.9 for 2 hr. Possemiers et al. (2010) found that *L. helveticus* I-1722 and *B. longum* I-3470 when microencapsulated in stearate, yielded nearly 100% survival when ingested in either dark chocolate or milk chocolate.

Bezkorovainy (2001) reviewed several studies described by other researchers that evaluated the survival of different probiotic strains *in vitro* and *in vivo* during their passage through the upper GIT and observed that survival values ranged from 20% to 40%. The percent survival of *L. acidophilus* La-5 when ingested in ice cream in this study was above this range for all the samples. The variability in survival rate of probiotic bacteria in various studies depends on number of factors such as type of probiotic

bacteria and its response mechanism to stress, form of probiotic bacteria (harvested, freeze dried or free flowing powders), number of bacteria ingested, the composition of food vehicle in which they are ingested, physical protection of bacteria by food, buffering of gastric content, type of *in vitro* model used and difference in its components compared to other models and rate of gastric emptying (Gianella et al, 1972; Curto et al., 2011).

Comparison of our results with other studies that have conducted survivability tests using *in vitro* digestion models has revealed that it is important to study the combination of food product and probiotic bacteria of interest to understand any synergistic or antagonistic effects of food matrix on the viability of probiotic bacteria during *in vitro* digestion, effect of HCl and enzymes and to mimic real mixing and shearing caused due to peristaltic contractions as *in vivo*. In addition, the extent of protection provided by the food vehicle against harsh stomach conditions would also depend on whether the product is consumed before eating a meal, with the meal or after the meal as in a typical case of ice cream. Tompkins et al. (2011) showed that the survival of tested probiotic bacteria through the stomach and duodenum was highly dependent on the time of ingestion and the protective capacity of the meal or beverage. They reported that the bacterial survival was best when consumed within 30 min before or simultaneously with a meal or beverage that contained some fat content and poorest when taken after a meal or with products such as spring water or apple juice that does not contain fat.

5.0 Conclusions

Probiotic bacteria have been extensively incorporated in various fermented dairy products, especially yogurt and fermented milk. It is extremely important that the viable numbers of the probiotic bacteria are maintained above the recommended level of 10^6 cfu/g throughout the shelf life of the product. The low pH, presence of starter cultures and organic acids has been found to be detrimental on the viability of *Lactobacillus* and *Bifidobacterium* during the storage of fermented dairy products. In this thesis, potential of non-fermented low fat ice cream was investigated to deliver high amount of *L. acidophilus* La-5 to the host. The viability of *L. acidophilus* La-5 was not only studied throughout ice cream's shelf life of 90 days, but also during simulated digestion of ice cream in an *in vitro* model stomach called Human Gastric Simulator (HGS) to ensure high numbers of viable cells of *L. acidophilus* La-5 are maintained throughout storage and after gastric transit.

The viability of *L. acidophilus* La-5 was maintained throughout shelf life at $\sim 10^6$ cfu/g of 3.5 oz low fat ice cream incorporated with 60% and 100% overrun. The effect of freezing and different levels of overrun was found to be non significant ($p > 0.05$) on the survivability of La-5 when tested post freezing process. The storage of ice cream samples incorporated with 60% and 100% overrun at -10°C for 90 days did not result in a significant ($p > 0.05$) negative impact on the viability of *L. acidophilus* La-5 throughout shelf life.

The conventional acid resistance test of incubating the probiotic bacteria at pH 2.0 in the absence of ice cream mix was found to be extremely detrimental resulting in < 10 cfu/g of viable cells of *L. acidophilus* La-5 and *L. paracasei* 431 at the end of first hour

of two hour incubation study. Whereas, in the presence of ice cream mix, percent survival of *L. acidophilus* La-5 and *L. paracasei* 431 was found to be 65.34% and 43.04% respectively at the end of simulated digestion using a static model stomach. This shows that the acid tolerance in the presence of ice cream mix was species specific. This protective effect of ice cream mix due to its high buffering capacity was successfully validated during modeled digestion of frozen ice cream using shaking water bath model and HGS model.

The percent survivability of *L. acidophilus* La-5 was found to be 65.34%, 61.1% and 47.66% at the end of simulated digestion using static model stomach, shaking water bath model stomach and human gastric simulator, respectively. The mechanical shearing and mixing due to peristaltic contractions during ice cream's digestion in the HGS could have caused additional negative impact on the viability of *L. acidophilus* La-5 resulting in the lowest percent survivability of *L. acidophilus* La-5. The protective effect of ice cream components against harsh stomach conditions was mainly due to the high buffering capacity of ice cream. Difference in viscosity of ice cream samples did not appear to be the factor that contributed to the protective effect of ice cream. The lower initial inoculation level of *L. acidophilus* La-5 resulted in higher percent survival (64.53%) of *Lactobacillus acidophilus* La-5 at the end of 2 hr simulated digestion process compared to its survival at the end of digestion of ice cream sample supplemented with higher initial amount of La-5 (47.17%).

This study showed that human gastric simulator is a good platform for simulating *in vitro* conditions, to determine acid resistance capacity of probiotic bacteria and to evaluate protective effect of food vehicle on the survivability of probiotic bacteria in the

presence of harsh conditions like low pH, presence of enzymes and peristaltic contractions. Non-fermented low fat ice cream can be an excellent carrier of *L. acidophilus* La-5 to provide clinical benefits to the host.

6.0 Future Research

- Perform a study to determine the genetic response of *L. acidophilus* La-5 to stress caused during air incorporation and freezing that allowed it to survive the ice cream manufacture process extremely well.
- Conduct a sensory analysis comparing non-fermented and fermented low fat ice creams both supplemented with high and low amounts of *L. acidophilus* La-5.
- Conduct a study by supplementing other species of genus *Lactobacillus* along with *L. acidophilus* La-5 in an ice cream matrix to determine existence of any synergistic or antagonistic activity between the two species of *Lactobacillus*.
- Analyze the effect of bile present in the intestine on the survivability of *L. acidophilus* La-5 incorporated in ice cream matrix to determine whether after a period of acid stress in the human gastric simulator, *L. acidophilus* La-5 are able to resurrect their viability if they are exposed to more suitable conditions like those in the small intestine.
- Validate the protective effect of ice cream observed in the present study with an *in vivo* study.
- Compare the results obtained by plating method with confocal microscopy to take into account the presence of sublethally damaged or dormant cells that are unable to form visible colonies in the plating method.
- Conduct a study to determine whether *L. acidophilus* La-5 expressed any genes in response to acid stress that allowed its higher viability at the end of simulated digestion in this study.
- Use confocal microscopy to visualize interactions between *L. acidophilus* La-5 and Phospholipids present in the ice cream.

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Appendices

I: Ice cream formulation for determining the effect of 60% and 100% overrun on *L. acidophilus* La-5 survivability

Ingredients	Wt (lbs)	Fat (lbs)	NMS (lbs)	TS (lbs)
38% cream	3.97	1.51	0.22	1.73
3.5% fat milk	70.55	2.47	6.13	8.6
97% NMS	5.83		5.66	5.66
Sucrose	15			15
Corn Syrup Solids	4			4
Stab/Emulsifier	0.65			0.65
Total	100	3.98	12.01	35.64

II: Ice cream formulation for determining effect of shear and dilution on *L. acidophilus* La-5 survivability (bench top) and effect of HGS and shaking water bath on its survivability during simulated digestion of low viscosity, high concentration sample

Ingredients	Wt (lbs)	Fat (lbs)	NMS (lbs)	TS (lbs)
38% cream	6.83	2.60	0.38	2.98
3.5% fat milk	68.73	2.40	5.97	8.37
97% NMS	4.79		4.65	4.65
Sucrose	15			15
Corn Syrup Solids	4			4
Stab/Emulsifier	0.65			0.65
Total	100	5	11	35.65

III: Ice cream formulation for determining effect of shear and dilution on *L. acidophilus* La-5 survivability (bench top) and effect of HGS and shaking water bath on its survivability during simulated digestion of high viscosity, high concentration sample

Ingredients	Wt (lbs)	Fat (lbs)	NMS (lbs)	TS (lbs)
38% cream	6.89	2.62	0.38	3
3.5% fat milk	67.95	2.38	5.90	8.28
97% NMS	4.86		4.71	4.71
Sucrose	15			15
Corn Syrup Solids	4			4
Stab/Emulsifier	1.3			1.3
Total	100	5	11	36.29