Probiotic Application in the Development of Goat's Milk Products with Special Reference to *Propionibacterium jensenii* 702: Effects on Viability and Functionality

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Declaration

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Journal papers

Ranadheera, R.D.C.S., Baines, S.K., Adams, M.C. (2010). Importance of food in probiotic efficacy, *Food Research International*, 43, 1-7.

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Awards

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Abstract

Goat's milk and goat's milk products are widely consumed in developing countries, and are attractive to many consumers in developed countries due to their therapeutic and nutritional value. Although an increasing global demand exists, the variety of commercially available goat's milk products is currently limited. Goat's milk itself may be a suitable vehicle for delivering probiotics to humans, and the addition of probiotics to goat's milk may further enhance its health promoting value. This thesis examines the feasibility of using the novel probiotic *Propionibacterium jensenii* 702 in co-culture with established probiotic organisms (lactobacilli and bifidobacteria) in the manufacture of goat's milk products such as fermented milk, yogurt, ice cream and spray dried powder.

To provide beneficial health effects probiotic bacteria must survive passage through the gastrointestinal tract, then adhere to and colonise the gut epithelium. As these functional properties can be influenced by the food carriers used in probiotic delivery, a series of *in vitro* studies were performed to investigate the effect of goat's milk carrier foods on these functional aspects of probiotic efficacy. The viability of the probiotics during product shelf life, and the physico-chemical characteristics and sensory attributes of these products, were also analysed in order to assess both their general quality and appeal.

The first of the studies presented involved the examination of different mono-culture and co-culture combinations of *P. jensenii* 702 in fermented goat's milk with respect to viability during storage, physico-chemical and sensory attributes of the milk, and *in vitro* gastrointestinal tolerance and adhesion ability. Co-cultivation of *P. jensenii* 702 with *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 in goat's milk was not found to impact adversely on either the physico-chemical properties or sensory characteristics of the fermented milk preparations, or the functional properties of these bacteria. Thus it was found that combining *P. jensenii* 702 with lactobacilli and bifidobacteria could be

effectively utilised in the development of probiotic goat's milk products. Based on these findings, a triple co-culture of these three organisms was utilised in all subsequent studies.

In the second study, plain and stirred fruit yogurts were developed. *P. jensenii* 702 demonstrated high viability (10^8cfu/g) in all types of yogurt throughout storage, while the viability of the bifidobacteria also remained above the minimum recommended level (10^6 cfu/g). However, the results indicated that yogurt starter culture bacteria may have had an adverse effect on *L. acidophilus* LA-5 viability during the shelf life of the products, resulting in low viable counts in both types of yogurts ($<10^5$ cfu/g). In general the physico-chemical properties of the yogurts were within acceptable ranges and sensory tests revealed that the addition of fruit juice improved consumer acceptability of the yogurt.

The third study involved development of a chocolate flavoured probiotic ice cream using goat's milk. In addition to the performance of *P. jensenii* 702, the effect of packaging materials on both probiotic viability, and the physico-chemical and sensory properties of the product were also evaluated. Regardless of packaging type (glass, polyethylene or polypropylene) all three probiotics were able to maintain high viability (10^7-10^8 cfu/g) in the ice cream during 52 weeks of storage at -20^{o}C. While no significant effect on the sensory properties of the ice cream could be associated with the packaging materials, product stored for 12 weeks was more highly ranked for all sensory attributes than that stored for one week.

The relative influence of different food matrices on the gastrointestinal tolerance and adhesion of probiotics was examined *in vitro* using goat's milk ice cream, plain and 10% stirred fruit yogurts. In relation to the different food types, significant variation in the viability of all three probiotics was observed when exposed to simulated gastro-intestinal conditions of either low pH (2.0) or 0.3% bile. In general, ice cream was found to improve the acid and bile tolerance of the probiotics compared to plain and stirred fruit yogurts. In contrast, the fruit yogurt appeared to be the most favourable carrier in terms of *in vitro* adhesion of the probiotics to human Caco-2 intestinal cells, although a substantial number of viable bacteria (10^5-10^6 cfu/g) were found to be adherent in all cases.

The effects of micro-encapsulation and subsequent storage on the viability of the three probiotics was examined after spray drying the triple combination in reconstituted goat's milk. Although spray drying resulted in an initial reduction in the number of viable cells, the viability of all three probiotics remained unaffected (~10^6 cfu/g) under refrigerated storage for 24 weeks. Rehydration of spray dried probiotics in coffee and black tea at 85^{o}C lowered viable cell numbers, however, both *P. jensenii* 702 and *L. acidophilus* LA-5 retained satisfactory viability (~10^6 cfu/g) even after rehydration at this temperature.

This thesis provides evidence to suggest that the novel probiotic *P. jensenii* 702 may be successfully utilised in probiotic co-culture for the manufacturing of functional goat's milk products such as ice cream and yogurts. The findings also reveal the importance of careful selection of both the species to be used in co-cultured probiotic preparations, and a suitable carrier food matrix, in order to assure maximum benefit to consumers.

Chapter 1 : Probiotics, effect of carrier food on probiotic efficacy & goat's milk as probiotic carrier food

1. 1 Probiotic concept

The whole concept of health promoting microorganisms is not new, and in fact they have been consumed by human beings in the form of fermented foods, for thousands of years (Cross et al., 2001; Kopp-Hoolihan, 2001). Probiotic related health benefits have also been long known, with Hippocrates and other scientists in the early ages reporting that fermented milk could cure some disorders of the digestive system. Even Biblical scriptures mentioned the use of health promoting microorganisms in treating body ailments (Lourens-Hattingh & Viljoen, 2001). It was in 1907, that the Russian scientist Elie Metchnikoff (1845-1916) first proposed the concept of probiotics as it is known today (Metchnikoff, 1907). Metchnikoff observed that consumption of fermented milk products containing lactobacilli prevented intestinal putrefaction, promoted health and prolonged life (Metchnikoff, 1907). It was later in the 1960s that Lilly and Stillwell, proposed the term probiotics, the Greek meaning "for life", to these microbes (Lilly & Stillwell, 1965). The term "probiotics" has since been applied to those "microbes which transit the gastrointestinal tract and which, in doing so, benefits the health of the consumer" (Tannock et al., 2000). There are several definitions of probiotics including "probiotics are the live microbial feed supplements that exert beneficial effects for the host animal by improving its intestinal microbial balance" (Fuller, 1989) and the most recent definition "live microorganisms which when administered in adequate amounts confer health benefits on the host" (FAO/WHO, 2002).

1.1.1 Probiotics and human gastrointestinal flora

At birth the human gastrointestinal tract contains no microbes, but soon become colonized (Metchnikoff, 1907). The gut flora is influenced by many factors including composition of

the maternal gut microflora, diet, degree of hygiene, use of antibiotics or other medication, and the environment (Baines, 2010). The gastrointestinal tract of humans contains 100 trillion microbes (Ley et al., 2006; Sousa et al., 2008), comprising more than 500 different species (Malinen et al., 2005). The stomach and small intestine contain less numbers ($<10^3$/g of intestinal contents), possibly due to composition of the luminal medium with acid, bile and pancreatic secretions, which kill most ingested microbes and the phase propulsive motor activity towards the ileal end which impedes stable colonization of microbes. However, the large intestine contains a complex and dynamic microbial ecosystem with a high density of living bacteria which are adhered to the epithelia (Aureli et al., 2011; Del Piano et al., 2006; Guarner & Malagelada, 2003). The intestinal microflora exerts several health benefits to the host through metabolic, trophic, and protective functions (Guarner & Malagelada, 2003). Metabolic functions include fermentation of non-digestible dietary components and endogenous mucus, generation of short chain fatty acids, production of vitamin K and absorption of ions such as iron, zinc, copper and magnesium. Trophic functions are based on control of epithelial cell proliferation and differentiation, and development and homeostasis of the immune system. Protective functions are related to the barrier effect of preventing the colonization of pathogens in the gastrointestinal tract (Del Piano et al., 2006; Guarner & Malagelada, 2003; Rolfs & Hediger, 1999). Probiotics are also reported to prevent gastro-intestinal infections such as bacterial induced diarrhoea (de Vrese et al., 2010) and *Helicobactor pylori* infection (Shirasawa et al., 2010), although there are differences of opinion as which probiotics have beneficial effects towards these disorders (McNaught et al., 2005). Overall, it is generally accepted that intake of probiotics contributes to the improvement and maintenance of well balanced intestinal flora (D'Aimmo et al., 2007).

1.1.2 Common probiotic microorganisms

Microorganisms belong to genera *Lactobacillus* and *Bifidobacterium* mainly and some other species such as *Streptococcus* have been used as probiotics for hundreds of years in food manufacturing and therapeutic applications (Table 1.1). Some microorganisms such as *B. infantis* ATCC27920G and *L. acidophilus* ATCC4356) are derived from the intestinal

flora of healthy humans while others are from non-human sources (Boyle & Tang, 2006; Mainville et al., 2005). In general strains of the genera *Lactobacillus*, as well as *Lactococcus* and *Bifidobacterium*, are most commonly given the generally-recognized-as-safe status ie. safe to consume and less or no risk to the host compared to the benefits (Salminen et al., 1998).

Microorganisms used in starter cultures are of great industrial significance since they play a vital role in flavour and textural development of fermented dairy foods including fermented milk and yogurts (Cogan et al., 2007). Although some beneficial health promoting effects of yogurt starter cultures (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* spp *bulgaricus*) such as improved lactose utilization and enhancement of immune system (Guarner et al., 2005; Meydani & Ha, 2000) have been reported, it is still debatable as to whether the term "probiotics" can be used for these cultures due to their poor survival in the digestive tract (Senok et al., 2005).

Table 1.1 Microorganisms used as probiotic cultures

***Lactobacillus* spp.**	***Bifidobacterium* spp.**	**Other spp.**
L. acidophilus	*B. bifidum*	*Escherichia coli* Nissle
L. casei	*B. breve*	*Saccharomyces boulardii*
L. crispatus	*B. infantis*	*Streptococcus thermophilus*[a]
L. delbrueckii subsp. *bulgaricus*[a]	*B. longum*	*Enterococcus francium*[b]
L. fermentum	*B. lactis*	*Propionibacterium*
L. gasseri	*B. animalis*	*Pediococcus*
L. johnsonii	*B. adolescentis*	*Leuconostoc*
L. paracasei	*B. essensis*	
L. plantarum	*B. laterosporus*	
L. reuteri		
L. rhamnosus		
L. helveticus		
L. lactis		
L. sporogenes		

[a]There is still debate about the probiotic activity.

[b]Safety concerns remain because of potential pathogenicity and vancomycin resistance.

Adopted from Senok et al. (2005); Shah (2007); Sari et al (2011); Caplan & Frost (2011)

Regardless of the diversity of these organisms and different view points of their probiotic potentials, the main probiotic preparations currently available on the market belong to a group of bacteria designated as lactic acid bacteria (e.g. *Lactobacilli, Streptococci and Bifidobacteria*), which are important normal constituents of the human gastrointestinal microflora (Penner et al., 2005) and produce lactic acid as a major metabolic product (Reddy et al., 2007).

1.1.2.1 Lactobacilli as probiotic

Lactobacilli are Gram-positive, rod-shaped bacteria found in carbohydrate rich habitats, such as on the mucosal membrane of humans and animals, surfaces of plants and material of plant origin, in sewage, and in fermenting food such as yogurt (Bernardeau et al., 2007). Several *Lactobacillus* species have a long history of safe use in human food and nutrition (D'Aimmo et al., 2007). Their main application in the food industry has been in the manufacture of dairy products such as yogurts. Presently, 56 species within the genus *Lactobacillus* have been identified, with *L. acidophilus* being the most commonly recognized species. The optimal growth temperature for lactobacilli is in the range of 35-40^{o}C and pH range of 6.4-4.5 (Shah, 2007).

1.1.2.2 Bifidobacteria as probiotic

Bifidobacterium are Gram-positive, non-motile and non-spore forming bacteria. Generally, they are non-pathogenic and are part of the normal intestinal microflora of humans and animals. Like *Lactobacillus, Bifidobacterium* species are mostly considered safe for human consumption. Presently, 29 species of genus *Bifidobacterium* have been identified and the majority is used in production of probiotic food products. The optimal growth temperature for *Bifidobacterium* is in the range of 37-41^{o}C and pH of 6.0-7.0 (Delcenserie et al., 2007; Shah, 2007).

1.1.2.3 Propionibacteria as probiotic

Propionibacterium are Gram-positive, non-spore forming, non-motile, rod-shaped anaerobic bacteria. Their optimum growth temperature is 25-32^{o}C. Propionibacteria preferentially use lactate as a carbon substrate, but they can also use lactose (Gautier & Richard, 1999). Optimum pH depends on the growth medium, however it is usually between 6.5- 7.0. Some propionibacteria strains resist the gastrointestinal conditions and are able to reach a high population density within the digestive tract (Gautier & Richard, 1999). Although *Lactobacillus* and *Bifidobacterium* are the main probiotics that are used

extensively worldwide (Shah, 2007), some research has demonstrated that other genera, such as *Propionibacterium*, also possess potential probiotic characteristics. The probiotic characteristics of propionibacteria depend on their ability to produce propionic acid, bacteriocins, nitric oxide, folacin, vitamin B_{12}, CO_2, as well as having stimulatory effects on the growth of other beneficial bacteria such as *Bifidobacterium* (Gautier & Richard, 1999; Huang & Adams, 2004; Kotula, 2008). It has been reported that propionibacteria may confer beneficial health effects to the host by modulating immune functions (Suomalainen et al., 2006) and also exhibit antimicrobial activities towards pathogenic microorganisms (Lind et al., 2007).

The genus *Propionibacterium* is comprised of two different groups from different habitats; that is, the classical dairy group, which inhabits mainly raw milk and milk products, and the acnes group, which inhabits human skin. The dairy group includes four species including *P. freudenreichii*, *P. acidipropionici*, *P. jensenii* and *P. thoenii*. These dairy *Propionibacteria* have been used extensively in the dairy industry, especially in cheese production, and they have a long history of safe human consumption (Meile et al., 2007; Meile et al., 2008).

1.1.3 Beneficial health effects and therapeutic value of probiotics

There are several evidences supporting potential clinical applications of probiotics including the prevention and treatment of diseases of the gastrointestinal, respiratory and urogenital tracts (Gardiner et al., 2002; Saarela et al., 2002). The maintenance/modulation of healthy intestinal gut microflora (Lourens-Hattingh & Viljoen, 2001; Saarela et al., 2002), enhancement of the immune system, reduction of lactose intolerance (Dugas et al., 1998; Gilliland, 1990; Kim & Gilliland, 1983; Rasic, 2003), reduction of serum cholesterol level and blood pressure (Rasic, 2003), anti-carcinogenic activity (Commane et al., 2005; Gilliland, 1990; Lidbeck et al., 1992; Ouwehand et al., 1999; Rafter, 2003; Rasic, 2003) and nutrient metabolism (Lourens-Hattingh & Viljoen, 2001) are some of the many reported health benefits. Table 1.2 outlines some examples of probiotic potential for therapeutic applications.

Table 1.2 Examples of beneficial effects of therapeutic probiotic application in humans

Disorder	Probiotic strain	Mode of delivery	References
Antibiotic-associated diarrhoea in adults	Mixture of *L. casei* *L. bulgaricus* *S. thermophilus*	Drinking yogurt	(Hickson et al., 2007)
Traveler's diarrhoea	Single strain of *Lactobacillus* GG	Powdered form dissolved in cold water	(Oksanen et al., 1990)
Irritable bowel syndrome symptoms	Mixture of *B. longum, B. infantis, B. breve, L. acidophilus, L. casei, L. delbrueckii, L. plantarum, S. salivarius*	Lyophilized powdered form	(Kim et al., 2005)
	Mixture of *L. rhamnosus, B. breve* & *P. freudenreichii* subsp. *shermanii*	Capsules	(Kajander et al., 2005)
	Mixture of *B. animalis, L. bulgaricus* & *S. thermophilus*	Fermented milk	(Guyonnet et al., 2007)

	Mixture of *B. longum, B. infantis, B. breve, L. acidophilus, L. casei, L. delbrueckii, L. plantarum, S. salivarius*	Lyophilized powdered form	(Brigidi et al., 2001)
	Single strain of *L. plantarum*	Rose-hip drink with oat flour	(Nobaek et al., 2000)
	Single strain of *B. animalis*	Fermented semi skimmed-milk	(Marteau et al., 2002)
Crohn's disease	Mixture of *B. longum, B. infantis, B. breve L. acidophilus, L. casei, L. bulgaricus, L. plantarum, S. thermophilus*	Lyophilized form	(Campieri et al., 2000)
Ulcerative colitis	Single strain of *E. coli* Nissle	Capsules	(Kruis et al., 2004)
Pouchitis	Mixture of *L. acidophilus* La-5, *Bifidobacterium* Bb 12	Fermented milk	(Laake et al., 2005)

Bacterial vaginosis	Mixture of *L. rhamnosus* & *L. reuteri*	Gelatin capsules	(Anukam et al., 2006)
IgE associated eczema	Single strain of *L. reuteri*	Freeze dried form in coconut or peanut oil droplets	(Abrahamsson et al., 2007)
	Single strain of *L. rhamnosus*	Skim milk based freeze-dried form	(Wickens et al., 2008)
Atopic dermatitis	Mixture of *L. rhamnosus* & *L. reuteri*	Lyophilized powdered form	(Rosenfeldt et al., 2004)

1.1.4 Criteria for the selection of probiotics

Ibnou-Zekri et al. (2003) have reported that probiotic effects are strain specific. Thus, a beneficial effect attributed to one strain cannot necessarily be expected from another strain, even among the same species. A potentially successful probiotic strain is expected to have several desirable properties as outlined in Table 1.3, and these should be assessed during the development of new strains and novel probiotic foods. The source of origin is one of the important factors to consider since bacterial species that are present in the intestinal flora could have a better chance of survival in their native environment tolerating harsh gastrointestinal conditions (Vasiljevic & Shah, 2008). It is commonly noted that probiotics are host specific, and therefore micro-organisms of human origin may be desirable if they are intended for human use (Ouwehand et al., 1999). The bacterial strain must tolerate and survive gastric and bile secretions during transit through the upper gastro intestinal tract and then proliferate and/or colonize in the intestine. Fermentation products or cell components of the strain should not have any pathogenic, toxic, mutagenic, or carcinogenic reactions to the host organism. Further, it is desirable to have antagonistic effects towards gastrointestinal pathogenic microorganisms. It must be genetically stable with no plasmid transfer mechanism. During food processing and storage, it should survive and should have good technological properties such as withstand freezing temperatures and an adequate level of viability at the time of consumption. Furthermore, the potential probiotic should not have negative effects on organoleptic properties when applied to food (Lee & Salminen, 1995; Saarela et al., 2002; Vasiljevic & Shah, 2008; Ziemer & Gibson, 1998) and the health effects should be clinically validated in order to be considered as a suitable probiotic. Some researchers suggest that a strain not possessing all of these criteria may still have potential for probiotic use; however strains that fail to meet minimum selection criteria are likely not to be competitive products within the market (Gilliland, 2003; Ouwehand et al., 1999).

Table 1.3 Key and desirable criteria for the selection of probiotics in commercial application

Criteria	Property	Target and methods to be assessed
Safety criteria	Origin Pathogenicity and infectivity Virulence factors-toxicity, metabolic activity and intrinsic properties , i.e., antibiotic resistance	Source or origin should be assessed: be isolated from the same species as its intended host is desirable due to higher efficacy in the same species Pre-market clearance and post-market surveillance
Technological criteria	High viability retention during processing and storage Good sensory properties Ability to produce at large-scale Phage resistance	*In vitro* studies and food product development Sensory testing of model and final products and consumer studies on product formulations
Functional criteria	Tolerance to gastric acid and juices Bile tolerance Adhesion to mucosal surface Validated and documented health effects	Model systems for gastric and bile effects (e.g. *in vitro*, animal and human studies) *In vitro* adhesion models (e.g. intestinal segments, mucus, cell culture), animal and human studies Health effects confirmed by clinical studies
Desirable physiological criteria	Immunomodulation Antagonistic activity towards gastrointestinal pathogens Antimutagenic and anticarcinogenic properties	*In vitro*/*In vivo* animal and human studies. Adhesion and competitive exclusion of pathogens in *in vitro* and *in vivo* model systems

Adapted from McNaught and MacFie (2001); Saarela et al. (2002); Morelli (2007); Vasiljevic and Shah (2008); Aureli et al. (2011)

1.1.4.1 Gastrointestinal survival of probiotics

The high acidity in the stomach and the high concentration of bile components in the proximal intestine are the first host factors to consider when selecting microbial strains as potential probiotics (Hyronimus et al., 2000; Pennacchia et al., 2004). Survival of probiotics in the gastric juice depends on their ability to tolerate low pH (Pennacchia et al., 2004). The pH of the secreted HCl in the stomach is 0.9 (Erkkilä & Petäjä, 2000). However, the concentration of secreted acid varies with the rate of flow and the acidity often reaches pH 2.0 (Smith & Morton, 2001), but the presence of food matrix may raise the pH value to 3.0 (Erkkilä & Petäjä, 2000). Viability of probiotic is often observed to be significantly reduced at pH 2.0 or below (Huang & Adams, 2004; Masco et al., 2007). In contrast Masco et al (2007) reported higher survival rate for *B. animalis* ssp. *lactis* compared to *B. bifidum* after 180 minutes of exposure to gastric juice (pH 2.0) *in vitro*. This shows that resistance to the low pH of the stomach varies between genera and species, and even vary between different strains of the same species (Favaro-Trindade & Grosso, 2002; Huang & Adams, 2004; Masco et al., 2007).

Probiotics that survive acidic conditions of the stomach are further challenged by exposure to bile and bile salts in the duodenum, although pH of the small intestine (7.0-8.5) is more favourable towards the bacterial survival (Masco et al., 2007). Bile salts play an important role in food digestion and they emulsify dietary fat due to their detergent-like functions (Erkkilä & Petäjä, 2000). The bile salts may be detrimental to the microorganisms since their cell membrane is composed of lipids and fatty acids. However, some microbes possess bile salt hydrolase and therefore they are able to hydrolyse bile salts and limit the effect of bile (Erkkilä & Petäjä, 2000; Hofmann & Mysels, 1992). Although the bile concentration varies, many reports suggest that the mean intestinal bile concentration for the *in vitro* screening of resistant probiotic strains is 0.3% w/v (Erkkilä & Petäjä, 2000; Gilliland et al., 1984; Huang & Adams, 2004; Pennacchia et al., 2004). Bile resistance of probiotics is also reported to be strain dependent (Huang & Adams, 2004; Masco et al., 2007). For example, Huang and Adams (2004) observed significant reduction in cellular viability of *P. freudenreichii* CSCC2207 in the presence of 0.3% bile salts after 240 minutes of exposure

in vitro while *P. freudenreichii* CSCC2216 was able to maintain a high viability without any significant cell loss under the same conditions.

Although it is mandatory to perform preliminary *in vitro* assessments to evaluate the functional properties of probiotics (FAO/WHO, 2001, 2002; Morelli, 2007), in some cases, specially with respect to the gastrointestinal tolerance, *in vitro* conditions may not exactly represent the *in vivo* conditions. A low tolerance to acid by *L. paracasei* strains *in vitro* has been demonstrated, but the same strains have shown excellent *in vivo* gastric tolerance (Charteris et al., 1998a; Mishra & Prasad, 2005; Morelli, 2007). Although *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* have been reported, to be extremely sensitive to human gastric juice under *in vitro* conditions (Conway et al., 1987), some reports have shown the recovery of considerable number of viable cells from the faecal samples of consumers indicating their better gastrointestinal sensitivity under *in vivo* conditions (Elli et al., 2006; Mater et al., 2005).

1.1.4.2 Adhesion/colonization properties of probiotics

Probiotic adhesion/colonization in the gut is generally considered a prerequisite since adherent strains have the capacity to prevent pathogen adhesion and activation (Holzapfel et al., 1998; Ouwehand et al., 1999; Saarela et al., 2000; Ziemer & Gibson, 1998), In most cases, probiotics do not appear to colonize a host permanently and will disappear gradually after administration ceases, indicating only transit colonisation (Kullen et al., 1997; Ouwehand et al., 1999). Adhesion provides an interaction of probiotics with the mucosal surface and stabilises the intestinal mucosal barrier. Probiotic strains that can adhere to the intestinal epithelium may also provide better probiotic effects to the host compared to strains with less adhesive capacity (Saarela et al., 2000). A few studies have shown that even some bacterial DNA sequences (non viable bacteria) may have similar effects as the live bacteria and thus, intestinal colonization may not be a prerequisite for the action of probiotics (Jijon et al., 2004; Rachmilewitz et al., 2002). However, there are significant species and strain differences between the probiotic microorganisms, and therefore, they do not all necessarily share the same characteristics (Baines, 2010).

1.1.5 Probiotic food products

The popularity of dairy products among consumers has led to the extensive use of probiotic cultures as a tool for the development of novel functional products (Vasiljevic & Shah, 2008) and also because dairy products are considered to be good vehicles for the delivery of probiotics to humans (Lavermicocca, 2006; Lourens-Hattingh & Viljoen, 2001; Ross et al., 2002). Dairy products, including yogurts, fermented milk and cheese remain at the forefront of probiotic food development (Senok et al., 2005). A range of food products fortified with these probiotic bacteria is shown in Table 1.4 demonstrating range and diversity. Probiotics used in foods have been primarily added as part of the fermentation process, however increasingly they are added as supplementary components. Other forms of probiotics, such as tablets and powders, are now widely available in most supermarkets and health food outlets, especially in the developed countries (Fuller & Perdigon, 2003). Although there is no general agreement on the recommended levels (Vasiljevic & Shah, 2008) to achieve the claimed health benefits, a relatively high viability of probiotics in the final product, at least 10^6-10^7 cfu/g or ml, is recognized as a prerequisite (Lourens-Hattingh & Viljoen, 2001; Martin-Diana et al., 2003; Ravula & Shah, 1998b). These criteria have been made to compensate for the losses in viability of probiotics during processing and storage of the products as well as probiotic survival through the gastrointestinal tract upon consumption (Vasiljevic & Shah, 2008).

Table 1.4 Food products containing viable cells of probiotic strains during storage

Product type	Product	Probiotic strain	Viability at the end of storage	Total Storage time	References
Milk based	Fermented cow's milks	*L. acidophilus* *L. rhamnosus*	10^7 cfu/g	7 days	(Oliveira et al., 2001)
	Fermented goat's milk	*L. acidophilus* *Bifidobacterium* BB-12	$<10^6$ cfu/g 10^6-10^7 cfu/g	21 days	(Martin-Diana et al., 2003)
	Cow's milk yogurt	*L. acidophilus* *B. longum* *B. psedolongum* *B. infantis* *B. bifidum*	$>10^6$ cfu/g	42 days	(Shah & Lankaputhra, 1997)
		P. jensenii	10^5 cfu/g	15 days	(Ekinci & Gurel, 2008)
	Cow's milk fruit yogurt	*L. acidophilus* *B. animalsi* ssp.	10^6-10^7 cfu/g	35 days	(Kailasapathy et al., 2008)

	lactis			
Goat's milk yogurt	*L. acidophilus* *B. bifidum* *L. paracasei* subsp. *casei*	10^7 cfu/g	14 days	(Guler-Akin & Akin, 2007)
Ewe's milk yogurt	*L. acidophilus* *B. bifidum* *L. casei*	10^7 cfu/g	14 days	(Guler-Akin, 2005)
Ice cream	*L. johnsonii*	10^7 cfu/g	8 months	(Alamprese et al., 2002)
	L. acidophilus *B. lactis*	10^5-10^6 cfu/g	90 days	(Akin et al., 2007)
Cheddar cheese	*L. paracasei*	10^7 cfu/g	90 days	(Gardiner et al., 2002)
Fresh Minas cheese	*L. paracasei*	10^8 cfu/g	21 days	(Buriti et al., 2005)

White Turkish cheese	*L. acidophilus*	10^7 cfu/g	90 days	(Kasimoglu et al., 2004)
Semi hard Argentinian cheese	*L. paracasei* *L. acidophilus*	10^8 cfu/g	60 days	(Bergamini et al., 2005)
Argentinian Fresco cheese	*B. bifidum* *B. longum* *L. acidophilus* *L. casei*	10^6 cfu/g	60 days	(Vinderola et al., 2000b)
Requeijao-cheese (Portuguese-whey cheese)	*L. animalis* *L. acidophilus* *L. paracasei* *L. brevis*	10^7 cfu/g	28 days	(Madureira et al., 2005)
Semi hard goat's cheese	*L. acidophilus* *B. lactis*	10^6 cfu/g	70 days	(Gomes & Malcata, 1998)
Crescenza cheese (soft Italian cheese)	*B. bifidum* *B. infantis*	10^5 cfu/g	14 days	(Gobbetti et al., 1998)

		B. longum			
Soya based	Soya frozen dessert	*L. acidophilus* *L. paracasei* *B. lactis* *L. rhamnosus* *S. boulardii*	10^7 cfu/g ~10^5 cfu/g	28 weeks	(Heenan et al., 2004)
	Soy milk	*B. breve*	10^9 cfu/ml	20 days	(Shimakava et al., 2003)
Cereal based	Oat bars	*B. lactis*	10^9 cfu/25 g bar	7-14 days	(Ouwehand et al., 2004)
	Milk based maize/ rice pudding	*B. animalis* *L. acidophilus* *L. rhamnosus*	10^8-10^9 cfu/g	21 days	(Helland et al., 2004)
	Oat meal gruel mixed with fruit drinks (i.e: rose hip, strawberry)	*L. plantarum*	10^{10} cfu/ml	30 days	(Molin, 2001)

Fruit and fruit juice	Blackcurrant	*L. plantarum*	Not reported		(Luckow & Delahunty, 2004b)
	Dried apple fruits	*L. casei*	10^6 cfu/g	15 days	(Betoret et al., 2003)
	Orange	*Lactobacillus* GG	Not reported		(Luckow & Delahunty, 2004a)
Vegetable based	Table olives	*L. rhamnosus* *L. paracasei* *B. bifidum* *B. longum*	10^6-10^8 cfu/g	90 days	(Lavermicocca et al., 2005)
	Tomato juice	*L. plantarum* *L. acidophilus* *L. casei* *L. delbrueckii*	10^4-10^8 cfu/g	30 days	(Lavermicocca, 2006)
	Beet juice	*L. plantarum* *L. acidophilus* *L. casei* *L. delbrueckii*	10^6-10^8 cfu/ml	4 weeks	(Yoon et al., 2005)

	Cabbage juice	*L. plantarum* *L. delbrueckii*	10^7 cfu/ml 10^5 cfu/ml	4 weeks	(Yoon et al., 2006)
	Artichokes	*L. plantarum* *L. paracasei*	10^7-10^8 cfu/g	90 days	(Valerio et al., 2006)
Miscellaneous	Dry sausages-beef +pork	*L. rhamnosus*	10^8 cfu/g	28 days	(Erkkilä et al., 2001)
	African beverages made from maize and milk	*B. lactis*	10^7 cfu/ml	21 days	(McMaster et al., 2005)

Adapted from Lavermicocca (2006)

1.2 Effect of food carriers on probiotic efficacy

Food substrate/diet is considered as one of the major factors in regulating colonization of microorganisms in the gastrointestinal tract. Food helps to buffer the bacteria through the stomach and may contain functional ingredients such as prebiotics that could interact with probiotics to alter their functionality. Prebiotics are "non digestible food ingredients that beneficially affect the host by selectively stimulating the growth, or activity, or both, of one or a restricted number of bacteria in the colon" (Gibson & Roberfroid, 1995; Guarner & Malagelada, 2003). Oligosaccharides such as lactulose, galacto-oligosaccharides, inulin and fructo-oligosaccharides are some of the well known examples of prebiotics. There is an obvious potential for a synergistic effect when combining probiotics and prebiotics appropriately, since prebiotics promote the growth and activities of probiotics (Table 1.5). Furthermore, many types of foods such as dairy and meat products, cereals, beverages and infant milk formulates can be fortified with prebiotics during the manufacturing process to increase probiotic efficacy (Gibson et al., 2004). In addition a number of other suitable food components including plants and their extracts, and metabolites of microorganisms may also be important in probiotic efficacy (Bomba et al., 2006). Physico-chemical properties of food carriers used for probiotic delivery, such as buffering capacity, water activity, redox potential, pH and temperature are significant factors that influence survival of the probiotic during gastric transit. Fat, protein and sugar content of the product are also some other factors that could affect probiotic growth and survival in food. Many different stress factors involved in the manufacture of a product and its subsequent ingestion and transit in the gastrointestinal tract can adversely affect the viability and functional properties of probiotic cultures (Table 1.6). Better growth and survivability during food manufacturing and storage as well as in the stomach, protection against acid, bile and gastrointestinal enzymes, adhesion to intestinal epithelium, antimicrobial properties and antibiotic resistance could be considered as factors that might be important in maintaining probiotic efficacy.

Table 1.5 Beneficial effects of prebiotics and probiotic bacteria in foods

Food	Prebiotics	Probiotics	Effect	Reference
Yogurt	Hi-maize/Resistant starch	*L. acidophilus* *L. casei*	↑Growth & viability	(Donkor et al., 2007)
	Inulin	*L. acidophilus* *L. casei* *L. rhamnosus* *L. reuteri* *Bifidobacterium*	↑Growth & viability	(Hekmat et al., 2009) (Aryana & McGrew, 2007) (Donkor et al., 2007) (Capela et al., 2006)
	Fructooligosaccharides	*L. acidophilus* *L. casei* *L. rhamnosus* *Bifidobacterium* *B. animalis* *B. longum*	↑Viability & fatty acid production	(Akalin et al., 2007b) (Akalin et al., 2004) (Capela et al., 2006)
Fermented milk	Polydextrose	*L. acidophilus* *L. rhamnosus* *B. animalis* *subsp. lactis*	↑Growth, viability & fatty acid production	(Oliveira et al., 2009b)
	Oligofructose	*L. acidophilus* *L. rhamnosus* *B. animalis* *subsp. lactis*	↑Growth, viability & fatty acid production	(Oliveira et al., 2009b)
Ice-cream	Inulin	*L. acidophilus* *B. lactis*	↑Viability	(Akin et al., 2007)

Cheese & cheese based products	Oligofructose	*L. acidophilus* *B. animalis* subsp. *lactis*	↑Growth, viability, sensory & fatty acid production	(Cardarelli et al., 2007) (Cardarelli et al., 2008)
	Inulin	*L. acidophilus* *B. animalis* subsp. *lactis*	↑Growth, viability, sensory & fatty acid production	(Cardarelli et al., 2007) (Cardarelli et al., 2008)
	Carboxymethyl cellulose	*P. freudenreichii* subsp. *shermanii*	↑Growth	(Buriti et al., 2005)

Table 1.6 Stress vectors affecting probiotic viability during food processing and gastrointestinal transit

Processing step	Stress vector
Production of probiotic preparations	Presence of organic acids during cultivation
	Concentration-high osmotic pressure, low water activity, higher concentration of particular ions
	Temperature-freezing, vacuum and spray drying
	Drying
	Prolonged storage-oxygen exposure, temperature fluctuation
Production of probiotic containing products	Nutrient depletion
	Strain antagonism
	Increased acidity
	Positive redox potential (presence of oxygen), i.e., hydrogen peroxide and bacteriocins
	Presence of antimicrobial compounds
	Storage temperature
Gastrointestinal transit	Gastric acid and juices
	Bile salts
	Microbial antagonism

Adopted from Vasiljevic and Shah (2008)

1.2.1 Yogurt as a probiotic carrier food

Usually yogurt is prepared by allowing milk to ferment by pure cultures of specific lactic acid bacteria (*S. thermophilus* and *L. bulgaricus cultures*). Increasingly, yogurts have been prepared with probiotic microorganisms such as *L. acidophilus* with varying viability over a range of shelf lives (Lourens-Hattingh & Viljoen, 2001).

In 2000, Birollo et al observed that in whole-set-yogurt the level of streptococci decreased approximately 1 log cycle at 6^{o}C in 60 days of shelf life where as the microorganisms remained viable and even reproduced in skimmed-set-yogurt under the same conditions. However, addition of a concentrated product of heated milk and sugar into whole set-yogurt caused reduction in cell viability (1.5 log cycles) (Birollo et al., 2000). Plain-yogurts demonstrated a significant ability in retaining higher levels of *L. acidophilus* over the shelf life compared to yogurts containing mixed berry or passion fruits whereas, yogurts containing mango or strawberry contained higher level of *L. acidophilus* than the plain-yogurts (Kailasapathy, 2008). These findings demonstrate the effect of different physico-chemical properties of various fruit mixtures such as pH on the viability of probiotics in yogurt (Kailasapathy et al., 2008). Therefore, fruit mixtures or any other added ingredients that contribute to a lowering of pH in yogurt may contribute in reducing the viability of *L. acidophilus*. Kailasapathy et al. (2008) further reported rapid loss of viability of *B. animalis* subsp. *lactis* with increasing percentage of fruit pulp added into yogurt base. Acidity of stirred fruit yogurt could be increased with the addition of more fruit pulp into yogurt base resulting in rapid viability losses. Therefore, addition of substances such as whey protein into yogurt may enhance the viability of some probiotics due to maintaining the buffering capacity of yogurt. Different nutrient compositions such as vitamin levels of fruit juice may also have an influence on viability.

Fortification with ascorbic acid was reported to improve viability of *L. acidophilus* in yogurts although there was no effect on bifidobacteria (Dave & Shah, 1997b). Yogurt starter culture bacteria are also identified as oxygen scavengers and thus may be beneficial in improving the growth and viability of anaerobic probiotics. These starter cultures were

demonstrated to complete the fermentation of milk within 5-10 hours and utilised most of the oxygen in milk (Dave & Shah, 1997b). Therefore, incubation time of the product also affects the viability of probiotics in yogurt. In the case of a prolonged incubation period, added oxygen scavengers may not cause an advantage in improving viability of anaerobic probiotics. In addition to incubation time period, incubation temperature and storage time of yogurts appeared to be affect cell viability (Guler-Akin & Akin, 2007). On the other hand stirred fruit yogurts might result in low viability levels compared to plain-yogurts if the probiotic strain is less tolerant to oxygen, because oxygen is incorporated into yogurts while stirring fruit pulp/juice into yogurt base (Kailasapathy et al., 2008; Talwalkar & Kailasapathy, 2004b).

Higher viability of *L. acidophilus* was demonstrated in response to added cysteine at 250-500mg/l in yogurt during manufacture and storage while viability of bifidobacteria was adversely affected by the same levels although, bifidobacteria demonstrated better viability in a concentration of 50 mg/l of cysteine. The reduction in pH (from 4.5-4.4 to 4.3-4.0 after 35 days at 4^{o}C), observed in yogurts with the higher cysteine concentrations, may have had an adverse effect on bifidobacteria, while the availability of amino nitrogen from cysteine may have caused positive effects on growth of both lactobacilli and bifidobacteria, but in different concentrations as outlined above. Variations in the starter culture combinations used in these experiments may also have had an effect on viability levels due to antagonistic or symbiotic relationships (Dave & Shah, 1997b; Dave & Shah, 1997d). Likewise, addition of 5% cysteine greatly improved the growth and viability of *L. acidophilus, L. paracasei* subsp. *casei* and also *B. bifidum* in goat milk yogurts during fermentation and storage (Guler-Akin & Akin, 2007) confirming the favourable effect of cysteine towards the lactobacilli and bifidobacteria.. Addition of “Hi-maize” or amylase maize starch (a natural dietary fibre/resistant starch) and inulin demonstrated significant improvement on growth, viability and organic acid production of *L. acidophilus* and *L. casei* in set-yogurts. Interestingly, in the presence of inulin, both cultures showed better retention of viability compared to Hi-maize. However, proteolytic activity of these cultures was significantly improved in the presence of Hi-maize in comparison to that in the presence of inulin or without any supplementation (Donkor et al., 2007). This demonstrates

some prebiotics are a much better medium compared to other prebiotics and that they influence different functional properties of probiotics in yogurts. Positive effects of adding Hi-maize, inulin and fructooligosaccharides on improving viability of *L. acidophilus*, *L. casei*, *L. rhamnosus* and *Bifidobacterium* spp. in yogurts were also observed by Capela et al (2006) in yogurts. These authors reported fructooligosaccharides as the most effective prebiotic in helping to retain the viability of probiotics in yogurts. Variations in viability levels of *L. casei* have also been reported in commercial probiotic yogurts depending on the physical and chemical compositions of yogurts (Ravula & Shah, 1998b). Thus, it is clear that viability and some functional properties of probiotic bacteria in yogurt are related to the characteristics of the carrier food product such as chemical composition.

1.2.2 Ice cream and frozen products as probiotic carrier food and effect on efficacy

Ice cream and frozen dairy deserts have demonstrated great potential for use as vehicles for probiotic cultures. Ice cream is considered favourably as a probiotic carrier due to the lower storage temperature and less risk of temperature abuse during frozen storage which leads to higher viability of probiotics at the time of consumption (Cruz et al., 2009).

It has been reported that viability of *L. acidophilus* and *B. lactis* may vary depending on the sugar levels of ice cream (Akin et al, 2007). For example, viable *B. lactis* counts in ice cream produced with 15% (w/w) sugar at 90 days of frozen storage was ~ 4.5 log cfu/g while same probiotic was able to maintain higher viability (~6.2 log cfu/g) in ice cream with 21% sugar under the same storage conditions. In contrast ice cream made from different levels of fat and sugar (15/5, 15/10, 22/5 and 22/10% - sugar and fat respectively) have shown different survival rates of probiotic strain *L. johnsonii* La 1 at 30 days of storage at -28^{o}C (the lowest survival percentage was 85% in 15/10 mixture – the highest was 102% in 22/5 mixture). Furthermore, in the presence of 0.4% (w/v) bile, non frozen *L. johnsonii* La 1 cells demonstrated better survival compared to the frozen-thawed cells (Alamprese et al., 2002). Therefore, when a probiotic strain is used to produce frozen food product their efficacy in the small intestine may vary when compared to the non frozen food product fortified with the same strain. Addition of inulin (1-2% w/w) caused

significantly higher viability of *L. acidophilus* and *B. lactis* in ice cream due to prebiotic effect of inulin (Akin et al., 2007). Probiotics incorporated into frozen food products have demonstrated better viability during shelf life due to the positive effect of low storage temperature (Heenan et al., 2004; Kebary, 1996), however, freezing process may cause negative effects on probiotic viability during the manufacturing of frozen dairy products (Alamprese et al., 2002; Ravula & Shah, 1998b) due to the cell damages and mechanical stresses of mixing.

1.2.3 Cheese as probiotic carrier food and effect on efficacy

Cheese is a versatile food product, appealing to many palates and provides a valuable alternative to yogurt and fermented milk as a vehicle in probiotic delivery.

Production of *petit-suisse* cheese with oligofructose and/or inulin was reported to be excellent in terms of viability of both *L. acidophilus* and *B. animalis* subsp. *lactis* while addition of eucalyptus honey reduced the viability level of *L. acidophilus* and *B. animalis* subsp. *lactis* in the same cheese. The low oligosaccharide content of honey may have led to the poor growth and this could explain in part the reduction in viability. Interestingly cheese produced with oligofructose and inulin demonstrated better consumer acceptability compared to the cheese produced with honey indicating advantages of selected prebiotics not only in probiotic growth, viability and stability, but also in improving sensory qualities (Cardarelli et al., 2008).

Cheddar cheese ripening temperature significantly affected only the viability of starter culture lactococci and some physico-chemical properties such as moisture content and pH of the final product when combined with certain bifidobacteria and lactobacilli strains such as *B. longum, B. animalis* and *L. casei*. Viability of lactococci was significantly lower when cheese was ripened for 24 weeks at 8^{o}C compared to 4^{o}C. This may be due to the high concentration of butyric acid produced in cheese ripened at 8^{o}C (Ong & Shah, 2009). Tharmaraj and Shah (2004) studied the suitability of cheese based French onion dips as a delivery vehicle for probiotic *L. acidophilus, L. paracasei* subsp. *paracasei, L. rhamnosus*,

B. animalis and *P. freudenreichii* subsp. *shermanii* and the effect of organic acids, canola oil and gums, $_{L}$-cysteine and $NaHCO_3$ on the survival of these probiotics for a period of 10 weeks at refrigerated storage. In these cheese onion dips, addition of acetic acid has shown a negative effect on *L. acidophilus* viability compared to lactic acid or citric acid while *B. animalis* performed better in acetic acid than other two acids. This might be due to the reduced antagonistic effect of the other probiotics (*L. acidophilus*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus*, and *P. freudenreichii* subsp. *shermanii*) that were inhibited by acetic acid or more resistance of *B. animalis* to acetic acid compared to other bacteria since acetic acid is one of their major metabolites. Addition of $NaHCO_3$ caused a greater positive influence on viability retention of all probiotics compared to $_{L}$-cysteine possibly due to reduced acid effect as a result of buffering while addition of oil and gums did not demonstrate major effect on probiotic viability in general. These acids are widely used in the food industry to enhance organoleptic properties and safety aspects of food (Tharmaraj & Shah, 2004). Therefore, combination of suitable strains, type of acid/s and other food additives to be included in the final product should be carefully assessed when developing functional probiotic foods.

1.2.4 Fermented drinking milk as probiotic carrier food and effect on efficacy

Better growth (>10^9 cfu/ml) of *L. acidophilus* was reported in ovine (sheep) milk compared to the goat's milk or cow's milk (~10^8 cfu/ml) after 12 hours of incubation at 37^{o}C, indicating the effect of variations in composition between different types of milk on probiotic growth. The higher acidity development in goat's milk during fermentation (~0.9% lactic acid in goat's milk compared to 0.8% in ovine milk) may have created a hostile environment for the survival of *L. acidophilus* in goat's milk (Drakoularakou et al., 2003). According to Oliveira et al. (2001) different milk supplementation such as sweet whey, casein hydrolysate and milk proteins had various effects on microbiological stability of fermented drinking milk containing probiotic *L. acidophilus* and *L. rhamnosus* with or without *S. thermophilus*. For example viability levels of *L. acidophilus* and *L. rhamnosus* (~10^9 cfu/ml) were significantly higher in fermented milk supplemented with sweet whey than that of supplemented with casein hydrolysate (~10^8 cfu/ml) at one week of refrigerated

storage (4^{o}C). Addition of whey protein concentrate (3-5% w/v) has further been reported to significantly improve the viability of *Bifidobacterium* BB-12 in the fermented goats' milk during 21 days of refrigerated storage at 4^{o}C (Martin-Diana et al., 2003). Incorporation of micronutrients into the milk, such as peptides and amino acids, with these ingredients, may be helpful in reducing fermentation time and delay over acidification during fermentation and thereby improve the viability of probiotic microorganisms (Martin-Diana et al., 2003). Various viability levels of *L. casei* (6.98-8.22 log cfu/g) have also been shown in commercially available fermented drinking milks depending on the type of product based on the presence of other probiotics and presence of food ingredients such as fruit juice (Ravula & Shah, 1998b). A recent study (Vinderola et al., 2011) revealed that the gastric acid resistance of *L. casei* in commercial fermented milks may vary in relation to the flavouring and storage conditions of the product. For example *L. casei* in fermented milk with "fruit of the forest" flavour (stored at 5^{o}C for 10 days) demonstrated a significantly improved gastric acid tolerance (>7 log cfu/ml) *in vitro* compared to fermented milk with "natural" flavour (5 log cfu/ml). Addition of soy germ powder has shown positive effects on producing fermented milk with *L. reuteri*, due to improved bile tolerance ability. *In vitro* survival of *L. reuteri* was significantly higher in the presence of 2-3 mmol/l bile salts when 4g/l of soy germ powder was added to milk. Soy germ powder may release important bioactive isoflavones during fermentation that could protect *L. reuteri* from bile salt toxicity in the small intestine (De Boever et al., 2001). Addition of soy protein to fermented soymilk has also been reported to lower the probiotic inhibition activity of bile due to binding and aggregating bile salts (Shimakava et al., 2003; Sugano et al., 1990). Thirteen different strains of dairy propionibacteria including *P. freudenreichii, P. jensenii and P. acidipropionici* demonstrated significantly higher capacity of *in vitro* upper gastrointestinal transit tolerance in the presences of dairy based commercial liquid breakfast mixture and soy milk compared to those in a saline solution. Addition of these milk foods caused increase of pH in simulated gastric juice and this was one of the factors that contributed to the improvement of the viability of tested dairy strains (Huang & Adams, 2004).

1.2.5. Chocolate as probiotic carrier food and effect on efficacy

Dairy desserts such as chocolate mousse have also been considered as potential probiotic delivering agents (Aragon-Alegro et al., 2007). A recent *in vitro* study (Possemiers et al., 2010) revealed that chocolate (~36% total fat) was a significantly better carrier matrix in surviving *L. helveticus* and *B. longum* during gastrointestinal transit compared to half skimmed-milk possibly due to the protection provided by the lipid fraction of cocoa butter in chocolate. However viability of *L. paracasei* subsp. *paracasei* LBC 82 in Minas fresh cheese manufactured through direct acidification with lactic acid increased from 6.61 up to 8.22 log cfu/g during 21 days of storage at 5^{o}C (Buriti et al., 2005), whereas, viability of the same strain in chocolate mousse increased slightly from 7.36 up to 7.66 log cfu/g under the same storage conditions during 21 days (Aragon-Alegro et al., 2007). Although inulin can help to improve the growth and viability of various probiotic species such as *L. acidophilus*, *L. casei* and *B. longum* in a number of different dairy products such as cheese (Capela et al., 2006; Cardarelli et al., 2008), addition of inulin did not influence the viability of *L. paracasei* subsp. *paracasei* in chocolate mousse.

1.2.6. Cereals as probiotic carrier food and effect on efficacy

Significantly lower viable counts of *L. acidophilus, L. rhamnosus* GG and *Bifidobacterium animalis* Bb 12 were obtained during 12 hours of fermentation at 37^{o}C and storage (at 4-6^{o}C for 21 days) of water based cereal puddings (75% rice flour + 25% maize flour) compared to milk based cereal puddings, indicating the effect of different foods on the growth and stability of these probiotics. The pH levels of the products depended on the strain used and whether the products were based on milk or water. Water based puddings obtained significantly lower pH levels and faster reduction in pH during storage which could adversely affect probiotic growth and viability compared to milk based puddings (Helland et al., 2004). Malt, wheat and barley extracts have been reported to exert a positive influence in increasing bile tolerance of *L. acidophilus*, *L. reuteri* and *L. plantarum* (Michida et al., 2006; Patel et al., 2004). Immobilization of *L. plantarum* within malt and barley fibre seems to play a major positive role in improving gastrointestinal tolerance (by

providing a physical protection) of these bacteria (Michida et al., 2006). Furthermore, malt medium has demonstrated better support for the growth of *L. acidophilus, L. fermentum, L. reuteri* and *L. plantarum* than wheat or barley mediums due to its favourable chemical composition such as availability of considerable amount of maltose, sucrose, glucose, fructose and free amino nitrogen (Charalampopoulos et al., 2002).

1.2.7. Vegetable and vegetable products as probiotic carrier food and effect on efficacy

High survival rates (10^6-10^8 cfu/g for 90 days at room temperature) of probiotics such as *L. paracasei, L. plantarum* and some other probiotics in table olives and artichokes have been reported during storage as well as *in vitro* and *in vivo* gastrointestinal conditions. These survival rates are quite comparable or even higher than those of milk-based probiotic products. High viability of probiotics in these vegetable products can be caused by the micro-architecture of these vegetables which may protect the probiotics from harsh environmental conditions and presence of prebiotic substances (Lavermicocca, 2006; Lavermicocca et al., 2005; Valerio et al., 2006; Valerio et al., 2011). Therefore, not only chemical composition of foods but their physical structures are important in probiotic efficacy. More intensive lactic acid production was reported in carrot juice fermented with brewer's yeast autolysate and *L. acidophilus* compared to beetroot juice fermented with brewer's yeast autolysate because of the high content of minerals (such as Ca, P and Fe) influencing lactic acid fermentation in carrot juice. However, the number of *L. acidophilus* was higher in the beetroot juice (10^8 cfu/ml) at the end of the fermentation (at 37^{o}C for 8 hours) compared to the carrot juice (10^7 cfu/ml) confirming the favorable effect of beetroot juice towards the growth of *L. acidophilus* (Rakin et al., 2007). *L. plantarum*, *L. delbrueckii* and *L. casei* grew well and reached nearly similar cell concentrations (~ 10^9 cfu/ml) in cabbage juice after 48 hours of fermentation at 30^{o}C. However, only *L. plantarum* and *L. delbrueckii* have shown satisfactory viability rates in fermented cabbage juice up to 4 weeks of cold storage (4.1 x 10^7 and 4.5 x 10^5 cfu/ml respectively) while *L. casei* lost cell viability completely after 2 weeks under same conditions (Yoon et al., 2006). This may be partly due to the negative impact of some physico-chemical properties of cabbage juice such as pH (3.4) on the viability of *L. casei* compared to *L. plantarum* and *L. delbrueckii*.

1.2.8. Fruit juices as probiotic carrier food and effect on efficacy

Fruit juices have shown to be advantageous for the survival of probiotics during storage as they contain high amount of sugars such as glucose, fructose and sucrose (Ding & Shah, 2008; Rambla et al., 1997). Viability of *B. longum* in pomegranate and strawberry juices during 6 weeks at refrigerated storage was significantly lower (<1 log cfu/ml) compared to their viability levels in orange and pineapple juices (7-8 log cfu/ml) (Nualkaekul et al., 2011). Higher survival of *L. plantarum* (only less than 0.4 log decrease from 1 x 10^8 cfu/ml of initial counts) was observed by Nualkaekul and Charalampopoulos (2011) after 6 weeks at refrigerated storage in orange (pH 3.76), blackcurrant (pH 3.74) and pineapple (pH 3.76) juices compared to pomegranate (pH 3.25) and cranberry juices (pH 2.53) (<1 log cfu/ml). These authors concluded that the viability of probiotics in fruit juices during storage could be governed by the pH of the individual fruit juice as well as other compounds present in the fruit juice such as protein, dietary fibre and antimicrobial compounds (phenolic compounds). Certain fruits such as cranberry contain high levels of benzoic acid which can be detrimental for the probiotic cells (Nualkaekul & Charalampopoulos, 2011). *B. lactis* Bb 12 could survive in orange juice for 10 weeks at refrigerated storage (4.8 log cfu/ml) while no cells were recovered at week 10 when *B. lactis* Bb 12 stored in pineapple juice under similar conditions. In the same study, similar results were observed for *L. salivarius* confirming the protective effect of orange juice for these probiotics compared to pineapple juice (Sheehan et al., 2007). Therefore, it seems likely that the survival of probiotics in fruit juice during storage is strain as well as fruit juice specific.

1.2.9. Food carriers with microencapsulated probiotics and their effect on efficacy

Microencapsulation is defined as a technology of packaging solids, liquids or gaseous materials in miniature, sealed capsules that can release their contents at controlled rates under the influence of specific conditions (Anal & Singh, 2007; Anal & Stevens, 2005). Protection of probiotics by microencapsulation is an important method of improving their viability in functional foods (Ding & Shah, 2008). Microencapsulated *B. lactis* in traditional maize and milk based African fermented beverages (“mehewu”) has shown

significantly higher survival rates in the presence of stimulated gastric juice and considerable higher viability over the 21 days of shelf life at 4^{o}C as well as at 22^{o}C compared to free cells (McMaster et al., 2005). Microencapsulation with sodium alginate was also found to improve the viability of probiotics such as *L. casei* in other products such as freeze-dried yogurt after 6 months of storage at 4 and 21^{o}C (Capela et al., 2006). This is because microencapsulation helps to reduce cell injury and cell loss by retaining cells within the encapsulating materials (Ravula & Shah, 2003). After two months of refrigerated storage, viability of *L. gasseri* was reported to be reduced by 0.5 log cfu/g in spray dried milk powder and 1 log cfu/g in spray dried kudzu powder, a starch derived from roots of *Pueraria lobata* which has traditionally been used as a food ingredient in East Asia. Survivability of *L. gasseri* in the presence of simulated small intestinal juice was also varied depending on the carrier material. For example their survival in the presence of simulated small intestinal juice was significantly higher when spray dried with cow's milk compared to kudzu (5 log cfu/g vs. <1 log cfu/g after 180 minutes of exposure) (Ho, 2008). Use of different cryoprotectants such as pectin and sucrose in freeze drying also altered the probiotic viability in food products (depending on the type of cryoprotectant used) due to their variations in inhibition of intracellular or extracellular ice formation by binding to the water (Capela et al., 2006; Ravula & Shah, 2003). Saarela et al (2006) used sucrose and reconstituted skim milk in freeze drying probiotic *B. animalis* subsp. *lactis*. The spray dried product was incorporated into either fruit juices (orange, grape and passion fruit, pH 3.7) or pasteurized milk (pH 6.6 - 6.7) and stored at 4^{o}C for 6 weeks. Sucrose protected freeze dried *B. animalis* subsp. *lactis* exhibited significantly higher survival rate than skim milk protected cells in fruit juices during storage. However, *in vitro* acid and bile tolerance of skim milk protected *B. animalis* subsp. *lactis* was significantly better in the presence of pasteurised milk than in fruit juices. This may be due to additional protective effect of milk besides its buffering capacity (Saarela et al., 2006).

1.3 Goat's milk

Many animals are exploited to produce milk for human consumption. Cows, goats, buffaloes, sheep, and camels are the main milk producers in the various regions of the

world (Devendra, 1980; Hashim et al., 2009; Konuspayeva et al., 2009; Morand-Fehr & Boyazoglu, 1999; Sanz Sampelayo et al., 2007). The dominant milk producing animal in a particular region depends on the geographical and climatic conditions of the region (Devendra, 1980; Hashim et al., 2009; Pandya & Ghodke, 2007). Cows are the most common milk producer in many countries of the world (Devendra, 1980; Nardone & Valfrè, 1999). World goat's milk production ranks third after bovine (cow) and buffalo milk (Guo & Benjamin, 2003). Although majority of milk consumed throughout the world is bovine in origin (Konuspayeva et al., 2009; Malau-Aduli & Anlade, 2002), goat's (caprine) milk is also available in many countries and a considerable number of people consume goat's milk and goat's milk products (Guo & Benjamin, 2003; Malau-Aduli & Anlade, 2002; Morand-Fehr et al., 2004; Silanikove et al., 2010).

Although world production of goat milk is small, FAO (2001) official statistics revealed that the goat milk production tonnage had the largest increase (58%) compared to other mammalian farm animals during the past two decades (Guler, 2007; Haenlein, 2004; Stelios & Emmanuel, 2004). Milk production from goats is likely to be much greater than these official statistics, because of the large amounts of unreported home consumption, especially in developing countries (Guler, 2007; Haenlein & Abdellatif, 2004). Goat's milk differs from cow's and human breast milk in digestibility, alkalinity, buffering capacity, and it has certain therapeutic values making it useful in medicine and human nutrition (Park, 1994b, 2000; Park & Chukwu, 1988; Slacanac et al., 2010).

1.3.1 Physico-chemical characteristics of goat's milk

Milk contains all the basic nutritional components for the human diet such as protein, fat, carbohydrates, vitamins, and minerals. These nutrients are important in the body building processes and physiological functions, especially in young mammals. Composition and physico-chemical characteristics of milk vary from species to species (Edgar, 1998; Malau-Aduli & Anlade, 2002). There are some differences in physico-chemical characteristics between goat's and cow's milk which can influence their technological properties (Park, 1994b; Park et al., 2007; Slacanac et al., 2010) such as acidification ability (Morgan et al.,

2003). Further, these compositional differences affect the textural and organoleptic characteristics of milk products including consistency, flavour, odour, colour, stability during storage and syneresis (Slacanac et al., 2010; Stelios & Emmanuel, 2004).

The density of goat's milk is in the same range as that of cow's milk, but slightly higher. As a result of higher density, goat's milk has a higher viscosity, but lower refractive index and freezing point than cow's milk (Park, 1994b; Park et al., 2007; Slacanac et al., 2010). The titratable acidity of fresh as well as heat-treated goat's milk has been consistently higher compared to cow's milk. Consequently, fresh goat's milk habitually has a lower pH (6.50-6.80) value than fresh cow's milk (6.65-6.71) (Guo & Benjamin, 2003; Park et al., 2007; Slacanac et al., 2010).

Fat content

Of all the basic nutrients present in milk, perhaps the greatest difference between goat's milk and cow's milk is in the composition and the structure of the milk fat or lipids (Slacanac et al., 2010). Fat component is an important determinant of the technological and nutritional quality of goat's milk (Chilliard et al., 2003; Park et al., 2007). It influences the physical and sensory characteristics including yield, texture, consistency colour and flavour of dairy products, besides its quantitative contribution to the amount of dietary energy (Bozanic et al., 2002; Chilliard et al., 2003; Slacanac et al., 2010). The lipids in goat's milk consist mainly of triacylglycerols (98% of total lipids), phospholipids (1%), and cholesterol and its esters (1%) (Guo & Benjamin, 2003). The structure, size and the arrangement of the fat globules in goat's milk are different to that of cow's milk (Slacanac et al., 2010). The average size of fat globules of goat milk is about 2 μm in diameter and smaller than that observed for cow, which is about 3 μm in diameter (Guo & Benjamin, 2003). Fat globules in goat's milk are better distributed in the milk lipid emulsion compared to the cow's milk (Attaie & Richter, 2000; Slacanac et al., 2010). Goat's milk also possesses higher number of fat globules per ml of milk in comparison with cow's milk (Slacanac et al., 2010). These properties may have a technological impact other than the impact in human nutrition (Park, 1994a; Slacanac et al., 2010). Because of the small fat globules and lack of agglutinin (a

clustering agent which helps fat globules in milk to cluster upon cooling), goat milk has a poor creaming ability compared to cow's milk (Guo & Benjamin, 2003).

When compared with cow's milk, goat's milk has a higher level of short chain fatty acids (C4:0-C12:0). Furthermore, free fatty acid levels are also higher in goat's milk (Guo & Benjamin, 2003). Caprylic acid (C8:0) and capric acid (C10:0) levels are remarkably higher in goat's milk (Chilliard et al., 2003; Guo & Benjamin, 2003; Haenlein, 2004; Slacanac et al., 2010). Those short chain free fatty acids, especially C6:0 (caproic) and C8:0 (caprylic), are responsible for the specific "goaty" flavor of the goat's milk (Chilliard et al., 2003; Guo & Benjamin, 2003). The long chain fatty acid profile of goat's milk is similar to cow's milk. Goat's milk as well as cow's milk contain adequate amounts of essential fatty acids for human consumption (Guo & Benjamin, 2003). However, goat's milk exceeds cow's milk in monounsaturated fatty acids, polyunsaturated fatty acids, and medium chain triglycerides, which are considered as beneficial in human health (Chilliard et al., 2003; Haenlein, 2004; Park et al., 2007; Slacanac et al., 2010). Goat's milk also posses higher proportion of conjugated linoleic acid, which has been attributed diverse benefits in human health (Ceballos et al., 2009; Haenlein, 2004; Slacanac et al., 2010).

Protein content

The protein content of goat's milk consists of two distinct types of proteins known as casein and soluble whey proteins (Haenlein, 2004; Slacanac et al., 2010). Casein, the basic protein in milk constitutes over 80% of the total protein of milk (Slacanac et al., 2010). Caseins in the goat's milk are about the same as in the cow's or sheep's milk: α_{s1}, α_{s2}, β, and κ-caseins (Haenlein, 2004; Slacanac et al., 2010). The whey proteins are groups of compounds of albumin and globulin. β-lactoglobulin, α-lactalbumin are the two major whey proteins of goat's milk and has higher amounts compared to the cow's milk (Guo & Benjamin, 2003; Park, 1994a; Slacanac et al., 2010).

Although the protein profile of goat's milk is generally similar to cow's milk (Guo & Benjamin, 2003; Park et al., 2007), there are unique differences in the comparative

composition of proteins and their components among goat's and cow's milk. In general goat's milk contains higher amounts of β-caseins, lower amount of α_s-casein and approximately equal amounts of κ-casein fraction with cow's milk. In contrast to bovine milk, β-caseins is the major protein in goat's milk (Slacanac et al., 2010). It was reported that goat's milk has a slightly lower level of casein content than cow milk with a very low proportion or absence of α_{s1} casein. Since the level of α_{s1} casein affects the coagulation properties of milk, this is one of the main reasons for the poor coagulating properties of goat's milk, which ultimately responsible for the low cheese yield and weak yogurt structure (Guo & Benjamin, 2003; Slacanac et al., 2010). However, some studies have demonstrated that this protein does exist in some goat's milk depending on genetic variations among the goat breeds (Guo & Benjamin, 2003; Haenlein, 2004; Slacanac et al., 2010). Five major proteins of goat milk are α_{s2}, β, κ-caseins, β-lactoglobulin, and α-lactalbumin (Guo & Benjamin, 2003; Jenness, 1980). Most of these proteins have different numbers of amino acid residues and chain structures in comparison to the bovine milk protein and bring nutritive differences between goat's and cow's milk (Haenlein, 2004; Slacanac et al., 2010). In comparison with cow's milk, goat's milk shows higher amounts of 6 amino acids out of the 10 essential amino acids: threonine, lysine, isoleucine, cystine, tyrosine and valine. Overall, the adult daily dietary nutrient recommendations for essential amino acids would be met equally or exceeded by a 0.5 liter goat's milk consumption compared to cow's milk (Haenlein, 2004).

Lactose content

Lactose content, which is the major carbohydrate in both cow's and goat's milk, is slightly lower in goat's milk (4.5 g/100 ml) compared to the cow's milk (5 g/ 100 ml) (Guo & Benjamin, 2003; Silanikove et al., 2010; Slacanac et al., 2010). Goat's milk is significantly rich in lactose derived oligosaccharides than cow's milk, which has beneficial effects in human nutrition due to their prebiotic and anti-infective properties (Kunz et al., 2000; Slacanac et al., 2010). Lactose also has a technological importance during milk fermentation, in which it serves as the substrate for the lactobacilli bacteria (Edgar, 1998).

Mineral content

Goat's milk contains higher amounts of Ca, P, K, Mg and Cl, and lower levels of Na and S than cow's milk (Guo & Benjamin, 2003; Slacanac et al., 2010). Because of these differences, particularly, higher content of K and also Na, goat's milk has a specific slightly salty taste (Bozanic et al., 2002; Slacanac et al., 2010). The P_2O_5/CaO ratio of goat's milk, which has an important significance in nutrition is nearer to that of human milk than cow's milk (Slacanac et al., 2010). Unlike the major minerals, the concentration of trace elements in goat's milk is affected by the diet, breed, individual animal variations and stage of lactation (Guo & Benjamin, 2003).

Vitamins

Goat's milk contains higher level of vitamin A, because these animals convert all dietary carotene into vitamin A. Therefore, goat's milk is more whitish in color than cow's milk (Guo & Benjamin, 2003; Slacanac et al., 2010). In addition to vitamin A, goat's milk supplies adequate levels of niacin, and excesses of thiamin, riboflavin and pantothenate (Guo & Benjamin, 2003). Goat's milk however is a poor source of vitamin B_{12} and folic acid, and it contains only 20% of the amount of folic acid that cow's milk has. In addition, bioavailability of folic acid in goat's milk is lower than cow's and human breast milk (Guo & Benjamin, 2003; Slacanac et al., 2010). Both goat's and cow's milk are deficit in vitamin B_6 and D in addition to vitamin C (Guo & Benjamin, 2003; Park et al., 2007).

1.3.2 Therapeutic and nutritional value of goat's milk

Similar to cow's milk, goat's milk has been traditionally considered as a fundamental dairy food in the diets of many cultures (Silanikove et al., 2010). There is little difference in nutritional value between goat's milk and cow's milk. Goat's milk, however has been identified as a good nutritional source with lower allergenic properties compared to cow's milk (Guo & Benjamin, 2003; Martin-Diana et al., 2003). Goat's milk has been recommended as a substitute for the patients allergic to cow's milk (Dabrowska et al.,

2010; Park, 1994a). Fifty percent (50%) of human population who has allergic reaction to cow's milk may be able to tolerate goat's milk (Park, 1994a; Slacanac et al., 2010). Although there are some rare incidences of allergic reactions to goat's and sheep's milk without allergy to cow's milk (Bellioni-Businco et al., 1999), the reason for hypoallergenic value of goat's milk compared to cow's milk is the difference in their protein structures (Imafidon et al., 1991). Goat's milk proteins were reported to be digested by human gastrointestinal enzymes faster than that of cow's milk proteins *in vitro* (Almaas et al., 2006). However, it is recommended that any new food, including goat's milk, should only be introduced into the diet of individuals who are highly reactive to cow's milk, particularly infants, in consultation with appropriate medical professionals (Silanikove et al., 2010).

1.3.3 Goat's milk products

Application of goat's milk for cheese making is well known and cheese is arguably the most popular goat's milk product (Morghan & Gaborit, 2001; Pandya & Ghodke, 2007). Other goat's milk products include pasteurized fresh goat's milk, UHT (ultra high temperature) milk, evaporated milk, yogurt, ice cream, powdered milk, butter, and traditional South Asian goat's milk food products such as chana and paneer and even sweets, cosmetics such as soaps, creams, shampoos and body lotions (Pandya & Ghodke, 2007; Ribeiro & Ribeiro, 2010).

Goat's milk is suitable for the production of functional probiotic products by enhancing the functionality of goat's milk (Martin-Diana et al., 2003; Slacanac et al., 2010). Studies of bifidobacteria and lactobacilli have demonstrated satisfactory but variable growth and viability levels in various goat's milk products including cheese (Gomes & Malcata, 1998; Nikolic et al., 2008), yogurt and fermented milk (Bozanic & Tratnik, 2001; Bozanic et al., 2004; Farnsworth et al., 2006; Guler-Akin & Akin, 2007; Martin-Diana et al., 2003). However, use of propionibacteria as a probiotic in goat's milk products such as yogurts and ice cream has not been studied thoroughly and there is a possibility of masking unpleasant odour of goat's milk and improve its flavours through fermentation (Bozanic et al., 2004; Bozanic et al., 2003; Slacanac et al., 2010).

1.4 The research problem

Currently, the majority of commercially available probiotic products utilise *Lactobacillus* spp. and *Bifidobacteria* spp. Dairy propionibacteria have been examined as probiotics recently, however, at this time only a few probiotic foods containing *Propionibacterium* spp. are available for human consumption (refer Table 1.4). The novel probiotic *P. jensenii* 702 has already demonstrated a number of potential health promoting characteristics such as improving the growth of indigenous bifidobacteria in the gut (Adams et al., 2008; Huang & Adams, 2003, 2004; Huang et al., 2003; Kotula, 2008).

According to the reviewed literature, the nature of the food matrix used as a carrier to transport the probiotics can greatly influence their viability during storage. It seems likely however, that viability assessment through simple cell enumeration during storage may not be a sufficient measure to evaluate the efficacy of probiotics in foods. While cell viability is important for cell functionality such as acid and bile tolerance, other functional properties may also be highly influenced by the carrier food matrix. However, there is little information in the published literature that reveals the effect of different carrier food types on functional properties of probiotic bacteria. It is therefore important to determine the most suitable carrier food type/s for a particular probiotic or probiotic combinations based on viability and other functional properties, in order to assure maximum probiotic efficacy for the consumer.

At present, cheese is the only widely available goat's milk product in the market although a limited number of other goat's milk products are also available. When the potential beneficial health effects of probiotics and goat's milk are considered together, developing different types of goat's milk products by incorporating probiotic bacteria with satisfactory viability and functionality may prove valuable in expanding the market potential of goat's milk and fulfilling potential consumer needs.

1.5 Thesis aims and scope

The overall aims of this thesis were to evaluate aspects of the performance of the novel probiotic *P. jensenii* 702 by assessing their ability to withstand the processing and storage of different dairy foods such as fermented milk, yogurt, ice cream and spray dried milk powder, and to determine their functional properties with special reference to gastrointestinal tolerance and adhesion *in vitro* as affected by the carrier food type. Goat's milk was selected as the main ingredient in developing these probiotic foods based on the therapeutic and nutritional value of the goat's milk, and consumer desire for novel taste. Since *Propionibacterium* is widely utilized and well established in cheese manufacturing, this thesis mainly aimed to evaluate the feasibility of manufacturing other popular dairy products such as yogurt and ice cream with *P. jensenii* 702. The physico-chemical properties and sensory attributes of these products were analysed to establish the technological capabilities of *P. jensenii* 702 other than their viability during processing of different foods and subsequent storage. The experimental studies were mainly aimed at assessing technological and/or functional properties of *P. jensenii* 702 together with the common and widely used probiotics *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12, with additional assessment of various other parameters such as effect of fruit juice in yogurt, effect of packaging materials of ice cream on these technological and functional properties.

1.6 Thesis outline

Chapters 3, 4 5 and 7 of this thesis include the development of various probiotic carrier food types including fermented goat's milk, yogurt, ice cream and spray dried milk powder respectively. Chapter 3 aimed to explore both the technological and functional capabilities of *P. jensenii* 702 compared to *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 in both mono-culture and co-culture preparations. One of the main objectives of this chapter was to identify the most suitable way of delivering *P. jensenii* 702 (either monoculture or as a co-culture with other probiotics) in terms of their viability during processing and storage and physico-chemical, sensory and *in vitro* functional properties. The remaining chapters

are based on goat's milk products containing a co-culture of above three probiotics (*P. jensenii* 702, *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12). Chapters 4, 5 and 7 specifically aimed to assess the technological capabilities of these probiotics in selected probiotic foods, while Chapter 6 focuses on the gastro-intestinal tolerance and adhesion properties of the probiotics. These five experimental chapters are preceded by the materials and methods chapter (2), in which details of all procedures and protocols, techniques, equipment and materials used are provided, and followed by closing remarks in the final discussion and overall conclusions (Chapter 8).

Chapter 2 : Materials and methods

2.1 General

The studies presented in this thesis comprised various determinations of bacterial viability, acid and bile tolerance assays, cellular adhesion assays, and measures of the physico-chemical and sensory properties of probiotic food products. All experimental procedures involving the cultivation and analysis of micro-organisms were conducted in a sterile PC2 laboratory environment using aseptic techniques. Unless otherwise specified these experiments were repeated twice. Foods for sensory studies were in all cases prepared in a hygienic kitchen environment.

2.2 Probiotic bacteria

Pure freeze dried probiotic cultures of *L. acidophilus* LA-5 and *Bifidobacterium animalis* subsp. *lactis* BB-12 were obtained from CHR Hansen Pty Ltd (Bayswater, VIC, Australia). The *Propionibacterium jensenii* 702 used in these studies was obtained from a stock culture maintained at the School of Environmental and Life Sciences, University of Newcastle, Australia.

2.3 Media

Due to the varying growth requirements and need for selective enumeration of each of the probiotic organisms examined, a variety of different growth media were utilised in these studies. Each of the media and relevant details of their origin and/or preparation are provided below.

Unless otherwise specified, all media were prepared as per the manufacturer's instructions, and sterilised prior to use by autoclaving at 121°C for 20 minutes. Where relevant, agar

plates were prepared by pouring autoclaved media (cooled to approximately 50°C) into sterile Petri dishes (Sarstedt Australia Pty Ltd).

2.3.1 M-17 agar

M-17 agar (pH = 6.9±0.2) was obtained from Oxoid Australia Ltd.

2.3.2 MacConkey agar

MacConkey agar (pH = 7.4±0.2) was obtained from Oxoid, Australia Ltd.

2.3.3 MRS (deMan-Rogosa-Sharpe) broth/agar

MRS broth and MRS agar (pH = 6.2±0.2) were obtained from Oxoid Australia Ltd. pH modified MRS agar was prepared by adjusting the pH to 4.58 using concentrated HCl.

2.3.4 MRS-NNLP (Nalidixic acid, Neomycine sulphate, Lithium chloride, Paromoycine sulphate)

The MRS agar base was obtained from Oxoid, Australia Ltd. and was prepared following the manufacturer's instructions (pH = 6.2±0.2).

NNLP solution was prepared with nalidixic acid (Sigma-Aldrich, Australia), neomycine sulphate (Oxoid Australia Ltd.), lithium chloride (Sigma-Aldrich, Australia) and, paromoycine sulphate (Sigma-Aldrich, Australia) as per the method of Tamime and Robinson (1999). Filter sterile (0.22 μm filter, Millex Millipore, Ireland) solution was mixed with the autoclaved MRS agar base at approximately 50 ^{o}C.

2.3.5 MRS–sorbitol agar

MRS-sorbitol agar was prepared by first combining each of the separate ingredients of MRS agar, with the exception of glucose. After autoclaving the basal medium and allowing to cool to approximately 50°C, a sterile membrane filtered (0.22 μm filter, Millex Millipore, Ireland) solution of 10% (w/v) D-sorbitol (Oxoid Australia Ltd.) was added to a final concentration of 1%. The pH of the final preparation was 6.2±0.2.

2.3.6 RCM (Reinforced Clostridial Medium)

RCM (pH = 6.8±0.2) was obtained from Oxoid Australia Ltd. RC agar was prepared by combining RCM with bacteriological agar (15 g/L) (Oxoid Australia Ltd.).

2.3.7 Rose Bengal-chloramphenicol agar

One vial of chloramphenicol selective supplement (Oxoid Australia Ltd.) was added to a Rose-Bengal agar (Oxoid Australia Ltd.) solution, according to the manufacturer's instructions, to prepare 1 L of Rose Bengal-chloramphenicol agar (pH = 7.2±0.2).

2.3.8 Sodium Lactate (SL) broth and agar (SLA)

SL broth consisting of 10 g/L tryptone (Oxoid Australia Ltd.), 10 g/L yeast extract (Oxoid Australia Ltd.), 16.5 ml/L sodium DL-lactate (Sima Aldrich, Australia), 0.25 g/L K_2HPO_4 (Ajex Finechem Pty Ltd, Australia), 0.05 g/L $MnSO_4$ (APS Finechem Pty Ltd, Australia) was prepared and the pH of the medium was adjusted to 7.0 ± 0.2. SL agar was prepared by adding 15 g/L bacteriological agar (Oxoid Australia Ltd.) to SL broth.

2.4 Recovery and preservation of bacterial strains

L. acidophilus LA-5 and *B. animalis* subsp. *lactis* BB-12 were recovered from freeze dried cultures in MRS broth and RCM respectively by anaerobic incubation (37°C for 24 hours)

in 2.5 L anaerobic jars containing AnaeroGen™ sachets (Oxoid Australia Ltd.). *P. jensenii* 702 was inoculated into SL broth and incubated anaerobically at 30^{o}C for 72 hours. After recovery in liquid medium, all bacterial strains were streaked onto the appropriate agar plates (*L. acidophilus* LA-5 on MRS agar plates, *B. animalis* subsp. *lactis* BB-12 on RC agar plates and *P. jensenii* 702 on SLA plates) and subcultured three times in order to establish the purity. Once the pure cultures were obtained, cell morphology was examined via Gram staining and scanning electron microscopy. Probiotic bacterial identifications were confirmed by DNA extraction and PCR analysis using 16S rRNA gene targeted species-specific primers (refer appendix A for the list of the primer sets). Once the purities of cultures were established, probiotic bacteria were again grown in appropriate broths. Bacterial cells were harvested from the broths by centrifugation (2500 x g, 10 minutes, 4^{o}C) (Eppendorf centrifuge 5810R, Germany), washed three times with 0.1% sterile saline solution, resuspended in appropriate broths containing 20% glycerol (Sigma-Aldrich), divided into 0.5 ml aliquots and stored at -80^{o}C in cryovials (Nunc^{TM}, Denmark), to be used as stock cultures for subsequent experimentation.

For the production of fermented goat's milk and for microencapsulation, aliquots of each probiotic bacteria stored at -80^{o}C were grown in appropriate liquid media, harvested by centrifugation in their stationary phases, washed three times with 0.1% sterile saline solution, and resuspended in pasteurized goat milk as the inoculum. The total viable count of washed bacterial suspension was determined prior to inoculation by spread plate techniques.

A freeze dried yogurt culture (ABY-1) containing *Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus, L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 was obtained from CHR Hansen Pty Ltd (Bayswater, VIC, Australia) and directly used as starter culture in yogurt production as per manufacturer's instructions. Freeze dried *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 cultures were used for ice cream production. Spray dried *P. jensenii* 702 was used when producing fermented milk, yogurt and ice cream for sensory analysis. The probiotic inoculums were prepared fresh before each experiment.

2.5 Growth conditions and selective enumeration of microorganisms

Based on the findings of Dave and Shah (1996), MRS–sorbitol agar was chosen for the selective enumeration of *L. acidophilus* LA-5 from goat's milk products. *B. animalis* subsp. *lactis* BB-12 was enumerated by plating onto MRS-NNLP agar (Tamime & Robinson, 1999) and SLA was chosen, based on the findings of Tharmaraj and Shah (2003) for the selective enumeration of *P. jensenii* 702. *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* were enumerated on pH modified MRS agar (4.58) and M-17 agar respectively. All the bacteria were incubated anaerobically in anaerobic jars except *S. thermophilus* which was incubated under aerobic conditions. Incubations were conducted at 30^{o}C for 5-7 days (*P. jensenii* 702), 37°C for 72 hours (*B. animalis* subsp. *lactis* BB-12 and *L. acidophilus* LA-5), 37^{o}C for 24 hours (*S. thermophilus*), and 45^{o}C for 72 hours (*L. delbrueckii* subsp. *bulgaricus).*

Coliform (*E. coli*) counts were estimated by counting red/pink colonies after plating onto MacConkey agar with incubations conducted aerobically for 18 hours at 37^{o}C (Gagnon et al., 2004). Yeast and mould enumeration was carried out on Rose Bengal-chloramphenicol agar, incubated aerobically at 25^{o}C for 5 days (Welthagen & Viljoen, 1997).

Spread plate techniques were used for plate counting of all bacteria with triplicate agar plates made from the appropriate dilutions.

2.6 Product manufacturing

2.6.1 Production of fermented goat's milk

The milk used in these experiments was reconstituted (12% total solids) from spray dried skim goat's milk powder (Healtheries of New Zealand Ltd., Auckland, New Zealand) and heat treated at 85^{o}C for 30 minutes before being cooled to the inoculation temperature (37^{o}C). The approximate inoculation levels of both *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 were 10^{7} cfu/ml. Since there is no recommended inoculation level for

novel probiotic *P. jensenii* 702 in manufacturing dairy foods, two different inoculation levels (10^6 cfu/ml or 10^8 cfu/ml) were examined for *P. jensenii* 702. After inoculation milk was fermented for 10 hours at 37^{o}C. Seven different types of fermented milks were produced based on the different combinations of the probiotic bacteria as follows:

L. acidophilus LA-5 (L)
P. jensenii 702 (P)
B. animalis subsp. *lactis* BB-12 (B)
L. acidophilus LA-5 + *P. jensenii* 702 (L + P)
L. acidophilus LA-5 + *B. animalis* subsp. *lactis* BB-12 (L + B)
P. jensenii 702 + *B. animalis* subsp. *lactis* BB 12 (P + B)
L. acidophilus LA-5 + *P. jensenii* 702 + *B. animalis* subsp. *lactis* BB-12 (L + P +B)

Milk samples were stored in sterile glass containers at 4^{o}C for 3 weeks.

2.6.2 Production of plain and stirred fruit yogurt

Homogenized and pasteurized goat's milk (Parmalat Australia Ltd.) was heated to 45^{o}C and total solids were adjusted to 18 g/100 g by adding skim goat's milk powder (Healtheries of New Zealand Ltd., Auckland, New Zealand). The mixture was heated to 85^{o}C and held for 25-30 minutes. When the mixture reached 40-45^{o}C, 2 g/100 g freeze dried ABY-1 yogurt culture containing probiotics was inoculated according to the producer's recommendation. *P. jensenii* 702 was also added to the yogurt mixture to a final concentration of approximately 10^8 cfu/ml. Plain yogurts were produced by placing a portion of yogurt mixture into 50 ml sterile plastic containers followed by incubation at 42±1^{o}C until a pH of 4.4- 4.5 was reached. The rest of the mixture was bulk fermented at 42±1^{o}C until a pH of 4.4- 4.5 was reached, stirred with 5, 10 or 15 g/ 100 g (w/w) mixed fruit juice, distributed in 50 ml sterile plastic containers and sealed. Both types of yogurts were refrigerated at 4^{o}C.

The commercial mixed fruit juice consisted of 45% apple juice, 44% orange juice, 5% banana puree, 4% pineapple juice, 1.3% mango puree and 0.6 % passionfruit juice without

any added water, added sugars, colourings, flavours, concentrates or preservatives. Nutrition information: quantity per ml is as follows: energy 185KJ, protein less than 1g, total fat less than 1g, saturated fat 0 g, carbohydrate 10.7 g, sugars 10.5 g, dietary fibres total less than 1 g, sodium 4 mg, vitamin C 40 mg (Berri Ltd. Melbourne, Australia). The shelf life of the fruit juice was 30 days in unopened containers, as confirmed by the manufacturer.

2.6.3 Production of ice cream

The ice cream recipe was adopted from Akin et al. (2007). Homogenized and pasteurized goat's milk (Parmalat Australia Ltd.) was used in the manufacture of the ice cream. Goat's cream was supplied from the Sherallee Goat Dairy, Cooranbong, NSW, Australia. Xanthan gum (Lotus Foods Pty Ltd, Australia), guar gum (Melbourne Food Ingredient Depot, Australia) and dextrose (Melbourne Food Ingredient Depot, Australia) were used as stabilizers. Commercial sugar (Woolworths, Australia) was used as a sweetener. Cocoa powder (Woolworths, Australia) was used to develop chocolate flavour and mask the characteristic "goaty" flavour of the goat's milk. Vanillin (Queen Fine Foods Pty Ltd, Australia) was incorporated for further aroma development. Ice cream was formulated with the following composition (percentage by weight) to make 37-39 g/100 g total solids in the final product.

Homogenised & pasteurized milk	64.5
Cream	15
Sugar	12
Cocoa powder	8
Stabilizer	0.4
Vanillin	0.1

All the ingredients were mixed thoroughly using a food blender and pasteurized at 85^{o}C for 30 minutes. The mixture was then immediately cooled on ice before aging in a refrigerator (4^{o}C) for 12 hours. A portion of milk (15% w/w of total milk) was separately pasteurized

(85^{o}C for 30 minutes), allowed to cool to 40^{o}C and inoculated with probiotic cultures: *L. acidophilus* LA-5, *B. animalis* subsp. *lactis* BB-12 and *P. jensenii* 702. Probiotic inoculated milk was incubated for one hour at 37^{o}C under anaerobic conditions. After incubation the fermented milk was aged for approximately 12 hours at 4^{o}C. The aged ice cream base and fermented milk were then well mixed immediately prior to freezing, to produce the final product.

The mixture was frozen in a Krups GVS2 ice cream maker (1.6 L volume, Krups International, China). After a freezing time of 30-40 minutes, 50 g portions were drawn and placed into polypropylene (Sistema, New Zealand & Sarstedt Australia Pty Ltd.), polyethylene (Glad Products, Australia), or glass containers (Pyrex, USA), immediately sealed and stored at -20^{o}C. Several batches were made to measure microbial, physico-chemical, sensory, and functional properties.

2.6.4 Microencapsulation of probiotics by spray drying

The milk used for spray drying was reconstituted (20% w/v) from spray dried skim goat's milk powder (Healtheries of New Zealand Ltd., Auckland, New Zealand) and heat treated at 85^{o}C for 30 minutes before being cooled to the inoculation temperature (37^{o}C). Inoculation levels of both *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 were 10^{8} cfu/ml and for *P. jensenii* 702 inoculation level was 10^{9} cfu/ml. Higher inoculation levels were maintained in spray drying in order to achieve satisfactory probiotic viability in spray dried powder. Immediately after inoculation the probiotic bacterial suspensions in reconstituted milk were processed using a laboratory scale spray dryer (Buchi mini spray dryer B-290, Flawil, Switzerland). Samples were processed at a constant feed rate (pump feed rate 40%), air spray flow of 600 litre per hour, 100% aspirator setting and at 195^{o}C air inlet temperature. The resultant outlet air temperature was maintained at 85±2^{o}C. The spray dried powder was stored in air tight glass jars at 4^{o}C and 30^{o}C for 24 weeks. Several batches were made to allow measurement of microbial, physico-chemical and functional properties.

2.7 Microbiological analyses

Samples from each type of dairy product were used to enumerate probiotic bacteria, yogurt culture bacteria, and to estimate coliform, yeast and mould counts. Dairy product samples (1 ml or 1 g) were measured directly into sterile test tubes and mixed with 9 ml of maximum recovery diluents (MRD) (Oxoid Australia Ltd). Samples were serially diluted using maximum recovery diluents, with 0.1 ml aliquots of the respective dilutions plated over the relevant culture media and incubated as described previously. Colonies were counted using a colony counter (Ratek Instruments Pty Ltd, Boronia, Australia) and expressed as cfu/ml or cfu/g.

2.8 Physico-chemical analyses

Except where stated otherwise, all physico-chemical analyses were performed in triplicate.

2.8.1 pH measurements

The pH of well mixed dairy food samples were measured using a calibrated Cyberscan 510 digital pH meter (EUTEOH Instruments, Singapore).

2.8.2 Titratable acidity

The titratable acidity of probiotic dairy products was measured by titrating 9 g of dairy food samples with 0.1N NaOH solution using phenolphthalein as indicator. The titratable acidity of the fruit juice was measured using 10 ml of filtered fruit juice as described by James (1995).

2.8.3 Total solids and moisture contents

The total solids content of samples was determined by oven-drying samples to constant weight at 105 ± 1^{o}C in pre-dried porcelain crucibles, as described by James (1995). The percentages of total solids and moisture content in samples were determined as follows,

Total solids (%) = (dried weight / fresh weight) x 100

Moisture content (%) = 100 - % total solids

2.8.4 Ash content

The ash content was measured by ignition of dairy food materials at 550^{o}C overnight in an electric muffle furnace (Labec Laboratory Pty Ltd, Marrickville, NSW, Australia) until a white/light gray ash resulted (James, 1995). The samples, placed in porcelain crucibles, were evaporated to dryness in an air oven at 100^{o}C before transfer into the muffle furnace. Total ash was calculated as a percentage of the original sample as follows,

Ash (%) = (weight of ash / original sample weight) x 100

2.8.5 Fat content

The fat content of fermented milk was determined by the Gerber method using a milk testing butyrometer as described by James (1995).

The fat content of yogurt was also estimated via this procedure, using 11.3 g of yogurt in place of the milk.

The fat content of ice cream was estimated by measuring 2.65 g of ice cream sample and 12 ml of Neusal solution into a cheese butyrometer. Neusal solution was prepared as follows: 100 g of trisodium citrate (Chem Supply, Australia) and 100 g of sodium salicylate (Sigma-

Aldrich, USA) were dissolved in 400 ml distilled water, to which 172 ml of isobutyl alcohol (Sigma-Aldrich, USA) and 750 ml of distilled water containing 0.2 g of powdered methylene blue (Sigma-Aldrich, USA) were added, to make a total solution volume of 1500 ml. Distilled water was added to the butyrometer with the ice cream sample and Neusal solution until the fat level came to a suitable level on the graduated scale. The stopper was inserted and the butyrometer was placed in a water bath at 65^{o}C for 2 minutes, shaken well, and returned to the water bath again. The procedure was repeated until the ice cream was completely dissolved. The tube was centrifuged at 1100 rpm for 4 minutes and the reading taken after placing the tube in a water bath for 3 minutes at 65^{o}C.

2.8.6 Protein content

The protein content of probiotic dairy products was estimated at Sanitarium Food Laboratories (Cooranbong, NSW, Australia) using Buchi 324 distillation unit (Buchi Laboratoriums Technik AG, Flawil, Switzerland) according to estimation of standard total nitrogen by Kjeldhal method (AOAC, 1990).

2.8.7 Susceptibility to syneresis

The yogurt/fermented milk's 'susceptibility to syneresis' (STS) was determined by the method reported by Isanga and Zhang (2009). This involved placing a 100 ml yoghurt sample in a funnel lined with a Whatman filter paper number 1 (Whatman International Ltd, Maidstone, England). After 6 hours of drainage, the volume of whey collected in a beaker was measured and used as an index of syneresis. The following formula was used to calculate STS:

STS (%) = V1/V2 x 100

where: V_1 = Volume of whey collected after drainage; V_2 = Volume of yoghurt sample.

2.8.8 Water holding capacity (WHC)

The WHC of samples was measured by placing five grams of sample in a centrifuge tube (BD Falcon^{TM}, Australia) spun at 4500 rpm for 30 minutes at 10°C. After centrifugation, the supernatant was collected and the weight recorded. The WHC was calculated as follows:

WHC (%) = (1-W1/W2) x 100

where: W_1 = Weight of whey after centrifugation, W_2 = Yoghurt weight (Isanga & Zhang, 2009)

2.8.9 Lactic acid content

To determine the lactic acid content, 1.5 g of each fermented milk sample was diluted with 0.5 ml of 2.5 mM methanesulfonic acid (Fluka Analytical, Switzerland) and centrifuged at 14000 rpm for 30 minutes using an Eppendorf 54145C centrifuge (Crown Scientific Pty Ltd, Minto, NSW, Australia). After centrifugation, the supernatant was filtered through 0.22 µm membrane filters (Millipore Corporation, Bedford, MA, USA) and stored at -20^{o}C prior to analysis. The quantification of lactic acid was achieved by High Performance Liquid Chromatography (HPLC) on a Hewlett Packard (Series 1100) instrument fitted with a pyrospher RP-18 (125x4 mm, 5 µm) column (maintained at 30 ^{o}C) and a UV detector, using 2.5 mM methanesulfonic acid with a flow rate of 1 ml/min as the mobile phase and HPLC grade 100% lactic acid. Detection of lactic acid was based on absorbance at 210 nm and a retention time of ~1.6 minutes. Quantification of lactic acid was performed from the standard curve obtained using solutions of pre-determined concentrations.

2.8.10 Overrun

The overrun of ice cream samples (an indicator of the amount of air incorporated) were determined using the following formula (Akin et al., 2007). The weight of ice cream mix was determined just before freezing.

Overrun = $(W_1-W_2) / W_2 \times 100$.
where: W_1 = Weight of unit mix; W_2 = Weight of same volume of ice cream

2.8.11 First dripping time

First dripping times were measured according to the method of Akin et al. (2007), whereby 25 g of ice cream was left to melt at room temperature (20^{o}C) on a 0.2 cm wire mesh screen above a beaker, with the time of the first drip was recorded in minutes.

2.8.12 Complete melting time

Complete melting times were also measured according to the method of Akin et al. (2007), with 25 g of ice cream left to melt at room temperature (20^{o}C) on a 0.2 cm wire mesh screen above a beaker, and the time taken to completely melt recorded in minutes.

2.8.13 Viscosity measurements

Viscosity of the samples was determined at 15^{o}C using a digital Viscometer, Model DV-II+ Pro (Brookfield Engineering Laboratories, Middleboro, MA, USA) and spindle number LV 2. The spindle was rotated at 0.5 rpm. The readings were recorded at the 15th second of the measurement period as centipoises (cP).

2.8.14 Brix value

Brix value of fruit juice was measured using a refractometer (Baclo Laboratories Pty Ltd, Australia) before stirred into yogurt.

2.9 Evaluation of functional properties of probiotics in different products

2.9.1 *In vitro* upper gastrointestinal tolerance

2.9.1.1 Preparation of simulated gastric and small intestinal juices

Simulated gastric and small intestinal juices were prepared following the methods previously described by Huang & Adams (2004) with some modifications. In order to prepare simulated gastric juices, pepsin (1:10 000, ICN) (Chem Supply, Australia) was suspended in sterile filtered 0.5% (w/v) NaCl solution to a final concentration of 3 g/L, with the pH adjusted to 2.0, 3.0 and 4.0 with concentrated HCL or sterile 0.1 mol/L NaOH with using a Cyberscan 510 digital pH meter (EUTEOH Instruments, Singapore).

Simulated small intestinal juices were prepared by suspending pancreatin USP (P-1500, Sigma-Aldrich, USA) in filter sterile 0.5% (w/v) NaCl solution to a final concentration of 1 g/L, with or without 0.3% bile salts (Oxoid, Australia), and adjusting pH to 8.0 with sterile 0.1 mol/L NaOH.

Simulated gastric and small intestinal juices were prepared fresh for each experiment.

2.9.1.2 *In vitro* upper gastrointestinal transit tolerance assay

To determine the acid and bile tolerance of probiotics in dairy products, 1 g of yogurt, ice cream or spray dried sample (or 1 ml of milk in the case of fermented milk) was transferred into a 10 ml screw cap Eppendorf tube (Sarstedt Australia Pty Ltd., Australia) containing either 9 ml of gastric (pH 2.0, 3.0 or 4.0) or small intestinal juices (pH 8.0) with or without bile salts. The mixture was then homogenised using a vortex mixer (Ratek Instruments Pty Ltd., Australia) at maximum setting for about 10 seconds and incubated at 37^{o}C. Aliquots of 1 ml were removed from tubes containing simulated gastric juice and serially diluted with maximum recovery diluent (Oxoid Australia Ltd.) after 1, 30, 60 and 180 minutes to determine acid tolerance by total viable counts. To assess small intestinal transit tolerance,

aliquots of 1 ml were removed after 1, 120 and 240 minutes and viable bacterial counts were determined.

Samples were hand shaken periodically to simulate peristalsis during the gastric and small intestinal transit tolerance assays.

2.9.2 *In vitro* adhesion ability

2.9.2.1 Caco-2 cell line

The Caco-2 cell line ATCC HTB-37 (American Type Culture Collection) used for this experiment was kindly provided by Dr. Matthias Ernst (Ludwig Institute for Cancer Research, Melbourne, Australia). The cells were cultured to passage 15-16 in Nunc^{TM} tissue culture flasks (Thermo Fisher Scientific, USA) containing RPMI 1640 medium supplemented with 20% heat inactivated fetal bovine serum, 2% HEPES buffer, 2% sodium bicarbonate, 1% L-glutamine and 2% penicillin/streptomycin at 37^{o}C in a 5% CO_2/95% air atmosphere using a humidified HERAcell 150 CO_2 incubator (Thermo Electron, USA). All chemicals and cell culture media were from Gibco, Invitrogen, CA, USA. The cell culture medium was replaced with fresh medium every 2-3 days while culturing.

Confluent monolayers in tissue culture flasks were washed with PBS and were collected using trypsin/EDTA solution (Gibco, Invitrogen, CA, USA). The trypsin/EDTA cell suspensions were centrifuged at 800 rpm, at 21^{o}C for 5 minutes. After centrifugation the supernatant was discarded and harvested cells were re-suspended in the freezing medium (50% heat inactivated fetal bovine serum, 40% RPMI medium and 10% dimethyl sulphoxide (DMSO), divided into 0.5 ml aliquots into cryovials, transferred into a NALGENE “Mr. frosty” cryo freezing container (Nalgene, USA) and strored at -80^{o}C for 2- 3 days. Cryovials were subsequently removed from the cryo freezing container but maintained in storage at -80°C for use as the cell stock. For cell culture experiments aliquots of the Caco-2 cell stock were grown in appropriate liquid medium in 25 cm^2 and or 75 cm^2 tissue culture flasks.

2.9.2.2 *In vitro* adhesion assay

Confluent monolayers from tissue culture flasks were harvested by washing the cells with sterile PBS followed by trypsin/EDTA solution (Gibco, Invitrogen, CA, USA) and centrifugation (800 rpm, 21^{o}C, 5 minutes). Caco-2 cells were seeded at a concentration of 10^5 cells/well into 24 well Nunc^{TM} tissue culture plates (Thermo Fisher Scientific, Denmark), and incubated at 37^{o}C in the humidified CO_2 incubator until a confluent monolayer had formed (7-10 days). The formation of a complete cell layer was carefully observed by Motic AE31 microscope (Australian Instrument Services Pty Ltd, Australia). The cell culture medium was replaced with fresh medium every 2-3 days. At least 1 hour before the adhesion assay, the RPMI medium was replaced with the same medium without antibiotic (penicillin/streptomycin). Immediately prior to the assay, the post confluent monolayers of Caco-2 cells were washed three times with sterile PBS.

A 1 g aliquot of probiotic dairy products (1 ml in the case of fermented milk) was transferred to post confluent monolayers of Caco-2 cells in the 24-well tissue culture plates and incubated at 37^{o}C in 5% CO_2 95% air atmosphere for 2 hours. The remaining food particles were subsequently removed using sterile pasture pipettes and the cell layers washed 3 times with PBS in order to remove non-adherent bacteria. Cells were then detached from each well by addition of 1 ml of trypsin/EDTA (Gibco, Invitrogen, CA, USA) followed by incubation at 37^{o}C for 3-5 minutes. The suspension (1 ml) from each well was then transferred to a tube containing 9 ml of MRD, serially diluted, and plated on appropriate media.

2.9.2.3 Scanning electron microscopy (SEM)

Scanning electron microscopic imaging was used for the qualitative examination of probiotic adhesion. Before seeding with Caco-2 cells, sterile 13 mm coverslips (Sarstedt Inc., Newtown, NC, USA) were placed in the bottom of the each well of the tissue culture plates. Preparatory stages of coverslips were similar to the above section 2.9.2.1. After incubation of post confluent monolayers of Caco-2 cells on coverslips with dairy foods, the

coverslips were removed from the wells and washed three times with PBS to remove non-adherent probiotic bacteria. Afterwards, cell layers on coverslips were fixed with 3% formaldehyde solution (Merck Pty Ltd., Australia) for 30 minutes at room temperature. Specimens were then air dried for 1-2 hours at room temperature and stored at room temperature. Before scanning electron microscopy the specimens were mounted on stubs and coated with conductive material (gold particles) using a SPI Sputter Gold Coater (SPI Structure Probe Inc., West Chester, PA, USA). Specimens were examined with a Philips XL30 scanning electron microscope (Philips, Eindhoven, The Netherlands) equipped with the EDS link (Isis, Oxford Instruments, Concord, MA, USA).

2.9.3 *In vitro* analysis of cytokine production

2.9.3.1 Cell and probiotic preparation for cytokine assay

In vitro analysis of cytokine production by the Caco-2 cells was performed by the method of Amin et al. (2009) with some modifications. Preparatory stages for the cultivation of confluent Caco-2 cell layers in 24-well tissue culture plates were as described in sections 2.9.2.1 and 2.9.2.2. Three probiotic bacteria were grown in relevant broths and were harvested as described in section 2.4. Harvested bacterial cells were re-suspended in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen, CA, USA) and combined as required to provide bacterial combinations as listed in section 2.6.1. The bacterial concentration of each solution was adjusted to approximately 10^8 cfu of each bacterium/ml.

At least 1 hour before the adhesion assay, the RPMI medium was replaced with same medium without antibiotic (penicillin/streptomycin). Cell layers were washed three times with PBS before the start of the assay. Caco-2 cell layers were then exposed to above combinations of probiotic bacteria in DMEM (1 ml per well) and were incubated as described in section 2.9.2.2. After 2 hours of incubation, supernatants were collected from the cell layers and centrifuged for 10 minutes at 1300 rpm at 4^oC. Supernatants were frozen at -20^oC until cytokine determination by ELISA.

2.9.3.2 Cytokine determination by ELISA

Previously frozen supernatants were thawed and analysed for TNF-α and IL-6 concentrations using a commercially available enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (BD OptEIA^{TM} Human kits, BD Bioscience, Australia).

2.10 Sensory evaluation

2.10.1 Procedure

Sensory evaluation of different types of fermented milk, yogurt and ice cream was conducted using the products stored at 4^{o}C (fermented milk and yogurts) or -20^{o}C (ice cream). The tasting panel consisted of students and staff from the University of Newcastle, Australia (untrained panel). Each panellist received samples of dairy foods to taste and evaluate sensory characteristics at each serving. All the samples were presented in uniform plastic cups that did not impact on the flavour of the product during the sensory evaluation. The panellists were asked to evaluate the colour and appearance, aroma, body and texture, taste, and overall acceptability, based on a 9 point hedonic scale. The sensory scores included; Like extremely = 9, Like very much = 8, Like moderately = 7, Like slightly = 6, Neither like nor dislike = 5, Dislike slightly = 4, Dislike moderately = 3, Dislike very much = 2, Dislike extremely = 1. The panelists were also asked to make comments/recommendations regarding the sensory attributes of the dairy food samples 3(Appendix B - the score card).

2.10.2 Ethics approval

The sensory evaluation was conducted following approval by the Human Research Ethics Committee of the University of Newcastle, Australia (Ethics approval number: 2008-0212).

2.11 Statistical analysis

Data analyses were performed using SPSS/PASW statistical software version 17 (SPSS Inc., Chicago, IL, USA). Microbial viability data and some physico-chemical data were analysed using repeated measure ANOVA. One way ANOVA was used to analyse data on physico-chemical properties, adhesion properties and cytokine production. Where appropriate, T-tests were performed for comparison of two means. Sensory data were statistically tested using nonparametric tests (Friedman and Wilcoxon signed rank test) to determine statistical differences and the Bonferroni post hoc test was performed for means comparison.

A p value <0.05 was considered statistically significant for all analysis unless stated otherwise.

Chapter 3 : The *in vitro* functional efficacy of probiotic combinations and their effect on the microbial, physico-chemical and sensory characteristics of fermented goat's milk

3.1 Introduction

Cultured dairy foods might be considered more nutritious than the milk they are made from due to the increased production or availability of certain nutrients such as vitamin B, and increased digestibility through pre-hydrolysis of the major milk components by lactic starter cultures (Khem & Ramesh, 1979; Lee et al., 1988). Due to the increasing popularity of fermented dairy products, manufacturers are continually investigating the value of added ingredients such as probiotics and prebiotics to entice health-conscious consumers (Allgeyer et al., 2010). Fermented milk is a growing area of interest among producers of fermented dairy foods, because of the convenience, portability and the ability to deliver all the health and nutritional benefits of set yogurts (Allgeyer et al., 2010; Thompson et al., 2007). In addition probiotic dairy drinks are consumed in larger quantities than other functional probiotic beverages such as probiotic fruit juice (Ozer & Kirmaci, 2010). Development of probiotic food and beverage products that are popular among consumers is one means of ensuring probiotic intake above minimum therapeutic levels. The production of drinkable yogurt with goat's milk and probiotic bacteria may also be likely to further enhance its health promoting value (Slacanac et al., 2010; Uysal-Pala et al., 2006).

Another recent trend in the manufacturing of probiotic products is to combine two or more strains in order to achieve possible additional health benefits (Collado et al., 2007a). The inclusion of different probiotics in various combinations would not only expand the variety of products that can be formulated, but may also improve their sensory characteristics (Guler-Akin & Akin, 2007; Kneifel et al., 1993; Thierry et al., 2005). Dairy products manufactured with the A, B, C approach (a combination of *L. acidophilus*, *Bifidobacterium* spp. and *L. casei*) are well recognized and have good consumer acceptance (Phillips et al.,

2006). Bifidobacteria and lactobacilli, which can be considered the most commonly used probiotics (Allgeyer et al., 2010; Saxelin et al., 2005; Vesterlund et al., 2007), have demonstrated satisfactory but variable growth and viability levels in fermented goat's milk products (Farnsworth et al., 2006; Guler-Akin & Akin, 2007; Martin-Diana et al., 2003). Although other genera such as *Propionibacterium* have been extensively used in the cheese and dairy industry, and have a long history of safe human consumption (Collado et al., 2007a; Ekinci & Gurel, 2008; Huang & Adams, 2003; Meile et al., 2008; Rossi et al., 1999), *Propionibacterium jensenii* 702 has not previously been added or co-cultured with other probiotics in goat's milk. *P. jensenii* 702 is a novel probiotic strain, isolated from raw cow's milk (Huang et al., 2003), which has demonstrated beneficial probiotic characteristics in *in vitro* studies (Ho et al., 2009; Huang & Adams, 2003, 2004; Moussavi & Adams, 2009), in rat (Huang et al., 2003) and farm animal models (Adams et al., 2008; Luo et al., 2010), and in humans (Kotula, 2008). It is important however, that new probiotic strains be screened by evaluating not only their potential health benefits, but also their performance in terms of growth and stability in milk, impact on the physico-chemical and organoleptic characteristics of the final product (Minelli et al., 2004; Moayednia et al., 2009), and their functional properties.

Effects of probiotics on sensory characteristics

The commercial success of probiotic products ultimately depends on taste and appeal to the consumer (Heenan et al., 2004; Nousia et al., 2011), as consumers are unlikely to be interested in consuming a functional food, regardless of the potential health benefits, if the added ingredients contribute disagreeable flavors to the product (Cruz et al., 2010a). Sensory properties of probiotic products can be influenced by the probiotic cultures used in product manufacturing. For example, incorporation of activated cells of *L. acidophilus* LMGP-21381 into an ice cream mix was found by Nousia et al (2011) to significantly improve the organoleptic characteristics such as aroma, taste and overall acceptance of the final product (Nousia et al., 2011). Drinkable goat's milk yogurts made with the probiotic cultures *B. bifidum* BB 12 and *L. acidophilus* LA-5, have also resulted in lower intensities of the unpleasant "goaty" odor compared to the same yogurts produced by regular yogurt

cultures (Uysal-Pala et al., 2006). Sensory properties of a frozen soy dessert were observed by Heenan et al (2004) to vary depending on the type of probiotic strains included in the manufacturing of the product. For example, a frozen soy product fermented with *L. acidophilus* MJLA1 could not be distinguished from the control samples in terms of the sensory attributes, while the product with *S. boulardii* 74012 differed from the control and the product fermented with *L. acidophilus* MJLA1. Storage time of the same product was also shown to have a significant effect on consumer preference. In further studies, different strains of *S. thermophilus* and *L. delbrueckii* spp *bulgaricus* were found to have a significant effect on sensory properties in set yogurt (Pourahmad & Assadi, 2007), while *B. lactis* BB 12 and *L. acidophilus* LA 5 were reported to alter the sensory attributes in a drinkable yogurt (Allgeyer et al., 2010). It is therefore important to consider changes in consumer preference for a functional food product manufactured with different probiotic combinations throughout the storage period, especially when attempting to incorporate a novel probiotic strain.

Growth and viability of probiotics in fermented foods

There is also a growing industry interest in developing techniques to ensure that the numbers of probiotic bacteria remain adequate throughout the shelf life of fermented milk products (Farnsworth et al., 2006). The probiotic strains used and interactions between the species present, are two important factors that determine probiotic viability in fermented milk products (Kailasapathy & Rybka, 1997; Shah, 2000). Hence in order to ensure high product quality, care should be taken in selecting the strains and species to be incorporated when producing such foods (Lourens-Hattingh & Viljoen, 2001; Phillips et al., 2006). The presence of other strains may affect probiotic growth and viability due to synergistic and or antagonistic relationships (Kaneko et al., 1994; Moussavi & Adams, 2009). Production of growth stimulators for *Bifidobacterium* spp. by *Propionibacterium* spp. has been confirmed by Kaneko et al. (1994), while the study of Gardner and Champagne (2005) revealed that bifidobacteria may also stimulate the growth of propionibacteria. A significant synergistic influence on growth of *P. jensenii* 702 and *B. lactis* BB 12 when co-cultured in a liquid growth medium has also been observed (Moussavi & Adams, 2009).

Bacteriocins are bacterial protein compounds known to kill or inhibit closely related strains (Willey et al., 2008) but which may also exhibit bacteriocidal activity beyond species that are closely related (Dave & Shah, 1997a; Schillinger et al., 1993). Based on a study of the acidophilicin LA-1, a bacteriocin produced by *L. acidophilus* LA-1, Dave and Shah (1997a) concluded that acidophilicin LA-1 was active against seven strains of *L. delbruecki* sp *bulgaricus*, one strain each of *L. casei*, *L. helveticus* and *L. jugurti*, but not against other lactic acid bacteria. In addition to bacteriocin, other substances such as hydrogen peroxide produced by certain microorganisms, have been found to be inhibitory to other microbes (Dave & Shah, 1997d). Hydrogen peroxide produced by *L. delbruecki* sp *bulgaricus* during the manufacturing and storage of yogurt can be considered one of the main substances responsible for antagonism towards *L. acidophilus* (Lourens-Hattingh & Viljoen, 2001).

Lactobacilli and propionibacteria have demonstrated different interaction effects including inhibition, stimulation and no effect (Liu & Moon, 1982; Parker & Moon, 1982; Piveteau et al., 1995; Tharmaraj & Shah, 2004). Piveteau et al. (1995) demonstrated that growth stimulation of lactobacilli by propionic acid bacteria was strain specific. Growth stimulation of propionic acid bacteria by *S. thermophilus* in whey was also reported by the same researchers. *L. acidophilus*, *B. animalis*, *L. paracasei* subsp. *paracasei* and *L. rhamnosus* have demonstrated varied levels of antagonism and synergistic effects, while *P. freudenreichii* subsp. *shermanii* showed no effect in cheese based French onion dips (Tharmaraj & Shah, 2004). The survival of *B. animalis* was reported to be affected by both the bacteria with which it was combined and storage time, with *L. acidophilus* demonstrating a positive effect on the viability of *B. animalis*. However, the findings also suggested that *L. paracasei* subsp. *paracasei* and *L. rhamnosus* may inhibit *B. animalis* in dips (Tharmaraj & Shah, 2004). Synergistic growth promoting effects between *L. acidophilus* and *B. bifidum* have also been observed (Kneifel et al., 1993). Generally, bifidobacteria are weakly proteolytic, therefore co-culturing bifidobacteria with proteolytic lactobacilli species such as *L. acidophilus* may benefit bifidobacteria through provision of necessary growth stimulants (Lourens-Hattingh & Viljoen, 2001). The oxygen scavenging ability of *S. thermophilus* may also have beneficial effects on *Bifidobacterium* spp under co-culture conditions (Lourens-Hattingh & Viljoen, 2001) due to bifidobacteria's strict

anaerobic nature (Kailasapathy & Rybka, 1997; Mombelli & Gismondo, 2000; Tharmaraj & Shah, 2003).

The carrier food may also play an important role in probiotic efficacy during manufacturing and storage. For example, probiotic survival in cheddar cheese was reported by Phillips et al (2006) to be species specific. Thus certain species of probiotic might grow and survive better in a particular media or type of carrier food, than other probiotic species. It is therefore of considerable value to assess which probiotic or probiotic combination may be best suited to the particular carrier food when developing new food products, particularly when attempting to incorporate a novel probiotic.

Effects of probiotics on physico-chemical properties of fermented foods

It is also very important to select a suitable combination of probiotic strains and starter culture bacteria when different types of yogurt are formulated (Vinderola et al., 2000a). The yogurt starter bacteria *L. delbruecki* sp *bulgaricus* is known to accelerate post-fermentation acidification of yogurt in storage (i.e. a decrease in pH after fermentation). In overcoming the problem of further acidification of yogurt during storage, a recent trend is to use starter cultures that are devoid of *L. delbruecki* sp *bulgaricus* such as ABT (*L. acidophilus*, *Bifidobacterium* and *S. thermophilus*) (Kailasapathy et al., 2008; Lourens-Hattingh & Viljoen, 2001). The lower buffering capacity of goat's milk compared to cow's milk may also lead to over acidification of the final product during fermentation (Lutchman et al., 2006; Martin-Diana et al., 2003; Rysstad & Abrahamsen, 1983; Vegarud et al., 1999). Martin-Diana et al (2003) previously reported unpleasant acidity development when using cultures containing *L. delbruecki* sp *bulgaricus* in manufacturing fermented goat's milk. Therefore, manufacturing a fermented goat's milk product without yogurt starter cultures could be beneficial in receiving higher consumer acceptability due to lower acidity development during fermentation and storage. This procedure could also be beneficial in minimizing certain antagonistic reactions between yogurt starter cultures and probiotics (Guler-Akin & Akin, 2007) and facilitate greater understanding of interactions among probiotics only, without any interference from yogurt starter culture bacteria or their

metabolic by-products. However, while probiotic cow's milk products manufactured without yogurt starter cultures are already commercially available (Bozanic & Tratnik, 2001; Ozer & Kirmaci, 2010), the production of fermented goat's milk through probiotic fermentation only, has not yet been well developed (Slacanac et al., 2010).

Physico-chemical properties of fermented milk such as pH, acidity, and the concentration of lactic and acetic acids, may all affect the viability of probiotics in these products (Shah, 2000). Conversely, the physico-chemical characteristics of the products may also be influenced by the probiotic combinations (Ekinci & Gurel, 2008; Liu & Moon, 1982). *P. jensenii* has already been identified as a species which produces extracellular slime in liquid media which may affect some physico-chemical properties of the final product (Ekinci & Barefoot, 2006; Ekinci & Gurel, 2008). Starter culture bacteria *S. thermophilus* and *L. delbrueckii* spp. *bulgaricus* have also been confirmed to produce exopolysaccharides during the manufacturing of fermented milk, that are essential for proper consistency and texture in this product (Cerning, 1995). Co-culturing *L. acidophilus* LA-5 with a starter culture of *S. thermophilus* has resulted in better quality Minas fresh cheese during storage (Souza & Saad, 2009). In the same study Souza & Saad (2009) observed significantly lower pH values and higher titratable acidity at the end of the storage period, for the cheese co-cultured with *S. thermophilus* and *L. acidophilus*, compared to cheese supplemented with *L. acidophilus* after fermentation. Significantly different acidity and pH values were recorded by Pourahmad et al. (2007) for yogurt produced with different strains of *S. thermophilus* and *L. delbrueckii* spp. *bulgaricus*. Yogurt made with a single strain of lactobacilli exhibited significant differences in syneresis compared to the same product made with mixed cultures of lactobacilli and streptococci (Hassan et al., 1996). Acidophilus milk products containing *L. acidophilus* DDS1 have demonstrated a significantly lower net protein ratio and lower computed protein efficacy ratio values compared to yogurt produced with *L. bulgaricus* and *S. thermophilus* (Lee et al., 1988).

The physico-chemical properties of many probiotic products have also been reported to change over their shelf life. For example, decreases in pH over shelf life has been recorded in cow's milk yogurt (Antunes et al., 2005; Aryana & McGrew, 2007; Kailasapathy et al.,

2008; Pourahmad & Assadi, 2007; Sahan et al., 2008), ewe's milk yogurt (Guler-Akin, 2005) cheese based French onion dips (Tharmaraj & Shah, 2004), buffalo curd (Jayamanne & Adams, 2004) and goat's milk yogurt (Bozanic & Tratnik, 2001). An increase of titratable acidity and water holding capacity during refrigerated storage in cow's milk based fruit yogurt was reported by Singh & Mathukumarappan (2008), who also confirmed a significant effect of storage on apparent viscosity. These physico-chemical characteristics could influence the quality and consumer acceptability of the final product. For example, adequate firmness without syneresis is essential, while curd texture or firmness is also important in determining yogurt quality (Park, 2007). Lactic acid bacteria and propionibacteria are often grown together in cheese production, because the action of both microorganisms is necessary for proper final product quality (Gardner & Champagne, 2005). Cheese has been a predominant and a popular goat's milk product with high consumer acceptability. Therefore, incorporation of propionibacteria with lactic cultures in manufacturing other dairy foods from goat's milk may be helpful in achieving a higher quality final product.

Gastrointestinal tolerance of probiotics

Resistance to gastrointestinal conditions is a critical factor in maintaining probiotic efficacy. Most of the studies evaluating the resistance of probiotics to gastric, bile and pancreatic juices have been conducted *in vitro* using simulated gastric juice, bovine or pig bile, and various types of animal pancreatic extracts (Del Piano et al., 2006). It is well known that most bacteria are sensitive to gastric juice but have high rates of isolation from feces. This may be due to the protective effect of food during gastric passage (Del Piano et al., 2006). While Huang & Adams (2004) observed that different food matrices have a significant influence on the acid tolerance of propionibacteria strains in simulated gastric juice, there has been little research focus on evaluating the gastric resistance of probiotics and different probiotic combinations once incorporated into the carrier food.

Adhesion properties of probiotics

The adhesion of probiotics to intestinal epithelium, one of the more important criteria in the selection of probiotics (Alander et al., 1999), has been reported to be strain as well as species specific. *L. acidophilus* ADH has demonstrated better adherence to human and swine intestinal epithelial cells compared to other lactobacilli strains while *S. thermophilus* adhered poorly (Conway et al., 1987). Moussavi and Adams (2009) further demonstrated the influence of probiotic combinations on adhesion ability *in vitro*. Adhesion percentages of *L. casei* 01 and *L. rhamnosus* GG both decreased significantly in the presence of *P. jensenii* 702 compared to their adhesion levels when alone, while adhesion of *L. reuteri* ATCC 55730 increased in the presence of *P. jensenii* 702. Probiotic combinations were previously shown to enhance the adhesion of *L. rhamnosus* GG, *L. rhamnosus* LC705 and P. *freudenreichii* ssp. s*hermanii* JS to immobilized intestinal mucus (Collado et al., 2007a). Different probiotic strains may have different adhesion sites on the intestinal epithelial cells, thus it may be beneficial to produce probiotic products with suitable probiotic combinations in order to maximize utilization of available binding sites (Collado et al., 2007a). Conversely, the presence of other strains may adversely affect adhesion due to competition for the same binding sites. Only a few studies have investigated the effect of the presence of other probiotic strains on the intestinal epithelial cell adhesion properties of individual probiotics (Collado et al., 2007a; Moussavi & Adams, 2009).

Probiotic foods and immunomodulation

In general, cow's milk can be considered as the main vehicle in delivering probiotics to humans. However, hypersensitivity to cow's milk is one of the major food allergies, affecting mostly infants although it may also persist through adulthood (El-Agamy, 2007). Normally, the total elimination of milk from the diet prevents the allergy, but in the majority of cases the problem can be avoided simply by replacing cow's milk with the milk of some other species such as goat (Dabrowska et al., 2010). Although goat's milk has been reported not to be an appropriate substitute for children with proven IgE-mediated cow's milk allergy (Bellioni-Businco et al., 1999) rare incidences of allergy to goat's and sheep's

milk, without allergy to cow's milk, have also been reported (Ah-Leung et al., 2006; Tavares et al., 2007).

Probiotic bacteria may provide important local and systemic immunoregulatory signals (D'Arienzo et al., 2009; Pohjavuori et al., 2004). It has been proposed that probiotics could potentially restore intestinal homeostasis and prevent allergy through interaction with the intestinal immune cells (Hol et al., 2008; Rosenfeldt et al., 2004). The suggested probiotic mechanisms include stimulation of epithelial mucin production (Mack et al., 2003), enhanced production of secretory IgA (Malin et al., 1996) and alleviation of intestinal inflammation by stimulation of anti-inflammatory cytokines (Pessi et al., 2000; Pohjavuori et al., 2004; Rosenfeldt et al., 2004). Ingestion of probiotic yogurts has been reported to stimulate cytokine production in blood cells and enhance the activities of macrophages (Shah, 2007). Some investigation of the immune-modulation aspects of probiotics and probiotic combinations, with special reference to the novel probiotic *P. jensenii* 702, is therefore warranted.

3.1.1 Objectives and study design

This study can be defined in terms of two broad objectives. The first, to investigate the performance of the novel probiotic *P. jensenii* 702 in manufacturing fermented goat's milk products in terms of its growth, stability, and functional properties. The second, to identify the best probiotic combination for goat's milk fermentation using *L. acidophilus* LA-5, *B. animalis* subsp. *lactis* BB-12 and *P. jensenii* 702, in terms of viability, physico-chemical properties, sensory characteristics, *in vitro* adhesion ability, acid and bile tolerance, and influence on cytokine production by intestinal epithelial cells.

The performance of the novel probiotic *P. jensenii* 702 in food, especially with respect to sensory and functional properties, has not been extensively studied. Assessment of the probiotic potential of *P. jensenii* 702 in product development by means of a comparison with two of the most widely used and well accepted probiotic species - *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 - was therefore considered pertinent. The study was

designed to produce fermented goat's milk with *P. jensenii* 702, *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12, both separately and in various co-culture combinations, and to examine the microbial, physico-chemical, sensorial, and functional properties in the product over 3 weeks of refrigerated storage.

While high inoculation levels of *L. acidophilus* in yogurt have previously been associated with inferior product quality (Olson & Aryana, 2008), it is important to ensure a sufficient inoculum dosage of probiotics at the time of manufacture in order to meet the recommended therapeutic minimum at the end of the shelf life (Kailasapathy et al., 2008). The inoculum dosages for *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 applied in this study were therefore based on manufacturer's recommendations. Since there is no recommended/established inoculum dosage for *P. jensenii* 702 in manufacturing dairy products, two different inoculum levels (10^6 cfu/ml and 10^8 cfu/ml) were trialed for suitability. Approximately similar viable counts of each bacterium were maintained at the beginning of the study. Incubation temperatures ranging from 30^{o}C to 44^{o}C have previously been used in the manufacture of fermented goat's milk products (Guler-Akin & Akin, 2007; Martin-Diana et al., 2003; Minervini et al., 2009). In several of these studies 37^{o}C resulted in satisfactory viable counts (10^7-10^8 cfu/g) of probiotics during incubation. Thus 37^{o}C was considered an appropriate temperature for fermentation in this study.

It is important to determine physico-chemical parameters such as total solids, fat and ash contents when developing new products in order to provide the nutritional information of the product. Parameters such as total acidity, lactic acid content and syneresis may also be useful in evaluating the quality of the final product. Thus the study also includes measurement of these physico-chemical properties of the fermented goat's milk products.

The physical nature of the food can affect transit time through the stomach, with liquids generally transiting more quickly than solids. Once consumed, food normally remains in the stomach for 2-4 hours (Huang & Adams, 2004) and food transit time through the small intestine is generally from 1-4 hours (Charteris et al., 1998a; Conway et al., 1987; Davis et al., 1986; Huang & Adams, 2004). For the analysis of *in vitro* gastrointestinal tolerance of

probiotic organisms there are currently no universally agreed/specified time intervals. The protocol applied in this study was adapted from that of Huang and Adams (2004) with gastric tolerance estimated at 0, 1, 60 and 180 minutes after exposure to simulated gastric juice, and small intestinal tolerance estimated at 0, 1, and 240 minutes after exposure to simulated small intestinal juice with or without bile salt. There are also no agreed or most appropriate pH values for screening of gastric tolerance of probiotics, however a range of values, from pH 1.0 to 5.0, have been used to screen *in vitro* acid tolerance of lactobacilli, bifidobacteria and some dairy propionibacteria strains (Charteris et al., 1998a; Conway et al., 1987; Huang & Adams, 2004). A concentration of 0.15-0.3% of bile salt has been recommended for *in vitro* screening of probiotic bile tolerance (Goldin & Gorbach, 1992; Huang & Adams, 2004). In this study the pH value of simulated gastric juice was adjusted to pH 2.0 and simulated small intestinal juice was prepared with a 0.3% bile salts concentration.

In vivo testing of probiotic adhesion is expensive, time consuming and requires approval by ethical committees. Therefore, reliable *in vitro* methods for selection of promising strains are required (Pan et al., 2009). Intestinal epithelial-like Caco-2 cells have been successfully utilized for *in vitro* studies of the mechanism of cellular adhesion of probiotic *L. acidophilus*, *B. animalis* subsp. *lactis* BB-12 and *P. jensenii* 702 (Moussavi & Adams, 2009; Pan et al., 2009). Furthermore, Collado et al. (2007a) recommended that the efficacy of probiotic combinations should be tested *in vitro* prior to introducing such combinations in clinical intervention studies. Thus the adhesion properties of these probiotics were examined in this study using an *in vitro* Caco-2 cell model.

Characterising how the innate immune system responds to probiotic bacteria *in vitro* through the production of cytokines may provide an indication as to the likely immunomodulatory events that can be triggered following probiotic administration *in vivo* (Cross et al., 2004). Understanding the cytokine patterns elicited by probiotics may therefore help in the design of probiotics for specific prophylactic purposes and enable the development and optimal clinical use of these microbes as health promoting substances (Foligne et al., 2007; Foligné et al., 2010; Pessi et al., 2000). Activation of immune cells

and tissues requires close contact of the probiotic with the immune cells and tissue on the intestinal surface (Kalliomaki et al., 2001; Salminen et al., 2005). Hence a Caco-2 cell model was used to evaluate *in vitro* cytokine production. Interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) are important cytokines involved in the pathogenesis of gastrointestinal diseases such as inflammatory bowel disease and *Helicobactor pylori* infection related disease (Ding et al., 2000; Lee & Fedorak, 2010; Orlando et al., 2011; Yamaoka et al., 1997). Furthermore mucosal IL-6 levels have been found to be closely related to the mucosal TNF-α level (Ding et al., 2000; Yamaoka et al., 1997). Bacterial contact with Caco-2 monolayers was also previously observed to be an inducer of TNF-α (Amin et al., 2009) and IL-6 (Amin et al., 2009; Hosoi et al., 2003). Therefore this study includes evaluation of *in vitro* IL-6 and TNF-α production by Caco-2 cells under exposure to combinations of the 3 probiotic strains. Although the acid and bile tolerance and adhesion properties of these probiotics and their combinations were evaluated directly from fermented goat's milk samples, cytokine assays were conducted with the probiotics and combinations suspended in PBS. This variation was employed in order to avoid both the possibility of interference from proteins in the goat's milk, and difficulties in reading the colour changes during the assay. Bacterial numbers in the respective PBS samples were adjusted to be approximately similar to those in fermented goat's milk.

3.1.2 Research hypotheses

***Probiotic* viability:** It is widely accepted that most propionibacteria produce bifodogenic factors which stimulate the growth and performance of *Bifidobacterium* spp., while bifidobacteria may also stimulate the growth of propionibacteria (Gardner & Champagne, 2005). Furthermore, antagonism between *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 is not widely documented, while the proteolytic nature of lactobacilli is also likely to support the growth and viability of propionibacteria without mutual antagonism (Hugenschmidt et al., 2011). Thus due to likely synergistic interactions, it was hypothesized that compared with mono-cultures or paired combinations, the combining of all three probiotics in the manufacturing of fermented goat's milk would result in the highest viable counts of each at the end of the three week product shelf life.

Physico-chemical properties: Many of the physico-chemical properties of food are at least to some extent inter-related. For example, higher lactic acid levels may contribute to higher overall acidity in the product which may in turn accelerate syneresis (Tamime & Robinson, 1999). Clearly, the number of probiotic species/strains in the final product is a factor likely to influence the physico-chemical properties of the product. For example, by comparison with monocultures of the same species in a product, co-cultivation of two probiotics may result in higher acidity due to elevated release of metabolic by-products responsible for development of acidity. However if one or both organisms can effectively utilize the metabolic by-products of the other, co-cultivation may suppress acidity development. While potential relationships between the probiotic strains employed and other physico-chemical parameters were less obvious, it was hypothesized that the combining of all three probiotics in the manufacture of fermented goat's milk in this study would result in the lowest levels of lactic acid, total acidity, and syneresis compared to other preparations containing *L. acidophilus* LA-5, due to possible utilization by *B. animalis subsp. lactis* BB-12 and *P. jensenii* 702 of by-products of *L. acidophilus* LA-5 metabolism that might otherwise contribute to the development of acidity (Badis et al., 2004; Frohlich-Wyder et al., 2002; Gupta et al., 1996; Timmerman et al., 2004).

Sensory attributes: Probiotics have been widely utilized in the food industry to enhance flavours and improve other sensory characteristics of fermented foods, and products fortified with lactobacilli and bifidobacteria are well accepted by consumers (Allgeyer et al., 2010; Bonczar et al., 2002; Hemsworth et al., 2011). Dairy propionibacteria, which have been widely utilized in the cheese industry, are also known to produce flavour-active compounds such as carboxylic acid and esters (Thierry et al., 2004). It was therefore considered that *P. jensenii* 702 may contribute further improvement to the sensory properties of probiotic products fortified with lactobacilli and bifidobacteria. For this reason, along with the predicted reductions in acidity and syneresis, it was hypothesized that the combining of all three probiotics together in manufacturing fermented goat's milk would enhance the body and texture, taste, and overall acceptability, relative to the products containing monocultures or paired combinations.

Functional properties: Potential synergistic and or antagonistic relationships of probiotics could also influence their functional properties such as gastrointestinal tolerance and adhesion ability. Exopolysaccharide produced by one organism may act as a physical barrier and thereby influence the gastrointestinal tolerance of another organism. In addition, exopolysaccharide secretion may be a critical factor in the adhesion of probiotics to intestinal epithelial cells. Compared to the monoculture condition, adhesion of probiotics may be affected when co-cultured by competition between probiotic strains for available binding sites on the intestinal epithelium. On this basis it was hypothesized that the combining of all three probiotics would improve the *in vitro* acid and bile tolerance of each relative to the monoculture or paired combination preparations, but would also result in the lowest rates of adhesion for each strain.

Immunomodulation: Probiotic induction of cytokine production by intestinal epithelium cells is known to be both strain specific and dose dependent (Candela et al., 2008; Nemeth et al., 2006). For example, Nemeth et al. (2006) reported a gradual increase of cytokine IL-8 production from Caco-2 cells with increasing number of lactobacilli. Thus, due to the higher number of organisms present, it was hypothesized that in this study the combining of the three probiotics (i.e. the preparation with the highest number of bacterial cells) would increase *in vitro* production of the cytokines TNF-α and IL-6, compared to the monoculture and paired combination preparations.

3.2 Materials and methods

3.2.1 Microbiological, physico-chemical and sensory properties of fermented goat's milk

As described in Chapter 2 (2.6.1), seven different types of fermented milk were produced based on different combinations of the probiotic bacteria as follows:

L. acidophilus (L)

P. jensenii 702 (P)

B. lactis BB 12 (B)

L. acidophilus + *P. jensenii* 702 (L+P)
L. acidophilus + *B. lactis* BB 12 (L+B)
P. jensenii 702 + *B. lactis* BB 12 (P+B)
L. acidophilus + *P. jensenii* 702 + *B. lactis* BB 12 (L+P+B)

Samples of fermented goat's milk were used to enumerate probiotics, as described in Chapter 2 (2.7), from the day of manufacturing up to 3 weeks of refrigerated storage. Coliform, yeast and mould counts were assessed after incubation and again after 3 weeks of storage. Goat's milk samples were also evaluated for viable probiotic numbers before incubation.

Physico-chemical properties of fermented goat's milk samples were measured in duplicate on a weekly basis over the product shelf life, as described in Chapter 2 (2.8).

Sensory evaluation of the fermented goat's milk preparations was conducted by 7 (5 male and 2 female) untrained taste panellists over the product shelf life as described in Chapter 2 (2.10.1).

3.2.2 Functional properties of probiotics

Evaluation of *in vitro* gastrointestinal tolerance, adhesion ability, stimulation of cytokine production and scanning electron microscopy of probiotics were performed as described in Chapter 2 (2.9).

3.2.3 Statistical analysis

Data analyses were performed using SPSS/PASW statistical software version 17 (SPSS Inc., Chicago, IL, USA) as described in Chapter 2. Microbial viability, physico-chemical and acid bile tolerance data were analysed using repeated measure ANOVA. One way ANOVA was used to analyse data on adhesion properties and cytokine production. Nonparametric tests were performed to determine the statistical differences of the sensory

data. The Bonferroni post hoc test was performed for means comparison. Where appropriate, T-tests were performed for comparison of two means. A p value <0.05 was considered statistically significant for all analyses.

3.3 Results

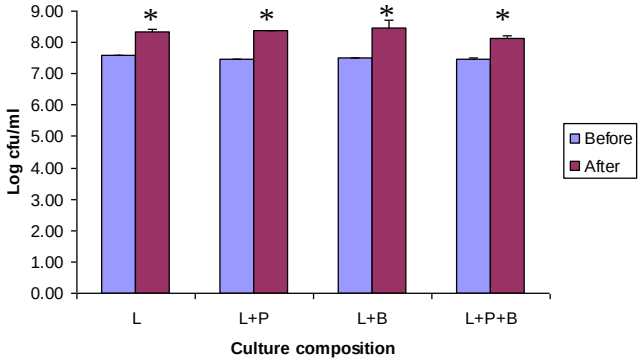
The presentation of data in this chapter begins with the basic microbiological aspects of the project, covering the growth and viability of probiotic mono- and co-cultures in reconstituted goat's milk during fermentation and storage, as well as the occurrence in the products of undesirable microorganisms (i.e. coliforms, yeasts and moulds). This is followed by an examination of changes in the physico-chemical characteristics and subsequent assessment of changes in the organoleptic attributes of these products over the shelf life. The final set of data is focussed on *in vitro* functional properties of these probiotics including tolerance to simulated gastric and small intestinal juices, cellular adhesion rates, and immune stimulation of intestinal cell cultures.

3.3.1 Growth and viability of probiotics

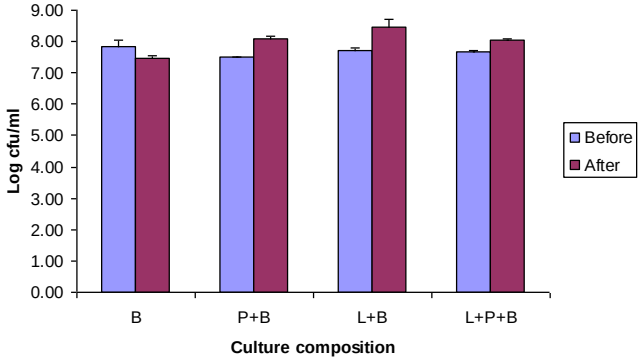
As explained previously (section 3.2) two different inoculum levels were trialled for *P. jensenii* 702 in the fermented goat's milk. At the lower inoculum level of 10^6 cfu/ml, it was found that viable counts of *P. jensenii* 702 were below the accepted minimum therapeutic level of 10^6 cfu/ml at the end of the shelf life. However, significant improvement in viability of *P. jensenii* 702 was observed when the inoculation level was increased from10^6 cfu/ml to10^8 cfu/ml, regardless of the overall culture composition. Therefore, fermented goat's milk samples with the higher inoculum level (10^8 cfu/ml) of *P. jensenii* 702 were utilized for the rest of the study. Inoculum level is one of the critical factors that determine the viability of probiotics in food during storage and it would appear based on these findings that an inoculum level of 10^8 cfu/ml could be recommended as appropriate for the novel probiotic *P. jensenii* 702 when manufacturing fermented dairy products.

During the fermentation period, viable numbers of both *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 appeared to increase in all preparations with the exception of the *B. animalis* subsp. *lactis* BB-12 mono-culture (Figures 3.1), however, the apparent increases were confirmed as statistically significant growth in the case of *L. acidophilus* LA-5 only. In contrast, although not statistically significant, an opposing trend was apparent in the case of *P. jensenii* 702 suggesting a lag phase of growth during the fermentation period. With regard to the effect of storage, all three probiotics were found to be relatively stable in goat's milk ($>10^7$ cfu/ml). Although the L+P+B combination did not result in the highest viability of each probiotic, all three species (*L. acidophilus* LA-5, *B. animalis* subsp. *lactis* BB-12 and *P. jensenii* 702) remained above the minimum therapeutic level at the end of storage (10^7-10^8 cfu/ml) (Figure 3.2).

A



B



C

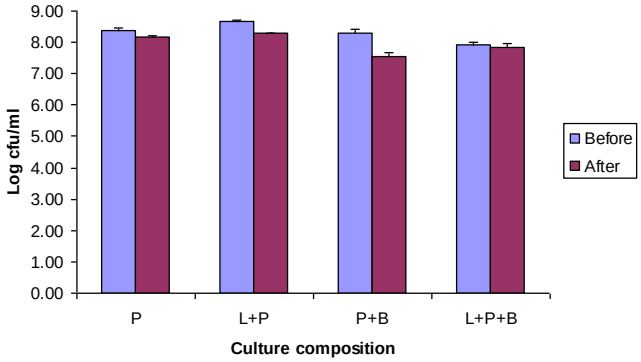


Figure 3.1 Numbers of viable bacteria (Log cfu/ml) in goat's milk before and after incubation at 37^{o}C in both monoculture and co-culture preparations. Viable counts of (A) *L. acidophilus* LA-5, (B) *B. animalis* subsp. *lactis* BB-12 and (C) *P. jensenii* 702. (*n* = 4, * indicates a statistically significant difference between post- and pre-incubation counts).

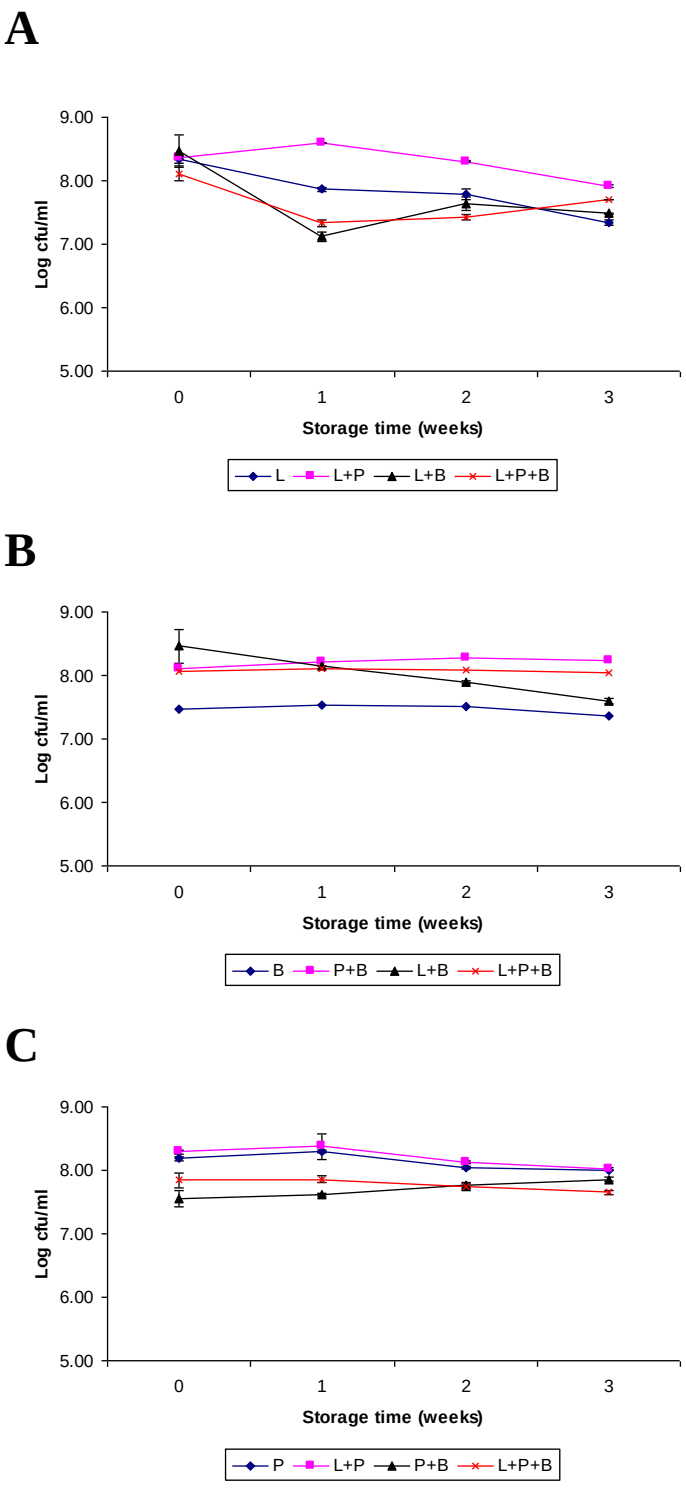


Figure 3.2 Viable counts of *L. acidophilus* LA-5 (A), *B. animalis* subsp. *lactis* BB-12 (B) and *P. jensenii* 702 (C) in both monoculture and co-culture preparations in fermented goat's milk during 3 weeks of storage at 4°C (*n* = 4).

3.3.2 Coliforms, yeasts and moulds

With regard to food safety it is necessary to assess the presence of undesirable micro-organisms such as coliform bacteria, yeasts, and moulds, especially in the case of novel product development. In the fermented goat's milk products prepared in this study, no coliform bacteria were detected in any of the samples, and yeast and mould counts were found to be <1 cfu/ml at the end of the storage period, indicating that these preparations were, from a microbial perspective, appropriately safe for human consumption.

3.3.3 Physico-chemical properties of fermented goat's milk

Details of the major physico-chemical properties of fermented goat's milk samples are provided in Tables 3.1-3.4. With the exception of fat content, significant differences among all the physico-chemical properties of different goat's milk samples were observed over the product shelf life. However, although there were statistically significant differences among the total solids and ash contents of the samples, the low magnitude of these differences does not necessarily imply any real significance in practical terms. Total solids, fat and ash contents of the samples fell within the ranges of 10.16-10.86 %, 2.65-3.45 % and 0.73-0.87 % respectively (refer appendix C for a complete list of the total solids, fat and ash contents of the fermented goat's milk preparations during storage).

Lactic acid contents were generally higher throughout storage in all preparations containing *L. acidophilus* LA-5 than in preparations without *L. acidophilus* LA-5. Fermented goat's milk with a monoculture of *L. acidophilus* LA-5 demonstrated the highest lactic acid content during storage, except at week 2 where samples containing co-cultures of *L. acidophilus* LA-5 and *P. jensenii* 702 showed the highest lactic acid levels (Table 3.1). During storage, titratable acidity in all preparations was found to increase, confirming the development of acidity in fermented goat's milk during refrigerated storage (Table 3.2). With the exception of preparations containing *P. jensenii* 702 and *B. animalis* subsp. *lactis* BB-12 monocultures and their co-culture (*P. jensenii* 702 + *B. animalis* subsp. *lactis* BB-12), the trend observed in acidity development was similar to that observed in relation to

pH changes during storage (Table 3.3). Although lactic acid content is often a major contributor in the development of acidity in fermented dairy products, no significant correlation was observed between titratable acidity or pH and lactic acid content during storage of the fermented goat's milk. In all preparations except *L. acidophilus* LA-5 + *P. jensenii* 702, lactic acid content appeared to decline during the initial 1 to 2 weeks of storage before increasing again at week 3. Among the different goat's milk samples, pH levels were lowest in the *L. acidophilus* LA-5 monoculture, and highest in the *P. jensenii* 702 monoculture, throughout the shelf life. At all time points, pH values were significantly lower in all preparations containing *L. acidophilus* LA-5 than in those without. In all preparations without *L. acidophilus* LA-5, pH values were not significantly different from each other at any time point. A significant reduction in the pH of fermented goat's milk containing *L. acidophilus* LA-5 monoculture was observed at week 3, however, there were no significant changes of pH in any other preparations during storage. In line with the lactic acid results, samples containing *L. acidophilus* LA-5 demonstrated higher titratable acidity, while preparations containing *P. jensenii* 702 demonstrated the lowest except when *P. jensenii* 702 was co-cultured with *L. acidophilus* LA-5 (L+P) (Table 3.2). Significant increases in titratable acidity were observed during storage in all preparations except the samples containing the monoculture of *P. jensenii* 702. Among the other preparations, samples containing the three probiotics together exhibited the least variation in titratable acidity across the product shelf-life.

In agreement with the increasing acidity during storage, syneresis values were generally found to increase during storage in all preparations except the preparations containing *L. acidophilus* LA-5 monoculture and co-cultures of *P. jensenii* 702 and *B. animalis* subsp. *lactis* BB-12 (Table 3.4). Higher acidity in the product can induce the syneresis, however, surprisingly the preparation containing the *L. acidophilus* LA-5 mono-culture demonstrated the lowest syneresis values during storage despite exhibiting the highest acidity levels. All preparations containing *B. animalis* subsp. *lactis* BB-12 demonstrated a dramatic increase in syneresis with increasing storage time except for the preparation in which it was co-cultured with *P. jensenii* 702, where syneresis values were at least 3-fold greater than in any other preparation initially, and remained high throughout the entire storage period.

Table 3.1 Changes in lactic acid content (mg/ml) of the fermented goat's milk preparations during 3 weeks of storage at 4°C (*n* = 2)

Storage time (wks)	L	P	B	L+P	L+B	P+B	L+P+B
0	3.73 ±0.69^{Aa}	0.42 ±0.03^{Ab}	0.58 ±0.01^{Ab}	1.81 ±0.11^{Abc}	2.79 ±0.05^{Aac}	0.56 ±0.09^{Ab}	1.35 ±0.49^{Abc}
1	3.65 ±0.12^{Aa}	0.19 ±0.19^{Ab}	0.43 ±0.17^{Abc}	2.06 ±0.26^{Ade}	2.67 ±0.13^{Aad}	0.44 ±0.04^{Abc}	1.39 ±0.32^{Ace}
2	2.66 ±0.01^{Aa}	ND	ND	3.26 ±0.08^{Bb}	2.04 ±0.08^{Bc}	ND	0.38 ±0.01^{Bd}
3	4.78 ±0.07^{Aa}	ND	0.52 ±0.17^{Ab}	3.72 ±0.16^{Bc}	3.20 ±0.10^{Ad}	ND	0.76 ±0.01^{ABb}

Mean value (±SE)

$^{A, B}$ Values in the same column having different superscripts for mean lactic acid contents differ significantly ($p<0.05$).

$^{a, b, c, d, e}$ Values in the same row having different superscripts for mean lactic acid contents differ significantly ($p<0.05$).

ND = Not detected

Table 3.2 Changes in titratable acidity (%) of the fermented goat's milk preparations during 3 weeks of storage at 4°C (*n* = 2)

Storage time (wks)	L	P	B	L+P	L+B	P+B	L+P+B
0	0.45 ±0.00^{Aa}	0.27 ±0.01^{Abc}	0.29 ±0.00^{Ab}	0.45 ±0.00^{Aa}	0.36 ±0.00^{Ad}	0.24 ±0.01^{Ac}	0.35 ±0.01^{Ad}
1	0.48 ±0.01^{Aa}	0.29 ±0.01^{Ac}	0.33 ±0.02^{Ab}	0.50 ±0.01^{Ba}	0.37 ±0.00^{Ad}	0.26 ±0.00^{ABc}	0.35 ±0.00^{Abd}
2	0.56 ±0.01^{Ba}	0.32 ±0.01^{Ab}	0.42 ±0.01^{Bc}	0.55 ±0.01^{Ca}	0.41 ±0.00^{Bc}	0.30 ±0.01^{Bb}	0.38 ±0.00^{Ac}
3	0.64 ±0.02^{Ca}	0.35 ±0.03^{Ab}	0.44 ±0.01^{Bc}	0.63 ±0.01^{Da}	0.50 ±0.01^{Cc}	0.33 ±0.00^{Bb}	0.44 ±0.01^{Bc}

Mean value (±SE)

$^{A, B, C, D}$ Values in the same column having different superscripts for mean titratable acidity levels differ significantly ($p<0.05$).

$^{a, b, c, d}$ Values in the same row having different superscripts for mean titratable acidity levels differ significantly ($p<0.05$).

Table 3.3 Changes in pH of the fermented goat's milk preparations during 3 weeks of storage at 4°C (*n* = 2)

Storage time (wks)	L	P	B	L+P	L+B	P+B	L+P+B
0	5.43 ±0.00^{Aa}	6.23±0.01^{Ab}	6.14±0.01^{Ab}	5.44 ±0.01^{Aa}	5.43 ±0.10^{Aa}	6.21 ±0.00^{Ab}	5.57 ±0.00^{Aa}
1	5.18 ±0.07^{Aa}	6.27±0.03^{Ac}	6.20±0.01^{Ac}	5.20±0.06^{Aab}	5.44 ±0.06^{Aab}	6.18±0.00^{Ac}	5.48±0.09^{Aab}
2	5.10±0.07^{Aa}	6.31±0.04^{Ac}	6.21±0.03^{Ac}	5.17 ±0.06^{Aab}	5.39 ±0.09^{Aab}	6.20±0.03^{Ac}	5.45±0.07^{Ab}
3	4.74 ±0.08^{Ba}	6.16 ±0.03^{Ac}	6.09±0.08^{Ac}	4.91±0.20^{Aab}	5.32±0.04^{Ab}	6.27 ±0.01^{Ac}	5.33 ±0.00^{Ab}

Mean value (±SE)

$^{A, B}$ Values in the same column having different superscripts for mean pH values differ significantly ($p<0.05$).

$^{a, b, c,}$ Values in the same row having different superscripts for mean pH values differ significantly ($p<0.05$).

Table 3.4 Changes in syneresis (%) of the fermented goat's milk preparations during 3 weeks of storage at 4^{o}C (*n* = 2)

Storage time (wks)	L	P	B	L+P	L+B	P+B	L+P+B
0	18.50 ±1.50^{Aa}	19.50 ±0.05^{Aa}	13.00 ±1.00^{Aa}	19.00 ±1.00^{Aa}	13.00 ±2.00^{Aa}	59.50±2.50^{Ab}	17.00 ±1.50^{Aa}
1	16.00 ±0.00^{Aa}	21.50 ±2.50^{Aa}	13.50±2.50^{Aa}	11.25±2.30^{Aa}	43.5 ±2.50^{Bb}	51.50±5.50^{Ab}	26.50±0.05^{ABa}
2	16.00 ±0.00^{Aa}	27.00±5.00^{Aab}	29.00 ±4.00^{Bab}	14.50 ±0.50^{Aa}	54.00 ±4.00^{Bc}	49.50±4.50^{Ac}	45.50±2.50^{BCbc}
3	12.50±0.50^{Aa}	27.50 ±0.50^{Aab}	41.50±0.50^{Bbcd}	36.00 ±2.00^{Bbc}	58.50 ±2.50^{Bd}	49.00±5.00^{Acd}	54.00 ±6.00^{Cd}

Mean value (±SE)

$^{A, B, C}$ Values in the same column having different superscripts for mean syneresis values differ significantly ($p<0.05$).

$^{a, b, c,d}$ Values in the same row having different superscripts for mean syneresis values differ significantly ($p<0.05$).

3.3.4 Sensory properties of fermented goat's milk

Although some differences in the average consumer response to the tested sensory characteristics were apparent among the fermented goat's milk preparations, these differences were found not to be statistically significant, possibly due to the relatively small number of panellists (n = 7) and substantial variance among their responses (Table 3.5). Among the attributes tested, the colour and appearance of the preparations were generally scored most highly, while taste and overall acceptability scored lowest. Preparations containing either the co-culture of *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 (L+B) or all three probiotics together (L+P+B), received the highest level of acceptability for taste during storage. In general, most of the preparations received lower scores for all the sensory characteristics at the end of the shelf life compared to the fresh samples.

3.3.5 Gastrointestinal tolerance of probiotics in fermented goat's milk

All three probiotics in fermented goat's milk have demonstrated significantly lower viability levels regardless of the combinations at the end of 180 minutes gastric juice exposure *in vitro* (Table 3.6). However, certain combinations (L+B and L+P+B) appeared to improve the simulated gastric juice tolerance of *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 ($p<0.05$). *P. jensenii* 702 has demonstrated the overall lowest gastric acid tolerance in fermented goat's milk, regardless of the combinations.

All three probiotics demonstrated significantly lower viability levels at the end of the 240 minutes exposure period *in vitro* in the presence of 0.3 % bile salt in simulated small intestinal juice regardless of the combinations (Table 3.7). Furthermore, bile salt had a significant impact on reducing viability of all probiotics in each preparation even 1 minute after exposure. However, monocultures of each probiotic performed better compared to their respective co-culture preparations in the presence of bile salts. There were no significant differences in the viable bacterial counts in all preparations at the end of the no bile assay except *L. acidophilus* LA-5 monoculture and *B. animalis* subsp. *lactis* BB-12 in

the triple co-culture preparation (L+P+B), however, their viability was significantly higher in these cases compared to their respective viability in the presence of bile salts.

3.3.6 Adhesion ability of probiotics in fermented goat's milk

All three probiotics either alone or in combinations in fermented goat's milk, were able to adhere to Caco-2 cells, although there were significant differences among the rates of adhesion of the various probiotic strains. Adhesion percentages varied from 0.03% (lowest among all the preparations) for *P. jensenii* 702 in combination with *L. acidophilus* to 2.78% (highest among all the preparations) for *P. jensenii* 702 in the presence of *B. animalis* subsp. *lactis* BB 12, representing an almost 100-fold variation across the range. While these adhesion rates appear low, it should be recognized that they represent actual cell counts of approximately 10^4-10^6 cfu/ml, thus from each preparation substantial numbers of each probiotic were able to attach to the Caco-2 cell layers. Attachment of probiotics to Caco-2 cell layers was further confirmed by scanning electron microscopy (Figure 3.4), from which it was noted that the Caco-2 cell layer surface was clearly not fully populated with bacterial cells. Where bacterial cells were attached there was evidence of considerable clumping in most cases, an example of which is clearly apparent in Figure 3.4D.

Table 3.5 Average responses of tasting panellists to the sensory properties of fermented goat's milk preparations over 3 weeks of shelf life (Number of participants = 7)

Characteristic	Storage time (wks)	L	B	P	L+P	L+B	P+B	L+P+B
Colour & appearance	0	7.00 ±0.38	7.00 ±0.44	6.57 ±0.57	6.86 ±0.40	6.57 ±0.65	6.86 ±0.40	6.57 ±0.65
	1	6.57 ±0.48	6.43 ±0.57	5.71 ±0.64	6.57 ±0.53	5.71 ±0.61	6.57 ±0.48	5.57 ±0.57
	2	6.00 ±0.58	6.14 ±0.51	5.71 ±0.71	5.86 ±0.77	5.71 ±0.78	6.29 ±0.42	6.43 ±0.48
	3	6.71 ±0.42	6.29 ±0.57	6.43 ±0.44	6.43 ±0.48	6.14 ±0.51	6.43 ±0.48	6.00 ±0.62
Aroma	0	5.71 ±0.89	5.00 ±0.69	5.00 ±0.72	6.00 ±0.62	5.86 ±0.40	5.86 ±0.46	5.86 ±0.40
	1	5.29 ±0.47	4.71 ±0.75	4.71 ±0.75	5.71 ±0.52	5.71 ±0.36	4.71 ±0.42	5.71 ±0.36
	2	5.71 ±0.64	5.71 ±0.47	4.43 ±0.84	5.43 ±0.53	5.71 ±0.36	5.57 ±0.20	5.71 ±0.42
	3	4.57 ±0.48	5.00 ±0.65	4.57 ±0.72	5.14 ±0.67	5.29 ±0.36	4.86 ±0.59	5.29 ±0.36
Body & texture	0	5.57 ±0.69	5.57 ±0.78	5.14 ±0.74	5.43 ±0.53	5.29 ±0.68	5.86 ±0.59	5.29 ±0.68
	1	5.29 ±0.47	5.29 ±0.52	5.71 ±0.36	5.29 ±0.42	5.14 ±0.44	4.86 ±0.51	5.29 ±0.42
	2	4.71 ±0.29	5.29 ±0.36	4.57 ±0.53	4.86 ±0.55	4.71 ±0.47	5.14 ±0.34	4.86 ±0.55
	3	4.57 ±0.72	5.29 ±0.42	4.29 ±0.61	4.57 ±0.43	5.43 ±0.48	4.57 ±0.75	5.00 ±0.44
Taste	0	4.43 ±0.61	3.57 ±0.75	3.86 ±0.91	4.29 ±0.52	5.14 ±0.40	4.71 ±0.61	5.43 ±0.53
	1	4.14 ±0.67	4.43 ±0.90	4.29 ±0.81	4.14 ±0.46	5.71 ±0.36	4.29 ±0.81	4.86 ±0.51
	2	4.57 ±0.53	4.86 ±0.26	3.71 ±0.87	5.00 ±0.49	5.57 ±0.43	4.86 ±0.26	5.29 ±0.52
	3	3.29 ±0.64	4.00 ±0.85	3.43 ±1.00	3.71 ±0.84	4.71 ±0.29	4.29 ±0.71	5.00 ±0.62
Overall acceptability	0	4.86 ±0.67	4.29 ±0.78	4.00 ±0.82	4.57 ±0.53	5.57 ±0.30	4.86 ±0.55	5.43 ±0.37
	1	4.57 ±0.75	4.43 ±0.84	4.43 ±0.81	4.86 ±0.56	5.86 ±0.14	4.29 ±0.64	4.64 ±0.47
	2	4.57 ±0.48	5.14 ±0.34	4.00 ±0.82	5.14 ±0.51	5.21 ±0.31	5.00 ±0.31	4.86 ±0.51
	3	3.14 ±0.70	4.14 ±0.80	3.43 ±0.84	3.86 ±0.70	4.71 ±0.36	4.14 ±0.83	5.00 ±0.44

Mean value (±SE)

(The sensory scores: Like extremely = 9, Like very much = 8, Like moderately = 7, Like slightly = 6, Neither like nor dislike = 5, Dislike slightly = 4, Dislike moderately = 3, Dislike very much = 2, Dislike extremely = 1).

Table 3.6 Effect of simulated gastric juice (pH 2.0) on the viability of probiotics and their combinations in fermented goat's milk during 180 minutes of exposure (counts are shown as log cfu/ml, *n* =2)

Probiotics & combinations		0 min	1min	60 min	180 min
L. acidophilus LA-5	L	8.61±0.04^{a}	8.45±0.00^{a}	<1	<1
	L+P	8.57±0.03^{a}	8.50±0.02^{a}	<1	<1
	L+B	8.49±0.06^{a}	8.35±0.05^{a}	6.29±0.51^{Aa}	4.45±0.03^{Aa}
	L+P+B	8.59±0.00^{a}	8.64±0.01^{b}	6.55±0.01^{Aa}	5.33±0.02^{Ab}
B animalis subsp. *lactis* BB-12	B	6.69±0.09^{a}	6.02±0.10^{a}	<1	<1
	L+B	6.5 ±0.21^{a}	6.46±0.16^{a}	4.81±0.12^{Aa}	3.00±0.30^{Aa}
	P+B	8.31±0.05^{b}	8.32±0.06^{b}	4.77±0.01^{Aa}	<1
	L+P+B	8.57±0.00^{c}	8.56±0.01^{c}	6.43±0.05^{Ab}	4.16±0.05^{Ab}
P. jensenii 702	P	8.42±0.01^{a}	8.20±0.11^{a}	<1	<1
	L+P	8.36±0.03^{a}	8.07±0.04^{a}	4.09±0.09^{A}	<1
	P+B	7.64±0.08^{b}	7.60±0.03^{b}	<1	<1
	L+P+B	7.90±0.03^{b}	7.72±0.00^{b}	<1	<1

Mean value (±SE)

$^{a, b, c}$ Values in the same column having different superscripts for each probiotic differ significantly ($p<0.05$)

^{A} Indicates a significant difference of mean viable counts in the simulated gastric juice compared to that at 0 min ($p<0.05$)

Table 3.7 Effect of simulated small intestinal juice (pH 8.0) on the viability of probiotics and their combinations in fermented goat's milk during 240 minutes of exposure (counts are shown as log cfu/ml, *n* =2)

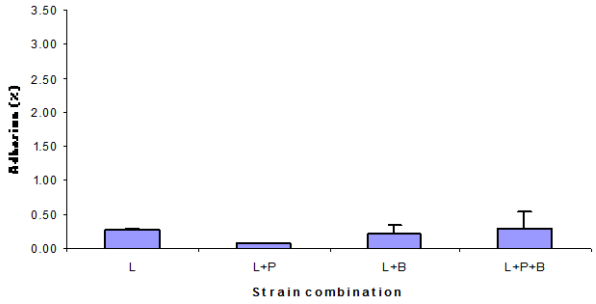
Probiotics & combinations		0 min		1 min		240 min	
		no bile	**0.3% bile**	**no bile**	**0.3% bile**	**no bile**	**0.3% bile**
L. acidophilus LA-5	L	8.24 ±0.02	8.16 ±0.02	8.42 ±0.01	7.16 ±0.01[b]	7.38 ±0.02[a]	6.72 ±0.04[b]
	L+P	8.53 ±0.00	8.23 ±0.03	8.57 ±0.03	7.31 ±0.03[b]	8.41 ±0.05	6.65 ±0.06[b]
	L+B	8.25 ±0.02	8.25 ±0.02	8.06 ±0.01	5.33 ±0.05[b]	7.70 ±0.04	4.39 ±0.09[b]
	L+P+B	8.40 ±0.00	8.36 ±0.00	8.43 ±0.02	6.98 ±0.03[b]	8.44±0.01	4.16 ±0.01[b]
B animalis subsp. *lactis* BB-12	B	8.01 ±0.01	8.02 ±0.01	8.08 ±0.03	7.55 ±0.09[b]	7.71 ±0.03	6.12 ±0.12[b]
	L+B	6.52 ±0.00	8.15 ±0.02	6.46 ±0.00	6.07 ±0.07[b]	6.19 ±0.06	4.10 ±0.02[b]
	P+B	8.18 ±0.04	8.47±0.04	8.26 ±0.00	7.88 ±0.05[b]	8.21 ±0.06	5.30 ±0.09[b]
	L+P+B	8.62 ±0.03	8.49 ±0.09	8.58 ±0.03	6.57 ±0.02[b]	8.03 ±0.03[a]	5.61 ±0.01$_b$
P. jensenii 702	P	8.50 ±0.19	8.78 ±0.14	8.22 ±0.15	7.40 ±0.06[b]	8.02 ±0.02	5.49 ±0.13[b]
	L+P	8.30 ±0.07	8.46 ±0.02	8.18 ±0.01	5.20 ±0.20[b]	8.03 ±0.03	2.39 ±0.09[b]
	P+B	8.08 ±0.08	8.29±0.13	8.27 ±0.00	6.80 ±0.02[b]	7.68 ±0.08	<1
	L+P+B	8.11 ±0.03	8.49 ±0.18	7.66 ±0.04	5.16 ±0.01[b]	7.68 ±0.02	<1

Mean value (±SE)

[a] Indicates a significant difference of mean viable counts in the simulated small intestinal juice (no bile) compared to that at 0 min .

[b] Indicates a significant difference of mean viable counts in the simulated small intestinal juice (0.3% bile) compared to that at 0 min.

A



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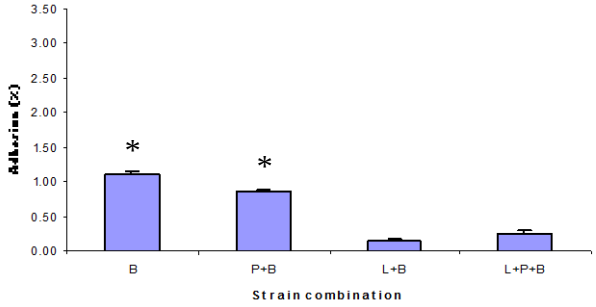


C

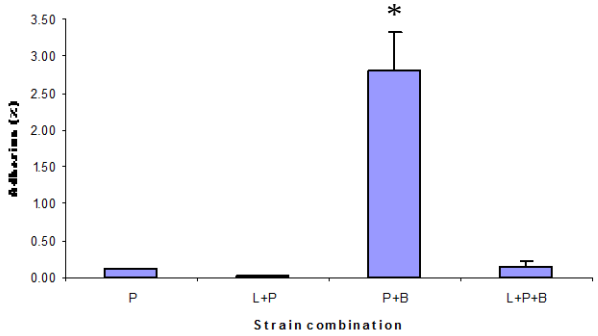


Figure 3.3 Percentage adhesion of different probiotics in goat's milk, either alone or in combination with other probiotics as indicated, to Caco-2 human intestinal epithelial cells. (A) *L. acidophilus* LA-5, (B) *B. animalis* subsp. *lactis* BB-12, and (C) *P. jensenii* 702. In combinations only the relevant bacterium has been counted (*n* = 3,* indicates a significant difference)

3.3.7 *In vitro* cytokine production

As indicated in Table 3.8, detectable cytokine levels were only evident in association with 3 of the probiotic cultures. IL-6 was detected only when cells were exposed to the *L. acidophilus* LA-5 monoculture, while TNF-α was only detected in the presence of *B. animalis* subsp. *lactis* BB-12 and the *B. animalis* subsp *lactis* BB-12 / *L. acidophilus* LA-5 combination, between which a 2-fold difference in mean concentration was observed ($p>0.05$).

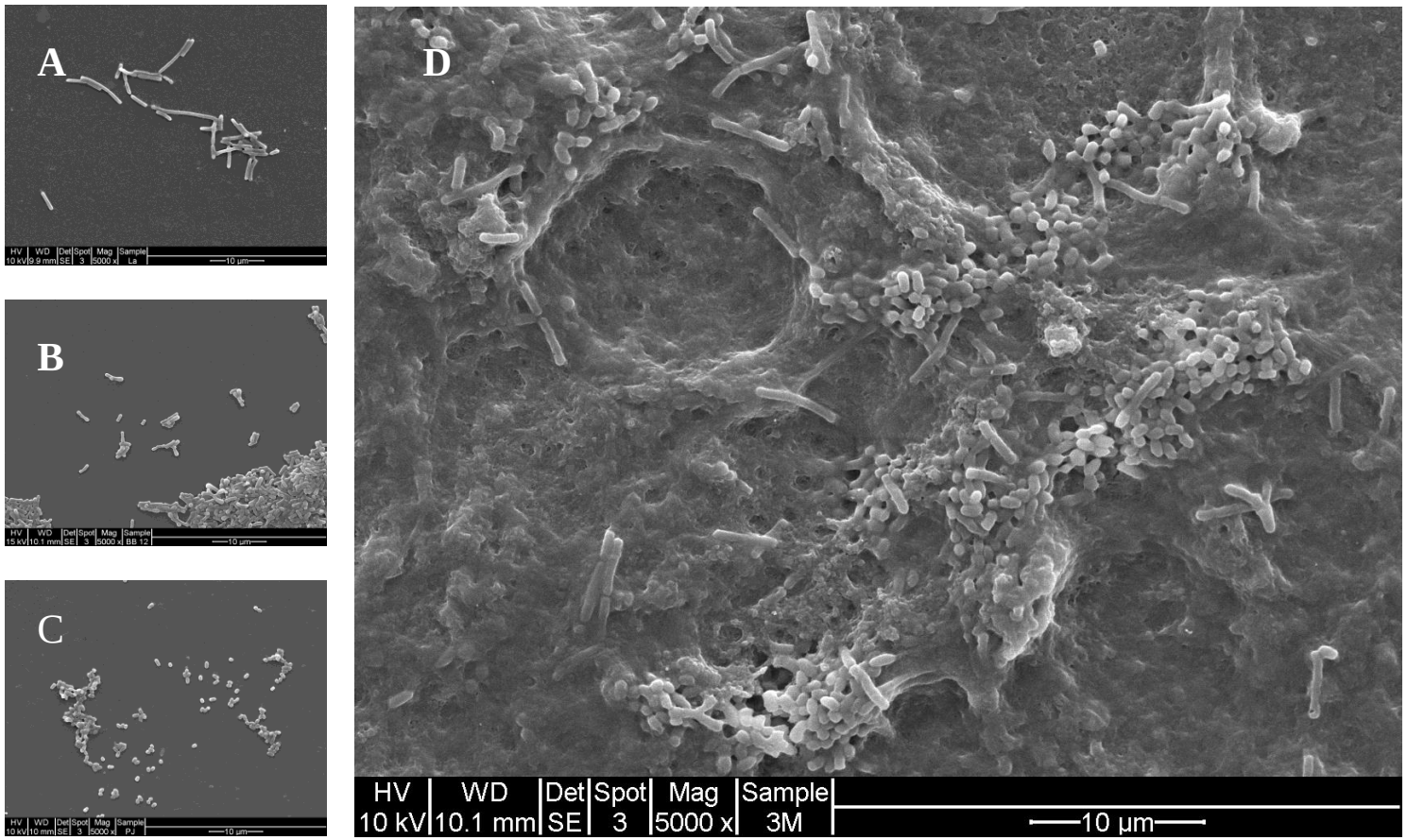


Figure 3.4 Scanning electron micrograph of (A) *L. acidophilus* LA-5, (B) *B. animalis* subsp. *lactis* BB-12, (C) *P. jensenii* 702 and (D) probiotic bacteria from fermented goat's milk containing L+P+B adhered to Caco-2 cell lavers.

Table 3.8 Levels of IL-6 and TNF-α secreted from Caco-2 cells exposed to the probiotic mono-cultures and combinations used in the goat's milk preparations (*n* = 4)

Probiotic & combinations	Cytokine Concentration (pg/ml)	
	IL-6	TNF-α
L	2.54 ± 1.46	ND
P	ND	ND
B	ND	4.17 ± 1.25
L+P	ND	ND
L+B	ND	1.99 ± 0.01
P+B	ND	ND
L+P+B	ND	ND

Mean Value (±SE)

ND: Not Detected

3.3.8 Summary of key findings

In general, the viable numbers of *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 appeared to increase during the incubation period in goat's milk regardless of the combinations, but declined in most cases with increasing storage time. Combination with other probiotics had a positive impact on the viability of all three probiotics resulting in viable counts > 10^7 cfu/ml over the shelf life of the goat's milk, while increasing the initial inoculation level to ~10^8 appeared to improve the viability of *P. jensenii* 702 during refrigerated storage.

While the acidity of the fermented milk samples was found to increase during storage, changes in lactic acid content of the preparations were not reflected in the overall trends in total titratable acidity. Syneresis values were widely varied among the different preparations as well as with the time of storage, however, most of the preparations demonstrated higher syneresis values at the end of the shelf-life compared to the fresh products.

Sensorial differences among different preparations were not found to be statistically significant. However in most cases, fresh fermented goat's milk samples were rated significantly more highly for the tested sensory characteristics than samples stored at 4^{o}C for 3 weeks.

All three probiotics and combinations in fermented goat's milk demonstrated significantly lower viability after exposure to simulated gastric and intestinal (with 0.3% bile) fluids *in vitro*. The ability of these probiotics to adhere to Caco-2 cells appeared to be influenced by the specific probiotic strains with which they were combined in the manufacturing of fermented goat's milk. Exposure to *P. jensenii* 702 did not induce IL-6 and TNF-α production from Caco-2 cells either in monoculture or when co-cultured with other probiotics, while the monocultures of *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 and the co-culture of those two probiotics, were able to induce low levels of IL-6 and TNF-α production respectively.

3.4 Discussion

The primary objectives of this study were to evaluate the performance of the novel probiotic *P. jensenii* 702 as a monoculture and co-culture with *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 in goat's milk, and to identify the most suitable combination of these probiotics for dairy fermentation. Findings of this study include some notable differences among the different goat's milk preparations with respect to microbial growth and viability, physico-chemical properties, sensory attributes and *in vitro* functional properties. Importantly, the findings suggested that *P. jensenii* 702 may have potential use as an adjunct culture in producing novel fermented dairy foods.

3.4.1 Probiotic growth and viability

It has been reported previously that probiotic viability in fermented milk can be influenced by the probiotic culture composition. For example, pure cultures of *L. acidophilus* (LA5) and *L. rhamnosus* (LC35) with *S. thermophilus* (ST7) were reported to be more stable in

terms of viability than their mixed cultures in fermented cow's milk during storage (Oliveira et al., 2001). In contrast, co-cultivation of *L. acidophilus*, *L. rhamnosus*, *L. bulgaricus* and *B. lactis* with *S. thermophilus* in skim milk was shown to result in better growth and viability compared to pure cultures of the same probiotics (Oliveira et al., 2009a). Species or strain specific synergistic or antagonistic interactions may cause these differences in their viability. In the current study high viabilities (10^7-10^8 cfu/g) of probiotics in combinations as well as in pure cultures were observed after 3 weeks of storage, possibly reflecting a degree of symbiosis among all three probiotics. Many strains of *L. acidophilus* and *Bifidobacterium* have previously demonstrated a high survival rate (>10^6 cfu/ml or g) in refrigerated storage during a similar storage time to that of the present study, in fermented goat's milk products (Farnsworth et al., 2006; Guler-Akin & Akin, 2007) as well as in many other probiotic products including cow's milk yogurts (Kailasapathy, 2006; Kailasapathy et al., 2008) and ewe's milk yogurts (Guler-Akin, 2005). Such evidence indicates a well established symbiotic relationship between *L. acidophilus* and *Bifidobacterium* in fermented foods. However, Phillips et al. (2006) observed poor viability (<10^4 cfu/ml) of 2 strains of *L. acidophilus* in the presence of *Bifidobacterium* in Cheddar cheese over 32 weeks in storage. Interestingly, *L. casei*, *L. paracasei* and *L. rhamnosus* were able to maintain higher viabilities along with *Bifidobacterium* (>10^7 cfu/ml) in cheese. This result may reflect either some antagonistic activity of other lactobacilli toward the *L. acidophilus* or an unsuitability of the cheese food matrix for these particular *L. acidophilus* strains. Similar to their observations, in the present study there was a reduction in viability of both *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 in the presence of each other during storage, however, interestingly this effect was less apparent when *P. jensenii* 702 was also included with them. These results highlight the potential use of *P. jensenii* 702 as an adjunct culture in dairy fermentation (Figure 3.2).

In this experiment both *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 have demonstrated a relatively higher growth during fermentation in the presence of each other, compared to their monocultures or co-cultures with *P. jensenii* 702, again perhaps indicative of their natural symbiosis (Figure 3.1). As explained previously, *Bifidobacterium* strains are weakly proteolytic (Shihata & Shah, 2000) and higher proteolytic activities of *L.*

acidophilus compared to bifidobacteria (Lourens-Hattingh & Viljoen, 2001) may support the growth of *Bifidobacterium* in these preparations. The *Bifidobacterium* has also demonstrated higher growth in goat's milk in the presence of *P. jensenii* 702 compared to *Bifidobacterium* monoculture in the present study, in accordance with the apparent growth stimulation of *B. lactis* Bb 12 by *P. jensenii* 702 previously observed by Moussavi and Adams (2009). Consistent with these findings, higher numbers of probiotic lactobacilli and bifidobacteria in goat's milk compared to cow's milk throughout the fermentation phase, have also been observed by other authors (Bozanic et al., 2004; Slacanac et al., 2010; Slacanac et al., 2005), indicating that goat's milk is a suitable medium for growth of lactobacilli and bifidobacteria.

A slow growth rate for *P. jensenii* compared to lactic acid bacteria has been previously reported (Ekinci & Gurel, 2008; Gardner & Champagne, 2005). Although incubation temperatures of 37-40^{o}C are generally considered optimal for probiotic growth (Guler-Akin & Akin, 2007; Tamime & Robinson, 1999), the optimum incubation temperature range for *Propionibacterium* is 25-32^{o}C (Gautier & Richard, 1999). All samples were incubated at 37^{o}C in the present study (to provide a common suitable growth temperature for all three bacteria) and this may have resulted in a lag in growth of *P. jensenii* 702 in the goat's milk during incubation. *P. jensenii* 702 was originally isolated from raw cow's milk (Huang & Adams, 2004), and differences between the physico-chemical properties of goat's and cow's milk (such as the lower pH of goat's milk) may have contributed to a lower growth in goat's milk in the present study.

In this study, except when paired with *P. jensenii* 702 (L+P), *L. acidophilus* LA-5 demonstrated the greatest loss and fluctuation of viability during storage at 4^{o}C. One possible explanation is loss of β-galactosidase (the enzyme responsible for lactose utilization) activity, which has been reported to be correlated with the loss of viability in *L. acidophilus* in cow's milk during refrigerated storage (Gilliland & Lara, 1988). Higher viability loss of *L. acidophilus* compared to *Bifidobacterium* in probiotic products during storage has been reported previously by a number of authors (Guler-Akin, 2005; Martin-Diana et al., 2003; Phillips et al., 2006).

Although the fermented goat's milk preparation produced by co-cultivation of the three probiotics together (L+P+B) has not resulted in the highest viability of each probiotic during storage, all three probiotics were able to maintain high viable counts (10^7-10^8 cfu/ml) in all preparations at the end of the shelf-life in the present study. Therefore, inclusion of *P. jensenii* 702 together with *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 in the manufacturing of fermented goat's milk products may be considered a beneficial approach, because multispecies probiotic products potentially provide a broader range of health benefits for the consumer compared to mono-species probiotic products (Timmerman et al., 2004). Some researchers have suggested that dairy propionibacteria possess a bifidogenic effect based on *in vitro* (Hiroharu et al., 1997; Kouya et al., 2007) as well as *in vivo* human studies (Bougle et al., 1999; Kotula, 2008). Consumption of freeze dried encapsulated *P. jensenii* 702 was reported by Kotula (2008) to significantly increase total fecal bifidobacteria in human subjects, apparently confirming the beneficial effects of propionibacteria toward bifidobacteria. An increase in the indigenous bifidobacteria population is potentially highly beneficial for the host, as they have a known capacity to interfere with the adhesion of pathogenic microorganisms to the intestinal epithelium cells and thereby enhance the host immune function (Hisako & Ohwaki, 1991; Martino et al., 2008; Salminen et al., 2005). 1,4-dihydroxy-2-naphthoil acid, a bifidogenic growth stimulator produced by *P. freudenreichii* ET-3 was observed to cause clinical and endoscopic improvements in patients with ulcerative colitis through improving the intestinal microflora (Suzuki et al., 2006).

3.4.2 Physico-chemical properties

It seems likely that the specific probiotic culture composition may influence the pH of the final product. Co-culturing *S. thermophilus* with each of *L. bulgaricus*, *L. acidophilus*, *L. rhamnosus* and *B. lactis* separately in milk was shown by Oliveira et al (2009b) to result in significant differences in fermentation time to reach pH 4.5. Likewise, different pH values were observed for the different fermented goat's milks after the incubation period at 37^{o}C in the present study. The acidity development in goat's milk was associated with the

probiotic species used in agreement with Beal et al. (1999). In the present study, fermented goat's milk with the monoculture of *L. acidophilus* LA-5 has demonstrated the lowest pH, highest titratable acidity and highest lactic acid levels (Table 3.1-3.3). Lactic acid and pH have been reported to be strongly related to each other, where higher lactic acid concentrations can cause lower pH values in fermented milk (Oliveira et al., 2009b). However, for some preparations lactic acid content was not correlated with respective acidity levels in the present study, thus it would seem that it was not simply the lactic acid content but also other unidentified parameters that governed the acidity of these goat's milk preparations. In most of the preparations, the lactic acid content appeared to decline over the storage before increasing again at week 3. This trend of changing lactic acid content is more difficult to explain. However, a gradual increase of lactic acid was observed in fermented goat's milk samples with co-cultures of *L. acidophilus* LA-5 and *P. jensenii* 702 (L+P) throughout the storage period. Similar observations in yogurts were previously reported by Akalin et al. (2007a).

The pH of the fermented goat's milk containing *L. acidophilus* LA-5 either as monoculture or co-culture was also found to be significantly lower than milk containing *P. jensenii* 702, *B. animalis* subsp. *lactis* BB-12 or both *P. jensenii* 702 and *B. animalis* subsp. *lactis* BB-12 after incubation period. This trend has been maintained throughout the storage. This may have been due to slower growth of bifidobacteria and propionibacteria in milk. Both bifidobacteria (Gomes et al., 1998; Walker et al., 2005) and propionibacteria (Ekinci & Gurel, 2008) have previously demonstrated slow growth in milk during incubation, because required nutrients are not always available in acceptable forms or in optimal concentration. For example the poor growth of bifidobacteria in milk is often associated with the lack of small peptides and free amino acids (Gomes et al., 1998). In the present study however, regardless of the culture composition, bifidobacteria has demonstrated higher viable counts after incubation under co-culture, compared to the bifidobacteria monoculture. Therefore slow growth of *P. jensenii* 702 and low metabolic activities of *B. animalis* subsp. *lactis* BB-12 may have resulted in the high pH and low acidity levels after incubation period due to slower release of metabolic byproducts such as propionate, acetate and CO_2 compared to *L. acidophilus* LA-5 (Biede & Hammond, 1979; Ekinci & Gurel, 2008; Ong et al., 2006), all

of which may contribute to acidity development in fermented milk products. Although *L. acidophilus* has been identified previously as a probiotic with relatively slow growth in milk (Gomes et al., 1998; Srinivas et al., 1990), its proteolytic system has an ability to generate its own nitrogen source (Gomes et al., 1998). The proteoletic activity of *L. acidophilus* LA-5 has shown a positive effect on its own and *B. lactis* BB 12 growth rates through release of amino groups as growth factors from milk proteins (Moayednia et al., 2009). Such an effect may have produced an increase in metabolic activity and hence a potential reason for higher acidity in the fermented milk containing *L. acidophilus* LA-5 in the present study. Furthermore, some *L. acidophilus* strains have exhibited higher acid production rates in milk (Badis et al., 2004; Gupta et al., 1996). Therefore according to the results obtained in this study it can be concluded that the acid production rate of *L. acidophilus* LA-5 was higher than *B. animalis* subsp. *lactis* BB-12 and *P. jensenii* 702 in goat's milk during fermentation.

Although pH decreased over the shelf life in every fermented goat's milk preparation in the present study, only the pH value of fermented milk containing the *L. acidophilus* LA-5 monoculture was significantly lower at week 3 than in the initial sample. It therefore appeared that *L. acidophilus* LA-5 greatly enhanced the lowering of pH in fermented goat's milk during storage compared to *P. jensenii* 702 and *B. animalis* subsp. *lactis* BB-12. This may also be attributed to the higher acid production rate of *L. acidophilus* (Gupta et al., 1996) during storage. According to a recent report, *L. acidophilus* induced the highest post-fermentation acidification and lactic acid release while *B. lactis* demonstrated the opposite in fermented cow's milk when co-cultured with *S. thermophilus* (Oliveira et al., 2009b). However, incorporation of probiotics with yogurt starter culture bacteria *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* has previously shown faster pH reduction during incubation period compared to the present study (Ekinci & Gurel, 2008; Guler-Akin & Akin, 2007; Martin-Diana et al., 2003; Minervini et al., 2009) due to higher acid production rate of yogurt starter cultures. Therefore a long incubation time is required when manufacturing fermented products without yogurt starter cultures in order to achieve desirable product qualities.

The decrease in the pH values of samples in the present study was concomitant with the increase in titratable acidity in fermented goat's milk as reported by many other researchers (Adhikari et al., 2000; Dave & Shah, 1998; Ekinci & Gurel, 2008; Ravula & Shah, 1998a; Souza & Saad, 2009). Fermented goat's milk with *L. acidophilus* LA-5 has demonstrated highest acidity compared to all the other milk types indicating their higher activities which accelerated post-fermentation acidification in monoculture throughout the shelf life in this study. An increase in the titratable acidity of milk containing *L. acidophilus* as monoculture or co-culture can occur due to the β-galactosidase activity of *L. acidophilus* upon refrigerated storage (Moayednia et al., 2009). Interestingly fermented milk with all three bacteria resulted in lower acidity values over the storage even with highest total microbial numbers compared to the fermented goat's milk with *L. acidophilus* LA-5 monoculture (Table 3.2). This may be due to the ability of *P. jensenii* 702 and *B. animalis* subsp. *lactis* BB-12 to utilize metabolic by products of *L. acidophilus* LA-5 which might otherwise increase acidification. Yogurts produced with yogurt starter cultures and two propionibacteria (*P. jensenii* B1264 and P126) have demonstrated lower acidity and higher pH after incubation, and over the storage period, compared to the yogurts produced with starter cultures and either one of the propionibacteria strains (Ekinci & Gurel, 2008). Thus a combination of *L. acidophilus* LA-5, *B. animalis* subsp. *lactis* BB-12 and *P. jensenii* 702 might also be beneficial in controlling post-fermentation acidification in yogurts. Guler-Akin and Akin (2007) observed lower pH values for goat's milk yogurt produced only with *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* after incubation and throughout the cold storage at 4±1^{o}C compared to bio yogurts which consisted of *L. acidophilus, B. bifidum* BB 12 and *L. paracasei* subsp. *casei* in addition to the yogurt starter cultures. In contrast, Ekinci et al. (2008) observed higher pH and lower titratable acidity over the storage period for yogurts produced with only yogurt starter cultures compared to yogurts produced with propionibacteria as an adjunct culture in addition to starter cultures. Therefore, only certain probiotic strains which can inhibit *L. delbrueckii* subsp. *bulgaricus* - the main organism responsible for the acid production in yogurt/fermented dairy products - may play a significant role in minimizing acidification (Dave & Shah, 1997a).

A recent study conducted with *L. acidophilus* LA-5, *B. lactis* BB 12 and *L. acidophilus* LA-5 + *B. lactis* BB 12 in fermented cow's milk production (Moayednia et al., 2009) reported a similar trend as the present study regarding post-fermentation acidification. Fermented milk produced with either both probiotics or a monoculture of *L. acidophilus* LA-5 underwent further acidification during 3 weeks of refrigerated storage while the fermented milk produced with a monoculture of *B. lactis* BB 12 did not demonstrate any significant difference in acidity levels over the shelf life (Moayednia et al., 2009). Although fermented goat's milk produced with a monoculture of *B. animalis* subsp. *lactis* BB-12 showed a significant acidification over the shelf life in the present study, it was relatively low compared to fermented milk produced with a monoculture of *L. acidophilus* LA-5 or a co-culture of both probiotics (Table 3.2). The lower buffering capacity of goat's milk (Martin-Diana et al., 2003; Rysstad & Abrahamsen, 1983; Vegarud et al., 1999) may have had an effect on acidification observed in the product containing the monoculture of *B. animalis* subsp. *lactis* BB-12 in the present study. However, the incubation time in this study was 10 hours while Moayednia et al. (2009) have incubated their product only for 5 hours at the same incubation temperature of 37^{o}C. The prolonged incubation time may also have had an effect on observed differences in post-fermentation acidification, as it may have facilitated the growth and activities of *B. animalis* subsp. *lactis* BB-12 for much longer time in the present study. The co-culture of *L. acidophilus* LA-5 with *P. jensenii* 702 in goat's milk also demonstrated a significantly higher acidity level compared to the product with a monoculture of *P. jensenii* 702 throughout the shelf life, and it is conceivable that *L. acidophilus* may exert benefits towards the growth and activities of *P. jensenii* 702 in goat's milk.

Development in acidity during storage may contribute to changes in the other physico-chemical parameters of dairy products such as whey separation. Excessive rearrangements of particles in the gel network before and during the gelation process have been implicated as being responsible for whey separation/syneresis (Lucey, 2001). This process may also be affected by the type of probiotics present in the product. In general there were significant differences of syneresis values among the samples depending on the probiotic culture composition as well as storage time in the present study. This trend was consistent with the

results described by Wang et al. (2010) who observed a significant effect of probiotic inoculation level as well as storage time on syneresis in yogurts. Farooq and Haque (1992) and Wang et al (2010) found that the amount of syneresis of yogurt increased during storage. In contrast, a non significant decrease in syneresis was observed in some fermented goat's milk samples (*L. acidophilus* LA-5 monoculture and co-culture of *P. jensenii* 702 and *B. animalis* subsp. *lactis* BB-12) in the present study. La Torre et al. (2003) and Guler-Akin (2005) obtained similar results (decrease in syneresis during storage) in fermented milk and yogurt. The gradual increase in syneresis of some fermented goat's milk samples (P, B, L+B, L+P+B) over time of storage in the present study may be attributed to the increased acidity of samples during storage, since higher acidity is known to stimulate the syneresis in fermented dairy products (Tamime & Robinson, 1999). However, the preparation made with the monoculture of *L. acidophilus* LA-5 in this study, which recorded the highest acidity over the shelf life compared to the other preparations, also provided the lowest syneresis values. Based on this and several other published reports, it would appear that not only the acidity, but also other parameters of the product such as specific culture composition (La Torre et al., 2003), incubation temperature (Guler-Akin, 2005) and inoculum level (Olson & Aryana, 2008), may influence syneresis in fermented dairy products.

3.4.3 Sensory evaluation

Some differences among scores for the sensory characteristics tested based on the strain/s used in manufacturing the fermented goat's milk products were observed in this study. Similar results were previously described by La Torre et al. (2003), Badis et al. (2004) and Guler-Akin (2005) for the yogurt/fermented milk produced by different starter cultures and probiotics. For example, fermented cow's milk made with *L. acidophilus*, *B. bifidum* and *B. lactis* (AB) cultures was rated more acidic in taste than the corresponding product made with *L. acidophilus*, *B. longum* and *S. thermophilus*. In addition, the flavour was rated significantly higher for products with AB cultures compared to the samples with a combination of *L. acidophilus*, *B. longum* and *B. infantis* (La Torre et al., 2003). According to the tasting test conducted by Badis et al. (2004) yogurt prepared with the

mixed cultures of *S. thermophilus* 16TMC and *L. helveticus* 20TMC were rated as more pleasant compared to the yogurt prepared with the mixed cultures of *S. thermophilus* 16TMC and *L. delbrueckii* subsp. *bulgaricus* 11TMC. Guler-Akin (2005) reported higher consumer acceptability for yogurt produced with starter cultures and probiotic *L. acidophilus, B. bifidum* and *L. casei* compared to yogurt produced only with starter cultures: *S. thermophilus* and *L. bulgaricus*. Different amounts of organic acid production such as lactic acid by different probiotic cultures (Table 3.1), different levels of acidity (Table 3.2) and variations in syneresis (Table 3.4) in the fermented goat's milk samples are likely to have contributed to these sensorial differences among the samples in the present study. Guler-Akin (2005) also observed different levels of lactic acid and acetaldehyde for a yogurt (comprising yogurt starter cultures only) and bio yogurts (comprising probiotics in addition to yogurt starter cultures) which were also rated differently in sensory tests. La Torre et al. (2003) found similar results for sensory characteristics for the fermented milk with different levels of organic acids including lactic and acetic acid.

Although considerable differences were apparent between the average scores of different preparations for certain sensory characteristics in the present study, these were not found to be statistically significant. It is possible however, that the existence of actual differences between the sensory qualities of the preparations were not identified due to the small number of untrained tasting panelists (n = 7) and hence, reduced statistical power of the analysis. It was originally intended that 25-30 participants would be recruited for the panel, however, due mainly to a reluctance of potential recruits to commit to tasting over 4 consecutive weeks, this number could not be obtained. The impact of small sample size was probably further compounded by the relatively high variance observed among the scores of the panelists, possibly reflecting the fact that they did not comprise trained food tasters or quality assessors.

According to the score card used in this study, the overall sensory scores for these preparations remained low. As a requirement of human ethics approval for the study panelists were pre informed about the type of milk used for the manufacturing of these preparations, and it is therefore possible that negative pre-conceptions of goat's milk may

have influenced the responses of some panelists. However, Martin-Diana et al (2003) also observed low sensory scores for fermented goat's milk containing *S. thermophilus, L. acidophilus* LA-5 and *Bifidobacterium* BB-12. Although goat's milk loses its characteristic "goaty" taste during fermentation (Haenlein, 2004; Tratnik et al., 2006), its specific composition results in many technological difficulties associated with the production of fermented goat's milk with good sensory properties (Slacanac et al., 2010). Those compositional characteristics include the slightly lower casein content with a very low proportion or absence of α_{s1}-casein in goat's milk compared to cow's milk, higher degree of casein micelle dispersion in goat's milk (Vegarud et al., 1999), weaker rheological properties of the curd and over acidification capacity of goat's milk compared to cow's milk (Drakoularakou et al., 2003; Slacanac et al., 2010). These characteristics may lead to development of undesirable characteristics such as development of acidity and semi-liquid curd in fermented goat's milk products and may cause a decrease in sensory acceptability. It is possible that in the present study, the lower rating for most of the sensory characteristics after 3 weeks of storage, compared to the respective fresh products at week 0, may have been related to acidity development in the products during storage.

3.4.4 Simulated gastric and intestinal juice tolerance

The acid and bile tolerance of probiotics can be species as well as strain specific (Gupta et al., 1996; Mustapha et al., 1997; Vinderola & Reinheimer, 2003) and according to the present study, co-cultivation of probiotics may also affect these functional properties. It has been reported previously that gastro-intestinal survival of potential probiotics *Enterococcus mundtii* ST4SA and *L. plantarum* 423 improved when used as a combined culture *in vitro* (Botes et al., 2008). It is known that in multispecies probiotic preparations, one may create a new niche that improves the survival of others under stressful conditions. For example probiotic species which can tolerate higher pH may display rapid growth in such environments which may in turn produce a local decline in pH and thereby create the optimal pH range for species which can not tolerate higher pH (Botes et al., 2008; Timmerman et al., 2004). With regard to the acid tolerance of both *L. acidophilus* LA-5 and *B animalis* subsp. *lactis* BB-12 in the present study, the hypothesized improvements in

survival of these organisms in the L+P+B combination throughout the *in vitro* gastric transit assay, were observed. Monocultures of both *L. acidophilus* LA-5 and *B animalis* subsp. *lactis* BB-12 in goat's milk have demonstrated poor acid tolerance in the present study, but were able to maintain significantly higher simulated gastric juice tolerance in the presence of each other, suggesting a synergistic effect. *L. acidophilus* (Badel et al., 2011) and *B. animalis* subsp. *lactis* (Salazar et al., 2009) can both produce exopolysaccharides. These exopolysaccharides can form capsules or "slimy" layers (Ruas-Madiedo & de los Reyes-Gavilán, 2005; Ruas-Madiedo et al., 2009) which thereby shield the bacterial surface against the environment (Badel et al., 2011). Exopolysaccharide produced by one species may act as a physical barrier/protective agent for the other species and vise-versa and may have provided protection against the acidic environment under co-culture conditions in the present study.

Poor *in vitro* gastric tolerance of *P. jensenii* 702 may be related to their dairy origin (Huang et al., 2003). Normally probiotics of intestinal origin demonstrate higher tolerance to gastric acid (Del Piano et al., 2006). Both *L. acidophilus* (Buck & Gilliland, 1994) and *B. animalis* subsp. *lactis* (Jalili et al., 2009) have been found in the human intestinal tract and therefore these two species may possess higher gastrointestinal tolerance compared to *P. jensenii* 702. Although *in vitro* gastric tolerance of *P. jensenii* 702 was found to be very low, according to Reid et al (2003) and Souza et al. (2009) microorganisms with substantial health effects administered alive should be considered as probiotic, regardless of their ability to survive intestinal transit. For example, a low bile resistance would not be a disadvantage for probiotic strains since the intracellular β-galactosidase might be released from the cells by lysis during passage through the gastrointestinal tract and may provide some benefits for efficient lactose hydrolysis to occur in the small intestine (Marteau et al., 1997; Vinderola & Reinheimer, 2003). Although it is best to incorporate probiotics with higher gastrointestinal resistance in manufacturing probiotic products, species with low gastric and small intestinal tolerance may still be important to a certain extent for some functions such as *in vivo* lactose digestion (Charteris et al., 1998b). Low viability of *P. jensenii* 702 in simulated gastric juice at pH 2.0 was previously observed by Huang et al. (2004). However, high numbers of *P. jensenii* 702 were recovered from human fecal

samples after consumption by study subjects of fermented soy milk containing *P. jensenii* 702 for 4 weeks (Kotula, 2008), demonstrating the bacteria's ability to tolerate human gastrointestinal conditions. Since the *in vitro* conditions employed here may not have exactly comprehensively represented all conditions of *in vivo* gastric transit, it may well be beneficial to include *P. jensenii* 702 together with *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 in manufacturing probiotic foods, despite the relatively unfavourable viability results observed in this study.

Bile salts have demonstrated a significant influence on lowering the viability of probiotics in every goat's milk preparation in the present study, however it appeared in the absence of bile salts (0 % bile) that pancreatin (a pancreatic secretion comprising several digestive enzymes) had little influence on their viability. Based on an *in vitro* study, De Carvlho et al. (2009) reported that digestive enzymes such as pepsin and pancreatin have little influence on gastrointestinal tolerance of probiotic *L. casei* LC01 and *L. casei* Shirota. It is well known that non-intestinal bacteria such as *L. bulgaricus* and *Lactococcus lactis* are more sensitive to bile compared to the natural gastrointestinal microflora (De Carvlho et al., 2009; Vinderola & Reinheimer, 2003). Despite its dairy origin, *P. jensenii* 702 was previously able to maintain relatively high viability in the presence of 0.3% bile (Huang et al., 2003). In this study *P. jensenii* 702 demonstrated a poor resistance to bile when used in combined cultures with other probiotics compared to monoculture. The same trend was also observed for *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis*-BB-12. The nature of this behavior is more difficult to explain, but may perhaps be related to potential antagonism towards each other in specific harsh environments. Bacteria usually respond to changes in their surrounding medium via metabolic reprogramming, leading to a cellular state of enhanced resistance (Pichereau et al., 2000). This process may be affected by the presence of other microorganisms under certain conditions such as bile salt stress.

One of the main limitations of the present work is the fact that this assay only examined the bile tolerance of probiotics in fermented goat's milk at 0, 1 and 240 minutes after exposure to simulated small intestinal juice. As explained in section 3.2 food transit times through the small intestine can vary from 1-4 hours and therefore, it may be worthwhile to take

another additional measurement in between 1 and 240 minutes for a more detailed interpretation of their behaviors. This issue has been addressed in the experiments presented later in this thesis, in which an additional measurement was taken at 120 minutes (in between the beginning and the end of the assay) after exposure to simulated small intestinal juice.

3.4.5 Adhesion properties

All three probiotics tested were found to adhere to the Caco-2 cell lines, however, adhesion percentages were widely varied between species. These results are similar to earlier observations demonstrating species and strain variations in adhesion (Collado et al., 2007b; Gueimonde et al., 2006; Schillinger et al., 2005; Tuomola et al., 1999). The probiotic adhesion process involves various bio-physical and bio-chemical properties of both probiotics and epithelial cell layers. These include electrostatic interactions, hydrophobicity, passive and steric forces, and specific cellular structures such as external appendages (Schillinger et al., 2005; Servin & Coconnier, 2003). These properties can vary among probiotics, and therefore it is not surprising to observe variations among adhesion ability of different probiotic strains/species.

In the present study culture composition of probiotics appeared to have an effect on the *in vitro* adhesion ability of probiotics in fermented goat's milk. Monocultures of *B. animalis* subsp. *lactis* BB-12 have demonstrated highest adhesion rates compared to their respective co-cultures possibly due to less competition for the adhesion sites and zero displacement by other probiotics (Collado et al., 2007b; Gueimonde et al., 2006). In contrast, the differences in the adhesion percentages in the case of *L. acidophilus* LA-5 preparations were not statistically significant. One possibility is that *L. acidophilus* LA-5 may have served as the primary biofilm colonizer in the present study in which case their adhesion ability is not likely to have been affected by the presence of *B. animalis* subsp. *lactis* BB-12 and or *P. jensenii* 702. It therefore seems likely that only certain species/strains may be affected by competition under co-culture conditions. In the present study, the highest adhesion percentage was observed for *P. jensenii* 702 when co-cultured with *B. animalis* subsp.

lactis BB-12. In this case it is possible that growth stimulators (Gardner & Champagne, 2005) or exopolysaccharide (Salazar et al., 2009) produced by *B. animalis* subsp. *lactis* BB-12 may also have a positive effect on improving the adhesion properties of *P. jensenii* 702. Adhesion properties of probiotics and enteric-pathogens have been previously reported to be influenced by the exopolysaccharides produced by probiotics (Patricia et al., 2006). Substances isolated from cell surface and culture supernatant fluid of *L. fermentum* 104R has been shown to be involved in the *in vitro* binding ability of this strain (Rojas et al., 2002). Likewise, growth stimulators and or exopolysaccharide secreted by *B. animalis* subsp. *lactis* BB-12 may provide suitable binding materials for *P. jensenii* 702 and may act as adhesion-promoting substances. However, it seems likely that *L. acidophilus* LA-5 may block this adhesion promoting ability of *B. animalis* subsp. *lactis* BB-12, since the adhesion rate of *P. jensenii* 702 was found in the present study to be significantly reduced in combination with *B. animalis* subsp. *lactis* BB-12 when *L. acidophilus* LA-5 was also present. Exopolysaccharides produced by *B. animalis* subsp. *lactis* BB-12 may bind to Caco-2 cells and *P. jensenii* 702 may in turn bind to these exopolysaccharides. If *L. acidophilus* LA-5 had any degree of inhibitory effect on the exopolysaccharide production of *B. animalis* subsp. *lactis* BB-12 this could lead to reduced binding of *P. jensenii* 702. These phenomena warrant further study. Both *P. jensenii* 702 and *L. acidophilus* LA-5 may have competed for adhesion sites and this may be another possible explanation for reduction in adhesion rate of *P. jensenii* 702 when co-cultured with both *B. animalis* subsp. *lactis* BB-12 and *L. acidophilus* LA-5.

Scanning electron micrographs (Figure 3.4) showed that attached bacteria were clumped into certain areas of the Caco-2 cell surface. The reason for this is not certain, but may be related to the binding ability of exopolysaccharide particles to Caco-2 cell layers containing clusters of agglutinated bacteria. The autoaggregation (adherence of bacteria to the each other which belong to the same strain) and or co-aggregation (adherence of bacteria of two or more different species to each other) abilities of probiotics may also have had an effect on adhesion behavior. Thus it is possible that in certain cases adherent cells were in fact bound to other bacterial cells rather than directly bound to Caco-2 cells, providing a possible explanation for the apparent observed bacterial clumping.

3.4.6 Cytokine Production

The findings of the cytokine assay did not support the hypothesis that preparations containing the highest numbers of bacterial cells would produce the greatest IL-6 and TNF-α response by the Caco-2 cells *in vitro*. In fact the results indicated that only certain probiotics and their combinations (L, B and L+B) were able to induce production of the target cytokines by Caco-2 cells to detectable levels. Such variation in the cytokine stimulation ability of probiotics depending on the species and strains involved has previously been documented (Hosoi et al., 2003; Salminen et al., 2005; Timmerman et al., 2007). In the present study, only the monoculture of *L. acidophilus* LA-5 has induced IL-6 production. Interestingly, combinations of *L. acidophilus* LA-5 with *P. jensenii* 702 and/or *B. animalis* subsp. *lactis* BB-12 were unable to induce IL-6 production. Although the *B. animalis* subsp. *lactis* BB-12 monoculture and the co-culture of *B. animalis* subsp. *lactis* BB-12 and *L. acidophilus* LA-5 were able to induce TNF-α production, neither *P. jensenni* 702 nor any combinations with *P. jensenii* 702 were able to stimulate TNF-α production by Caco-2 cells. Possible signal pathways which may be involved in cytokine expression by gastrointestinal epithelial cell lines include protein kinase, G-protein and nuclear transcription factor complex of NF-κB and IκB (Ding et al., 2000; Hosoi et al., 2003). However, the mechanism by which *P. jensenii* 702 and *B. animalis subsp. lactis* BB-12 were apparently able to inhibit IL-6 and TNF-α production under above co-culture conditions is not clear. Both IL-6 and TNF-α can be considered as pro-inflammatory cytokines (Schultz et al., 2003; Timmerman et al., 2007; Wohlgemuth et al., 2010) and since some gastrointestinal tract inflammatory conditions are known to be influenced by the presence of endogenous pro-inflammatory factors (Cross et al., 2004), decreased secretion of these cytokines may have potential therapeutic applications in human health (Schultz et al., 2003; Timmerman et al., 2007). In the present study levels of IL-6 and TNF-α were found to be low. Reasons for low levels of cytokine production could also be associated with the short incubation time of probiotic preparations in the presence of Caco-2 cells (2 hours). The findings of this study suggested that species specific mechanisms, rather than dosage levels, may be most influential in determining the cytokine response of intestinal cells, and further research is clearly needed to fully elucidate this phenomenon.

3.5 Conclusions

The data compiled from the study on probiotic growth and viability clearly indicated the potential for combining *L. acidophilus* LA-5, *B. animalis* subsp. *lactis* BB-12 and *P. jensenii* 702 together in the manufacturing of fermented goat's milk products without major antagonistic effects. All three probiotics were able to maintain high viability ($>10^7$ cfu/ml) throughout the shelf life regardless of the culture composition in goat's milk, however, *P. jensenii* 702 did require an initial inoculation level of 10^8 cfu/ml in order to maintain satisfactory viability at the end of storage. Therefore, co-cultivation of probiotics that demonstrate little or no antagonistic effect towards each other and satisfactory levels of inoculation can be considered as the critical factors involved in maintaining higher population levels of probiotic bacteria ($>10^7$ cfu/ml) in fermented goat's milk over the shelf life in the present study.

With the exception of fat content, significant differences were observed among the tested physico-chemical properties of samples over the storage period in relation to culture composition. In general, lowest pH, highest titratable acidity and highest lactic acid content were observed in fermented goat's milk with a monoculture of *L. acidophilus* LA-5. Higher pH, lower titratable acidity and lower lactic acid contents resulted for the samples with P, B, and P+B. It therefore appears that *L. acidophilus* LA-5 has greatly contributed to acidity development in the fermented goat's milk in this study. Syneresis values of all samples were increased during storage except those containing the monoculture of *L. acidophilus* LA-5 and co-cultures of *P. jensenii* 702 and *B. animalis* subsp. *lactis* BB-12. It therefore appears that factors other than acidity may influence the development of syneresis in fermented goat's milk.

Although some differences in the average consumer response to the tested sensory characteristics were apparent among the fermented goat's milk samples, these differences among samples were found to be statistically non significant. However, according to the score card overall sensory scores for these products remained low. The major objections of

the tasting panel to these products appeared to be those related to taste rather than the physical attributes of the products.

Co-cultivation of *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 in goat's milk with or without *P. jensenii* 702 remarkably improved the viability of *L. acidophilus* LA-5 and *B. lactis* BB 12 in the presence of simulated gastric juice. The effect of 0.3% bile was significant in reducing viability of all three probiotics and the combining of these strains in goat's milk did not improve their tolerance.

In contrast, the impact of combining probiotics on their capacity for adhesion to Caco-2 cells was clearly evident, with a 100-fold difference in the adhesion rate of *P. jensenii* 702 when co-cultured with *L. acidophilus* LA-5 as opposed to culturing with *B. animalis* subsp. *lactis* BB-12, the most obvious example. Only cultures containing *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 (L, B, L+B) were able to induce cytokine production by Caco-2 cells, while *P. jensenii* 702 appeared to suppress both IL-6 and TNF-α production from Caco-2 cells when co-cultured with these probiotics and combinations.

While the varying beneficial effects of different probiotics points to co-cultivation as an obvious strategy for providing a broad range of health benefits to the host, the identification of possible symbiotic or antagonistic interactions among probiotics with respect to microbial, physico-chemical, sensorial and functional properties, clearly deserves equal consideration in the development of probiotic foods.

The results of the present study provided no indication of any major disadvantages in using a cocktail of these three probiotics: *L. acidophilus* LA-5, *B. animalis* subsp. *lactis* BB-12 and *P. jensenii* 702, in fermented goat's milk. It was therefore determined that the triplet combination (L+P+B) would be utilised in producing goat's milk yogurts (chapter 4), ice cream (chapter 5) and in microencapsulation (chapter 7).

Chapter 4 : Probiotic viability and physico-chemical and sensory properties of plain and stirred fruit yogurts made from goat's milk

4.1. Introduction

Yogurt produced from cow's milk is widely consumed throughout the world. For example, recent market research has revealed that yogurt is the number one food eaten as a snack by children aged 2-17 in the USA (Nachay, 2010). However, there is also high demand for alternatives to cow's milk products for various reasons including a desire for novel tastes and problems associated with allergenicity and gastrointestinal disorders (Farnworth et al., 2007; Haenlein, 2004). As outlined in Chapter 1, goat's milk and goat's milk products are reported to have higher digestibility, lower allergenic properties and may provide additional therapeutic value to human nutrition compared to cow's milk and cow's milk products (Barrionuevo et al., 2002; Haenlein, 2004; Martin-Diana et al., 2003). Cheese and yogurt are the most popular and widely consumed goat's milk products (Pandya & Ghodke, 2007). Therefore, goat's milk yogurt may represent a suitable vehicle for delivering probiotics to humans.

Probiotic organisms have been extensively incorporated into dairy foods in recent years and yogurts containing *Lactobacillus acidophilus* and /or *Bifidobacterium* species are widely marketed (Gilliland, 2003; Shah, 2000). Lactobacilli and bifidobacteria can be considered the most commonly used probiotic genera in the yogurt industry, although probiotics from other genera such as propionibacteria may also possess characteristics that are desirable in the manufacturing of yogurt (Ekinci & Gurel, 2008). However, viability of probiotics in a yogurt matrix has been shown to be species specific. For example, *L. acidophilus* LAI has demonstrated a lower tolerance to the yogurt matrix than *B. bifidum* BBI in full fat and reduced fat yogurts (Vinderola & Reinheimer, 2000). Although the reduced fat yogurt in

that study demonstrated higher acidity development over the product shelf life, which is generally considered detrimental for probiotics (Dave & Shah, 1997d; Vinderola et al., 2002), full fat yogurt appeared to be an inhibitory medium for *B. bifidum* BBI compared to the reduced fat yogurt. This implies that the viability loss of probiotic bacteria may not only be governed by the acidity of the medium but also by other physico-chemical characteristics of the carrier food product (Vinderola et al., 2000a). These findings also indicate that some types of yogurt may not be a suitable vehicle for certain probiotic bacteria due to variations in physico-chemical properties. Therefore it is important to select a suitable combination of probiotic strains and starter culture bacteria when different types of yogurt are formulated. In addition, fortification of dairy products with different probiotic bacteria may potentially confer sensory advantages, and expand the variety of food and beverage products with additional health benefits to the host (Guler-Akin & Akin, 2007; Kneifel et al., 1993; Vinderola et al., 2000a). The growth and viability of the novel probiotic *P. jensenii* 702, when combined with yogurt starter cultures in either goat's milk or cow's milk, has not been studied previously.

Yogurts containing fruits are generally popular among consumers (Kailasapathy et al., 2008). As the characteristic "goaty" taste of goat's milk is unacceptable to many consumers (Slacanac et al., 2010), incorporation of fruit juice into the goat's milk yogurt may help to mask the characteristic unpleasant flavour of goat's milk and potentially increase acceptability. The effect of natural fruit juices on the growth of probiotics and yogurt starter culture has been reported to be species as well as strain specific (Vinderola et al., 2002). However, only a few studies have investigated the effect of added commercial fruit preparations on growth and survivability of probiotic bacteria (Kailasapathy et al., 2008; Nualkaekul & Charalampopoulos, 2011; Vinderola et al., 2002).

Besides being essential in the manufacturing of yogurt (Fonden et al., 2003; Giraffa et al., 1998), starter cultures have also been associated with better lactose digestion, absorption, and reduction of gastrointestinal symptoms in lactose intolerant subjects (Guarner et al., 2005; Pelletier et al., 2001; Rizkalla et al., 2000), positive effects in managing acute diarrheal disorders (Boudraa et al., 2001; Boudraa et al., 1990; Guarner et al., 2005) and

stimulation of the immune system (Guarner et al., 2005; Kitazawa et al., 2003; Meydani & Ha, 2000). While the application of the term "probiotic" to the starter cultures *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* is still strongly debated (Elli et al., 2006), due to certain factors such as their poor gastric acid tolerance (Conway et al., 1987) and moderate capacity for adhesion to intestinal epithelial cells (Greene & Klaenhammer, 1994), starter cultures may still be a significant factor in probiotic product development in terms of their potential effect on the growth and viability of other probiotic species under co-culture conditions.

When considered in total, the evidence outlined above suggests that the manufacturing of a novel yogurt made from goat's milk by incorporating *L. acidophilus* LA-5, *B. animalis* subsp. *lactis* BB-12 and *P. jensenii* 702, may have great commercial and nutritional value. In the present study the aim was to produce probiotic goat's milk yogurt with high consumer acceptability. The appropriate probiotic combination for goat's milk yogurt manufacture was chosen on the basis of the results obtained from the fermented milk study of Chapter 3. As explained previously (Chapter 2) the inoculation dosage for *L. acidophilus* LA-5, *B. animalis* subsp. *lactis* BB-12 and yogurt starter cultures applied in this study were based on manufacturer's recommendations. Results of the Chapter 3 study suggested that 10^8 cfu/ml was a suitable inoculation dosage for *P. jensenii* 702 in manufacturing fermented goat's milk. However, it was considered that the growth of *P. jensenii* 702 might improve during the incubation period of the yogurt due to the proteolytic activities of yogurt starter cultures. Therefore a preliminary test with two different incoculation levels (10^6 cfu/g and 10^8 cfu/g) was conducted in order to determine the optimum inoculation level for *P. jensenii* 702 in yogurt manufacturing.

While fruit juice was included in an attempt to improve the sensory acceptability of the goat's milk yogurt, it was recognized that the addition of fruit juice may impact negatively on other quality parameters of the yogurts such as acidity and syneresis. In an attempt to assess this impact, yogurts containing fruit juice in three different proportions (5, 10 and 15%) were produced and tested. The shelf life of the fruit juice was 30 days in unopened containers at refrigerated temperature, as confirmed by the manufacturer. Therefore,

viability of the probiotics in yogurts was tested only up to 4 weeks. With the exception of pH which was measured at 7 day intervals along with viable probiotic cell numbers, all physico-chemical properties and sensory characteristics of the products were analyzed once only. This measurement was made on day 7 of refrigerated storage, which had been identified previously by Iwalokun and Shittu (2007) as a suitable time for analyzing physico-chemical properties and sensory characteristics of plain and fruit flavoured yogurts.

4.1.1 Research hypotheses

It seems likely that the characteristic "goaty" taste of goat's milk, which is unacceptable to many consumers, may be lost during fermentation (Haenlein, 2004; Slacanac et al., 2010) and that the precise combination of included probiotics may determine the intensity of flavour development in the product during the fermentation process. In the previous experiment (Chapter 3) fermented goat's milk produced by co-culturing *P. jensenii* 702, *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12, was found to return relatively higher scores for aroma, taste, and overall acceptability, compared to similar products made with other probiotic combinations or monocultures.

Goat's milk is known to have a poor coagulum ability which often results in a semi liquid coagulum after fermentation (Martin-Diana et al., 2003), while *P. jensenii* has been characterized as a species which produces extracellular slime in liquid media (Ekinci & Barefoot, 2006; Ekinci & Gurel, 2008), which may in turn affect the physico-chemical properties of the final product. It is possible therefore, that the extracellular slime produced by *P. jensenii* 702 may strengthen the gel network of the coagulum and thereby improve certain physico-chemical properties of goat's milk yogurt. For example, beneficial effects in the controlling of post-fermentation acidification have been demonstrated in soy milk yogurt when *P. jensenii* 702 was incorporated with yogurt starter culture, by comparison with the same product manufactured without *P. jensenii* 702 (Kotula, 2008). A similar trend was observed in the previous experiment (Chapter 3) when *P. jensenii* 702 was co-cultured with *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 in manufacturing fermented

goat's milk. Given an inherently higher risk of over acidification due to the lower buffering capacity of goat's milk (Martin-Diana et al., 2003; Rysstad & Abrahamsen, 1983; Vegarud et al., 1999), incorporation of *P. jensenii* 702 may be beneficial in the manufacture of goat's milk yogurt. Development of higher acidity during fermentation and storage can lead to deterioration of the probiotic value and consumer acceptability of the final product (Dave & Shah, 1997d; Martin-Diana et al., 2003).

The results of the fermented goat's milk study (Chapter 3) have shown poor growth rates for *P. jensenii* 702 during the fermentation period regardless of the probiotic combinations. However, Kotula et al (2008) observed a significantly higher growth rate of *P. jensenii* 702 in the presence of starter cultures after 12 and 24 hours of incubation at 37^{o}C, followed by higher viability over 2 weeks of refrigerated storage in soy milk. On the basis of these findings it was hypothesized that:

1. The presence of yogurt culture bacteria *S. thermophilus* and *L. delbrueckii* subsp *bulgaricus* together with *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 in goat's milk yogurt would enhance the growth of the novel probiotic *P. jensenii* 702 during the incubation period, compared with that observed in fermented goat's milk (Chapter 3).
2. The presence of yogurt starter cultures together with *L. acidophilus* LA-5, *B. animalis* subsp. *lactis* BB-12 and *P. jensenii* 702 would result in a plain yogurt product with better consumer acceptability compared with the fermented goat's milk (Chapter 3).

In the previous experiment, all types of fermented goat's milk received low consumer acceptability for taste (Chapter 3), which may be largely attributable to a lack of sweetness as these products were all produced without any added sugars. Fruit juice would be an ideal additive for increasing the sugar content of the yogurt base, and hence the organoleptic properties of the yogurts. Fruit juices also contain simple sugars, vitamins, and minerals, which can be considered as potential growth promoters for the probiotics (Babu et al., 1992; Kailasapathy & Rybka, 1997; Zulueta et al., 2007). In a previous study, relatively higher sugar and fruit concentrations in frozen yogurts were reported to be more appealing

to consumers (Guven & Karaca, 2002). As such, within the context of the broader research hypotheses detailed above, two further hypotheses were tested:

1. That over the shelf life of the products, the viability of yogurt starter cultures and probiotic bacteria would improve in the fruit yogurts, relative to the plain yogurt, as the percentage of fruit juice added to the yogurt base was increased, and
2. That consumer acceptability of probiotic goat's milk yogurts would increase as the percentage of fruit juice in the product was increased.

Finally, via measurement of a range of relevant parameters, the study sought to address the question: In which ways does the addition of fruit juice affect the physico-chemical properties of goat's milk yogurt?

4.2. Materials and Methods

4.2.1 Microbiological, physico-chemical and sensory evaluation of goat's milk yogurts

A plain set type yogurt and stirred fruit yogurts were prepared as described in Chapter 2, (2.6.2).

Three samples of both the plain and each of the stirred fruit yogurts from refrigerated storage were used to enumerate probiotic and yogurt bacteria as described in Chapter 2 (2.7). The yogurt mixture was also assessed for yogurt starter and probiotic bacterial numbers before incubation. Coliform, yeast and mould counts were assessed after incubation and at 4 weeks after production.

Physico-chemical properties of plain and stirred fruit yogurts were measured as described in Chapter 2, (2.8).

Sensory evaluation of plain and stirred fruit goat's milk yogurts (stored at 4^{o}C) was conducted by examining the responses of 26 (15 male, 11 female) untrained taste panellists

aged 20-45 years, with tasting conducted one week after production as explained in Chapter 2, (2.10.1).

4.2.2 Statistical analysis

Data analyses were performed using SPSS/PASW statistical software version 17 (SPSS Inc., Chicago, IL, USA). Microbial viability data were analysed using repeated measure ANOVA. One way ANOVA was used to analyse data on physico-chemical properties. In both cases the Bonferroni post hoc test was performed for means comparison. Nonparametric tests were performed to determine the statistical differences of the sensory data. Where appropriate, T-tests were performed for comparison of two means. A p value <0.05 was considered statistically significant for all analyses.

4.3. Results

The presentation of data in this chapter begins with the basic microbiological aspects of this study covering the growth of probiotics and yogurt starter cultures in goat's milk during the fermentation/incubation period, their viability in plain and stirred fruit yogurts (5, 10 & 15% fruit juice) over 4 weeks of refrigerated storage, and the occurrence of undesirable microorganisms in the products with respect to coliform, yeast and mould counts. This is followed by an examination of changes in the pH over the shelf life, along with various other physico-chemical and organoleptic characteristics of these products, evaluated one week after production.

4.3.1 Microbiological analyses

Similar to the findings of Chapter 3, in this study a high inoculum level of *P. jensenii* 702 (10^8 cfu/g) was a crucial factor in maintaining higher viability at the end of the shelf life (10^8 cfu/g). The lower inoculum level of 10^6 cfu/g resulted in $<10^6$ cfu/g viable counts of *P. jensenii* 702 in goat's milk yogurts at the end of the shelf life. These results further confirmed the suggestion of Chapter 3 that a sufficient inoculation dosage of probiotic at

the time of manufacture must be ensured to obtain the recommended therapeutic minimum at the end of the shelf life. Therefore yogurt samples produced using the higher inoculum level of 10^8 cfu/g were analysed for physico-chemical properties and sensory attributes.

A significant level of growth was observed for both the starter cultures and *B. animalis* subsp. *lactis* BB-12 during the incubation period, however, significant change in the numbers of *L. acidophilus* LA-5 and *P. jensenii* 702 was not apparent (Table 4.1). These patterns of growth in the fermentation period were in some contrast with the trends observed in the viable counts recorded during refrigerated storage (Figure 4.1 and 4.2).

Table 4.1 Number of viable bacteria in goat's milk yogurt before and after incubation at 42°C (log cfu/g), (*n* = 6)

Organisms	Before incubation	After incubation
S. thermophilus	6.82± 0.07[a]	7.82± 0.07[b]
L. delbrueckii subsp. *bulgaricus*	7.40± 0.04[a]	8.58± 0.07[b]
L. acidophilus LA-5	7.51± 0.04[a]	7.55± 0.07[a]
B. animalis subsp. *lactis* BB-12	7.71± 0.06[a]	8.50± 0.07[b]
P. jensenii 702	8.63± 0.02[a]	8.51± 0.09[a]

Mean values (±SE)

[a, b] Values in the same row having different superscripts differ significantly ($p<0.05$)

Yogurt starter culture bacteria *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* demonstrated a better survival in plain yogurts compared to the stirred fruit yogurts. A slight reduction in the viability of the starter culture *S. thermophilus* was apparent in all types of yogurts by the end of the storage time while counts of *L. delbrueckii* subsp. *bulgaricus* were found to decrease significantly in the fourth week of storage from almost 10^8 cfu/g to approximately 10^5 cfu/g in all stirred fruit yogurts, and 10^6 cfu/g in plain yogurts. Although addition of fruit juice appeared to produce a positive impact on survival of *L. acidophilus* LA-5, viable numbers of *L. acidophilus* LA-5 fell below the accepted therapeutic threshold (10^6 cfu/g) regardless of the type of yogurt by the end of the shelf life.

Both *B. animalis* subsp. *lactis* BB-12 and *P. jensenii* 702 had survived well (10^7-10^8 cfu/g) at the end of the product shelf life regardless of the fruit juice level.

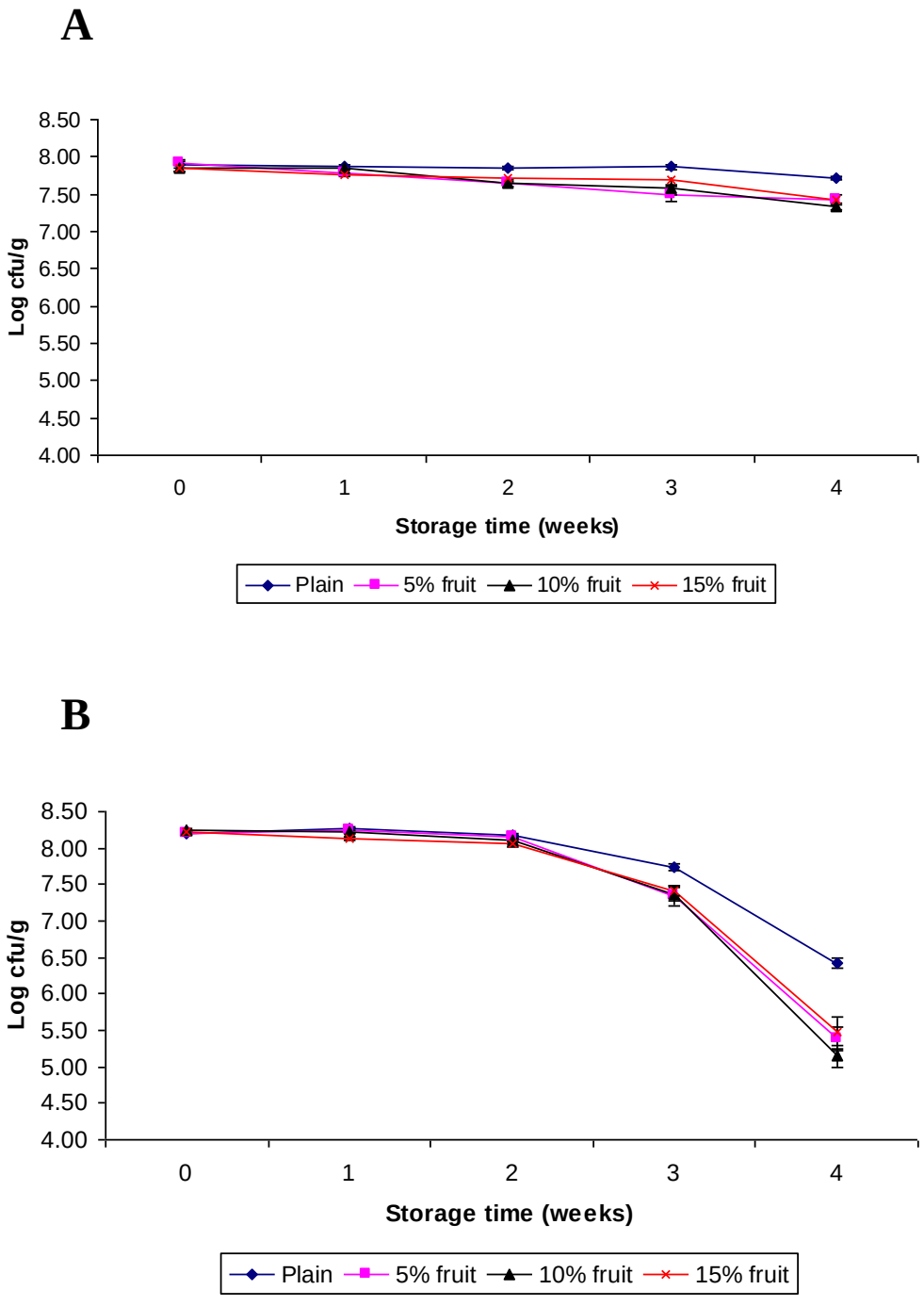
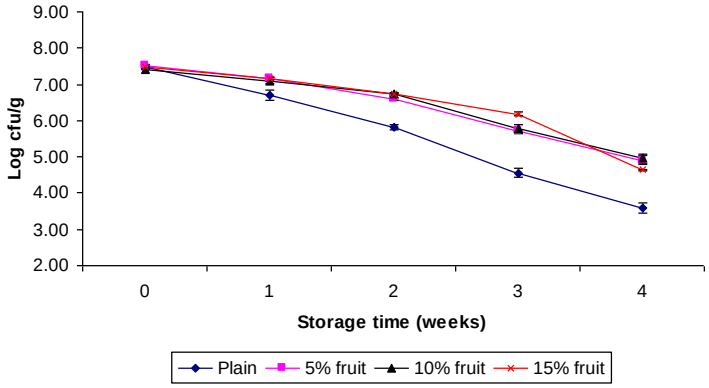
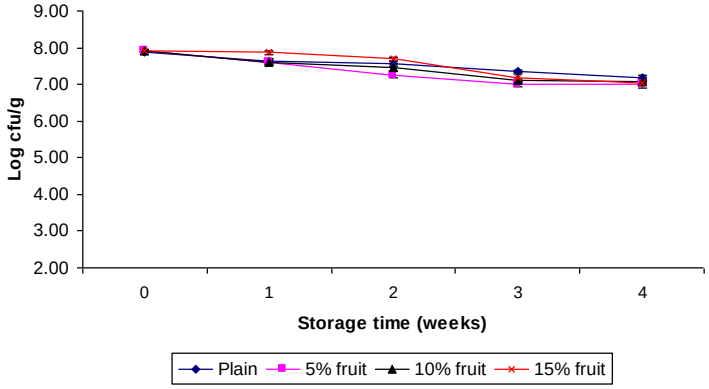


Figure 4.1 Viable counts of yogurt starter culture bacteria *S. thermophilus* (A) and *L. delbrueckii* subsp. *bulgaricus* (B) in plain and stirred fruit (5, 10, 15%) goat's milk yogurts during 4 weeks of storage at 4°C.

A



B



C

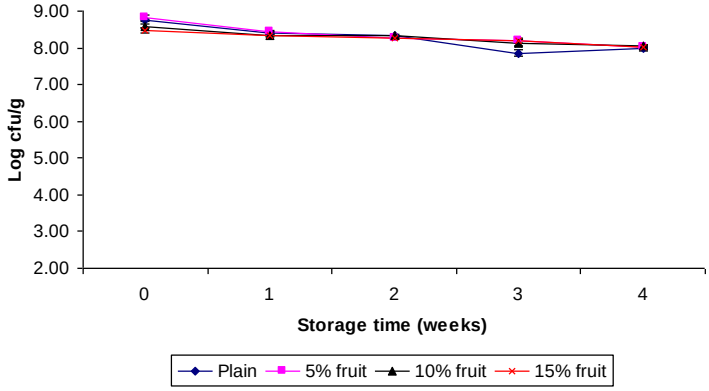


Figure 4.2 Viable counts of probiotic bacteria *L. acidophilus* LA-5 (A), *B. animalis subsp. lactis* BB-12 (B) and *P. jensenii* 702 (C) in plain and stirred fruit (5, 10 and 15%) goat's milk yogurts during 4 weeks of storage at 4°C.

Coliform counts of samples were zero and yeast and mould counts were <1 cfu/g at the beginning and at the end of the storage, in all preparations.

4.3.2 Physico-chemical properties

In general the pH of goat's milk was found to vary widely during both the manufacturing of yogurts and in subsequent storage. The pH of the goat's milk used in probiotic yogurt production was 6.64 (±0.01) and the titratable acidity was 0.15 % (± 0.01). The pH, titratable acidity and the brix values of the fruit juice used in stirred fruit yogurt production were 3.64 (±0.02), 1.18% (±0.01), and 12.13 (±0.07) respectively. The initial pH of the milk 6.64 (±0.01) was reduced to 4.38 (±0.01) ($p<0.05$) during yogurt production in approximately 3 ½ hours, in line with the growth of the starter culture and the probiotic bacteria during incubation (Table 4.1).

The average pH of plain yogurt was found to have further decreased to 4.24 (±0.02) by the end of the shelf life ($p<0.05$). The initial average pH of the 5, 10 and 15% stirred fruit yogurts were 4.27 (±0.01), 4.26 (±0.01), and 4.24 (±0.01) respectively. There was a significant difference in pH between plain yogurt and all of the stirred fruit yogurts at the end of the shelf life. The final pH values of 5, 10 and 15% stirred fruit yogurts were 4.15 (±0.01), 4.14 (±0.01) and 4.16 (±0.00) respectively (Figure 4.3).

There were also statistically significant differences between the mean levels of acidity of the yogurt samples, although it seems unlikely that the magnitude of these differences would be sufficient to produce distinguishable physico-chemical or sensory variation between the products. Total solids, protein, fat and ash values were all found to decrease as the fruit juice levels of the stirred fruit yogurts were increased. Water holding capacity and viscosity of plain yogurts were found to be significantly higher than that of all the types of stirred fruit yogurts while syneresis was significantly lower in plain yogurts (Table 4.2).

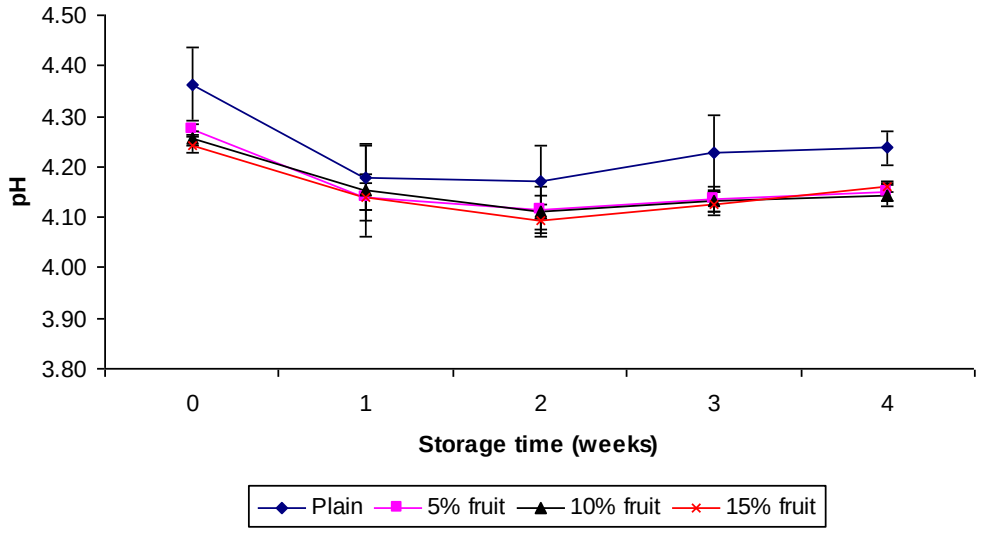


Figure 4.3 Changes of pH in plain and stirred fruit (5, 10 and 15%) goat's milk yogurts during 4 weeks of storage at 4°C.

Table 4.2 Mean values for physico-chemical characteristics of plain and stirred fruit (5, 10, 15%) goat's milk yogurts at one week after production (*n* = 3)

Characteristic	Plain	5%	10%	15%
Titratable acidity (%)	1.39± 0.01^{a,b}	1.43± 0.00^{a}	1.40± 0.00^{ab}	1.38± 0.02^{b}
Total solids (%)	16.12± 0.02^{a}	15.90± 0.04^{ab}	15.62± 0.00^{b}	15.30± 0.01^{c}
Protein (%)	5.39± 0.03^{a}	5.27± 0.02^{a}	5.03± 0.01^{b}	4.70± 0.06^{b}
Fat (%)	5.37± 0.17^{a}	4.90± 0.06^{a}	4.90± 0.10^{a}	3.70± 0.12^{b}
Ash (%)	1.12± 0.00^{a}	1.06± 0.01^{b}	1.04± 0.01^{b}	1.02± 0.01^{b}
WHC (%)	61.34± 0.35^{a}	57.87± 0.88^{b}	53.94± 0.53^{c}	53.64± 0.29^{c}
STS (%)	22.33± 0.32^{a}	32.00± 0.58^{b}	33.00± 1.53^{b}	33.33± 1.77^{b}
Viscosity (cP)	23834.92± 1181.77^{a}	13237.17± 121.63^{b}	12817.25± 121.63^{bc}	12257.39± 20.00^{c}

Mean value (±SE)

$^{a, b, c}$ Values in the same row having different superscripts differ significantly ($p<0.05$)

4.3.3. Sensory evaluation

All stirred fruit yogurts were scored higher on average by the panellists than plain yogurt in terms of colour and appearance, aroma, taste, body and texture, and overall acceptability, however, differences for colour and appearance, and aroma were not statistically significant. Among the tested sensory characteristics, taste received the lowest mean scores in all preparations. Colour and appearance of the yogurt samples was scored most highly for all four preparations, while among the various preparations addition of 10% fruit juice resulted in the highest overall scores for sensory attributes (Table 4.3).

Table 4.3 Mean scores of tasting panellists (*n* = 26) for sensory properties of plain and stirred fruit (5, 10, 15%) goat's milk yogurts at one week after production

Characteristic	Plain	5%	10%	15%
Colour & appearance	7.00± 0.28[a]	7.15± 0.26[a]	7.45± 0.20[a]	7.19± 0.26[a]
Aroma	5.58± 0.36[a]	6.04± 0.37[a]	6.35± 0.33[a]	6.04± 0.40[a]
Body & texture	5.23± 0.32[a]	5.69± 0.29[ab]	6.04± 0.28[b]	5.54± 0.39[ab]
Taste	2.96± 0.29[a]	3.65± 0.35[a]	4.65± 0.35[b]	4.85± 0.45[b]
Overall acceptability	3.62± 0.24[a]	4.38± 0.32[b]	5.19± 0.32[c]	5.12± 0.44[bc]

Mean value (±SE)

[a, b, c] Values in the same row having different superscripts differ significantly ($p<0.05$)

(The scale for sensory scores: Like extremely = 9, Like very much = 8, Like moderately = 7, Like slightly = 6, Neither like nor dislike = 5, Dislike slightly = 4, Dislike moderately = 3, Dislike very much = 2, Dislike extremely = 1).

Summary of key findings

Both the starter cultures and *B. animalis* subsp. *lactis* BB-12 have demonstrated significant growth during the incubation period. Data on the viable cell counts suggested that neither

P. jensenii 702 nor *L. acidophilus* LA-5 reached exponential growth phase by the end of the incubation period. Fruit juice had a negative influence on the viability of both starter cultures, but a positive influence on the viability of *L. acidophilus* LA-5, during storage. However, counts of *L. acidophilus* LA-5 were found to decrease significantly in the fourth week of storage to $< 10^6$ cfu/g in all types of yogurts. In contrast both *B. animalis* subsp. *lactis* BB-12 and *P. jensenii* 702 were able to maintain high viability (10^7-10^8 cfu/g) at the end of the shelf life in goat's milk yogurts regardless of the fruit juice levels. It appeared that the higher inoculum level of 10^8 cfu/g for *P. jensenii* 702 was pre-requisite to maintaining high viability of this organism in goat's milk yogurts throughout the shelf life.

Several significant differences were evident among the plain and stirred fruit yogurts with respect to their physico-chemical and sensory properties. Most importantly, the scores recorded for all sensory characteristics demonstrated that in general the addition of fruit juice enhanced the sensory appeal of the yogurt.

4.4 Discussion

The main objectives of this study were to examine the effect of added fruit juice on the viability of *P. jensenii* 702 and other probiotics in goat's milk yogurt, and the effect on physico-chemical properties and general consumer acceptability of these products. Of particular interest among the findings were apparent strain specific variations in viability, relationships between pH and probiotic viability over 4 weeks of storage, and specific relationships between physico-chemical parameters and the sensory appeal of the goat's milk yogurts.

4.4.1 Microbiological analyses

It was expected that significant growth of all five bacterial species would occur during the fermentation/incubation period, however, this was observed only for the two starter cultures and *B. animalis* subsp. *lactis* BB-12. In this experiment, both starter cultures have demonstrated an approximate10-fold increase in cell number during the fermentation. The

successful co-cultivation of *S. thermophilus* and *L. bulgaricus* during the fermentation of yogurts probably reflects a degree of symbiosis between them as reported previously (Lourens-Hattingh & Viljoen, 2001; Radke-Mitchell & Sandine, 1984). Generally, *S. thermophilus* grows quickly in milk at first by utilizing essential amino acids produced by *L. bulgaricus* due to its proteolytic nature. This is followed by production of lactic acid and carbon dioxide by *S. thermophilus*, which results in a reduction of the pH of the medium to an optimal level for *L. bulgaricus* growth, thereby stimulating further growth of *L. bulgaricus* (Giraffa et al., 1998; Lim et al., 2009; Lourens-Hattingh & Viljoen, 2001).

As outlined previously, probiotics generally possess slower growth in milk compared to starter cultures due to their weak proteolytic activity (Gautier & Richard, 1999; Janer et al., 2004; Shah, 2007). However, *B. animalis* subsp. *lactis* BB-12 demonstrated a significantly higher growth rate during fermentation than *L. acidophilus* LA-5 and *P. jensenii* 702 in the present study. The proteolytic nature of starter cultures, particularly *L. delbrueckii* subsp. *bulgaricus*, and growth stimulation by other probiotics, may have facilitated this growth of *B. animalis subsp. lactis* BB-12 during incubation. Growth stimulation of *B. animalis subsp. lactis* BB-12 by *L. acidophilus* LA-5 and *P. jensenii* 702 was suggested as a possible explanation for trends observed in the results of the previous experiment (Chapter 3). Production of growth stimulators for *Bifidobacterium* spp. by *Propionibacterium* spp. has been demonstrated by Kaneko et al. (1994).

The incubation temperature may also have contributed to the observed growth differential between *B. animalis* subsp. *lactis* BB-12 and the other probiotics. Generally incubation is conducted at 42-43^{o}C in order to achieve the desirable qualities of yogurts through growth stimulation of the starter cultures. This is above the optimum growth temperature ranges of lactobacilli (30-40^{o}C) (Batt & Richard, 1999) and propionibacteria (25-32^{o}C) (Gautier & Richard, 1999), but not so for bifidobacteria which can demonstrate optimum growth at temperatures as high as 41^{o}C (Shah, 2007). Thus the slower growth of *L. acidophilus* LA-5 and *P. jensenii* 702 observed, may also have been associated with the higher incubation temperature (42^{o}C) employed in the present study. In fact, similar to the findings of Chapter 3, *P. jensenii* 702 cell numbers were found not to have increased at all during the

incubation period, thus the findings did not support the hypothesis that yogurt starter cultures would stimulate the growth of *P. jensenii* 702 during incubation.

A slight reduction in the viability of the starter culture *S. thermophilus* was apparent in all types of yogurts by the end of the storage time. Several previous studies have reported a slight increase of *S. thermophilus* counts during storage up to one week, followed by a later decrease of about one log cycle, in goat's milk yogurt (Guler-Akin & Akin, 2007) and cow's milk yogurt (Birollo et al., 2000; Dave & Shah, 1997d). While the same trend was not observed in this experiment, this may possibly reflect differences in the yogurt manufacturing process, storage conditions, and use of different probiotic strains among these different studies.

Briollo et al. (2000) and Vinderola, et al. (2000a) have reported higher numbers of *S. thermophilus* than *L. delbrueckii* subsp. *bulgaricus* in yogurt. In the current study, there were higher viable counts for *L. delbrueckii* subsp. *bulgaricus* compared to *S. thermophilus* in all types of yogurts after incubation, however, in line with previous observations by Dave and Shah (1997d) and Tabasco et al. (2007) the viability of *S. thermophilus* remained well above that of lactobacilli at the end of the storage period. The plastic cups used for the storage of yogurts in the current study may have contributed to the observed higher viability of *S. thermophilus*. The use of plastic cups has been reported to improve the growth and viability of *S. thermophilus*, due to a high amount of oxygen permeation during storage of yogurt (Dave & Shah, 1997d). This is because *S. thermophilus,* which is not prone to extensive oxidative damage upon exposure to oxygen - due to possession of oxygen scavenging mechanisms such as catalase enzymes - relies heavily on aerobic respiration to power its metabolic activity (Talwalkar & Kailasapathy, 2004c).

By comparison, counts of *L. delbrueckii* subsp. *bulgaricus* were found to decrease significantly in the fourth week of storage, from almost 10^8 cfu/g to approximately 10^5 cfu/g in all stirred fruit yogurts, and 10^6 cfu/g in plain yogurts. This is in some contrast with the report of Vinderola et al. (2000a) who found no significant difference in *L. delbrueckii* subsp. *bulgaricus* numbers in yogurt after 4 weeks of storage time at 5^oC. During the

second and third weeks of storage in the present study *L. delbrueckii* subsp. *bulgaricus* was able to maintain a high viability (10^7-10^8 cfu/g) in yogurts regardless of the added levels of fruit juice. Similar results were reported by Guler-Akin et al. (2007). These authors further observed a higher number of *L. delbrueckii* subsp. *bulgaricus* in the yogurt incubated at 42^{o}C compared to 37^{o}C, suggesting stimulated growth of *L. delbrueckii* subsp. *bulgaricus* at 42^{o}C – the incubation temperature employed in the present study.

Significantly higher viability of both *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* was observed in plain yogurt compared to any of the stirred fruit yogurts at the end of storage. This apparent negative impact of added fruit juice on the starter culture bacteria may have been related to acid injury, as previously observed by Vinderola et al. (2002). Acid stress may inhibit bacterial growth by acidifying the cytoplasm (Russell, 1992; Shabala et al., 2006), increasing energy consumption required for maintenance of intracellular pH (O'Sullivan & Condon, 1999) and inhibiting important enzymatic reactions (Shabala et al., 2006). Lower acid tolerance of *S. thermophilus* (Iyer et al., 2010a; Vinderola et al., 2002; Zotta et al., 2009) and *L. delbrueckii* subsp. *bulgaricus* (Conway et al., 1987; Vinderola et al., 2002) was previously reported by other researchers. Some reports suggest that intracellular pH in neutrophilic bacteria is maintained at an almost constant level despite variations in the pH of the environment, but when the difference between intracellular pH and extracellular pH becomes too high, intracellular pH homeostasis cannot be maintained ultimately resulting in cell death (Christensen & Hutkins, 1992; Shabala et al., 2006). It seems likely that the pH drop in the yogurt base when fruit yogurts were produced (Figure 4.3) may have contributed to the lower viability of starter culture bacteria in stirred fruit yogurts.

Lower recovery of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* has also been reported in sugar based media containing maltose, galactose, sorbitol, mannitol and esculin. Interestingly, all these media were very effective in recovering *L. acidophilus* (Tharmaraj & Shah, 2003). Likewise, high sugar content in the fruit juice (Chapter 2, section 2.6.2) may have contributed to lowering the viability of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* while improving the viability of *L. acidophilus* LA-5 in fruit yogurts.

Bacteriocin produced by *L. acidophilus* LA-1 has also been previously confirmed to be active against several strains of *L. delbrueckii* subsp. *bulgaricus* (Dave & Shah, 1997a). Although a different *L. acidophilus* strain (LA-5) was used in this study, it is conceivable that this strain may also produce bacteriocins active against *L. delbrueckii* subsp. *bulgaricus*. Furthermore, Dave and Shah (1997a) observed higher bacteriocin production by *L. acidophilus* LA-1 in media containing various sugars. Due to the higher sugar content of stirred fruit yogurts such a phenomenon would be consistent with the observed lower viability of *L. delbrueckii* subsp. *bulgaricus* in the stirred fruit yogurts of this study, compared to the plain yogurt.

Even though *L. delbrueckii* subsp. *bulgaricus* does not require strict anaerobic growth conditions and can tolerate oxygen, it has been shown that the presence of oxygen in its environment can influence its physiology (Marty-Teysset et al., 2000). Marty-Teysset et al. (2000) observed that detoxification of oxygen by the bacteria led to an over production of hydrogen peroxide that caused oxidative stress, triggering an early entry of the culture into stationary phase. Oxygen incorporation through stirring, may therefore cause additional stress to *L. delbrueckii* subsp. *bulgaricus* and result in lower viability in stirred fruit yogurts compared to the plain yogurt which is not stirred. Superimposed on this effect would be oxygen permeation of the plastic cups used to store the yogurt, which might also contribute to the overall lower viability of *L. delbrueckii* subsp. *bulgaricus* at the end of the shelf life, regardless of the type of yogurt (Figure 4.1).

Although addition of fruit juice appeared to provide a significant positive impact on the survival of *L. acidophilus* LA-5, the viability of this organism was found to be unsatisfactory at the end of the shelf life ($<10^6$ cfu/g) in all yogurt types. In contrast to these findings Kailasapathy et al. (2008) reported high viability ($>10^7$ cfu/g) of *L. acidophilus* in stirred fruit yogurts made with commercial fruit mixes up to 5 weeks. However the study of Kailasapathy et al (2008) examined a different *L. acidophilus* strain (LAFTI L10), thus the contrasting results may simply reflect differences in acid tolerance of the two *L. acidophilus* strains. Different strains of *L. acidophilus* are known to vary in their ability to retain viability in fermented dairy foods during refrigerated storage (Nighswonger et al.,

1996). Furthermore, the starter culture used by Kailasapathy et al. (2008) consisted of *S. thermophilus* only. *L. delbrueckii* subsp. *bulgaricus* has been reported to be detrimental to *L. acidophilus*, due to production of hydrogen peroxide by *L. delbrueckii* subsp. *bulgaricus* in yogurt, which might in turn cause partial injury to the active cells of *L. acidophilus* (Dave & Shah, 1997d; Ng et al., 2011). However, *L. acidophilus* LA-5 was able to maintain the suggested minimum therapeutic level ($>10^6$ cfu/g) up to 2 weeks in storage in the present study. This improved viability of *L. acidophilus* LA-5 in stirred fruit yogurts may have been due to the availability of nutrients such as sugars, minerals, and vitamins supplied through the fruit juice although similar trends in relation to the fruit juice were not observed in the case of *B. animalis* subsp. *lactis* BB-12 or *P. jensenii* 702. Simple sugars mainly glucose and fructose, and minerals such as magnesium and manganese can be considered growth promoters for *L. acidophilus* (Babu et al., 1992; Kailasapathy & Rybka, 1997). The viability of *L. acidophilus* LA-5 was not influenced however, by the different fruit juice levels used in the manufacturing of stirred fruit yogurts in the present study, which is consistent with the findings of Kailasapathy et al. (2008). However, the type of fruit juice used in manufacturing stirred fruit yogurts was reported to affect the viability of both *L. acidophilus* and *B. animalis* ssp. *lactis* in that study (Kailasapathy et al., 2008), perhaps reflecting the effect of slight variations in the nutritional composition of different fruit juices on the growth of these microbes. Mineral (Hamurcu et al., 2010; Leterme et al., 2006; Wall, 2006), sugar (Rodriguez-Saona et al., 2001; Wall, 2006) and vitamin contents (Franke et al., 2004; Wall, 2006) which may all affect probiotic growth and viability, are known to vary among different fruits.

Vinderola et al. (2000a) have suggested that full fat yogurt (3.0% w/w) may be a more inhibitory medium for some probiotics than reduced fat yogurt (0.2% w/w), having observed a lower viability for *L. acidophilus* in full fat yogurts. It is generally accepted that the fat content is an integral part of yogurt microstructure. Therefore, changes in microstructure due to differing fat content may provide an unsatisfactory environment for certain probiotics. The higher fat content in plain yogurt compared to stirred fruit yogurts (Table 4.2) may have had a negative impact on the viability of *L. acidophilus* LA-5. However, in this study fat content was significantly lower only in 15% stirred fruit yogurts,

while significantly higher viability of *L. acidophilus* LA-5 was recorded in all stirred fruit yogurts compared to the plain yogurt during storage. This is more consistent with the conclusion of Micanel et al (1997) that varying fat content may not have any effect on probiotic viability during storage. Thus the viability loss of *L. acidophilus* LA-5 observed in the present study was perhaps more related to antagonistic interactions between *L. acidophilus* LA-5 and *L. delbrueckii* subsp. *bulgaricus*, rather than the effect of fat content. *L. acidophilus* LA-5 was able to maintain a relatively stable viability (10^7-10^8 cfu/ml) in fermented goat's milk preparations which were devoid of yogurt starter cultures during three weeks of storage (Chapter 3). The survival of *L. acidophilus* LA-5 was also found to be higher in stirred fruit yogurts compared to plain yogurt, while *L. delbrueckii* subsp. *bulgaricus* demonstrated higher viability in plain yogurt (Figure 4.2). Therefore, it might be concluded that the fruit juice may have affected the viability of *L. delbrueckii* subsp. *bulgaricus* in the stirred fruit yogurts while an antagonistic effect of *L. delbrueckii* subsp. *bulgaricus* towards the *L. acidophilus* LA-5 may have resulted in reduced viability of the latter in plain yogurt.

In this study the acidity of the yogurt samples increased when fruit juice was added, and continued to increase during the storage period, which may also have impacted on the viability of *L. acidophilus* LA-5. In agreement with the findings of Kailasapathy and Rybka (1997) and Ng et al. (2011), *L. acidophilus* LA-5 survived better in more acidic stirred fruit yogurts, confirming the apparent acid tolerance of this species. *L. acidophilus* has high cytoplasmic buffering capacity and membrane H^+ conductance which may allow them to resist changes in cytoplasmic pH and gain stability under acidic conditions (Rius et al., 1994). Viability of *L. acidophilus* was reported by Kailasapathy et al. (2008) to be higher in yogurt with pH between 4.1 and 4.5, a similar pH variation to that recorded in the present study. This is in contrast however to the findings of Vinderola et al. (2002) who reported a significantly higher loss in cell viability for *L. acidophilus* under acidic conditions (pH 4.0-5.0). These variations in acid tolerance ability of *L. acidophilus* could possibly be explained by the factors such as differences in carrier food matrices, food additives (sweeteners and flavouring agents) and strains used among these two studies.

In contrast to the implications described earlier for *S. thermophilus*, the use of plastic cups for the storage of yogurts may, due to their oxygen permeability and the importance of redox potential to the viability of *L. acidophilus*, have a negative effect on the viability of *L. acidophilus* (Dave & Shah, 1997d) and may therefore have contributed to the reduced viability of *L. acidophilus* LA-5 observed in the present study. It should also be recognized however, that the fruit mix used in the stirred yogurts contained a significant amount of the oxygen scavenging ascorbic acid (vitamin C) (Chapter 2, section 2.6.2) which would presumably help to counter any such effect and sustain the viability of *L. acidophilus* LA-5 in stirred fruit yogurts compared to plain yogurt. The oxygen scavenging ability of ascorbic acid has however been previously reported not to have a positive effect on viability of bifidobacteria (Dave & Shah, 1997b; Dave & Shah, 1997c). Incorporation of air when fruit juice was stirred into the yogurt mix may also have a negative impact on *L. acidophilus* LA-5 viability during storage. Such effects could be minimized by using vacuum lines in manufacturing stirred yogurt (Kailasapathy et al., 2008) and the use of glass containers, where oxygen permeation is minimal (Dave & Shah, 1997b). It should be recognized here that the fruit juice used in this study was also devoid of any preservatives that may have adversely affected the viability of *L. acidophilus* LA-5.

Acidity is also considered one of the most critical factors affecting the viability of bifidobacteria (Dave & Shah, 1997d). However, sensitivity of probiotics to lower pH in yogurt may be species and strain specific (Cruz et al., 2010b). Recent research discovered that *B. longum* was more susceptible to acidity compared to *B. animalis* ssp. *lactis* (Jayamanne & Adams, 2009). Similar differences in the acid tolerance of *B. animalis* subsp. *lactis* BB-12 and *L. acidophilus* LA-5 have been recorded in yogurts in the present study. Unlike *L. acidophilus*, the effect of hydrogen peroxide on bifidobacteria has been reported to be minimal (Dave & Shah, 1997d). Thus, bifidobacteria may survive better in the presence of *L. delbrueckii* subsp. *bulgaricus* compared to *L. acidophilus*. As previously explained, the highly proteolytic *L. delbrueckii* subsp. *bulgaricus* may also have provided the essential amino acids required for the growth of *B. animalis* subsp. *lactis* BB-12 during incubation (Dave & Shah, 1997b; Dave & Shah, 1997d; Guler-Akin & Akin, 2007) and this improved growth of *B. animalis* subsp. *lactis* BB-12 during incubation may have helped to

maintain a higher viability during storage in this study. However, it should also be noted that even without significantly improved growth during incubation, the viability of *B. animalis* subsp. *lactis* BB-12 in fermented goat's milk (Chapter 3), in which *L. delbrueckii* subsp. *bulgaricus* was not present, was just as stable as in the yogurt during this study.

Strain combination is clearly one of the factors that should be carefully considered in developing probiotic products (Kailasapathy et al., 2008; Vinderola et al., 2000a). In the present study, a combination of *P. jensenii* 702 with *Bifidobacterium* appeared to be very effective in the manufacturing of yogurt since both bacteria had survived well (10^7-10^8 cfu/g) at the end of the product shelf life.

4.4.2 Physico-chemical characteristics

The pH is a key physico-chemical parameter determining the quality of yogurt. In milk fermentation, the pH is allowed to drop to 4.3-4.5 (Fonden et al., 2003; Shah, 2000) in order to achieve desirable characteristics in final product. In the present study, the pH of goat's milk demonstrated wide variations during manufacturing of yogurts and in subsequent storage. The pH drop of the milk within about 3 ½ hours of fermentation was in line with satisfactory growth of the starter culture and the probiotic bacteria during incubation (Dave & Shah, 1997d). A similar incubation time for goat's milk yogurt production with the starter cultures *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* was previously observed by Guler-Akin and Akin (2007) at the incubation temperature of 42^{o}C. It is widely accepted that that the growth of microorganisms in milk will produce a reduction in pH. During fermentation, lactic acid bacteria ferment lactose, increase the lactic acid content and thereby lower the pH (Fonden et al., 2003; Kailasapathy & Rybka, 1997; Rybka, 1994). Among these microorganisms, *L. delbrueckii* subsp. *bulgaricus* could be greatly responsible for pH changes within such a short period of time, because cultures containing only *S. thermophilus*, *L. acidophilus* LA-5 and *Bifidobacterium* BB-12 have been shown to take about 10 hours of incubation to reach pH 4.5 in goat's milk (Martin-Diana et al., 2003). After 10 hours of fermentation time at 37^{o}C, the pH value of the fermented goat's milk produced with a combination of the probiotics *L. acidophilus* LA-5,

B. animalis subsp. *lactis* BB-12 and *P. jensenii* 702, without any starter cultures, was 5.57 (Chapter 3). *L. delbrueckii* subsp. *bulgaricus* showed higher growth during the incubation period (approximately one log increase) in the present study. Propionibacteria, as a sole starter culture have previously demonstrated slower growth in milk with a significant effect on increasing incubation time (~ 9 hours) to reach pH 4.6 in the final product (Ekinci & Gurel, 2008). Similarly, *P. jensenii* 702 demonstrated a slower growth in goat's milk during incubation in the present study (Table 4.1). It might therefore be concluded that *L. delbrueckii* subsp. *bulgaricus* was largely responsible for reducing the pH within a shorter incubation time in the present study.

Due to the higher acidity of the fruit juice compared to the yogurt base, declines in the pH of the yogurt base were noted immediately following production of stirred fruit yogurts. Posecion et al. (2005) observed the same trend in fruit-flavoured sundae-style goat's milk yogurts. Further, overall declines in the pH of all types of stored yogurts were also observed during the shelf life (Figure 4.3). Similar results have been reported by several other researchers for goat's milk yogurts (Guler-Akin & Akin, 2007) and cow's milk yogurts (Dave & Shah, 1997d; Ekinci & Gurel, 2008; Kailasapathy et al., 2008; Vinderola et al., 2000a). This pH decline is most likely due to continued fermentation by the lactic acid bacteria. Yogurts in this study were produced using a culture containing both *S. thermophilus* and *L. delbrueckii* spp. *bulgaricus* which accelerate post fermentation acidification in yogurt during storage compared to starter cultures which are devoid of *L. delbrueckii* spp *bulgaricus* (Kailasapathy et al., 2008; Lourens-Hattingh & Viljoen, 2001). Since a low pH environment generally decreases probiotic survival (Kailasapathy & Rybka, 1997), the pH of the product is not only critical in determining the physico-chemical and sensorial quality of probiotic products, but also viability of the probiotics. Therefore, it is very important to select a combination of cultures that do not over accelerate post-fermentation acidification when combining probiotics with both starter cultures. The pH of all types of yogurts in this study were around 4.2, which is recognized as the 'normal' pH for yogurt by Champagne et al. (2005) and Rompf et al. (1999), indicating the possibility of using *P. jensenii* 702 together with *L. acidophilus* LA-5, *B. animalis* subsp. *lactis* BB-12

and both starter cultures (including higher acid producing *L. delbrueckii* spp *bulgaricus*) in yogurt manufacturing.

Total solids, protein, fat and ash contents were all found to be highest in plain yogurts and lowest in 15% fruit yogurts (Table 4.2) reflecting the higher moisture content of the fruit yogurts due to addition of fruit juice. Such compositional changes in total solid, protein, fat and ash in manufacturing fruit flavoured stirred yogurts were previously reported by other researchers (Tarakci, 2010; Tarakci & Kucukoner, 2004). Changes in these parameters, especially total solids and fat content may affect certain other physico-chemical properties such as syneresis, water holding capacity and viscosity.

Syneresis was found to be significantly lower in plain yogurt than in the stirred fruit yogurts regardless of the added fruit juice levels, perhaps because it possessed the highest total solids, protein, fat, and ash content. Relatively higher total solids (Isleten & Karagul-Yuceer, 2006) and fat content (Isanga & Zhang, 2009; Keogh & O'Kennedy, 1998) in yogurt has been associated with lower syneresis values in previous experiments. As syneresis is essentially a function of the stability of the gel matrix of the product, it is feasible that in this study, disruption of the gel network through stirring of the yogurts with fruit juice may also have contributed to greater syneresis. The acidity of the yogurts may have been a further contributing factor, since higher acidity is known to stimulate syneresis in yogurt (Tamime & Robinson, 1999), although a comparison of the patterns in syneresis, pH and titratable acidity between the various yogurts after one week of storage, would suggest that acidity was not the driving force in this case.

Wu et al. (2001) demonstrated that water holding capacity was related to the ability of the proteins to retain water within the yogurt structure. These researchers further suggested that fat globules in the milk may also play an important role in retaining water. In this study, plain yogurts demonstrated significantly higher water holding capacity compared to stirred fruit yogurts, possibly reflecting the higher protein and fat content of the plain yogurt compared to fruit yogurt (Table 4.2).

The viscosity of the plain yogurt was also found to be higher than that of all the types of stirred fruit yogurts, in line with the higher level of total solids in plain yogurts as described by Isanga and Zhang (2009), Martin-Dianna et al. (2003) and Tamime et al. (Tamime & Robinson, 1999). Isanga and Zhang (2009) further reported that higher levels of fat may also contribute to a higher viscosity of yogurts where homogenized milk is used in production, as was the case in the study presented here, since homogenization facilitates copolymer formation between casein and the fat globules thereby strengthening the gel network (Shaker et al., 2000).

4.4.3 Sensory evaluation

The scores recorded for body and texture, taste and overall acceptability indicated that in general the addition of fruit juice positively influenced the sensory characteristics of the yogurt (Table 4.3). All stirred fruit yogurts were scored higher on average by the panellists than plain yogurt in terms of aroma and taste (although differences for aroma were not statistically significant), possibly reflecting a combined contribution from flavour compounds in fruit juice and higher viability of *L. acidophilus* LA-5 which may also produce flavour compounds. Acetaldehyde for example is recognized as a major flavour component in yogurt and the presence of lactobacilli in starter culture can influence the total content of acetaldehyde in final product (Ekinci & Gurel, 2008; Guler-Akin & Akin, 2007). It is also highly likely that in the present study, the incorporation of natural sugars into the yogurt base through addition of fruit juice was another key factor in the higher consumer acceptability of stirred fruit yogurts.

General comments by the panellists regarding sensory attributes were also evaluated. The most common criticisms were related to the higher acidity and the semi-liquid texture of the products and non-typical yogurt taste. Most of these defects are common in goat's milk products compared to cow's milk products, mainly due to the differences between these two types of milk regarding the structure, composition and size of the casein micelles, the proportion of individual protein fractions, and higher content of non-protein nitrogen and mineral contents (Domagala, 2009; Kucukcetin et al., 2011). For example a butter produced

from goat's milk yogurt has received lower consumer acceptability than butter produced from cow's milk yogurt (Senel et al., 2011). Significantly however, there was no complaint about the characteristic unpleasant "goaty" taste in 10 and 15% stirred fruit yogurts in the present study. Lower scores for organoleptic properties were previously reported for goat's milk yogurt with higher acidity compared to lower acidic products (Guler-Akin & Akin, 2007). Martin-Diana et al (2003) observed unpleasant acidity development when using cultures containing *L. delbrueckii* subsp. *bulgaricus* in manufacturing fermented goat's milk. Although stirred fruit yogurts demonstrated higher acidity levels compared to plain yogurts they were able to maintain higher consumer acceptability than plain yogurts in the present study, probably due to the sugar content of the added fruit juice.

Overall sensory scores for these products also remained low as reported in Chapter 3. Generally there were no remarkable differences in sensory characteristics among fermented goat's milk (Chapter 3) and goat's milk yogurts. A similar trend of higher scores for colour and appearance and lower scores for tastes was noted for both fermented milk (Chapter 3) and yogurts in this study. However, findings of this study suggested that through the improvement of flavour with fruits, probiotic goat's milk yogurt could become more acceptable and appealing to potential consumers.

4.5 Conclusions

This study is the first report the successful application of the recently identified potential probiotic *P. jensenii* 702 in goat's milk yogurt. The results suggest that *P. jensenii* 702 can be successfully utilised in combination with *B. animalis* subsp. *lactis* BB-12, *L. acidophilus* LA-5 and the yogurt starter cultures *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, to produce goat's milk yogurt with high potential probiotic efficacy. However, the data also indicated that an initial inoculation level of 10^8cfu/g was necessary to maintain adequate viable cell numbers of *P. jensenii* 702 throughout the shelf life of these yogurt products.

While the viable number of *L. acidophilus* LA-5 cells was found to decrease significantly in all of the products during 4 weeks of refrigerated storage, it was evident that the viability

of this probiotic in goat's milk yogurt can be greatly improved through the addition of fruit juice to the yogurt base. With the exception of this case however, the hypothesis that addition of fruit juice would enhance the viability of the probiotics and starter culture bacteria was not generally supported by the data presented.

In terms of physico-chemical and sensory properties it was hypothesised that the presence of starter cultures along with the three probiotics in combination, would improve the consumer appeal of the plain yogurt relative to the previously examined fermented milk, but again this was not found to be the case. Finally however, the responses of panellists to the sensory attributes of all the products tested indicated that the addition of fruit juice did significantly enhance consumer acceptability of goat's milk yogurt.

Chapter 5 : Production of probiotic ice cream from goat's milk and effect of packaging materials on product quality

5.1 Introduction

With the increased interest of the potential beneficial health effects of probiotics, a number of different types of products have been proposed as carrier foods. Ice cream is a well accepted food product by the public (Cruz et al., 2009) and could therefore be an ideal vehicle for delivering probiotics to humans (Alamprese et al., 2005; Turgut & Cakmakci, 2009). Ice cream is a frozen emulsion, consisting of a mixture of components such as milk, sweeteners, stabilizers, emulsifiers and flavouring agents in the continuous and dispersed phases (Cruz et al., 2009; Kambamanoli-Dimou & Benjamin, 2003; Marshall & Benjamin, 2003b). The continuous phase of ice cream consists of unfrozen syrup containing the dissolved substances, mostly sugars and minerals. The dispersed phase consists of air cells, globules of milk fat, ice crystals and insoluble substances, including proteins and hydrocolloids (Marshall & Benjamin, 2003b). Steps in the manufacture of ice cream include the deciding of composition, determining the availability and characteristics of ingredients needed to make the ice cream mixture, calculation of amounts of each ingredient needed, combination and processing of the ingredients, cold storage of the ice cream mixture, addition of liquid flavours, freezing of the ice cream mixture to a soft consistency while stirring and introducing of air to the soft-frozen product, addition of desired syrups or solid types of flavourings, packaging and hardening and storage of the packaged product (Marshall & Benjamin, 2003a). Goat's milk can be utilized to produce ice cream with a softer texture and desirable melting characteristics (Ribeiro & Ribeiro, 2010) which may result in even higher consumer acceptability compared to ice cream produced from cow's milk.

However, development of probiotic ice cream can be technologically challenging due to instability of probiotics in frozen products. The loss of viability of probiotic

microorganisms in frozen desserts such as ice cream may be due to undesirable increases in acidity, freeze injury and oxygen toxicity (Cruz et al., 2009; Ravula & Shah, 1998a). As explained in Chapter 3, higher acidity can cause injury to probiotic cells which may ultimately lead to cell death. Mechanical stress caused by agitation during mixing of the ice cream mixture and freezing, may also contribute to the lower viability of probiotics in ice cream products (Akin et al., 2007). Furthermore, it is important that the incorporation of probiotics into ice cream does not affect the overall quality of the product. Therefore, physico-chemical parameters involved in the quality control of ice cream such as melting rate, and the sensory features of probiotic ice cream, should be comparable with conventional ice cream (Cruz et al., 2009).

Air incorporation during manufacturing is essential to obtain the desired physico-chemical properties such as overrun in ice cream however, excess oxygen may affect the growth of microaerophilic *L. acidophilus* and anaerobic bifidobacteria (Kailasapathy & Sultana, 2003). Since oxygen tolerance of probiotics is strain dependent (Kawasaki et al., 2006), selection of oxygen-resistant strains is important to succeed in maintaining the satisfactory viability of the probiotic cultures in ice cream (Cruz et al., 2009). Oxygen permeation through the packaging material may also have an adverse affect on probiotic viability (Shah, 2000). The packaging of a food product is an integral part of the preservation system and functions as a barrier between the food and the external atmosphere (da Cruz et al., 2007). Dave and Shah (1997d) observed an improved viability of probiotic *L. acidophilus* and bifidobacteria in yogurt prepared and stored in glass containers rather than plastic containers, due to the low dissolved oxygen content in yogurt stored in glass containers. Jayamanne and Adams (2004) investigated the viability of *B. longum* NCTC11818 in buffalo curd, a popular fermented dairy product widely consumed throughout South-East Asia. Buffalo milk was fermented in three different packaging types: clay pots, plastic cups and glass bottles. It was found that the bifodobacteria survived well in the glass containers by comparison with the plastic cups and clay pots. These authors reported this pattern of viability loss to be related to the permeability of the packages, which allowed diffusion of oxygen into the product. Buffalo curd in plastic cups had a significantly higher redox potential suggestive of higher oxygen permeation compared with glass bottles throughout

storage (8 days). Plastic packaging materials such as polypropylene and polyethylene by nature possess high oxygen permeability while glass packages possess extremely low oxygen permeability (da Cruz et al., 2007). However, study of the influence of the packaging materials on viability of probiotics in ice cream has been limited to date.

Packaging also plays a fundamental role in maintaining the quality and shelf life of foods (da Cruz et al., 2007). Linssen et al. (1992) reported the absorption of aroma compounds from flavoured drink yogurts by high density polyethylene packaging materials. Polypropylene and polystyrene packaging materials have previously shown a greater impact on sensory and physico-chemical properties of 0% fat yogurts compared to 4% fat yogurts during storage (Saint-Eve et al., 2008). Sensory changes in food products may result from intended or unintended interactions with packaging materials and from failure of materials to protect product integrity or quality (Duncan et al., 2009). Packaging materials can significantly influence the physico-chemical properties of probiotic dairy foods such as acidity during storage (Jayamanne & Adams, 2004). These properties directly affect the quality and ultimately the consumer acceptability of the product however to our knowledge none of the previous studies have investigated the effect of food-packaging interactions with respect to sensory attributes of frozen probiotic dairy foods.

According to the results from Chapters 3 and 4 the overall sensory acceptability of different types of fermented goat's milk and plain and stirred fruit yogurts was low, although addition of fruit juice improved the sensory qualities of goat's milk yogurts. Therefore goat's milk ice cream may be an ideal product to use as a vehicle in delivering *L. acidophilus* LA-5, *B. animalis* subsp. *lactis* BB-12 and *P. jensenii* 702, because ice cream naturally possesses high consumer acceptability due to its sweetness, texture and flavours.

5.1.1 Experimental design and research hypotheses

Generally the pH of ice cream is close to neutral (pH ~7.0) however, the pH of probiotic ice cream could be much lower due to the growth and metabolic activities of probiotics and this low pH may in turn affect the survival of probiotic bacteria in ice cream (Kailasapathy

& Sultana, 2003; Wood, 2011). Consequently the possibility of incorporating probiotics without fermentation into the ice cream mix could help to reduce the negative impact of low pH on viability. However, use of fermented milk in producing ice cream would allow probiotics to increase their numbers and release their metabolic products that may be associated with sensorial and health benefits. Therefore use of milk subjected to a relatively short fermentation period (about 1-2 hours) in producing ice cream, may help to overcome higher acidity development as well as improve the probiotic quality of the final product. For this reason a portion of goat's milk (15% w/w of total milk) used in this study was fermented for 1 hour only.

A reduction in bacterial starter culture counts in the ice cream mix after freezing has been reported by many authors (Akin et al., 2007; Alamprese et al., 2002; Magarinos et al., 2007; Nousia et al., 2011). This decline in viability as a result of freezing may occur due to the freeze injury of cells, which may eventually lead to the death of cells (Akin et al., 2007). The freezing process may cause a thermal shock and an osmotic shock that inevitably affects the viability of the microorganisms (Magarinos et al., 2007; Ordonez et al., 2000). The freezing process may result in lethal injuries as well as non-lethal injuries to the probiotic cells, while some cells may withstand the freezing process without incurring any injuries (Thunell et al., 1984). Thus, the rate of viability loss during freezing in ice cream manufacture is dependent on the bacterial strain, ice cream production technologies (such as differences in the cooling and ageing of the ice cream mix), different ingredient formulations and the pH of the ice cream mix (Alamprese et al., 2002). As the novel probiotic *P. jensenii* 702 has not been utilized in manufacturing frozen desserts to date, it is not yet well known whether this organism possesses characteristics, such as tolerance to freezing that may be favourable in the manufacturing of ice cream. This study evaluates the capacity of *P. jensenii* 702, *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12, to withstand freezing and frozen storage.

Probiotic cells uninjured during the freezing process normally exhibit sustained viability during the storage period of frozen dairy products such as ice cream, possibly due to the effect of the low storage temperature (<-18^{o}C) in minimizing the biochemical reactions

(enzyme activity) of microorganisms (Cruz et al., 2009; Thunell et al., 1984). Many authors have reported sustained viability of probiotics (>10^6 cfu/g) in ice cream during frozen storage (Akin et al., 2007; Alamprese et al., 2005; Alamprese et al., 2002; Criscio et al., 2010; Haynes & Playne, 2002; Magarinos et al., 2007; Nousia et al., 2011), however the literature also contains reports of poor probiotic viability (<10^6 cfu/g) in ice cream during storage (Akalın & Erisir, 2008; Akin et al., 2007). Generally, it is difficult to define the shelf life of ice cream in frozen storage as it depends mainly on the exact conditions of storage such as temperature. Ice cream produced with probiotic lactobacilli and bifidobacteria can store for approximately up to one year while maintaining satisfactory probiotic viability (Alamprese et al., 2005; Haynes & Playne, 2002). Thus, viability measurements of probiotics were taken up to 52 weeks in the present study. The influence of packaging materials on the sensory characteristics of yogurt has previously been observed within 28 days of storage (Saint-Eve et al., 2008). In order to compare possible variation in the influence of packaging over short and longer term storage, the sensory evaluation in the present study was conducted at one week and 12 weeks of storage. All physico-chemical properties were measured at one week of storage as described by many other researchers (Akin et al., 2007; Alamprese et al., 2005; Guven & Karaca, 2002) while some important physico-chemical properties such as pH were also measured after 4,12 and 24 weeks of storage. Cocoa powder was used in the present study to improve the consumer acceptability of the product, both because chocolate flavours may aid in masking any unpleasant flavours associated with goat's milk, and because chocolate flavoured ice cream is generally popular among consumers.

This study can be defined in terms of two broad objectives. The first objective was to develop a chocolate flavoured ice cream made from goat's milk, containing *L. acidophilus* LA-5, *B.animalis* subsp. *lactis* BB-12 and the novel probiotic *P. jensenii* 702, exhibiting satisfactory probiotic viability, desirable physico-chemical properties and high consumer acceptability. The second was to evaluate the effect of packaging material on microbial, physico-chemical and sensory properties of the product during storage. To this end, probiotic ice cream was stored at -20^{o}C in three different types of packaging: glass,

polyethylene and polypropylene. On the basis of the factors and rationale described above, it was hypothesized that:

1. The freezing process during manufacturing of the goat's milk ice cream would result in a reduction in viability of all three probiotics.
2. Relative to polyethylene or polypropylene packaging, the low oxygen permeability of glass containers would result in improved viability retention of all three probiotics during storage.
3. Due to improved probiotic viability, and therefore possible flavour enhancement, packaging in glass containers would result in better sensory properties of probiotic goat's milk ice cream following storage, relative to packaging in polyethylene or polypropylene containers
4. Compared to polyethylene or polypropylene containers, packaging in glass containers would result in higher acidity in goat's milk ice cream due to improved viability retention of probiotics, but would not result in different total solids content, overrun, first dripping times or complete melting times.

5.2 Materials and methods

5.2.1 Microbiological, physico-chemical and sensory evaluation of goat's milk ice cream

Chocolate flavoured ice cream was produced as described in Chapter 2 (2.6.3), packaged in three different types of container (glass, polyethylene and polypropylene) and stored at -20^{o}C. Ice cream samples from storage were used to enumerate probiotics as described in Chapter 2 (2.7). Coliform, yeast and mould counts were assessed one week after production and at the end of the shelf life.

pH, titratable acidity, total solids, protein content, fat content, ash content, overrun, first dripping time, and complete melting time of ice cream samples were measured at various time points as described in Chapter 2 (2.8).

Sensory evaluation of goat's milk ice cream packed in glass, polyethylene and polypropylene was conducted by 29 taste panellists (19 male and 10 female) at 1 week and 12 weeks after production as described in Chapter 2 (2.10.1).

5.2.2 Statistical analysis

Data analyses were performed using SPSS/PASW statistical software version 17 (SPSS Inc., Chicago, IL, USA). Microbial viability data and certain physico-chemical data, including total solids, pH and titratable acidity were analysed using repeated measure ANOVA. One way ANOVA was used to analyse data for all other physico-chemical properties. The Bonferroni post hoc test was performed for means comparison. Nonparametric tests were performed to determine statistical differences within the sensory data. Where appropriate, T-tests were performed to compare two means. A p value <0.05 was considered statistically significant for all analyses.

5.3 Results

The data presented in this chapter includes the effect of freezing and packaging materials on the viability of probiotic bacteria *L. acidophilus* LA-5, *B. animalis* subsp. *lactis* BB-12 and *P. jensenii* 702 during manufacturing and storage of goat's milk ice cream. This viability analysis is followed by an examination of the effect of packaging materials on the physico-chemical properties of goat's milk ice cream such as pH, titratable acidity and total solids during storage. Other physico-chemical properties of goat's milk ice cream measured at one week after production are also presented. The study concludes with an assessment of the effect of packaging on organoleptic properties of probiotic goat's milk ice cream during storage.

5.3.1 Effect of freezing on probiotic viability

P. jensenii 702 demonstrated the highest survival rate with average viable cell numbers after freezing found not to be significantly reduced from those in the unfrozen mixture. By

comparison, a significant viability loss was observed for *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12, with viable numbers reduced after freezing by almost 34% and 44% respectively. However, most importantly all three probiotics were able to maintain viable numbers of 10^7-10^8 cfu/g after freezing (Table 5.1).

Table 5.1 Counts of probiotic microorganisms in goat's milk ice cream before and after freezing (*n* = 6)

Microorganism	Unfrozen mixture (log cfu/g)	Ice cream (log cfu/g)	Survival rate (%)
L. acidophilus LA-5	$7.98\pm0.04 \times 10^{7a}$	$4.48\pm0.44 \times 10^{7b}$	56.14
B. animalis subsp. *lactis* BB-12	$1.64\pm0.08 \times 10^{8a}$	$1.09\pm0.07 \times 10^{8b}$	66.46
P. jensenii 702	$5.23\pm0.05 \times 10^{8a}$	$4.64\pm0.03 \times 10^{8a}$	88.72

Mean value (±SE)

[a , b] Values in the same row having different superscripts differ significantly ($p<0.05$).

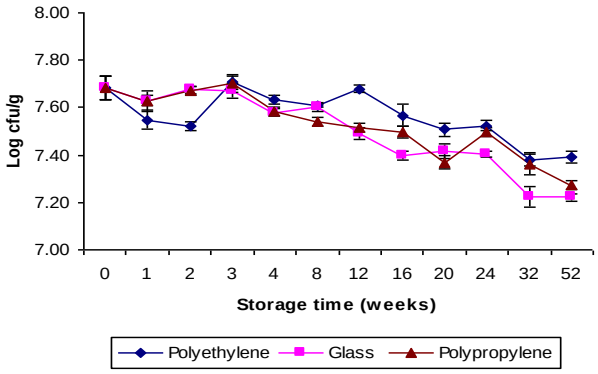
5.3.2 Probiotic viability during storage

It seems likely that the type of packaging materials did not affect the viability of probiotics during storage. High viability levels, > 10^7 cfu/g in the case of *L. acidophilus* LA-5 and *B. animalis subsp. lactis* BB-12 and >10^8 cfu/g in the case of *P. jensenii* 702, were observed at the end of 52 weeks storage at -20^{o}C in goat's milk ice cream regardless of the packaging type (Figure 5.1).

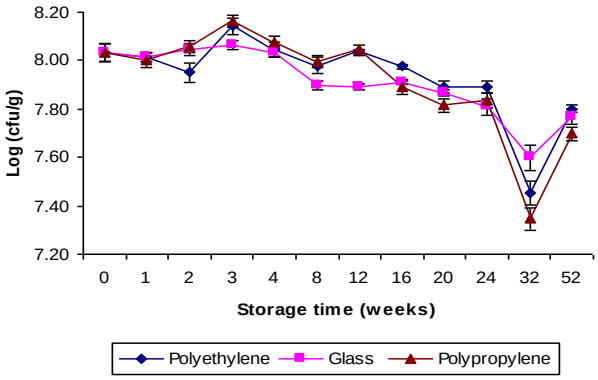
5.3.3 Coliform, yeast and moulds

Coliform counts of samples were zero and yeast and mould counts were <1 cfu/g at the beginning as well as at end of the storage period regardless of the differing packaging materials.

A



B



C

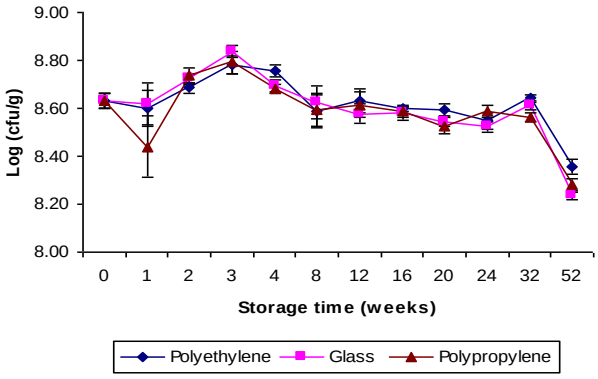


Figure 5.1 Viable counts of *L. acidophilus* LA-5 (A), *B. animalis* subsp. *lactis* BB-12 (B) and *P. jensenii* 702 (C) in different packaging materials during storage at -20^{o}C (Scale ranges (y-axis) were tailored to best highlight the trend in each figure).

5.3.4 Physico-chemical properties

The pH of the goat's milk used to produce the ice cream was 6.70 ± 0.00. The pH value fell to 6.65 ± 0.02 during ice cream manufacturing ($p>0.05$) when fermented goat's milk containing probiotics with pH value of 5.03 ± 0.01 were added to the ice cream mixture. Changes of pH and titratable acidity of goat's milk ice cream were non significant during storage regardless of the different packaging. While a statistically significant difference in the percentage total solids for product in polypropylene containers was recorded at week 4 of storage (Table 5.2), this result appears somewhat anomalous and more likely an artefact of the sample preparation and collection process (i.e. experimental variability) than any real effect associated with the packaging material.

Examination of the protein, fat and ash content of the goat's milk ice cream did not reveal any significant differences between the different packaging materials at one week after production. Similar results were observed for physical properties such as overrun and first dripping times. However, there were significant differences among the complete melting times of goat's milk ice cream depending on packaging materials. While the complete melting time of the ice cream stored in glass containers did not differ significantly from ice cream stored in polyethylene or polypropylene, the melting time for ice cream stored in polyethylene was significantly greater than for ice cream stored in polypropylene (Table 5.3).

Table 5.2 Mean values for the total solids, pH and titratable acidity of probiotic goat's milk ice cream in different packaging materials during storage at -20°C (*n* = 3)

Characteristic	Storage time (wks)	Polyethylene	Glass	Polypropylene
Total solids (%)	1	37.66 ± 0.24^{Aa}	38.56 ± 0.59^{Aa}	37.50 ± 0.16^{Aa}
	4	36.73 ± 0.96^{Aa}	37.28 ± 0.96^{Aa}	44.63 ± 0.19^{Bb}
	12	36.31 ± 0.39^{Aa}	36.11 ± 0.38^{Aa}	37.47 ± 0.68^{Aa}
	24	37.03 ± 0.41^{Aa}	37.00 ± 0.82^{Aa}	36.58 ± 0.35^{Aa}
pH	1	6.65 ± 0.02^{Aa}	6.63 ± 0.01^{Aa}	6.61 ± 0.01^{Aa}
	4	6.65 ± 0.08^{Aa}	6.64 ± 0.06^{Aa}	6.62 ± 0.11^{Aa}
	12	6.54 ± 0.02^{Aa}	6.60 ± 0.01^{Aa}	6.59 ± 0.00^{Aa}
	24	6.58 ± 0.01^{Aa}	6.57 ± 0.01^{Aa}	6.59 ± 0.00^{Aa}
Titratable acidity	1	0.19 ± 0.00^{Aa}	0.18 ± 0.00^{Aa}	0.16 ± 0.03^{Aa}
	4	0.18 ± 0.00^{Aa}	0.19 ± 0.00^{Aa}	0.21 ± 0.00^{Aa}
	12	0.17 ± 0.00^{Aa}	0.16 ± 0.01^{Aa}	0.16 ± 0.00^{Aa}
	24	0.16 ± 0.00^{Aa}	0.17 ± 0.01^{Aa}	0.16 ± 0.00^{Aa}

Mean value (±SE)

[A, B] Values in the same column having different superscripts for each characteristic differ significantly ($p<0.05$).

[a , b] Values in the same row having different superscripts differ significantly ($p<0.05$).

Table 5.3 Mean values for physico-chemical properties of probiotic goat's milk ice cream (stored at -20°C) one week after production (*n* = 3)

Characteristic	Polyethylene	Glass	Polypropylene
Protein (%)	4.44 ± 0.00[a]	4.41 ± 0.04[a]	4.46 ± 0.03[a]
Fat (%)	9.67 ± 0.33[a]	9.67 ± 0.33[a]	9.50 ± 0.87[a]
Ash (%)	1.27 ± 0.01[a]	1.29 ± 0.00[a]	1.29 ± 0.00[a]
Overrun (%)	33.83 ± 0.46[a]	26.17 ± 0.07[a]	33.83 ± 0.15[a]
First dripping times (minutes)	25.36 ± 2.09[a]	20.84 ± 1.70[a]	27.28 ± 0.72[a]
Complete melting times (minutes)	110.03 ± 1.67[a]	104.58 ± 1.48[ab]	98.92 ± 1.13[b]

Mean value (±SE)

[a, b] Values in the same row having different superscripts differ significantly ($p<0.05$).

5.3.5 Sensory characteristics

At one week after production the packaging materials appeared to have had no significant effect on any of the tested sensory characteristics of goat's milk ice cream, except for the melting quality. Ice cream stored in glass containers was significantly less preferred by the tasting panel in terms of the melting quality after one week of storage compared to ice cream stored in polyethylene and polypropylene (Table 5.4). However, ice cream stored in glass had significantly higher consumer acceptability for melting quality at week 12 compared to that stored for one week. In general consumer preference was found to be higher for the goat's milk ice cream that had been stored for 12 weeks compared to one week of storage regardless of the different packaging materials (Table 5.4).

Table 5.4 Mean scores of tasting panellists (*n* = 29) for sensory properties of goat's milk ice cream in different packaging materials after one week and twelve weeks of storage at -20°C

Characteristic	Storage time (wks)	Polyethylene	Glass	Polypropylene
Colour & appearance	1	6.90 ± 0.16^{Aa}	6.76 ± 0.22^{Aa}	6.66 ± 0.26^{Aa}
	12	7.24 ± 0.21^{Aa}	7.07 ± 0.28^{Aa}	7.14 ± 0.21^{Aa}
Aroma	1	7.00 ± 0.19^{Aa}	6.62 ± 0.23^{Aa}	6.66 ± 0.26^{Aa}
	12	7.10 ± 0.28^{Aa}	6.59 ± 0.23^{Aa}	6.86 ± 0.21^{Aa}
Body & texture	1	6.52 ± 0.27^{Aa}	6.14 ± 0.31^{Aa}	6.07 ± 0.31^{Aa}
	12	7.17 ± 0.20^{Ba}	6.83 ± 0.21^{Ba}	7.03 ± 0.25^{Ba}
Taste	1	5.90 ± 0.37^{Aa}	6.34 ± 0.34^{Aa}	6.38 ± 0.41^{Aa}
	12	6.69 ± 0.34^{Ba}	6.79 ± 0.33^{Ba}	6.83 ± 0.34^{Ba}
Melting quality	1	7.03 ± 0.25^{Aa}	6.10 ± 0.28^{Ab}	6.86 ± 0.25^{Aa}
	12	7.07 ± 0.23^{Aa}	6.79 ± 0.25^{Ba}	6.90 ± 0.23^{Aa}
Overall acceptability	1	6.28 ± 0.32^{Aa}	6.17 ± 0.31^{Aa}	6.59 ± 0.35^{Aa}
	12	6.86 ± 0.31^{Ba}	6.69 ± 0.25^{Aa}	6.83 ± 0.24^{Aa}

Mean value (±SE)

$^{A, B}$ Values in the same column having different superscripts for each sensory characteristic differ significantly ($p<0.05$).

$^{a, b}$ Values in the same row having different superscripts differ significantly ($p<0.05$).

(The scale for sensory scores: Like extremely = 9, Like very much = 8, Like moderately = 7, Like slightly = 6, Neither like nor dislike = 5, Dislike slightly = 4, Dislike moderately = 3, Dislike very much = 2, Dislike extremely = 1).

5.3.6 Summary of key findings

A significant reduction in the viable cell numbers of *L. acidophilus* LA-5 and *B. animalis subsp. lactis* BB-12 was observed following freezing of the ice cream mix, however this effect was not evident for *P. jensenii* 702. Although the viability of probiotic bacteria was reduced during the storage period, all three probiotics were able to maintain satisfactory

viability (10^7-10^8 cfu/g) at the end of 52 weeks of storage regardless of the packaging materials.

There was no packaging effect on physico-chemical properties of goat's milk ice cream except for the complete melting time. The complete melting time of goat's milk ice cream stored in glass containers did not differ significantly from ice cream stored in polyethylene or polypropylene but melting time for ice cream stored in polyethylene was significantly greater than for ice cream stored in polypropylene. Packaging materials had no apparent effect on the sensory attributes of goat's milk ice cream other than the melting quality. However, goat's milk ice cream stored for 12 weeks was highly ranked for all the sensory attributes compared to that stored for one week at -20^{o}C.

5.4 Discussion

With regard to the objectives of this study, several key findings have emerged in relation to the probiotic viability, the physico-chemical properties, and general consumer acceptability of chocolate flavoured goat's milk ice cream stored in different packaging materials. Overall *P. jensenii* 702 was noted to exhibit favourable characteristics regarding viability retention during ice-cream production and frozen storage.

5.4.1. Microbiological analysis

As reported previously by several authors, (Akalın & Erisir, 2008; Akin et al., 2007; Alamprese et al., 2002; Magarinos et al., 2007) the viable numbers of *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 bacteria were found to be significantly lower after freezing compared to the respective numbers prior to freezing (Table 5.1). This decrease in probiotic viability during freezing may have resulted from either freeze injury to the viable cells, mechanical stress of the mixing and freezing process, or incorporation of air into the ice cream mix leading to oxygen toxicity. Nonetheless, *P. jensenii* 702 did exhibit considerable tolerance to the freezing process, compared with *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12. *P. jensenii* 702 may be equipped with mechanisms enabling

survival during freezing that are not possessed by *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 such as an ability for example, to dehydrate themselves quickly and thus reduce the formation of intracellular ice crystals that can damage cytoplasmic membranes and lead to cell death (Magarinos et al., 2007; Nousia et al., 2011).

Although there were variations among the different probiotics examined in this study, all three strains exhibited satisfactory viability levels (10^7-10^8 cfu/g) after freezing. In this experiment, probiotics were allowed to grow for 1 hour in milk to facilitate the activation of cells which were subsequently used in the manufacturing of the ice cream. The observed reduction in the pH of the goat's milk during the 1 hour fermentation might be considered a good indicator of this growth and activation. According to Nousia et al. (2011) probiotic cultures activated in this way are better able than freeze dried cultures to survive during freezing, possibly because of increased cell sensitivity from stress incurred during the freeze drying process (Godward & Kailasapathy, 2003).

The physiological state of the probiotic at the time they are added into the food product during the manufacturing process may also be important, since bacteria are known to be much more susceptible to environmental stress during their growth stage than they are during their stationary period (Heller, 2001; Magarinos et al., 2008). Furthermore, growing the probiotics in milk before the freezing process may aid in adjustment to the environment and release of metabolic products such as exopolysaccharides which may also help to improve probiotic tolerance to freezing during ice cream manufacture. Exopolysaccharides can act as a thickening, stabilizing and emulsifying, or gelling agent (Kailasapathy, 2006), and may provide some protection by acting as a physical barrier during freezing.

During the manufacture of probiotic goat's milk ice cream in the current study, the ingredients were formulated with 12% (w/w) commercial sugar (sucrose) to achieve a sweet taste. Goat's milk it self contains lactose and casein. The casein, sucrose and lactose in the ice cream mixture may also have provided cryoprotector properties (Holcomb & Frank, 1991; Magarinos et al., 2007), thereby improving probiotic resistance to freezing. Higher levels of sugar and fat (22% and 10% respectively) in an ice cream mix have been

reported to result in higher survival rates of *L. rhamnosus* GG during freezing, compared to high sugar-low fat (22% and 5%), low sugar-low fat (15% and 5%), and low sugar-high fat (15% and 10%) mixtures (Alamprese et al., 2005). Therefore, it seems likely that fat content in ice cream mix may affect probiotic survival during the freezing process. Milk fat and air bubbles can act as insulators, reducing the transfer of heat through the frozen foam. In addition both components restrict the growth of ice crystals, minimizing the damage that could be caused to microbial cells (Magarinos et al., 2007). The probiotic goat's milk ice cream used in the present study contained a high fat content (~10%) (Table 5.3), and this high fat content may have contributed to the maintenance of satisfactory viability levels (10^7-10^8 cfu/g) of all probiotics during the freezing process

Independent of the packaging materials used in the current study, the maximum viable counts for each probiotic during storage were observed at week 3 (Figure 5.1). A similar trend of increase in cell concentration due to residual activity during storage in yogurts has been reported previously (Dave & Shah, 1997d). Although there is a possibility of slow cell growth under refrigerated storage, generally it is highly unlikely that probiotic cell growth would be observed at -20^{o}C. Therefore, the higher probiotic counts in ice cream samples at week 3 are more difficult to explain, and may simply be associated with natural variations in the data, due to non-uniform distribution of the bacterial cells within the ice cream mixture. Although all three probiotics were able to maintain satisfactory viability (10^7-10^8 cfu/g) throughout the storage period, regardless of the packaging material used, they each demonstrated a reduction in viable counts at the end of 52 weeks of storage at -20^{o}C. Viability loss of probiotics in ice cream during shelf life even when low storage temperature has been maintained is a common phenomenon and has been reported by other authors (Akin et al., 2007; Magarinos et al., 2007). Fluctuations in temperature during storage, causing ice crystal formation, may result in rupture of bacterial cells and reduced viability in frozen dairy foods such as ice cream (Davidson et al., 2000). Although it was difficult to examine any temperature fluctuations in the commercial scale freezer used to store the goat's milk ice cream in the present study, it is possible that minor temperature fluctuations which may affect probiotic survival might well have occurred during such a long period of storage (52 weeks).

Oxygen toxicity may be another major factor leading to cell death during storage. Since ice cream is a whipped product, oxygen is incorporated in large amounts during manufacturing (Akin et al., 2007). Certain probiotic cultures such as *B. bifidum* are very sensitive to oxygen and die in its presence, presumably due to the intracellular production of hydrogen peroxide (Champagne et al., 2005; Talwalkar et al., 2001). Furthermore, most probiotic bacteria do not produce catalase, an enzyme essential to the breakdown of hydrogen peroxide (da Cruz et al., 2007; Hung et al., 2003; Vasiljevic & Shah, 2008), and resulting in cell death due to the cellular injuries caused by toxic hydrogen peroxide (Dave & Shah, 1997d).

It is also possible that during the freezing process the product's water may not all be completely frozen, hence highly concentrated residual solutions may still be present in ice cream after freezing. The composition and concentration of this residual solution can change during storage and if the concentration increases sufficiently it may result in lethal osmotic effects for the probiotics (Magarinos et al., 2007). However, in general probiotics may survive better in ice cream compared to yogurt and other dairy products possibly due to the low temperatures employed in storage of ice cream. All three probiotics used in this study have demonstrated faster and higher viability loss in other goat's milk products such as fermented milk (Chapter 3), and plain and stirred fruit yogurts (Chapter 4). Although there are many differences between these products, such as physico-chemical properties (acidity, sugar level), culture composition and food ingredients which may possibly cause the variations in viability of probiotics in these products, lower storage temperatures may be considered as one of the critical factors that help these bacteria to maintain higher viability at the end of 52 weeks of storage in ice cream.

Use of oxygen impermeable containers such as glass has been recommended by Shah (2000) in order to improve the viability of probiotics in dairy products, thus it had been hypothesized that in this study the highest survival rates in the goat's milk ice cream would be observed in the samples stored in glass containers. However, no significant effect of packaging on the viability of *L. acidophilus* LA-5, *B. animalis* subsp. *lactis* BB-12 and *P. jensenii* 702 was observed. Some probiotic strains such as *L. acidophilus* have the ability to

respond to the detrimental effect of oxygen by producing enzymes that help to scavenge environmental oxygen such as NADH oxidase or NADH peroxidase (Champagne et al., 2008; Talwalkar & Kailasapathy, 2003; Talwalkar & Kailasapathy, 2004a), although it is unknown whether *L. acidophilus* LA-5, *B. animalis* subsp. *lactis* BB-12 and *P. jensenii* 702 also possesses this ability. Champagne et al (2008) evaluated the survival of *L. rhamnosus* R0011 in fruit juice stored in polyethylene containers under refrigerated storage. The containers were opened every seven days and shaken to facilitate oxygen permeation. It was found that *L. rhamnosus* R0011 can maintain a high viability ($>10^9$ cfu in 250 ml of fruit juice) over 3 weeks of refrigerated storage even if the containers have been opened and cells are exposed to oxygen. Therefore, the observation made in this study with respect to maintaining similar levels of probiotic viability despite the relative oxygen permeability of the packaging material is not unprecedented, and could be strongly linked to the physiological properties of the particular strains of probiotics used in the present study. Further research is needed to fully elucidate this phenomenon.

Interestingly, while oxygen permeation through packaging materials can be especially problematic for strictly anaerobic bacteria, packaging types did not appear to have a significant effect on the viability of anaerobic *B. animalis* subsp. *lactis* BB-12 in goat's milk ice cream during storage. In general, being strictly anaerobic, *Bifidobacterium* spp. are more sensitive to oxygen than *L. acidophilus*, however this sensitivity may be exclusively strain dependent (Talwalkar et al., 2004). *Bifidobacterium* as well as *L. acidophilus* have been found to be successfully adapted to high levels of dissolved oxygen in yogurts (Talwalkar & Kailasapathy, 2004a). The production of peroxide that is detrimental for bifidobacteria under aerobic conditions can be effectively suppressed in the presence of the bifidogenic growth stimulators produced by propionibacteria (Champagne et al., 2005). Therefore, the use of *P. jensenii* 702 as a co-culture in the present study may have helped maintain the viability of *B. animalis* subsp. *lactis* BB-12 regardless of the oxygen permeability of the packaging materials used. Talwalkar et al. (2004) reported that although dissolved oxygen in yogurts can be influenced by the type of packaging materials it may not affect the survival of *L. acidophilus* and *Bifidobacterium* spp. in yogurts.

Notwithstanding these possibilities, lower storage temperature may play a more important role in retaining viability of probiotics in ice cream than the packaging materials. In the case of frozen dairy desserts, even though a packaging material may have higher oxygen permeability, low storage temperature may minimize the biochemical reactions of microorganisms and thereby neutralize the packaging effect. The influence of packaging materials on probiotic viability may therefore depend on storage temperature. If so, inclusion of an oxygen removal step/use of vacuum lines before filling the product into retail containers in order to assure maximum probiotic viability may not be necessary in the case of probiotic ice cream, although vacuum lines can be utilized in commercial applications to minimize the incorporation of air into probiotic dairy products such as yogurts (Kailasapathy et al., 2008). Glass containers have previously been reported to prevent oxygen diffusion and thereby result in better probiotic viability in yogurt and yogurt like products (da Cruz et al., 2007; Dave & Shah, 1997d; Jayamanne & Adams, 2004). However, according to the results of present study, use of polyethylene or polypropylene as packaging materials for probiotic ice cream can be identified as an option to reduce the cost of production without deteriorating the probiotic quality of the final product rather than use of glass containers which can be more expensive and hazardous.

Although the pH of standard ice cream is approximately 7.0 (Hekmat & McMahon, 1992; Kailasapathy & Sultana, 2003; Wood, 2011), the pH of probiotic ice cream can vary depending on factors such as the amount of fermented milk used, fermentation time and the types of microorganisms used. As explained in Chapter 3, the pH of dairy products has also been shown to affect the survival of probiotics. High pH of the ice cream in the present study (Table 5.2), which could be attributed to the short fermentation time (1 hour) of probiotic cultures in the goat's milk before incorporation into the ice cream mix, may also have contributed to the relatively high rate of probiotic survival in the present study. Control of pH during the fermentation process (stopping the fermentation at pH values ranging from 5.0-5.5/short fermentation time) has been recommended as a solution to the problem of probiotic viability loss at low pH (Cruz et al., 2009).

5.4.2 Physico-chemical properties

There were no significant differences in terms of physico-chemical properties among ice cream samples stored in the different packaging materials with the exception of complete melting times after one week of storage and total solids after four weeks of storage (Table 5.2 and Table 5.3). Significantly higher levels of total solids in ice cream samples stored in plastic containers at week 4 was unexpected and difficult to explain. Given that all other total solids measurements recorded at all time points in all 3 preparations were similar to each other, it seems more likely that this apparently anomalous result was related to experimental factors (e.g. incomplete homogeneity of the product arising from the mixing process) than to any real effect of the packaging material. The longest complete melting time was observed in the samples stored in polyethylene while samples stored in polypropylene demonstrated shortest complete melting time. Since the chemical composition of the ice cream should be the same regardless of the packaging, these changes in melting properties may be related to the physical structure of ice cream. The three main structural components of ice cream are air cells, ice crystals and fat globules. Although the specific relationships have not yet been defined, the physical structure of the ice cream does affect its melting rate and hardness (Muse & Hartel, 2004). For example ice cream which has a high proportion of air cells tends to melt slowly as air cells act as an insulator (Marshall et al., 2003; Wood, 2011). Whether there is a real effect of packaging materials on the physical structure of ice cream (and hence the complete melting times in the present study) remain unclear at this stage, and further research is needed to elucidate this phenomenon.

During storage of ice cream a number of changes in the physical structure of the product may potentially occur, such as disproportionation and coalescence of air cells which may alter the overrun value of the product (Sofjan & Hartel, 2004), and ice recrystallization, whereby small ice crystals melt and large crystals grow simultaneously (Akalın & Erisir, 2008). Small crystals, with a slightly lower melting point, are more sensitive to temperature fluctuations than large crystals (Akalın & Erisir, 2008; Marshall et al., 2003), thus this phenomenon would be likely to effect the first dripping time of the product. Based on the

overrun and first dripping time values recorded in this study, it would appear that polypropylene, polyethylene, and glass packaging materials did not differ significantly in their effect on the air cell and crystal structure of the ice cream.

Changes in the pH of the goat's milk ice cream were non significant during storage regardless of the different packaging. Similarly, the titratable acidity of the ice cream was not influenced by the packaging materials or storage time (Table 5.2). Alamprese et al. (2002) observed a similar non significant trend in the acidity of ice cream across 12 weeks of storage, as did Kudelka (2005), whom reported that packaging materials - polypropylene, polystyrene and polyethylene - had no influence on the acidity of probiotic yogurts.

5.4.3 Sensory evaluation

In general ice cream has several attributes that make it a favored food for many people such as sweet flavor, smooth texture and a cold sensation that contrasts with the warmth of most other foods (Marshall & Benjamin, 2003b). It is likely that these characteristics of the goat's milk ice cream may have contributed to higher consumer acceptability of this product in the present study compared to the responses of tasting panelists to the fermented goat's milk (Chapter 3) and goat's milk yogurts (Chapter 4). Contrary to the hypothesis regarding the sensory attributes, packaging materials had no significant effect on any of the tested sensory characteristics of goat's milk ice cream except for the melting quality at one week of production, with ice cream stored in glass containers significantly less preferred by the tasting panel in relation to this property. Based on the results of their study, which compared 4% fat yogurts to 0% fat yogurts stored in glass, polypropylene and polystyrene as packaging materials, Saint-Eve et al. (2008) reported that the fat content of yogurt can act as an aroma solvent and reduce absorption into packaging. Likewise, high fat content of goat's milk ice cream (~10%) in the present study may have contributed to eliminating the effect of packaging on important sensory properties such as aroma and taste. In this study, compared to the goat's milk ice cream stored for one week, that stored for 12 weeks at -20^{o}C acquired higher consumer acceptability regardless of the packaging. As explained

previously, changes in the physical structure of the ice cream and development of flavours during storage may have contributed to this outcome. Significant positive changes in the body, texture, and taste of probiotic goat's milk ice cream during storage were observed in the present study. In contrast Nousia et al (2011) reported no significant association between storage time and changes in sensory characteristics, including texture and taste, for probiotic cow's milk ice cream at 15 and 45 weeks after production at -25^{o}C. The often reported unpleasant "goaty" flavour was not found to be particularly noticeable in the ice cream in this study, probably due to the inclusion of the chocolate flavor/cocoa powder.

5.5 Conclusions

According to the results of the present study, *L. acidophilus* LA-5, *B. animalis* subsp. *lactis* BB-12 and the novel probiotic *P. jensenii* 702 can survive in numbers (10^7-10^8 cfu/g) above the minimum recommended therapeutic level in goat's milk ice cream for up to 52 weeks at -20^{o}C, whether packaged in polyethylene, polypropylene or glass. However, it is important to further confirm whether these probiotic cultures are still able to maintain their functional properties such as gastrointestinal tolerance, adhesion and colonization in the gut after such a long storage period. Freezing can cause changes to the morphology, genetic stability, cell function and damage to the cell membrane of bacteria. Certain bacteria lose their ability to divide after freezing and thawing (Thunell et al., 1984), and since probiotic adhesion to intestinal epithelial cell layer is strongly associated with the cell membrane of these probiotic bacteria, these changes can affect their adhesion and colonization ability. Although probiotics such as lactobacilli and bifidobacteria can survive in high numbers (>10^7 cfu/g) in frozen desserts such as ice cream, such probiotics may be unable to adhere and colonize in the gut and therefore fail to confer health benefits such as maintaining of healthy gut flora after a certain storage period. Thus to ensure the efficacy and value of their inclusion in these products, it is essential that such functional properties of these probiotic microorganisms in frozen desserts be confirmed, at least via *in vitro* investigations, before commencement of any large scale commercial production.

This report also indicates the possibility of using *L. acidophilus* LA-5and *B. animalis* subsp. *lactis* BB-12 together with novel probiotic *P. jensenii* 702 in manufacturing probiotic ice cream without any antagonistic interaction in terms of their growth and viability. Many studies have been focused on the production of probiotic ice cream using cow's milk with common probiotic lactobacilli and or *Bifidobacterium* spp. Results of this experiment revealed both that propionibacteria has a great potential to be included in frozen dairy desserts such as ice cream, and that goat's milk may be considered as a suitable carrier food in manufacturing probiotic dairy desserts with high probiotic viability.

Packaging materials appeared to exert an influence on the complete melting time of goat's milk ice cream, but not the other physico-chemical properties such as overrun, and protein, fat and ash contents. The responses of panellists to the sensory attributes of probiotic goat's milk ice cream indicated that the glass packaging could significantly lower the melting quality of the product at week one of storage. It would also seem that goat's milk ice cream may not only be maintained in frozen storage for up to 12 weeks without any deterioration in sensory properties, but that certain organoleptic characteristics may in fact improve during storage over this period of time.

Chapter 6 : *In vitro* analysis of gastrointestinal tolerance and intestinal cell adhesion of probiotics in goat's milk ice cream and yogurt

6.1 Introduction

In order to provide beneficial health effects for the host animal, probiotic bacteria must survive through the gastrointestinal tract, tolerating acid, bile and gastric enzymes, and then adhere and colonize in the intestinal epithelium (Charteris et al., 1998a; Havenaar et al., 1992; Huang & Adams, 2004; Kailasapathy & Chin, 2000; Ouwehand et al., 2001). These functional properties can be influenced by the food carriers used in probiotic delivery (Ouwehand et al., 2001). Food formulations with appropriate pH (>5) and high buffering capacity would increase the pH of the gastric tract and thereby enhance the stability of probiotics (Kailasapathy & Chin, 2000; Mainville et al., 2005). Food components that have escaped digestion may also act as an energy source for bacteria in the intestine (Tyopponen et al., 2003) and facilitate their growth and stability in the gut. Food may also provide some protection to probiotics by reducing their physical exposure to the harsh gastrointestinal environment. In addition certain ingredients in the food substrate may interact with the probiotics to alter their functional performance. For example the high fat content of cheese has been reported to protect probiotics during passage through gastrointestinal tract (Stanton et al., 1998; Valerio et al., 2006). In the study of Stanton et al (1998), piglets receiving a total of 10^8-10^9 cfu of probiotic *L. paracasei* from cheddar cheese and those receiving 10^{11} cfu of the same probiotic from yogurt, had similar levels of *L. paracasei* in the small intestine (10^4-10^5 cfu/ml of small intestinal content). In this case cheddar cheese seemed a more efficient and protective carrier for *L. paracasei* than yogurt, indicating the importance of carrier food on probiotic efficacy. In a more recent study (Madureira et al., 2011) a whey cheese matrix was reported to protect lactobacilli and bifidobacteria during simulated gastrointestinal transit by comparison with MRS broth (Valerio et al., 2006).

Although probiotic delivery has traditionally been associated with foods, there is an increasing trend towards the use of different delivery systems such as capsules (Champagne et al., 2011; Gibson, 2004). It is possible however that this changing trend in delivering probiotics may lead to a reduction in their functional efficacy including gastrointestinal survival (Krasaekoopt et al., 2003) and ability to adhere to the intestinal epithelium (Conway et al., 1987; Ouwehand et al., 2001) due to the exclusion of the potential synergistic effect of the food, and that delivery of probiotics in a suitable food matrix is one of the most appropriate means of maximising probiotic efficacy.

The low pH and antimicrobial action of pepsin can be considered the main factors detrimental to the viability of probiotics in the stomach. The pH of the stomach generally ranges from 2.5 – 3.5 (Holzapfel et al., 1998), but can be as low as pH 1 or pH 2 at higher rates of gastric juice secretion (Conway et al., 1987; Kailasapathy, 2006; Maragkoudakis et al., 2006), or as high as pH 6 or more after food ingestion (Johnson, 2007). Therefore, to effectively colonize the gut probiotic bacteria must overcome the acidity and survive in the presence of gastric juices. Huang et al. (2004) observed that the addition of two different types of food matrix (soy milk and dairy based liquid breakfast) significantly enhanced the viability of dairy propionibacteria compared to the same strains in saline during gastric transit at pH 2.0 *in vitro*. In separate studies both milk, and a milk protein mixture, have previously been reported to enhance the viability of acid sensitive *Lactobacillus* and *Bifidobacterium* strains during simulated gastric tract transit (Charteris et al., 1998a; Conway et al., 1987), with skim milk shown to prolong the survival time of *L. acidophilus* and *L. bulgaricus* by increasing stomach pH by 4-5 units in humans (Conway et al., 1987). Improved survival of *L. acidophilus* and *L. gasseri* in skim milk, compared to a saline solution, upon exposure to simulated gastric juice at pH 2.0 has also been reported (Fernandez et al., 2003). It has been suggested that milk proteins may act both as buffering agents, and inhibitors of digestive protease activity (Charteris et al., 1998a).

Having passed through the stomach, ingested probiotics are faced with surviving in the small intestinal environment, where they are exposed to pancreatin, bile salts and a pH of around 8.0. As with gastric tolerance, the tolerance to small intestine conditions of probiotic

bacteria may also be influenced by the carrier food. Possemiers et al. (2010) reported significantly higher number of *L. helveticus* and *B. longum* after exposure to the conditions simulating the small intestine when embedded in the chocolate compared to the half skimmed-milk matrix. In this case, it seems likely that the chocolate matrix may have provided an additional protection (possibly due to its high fat content) towards probiotic survival during intestinal transit.

Probiotics surviving within the gastrointestinal tract must then adhere to the intestinal epithelium. Guglielmetti et al. (2009) reported that the *in vitro* adhesion ability of *B. bifidum* MIMBb75 was strongly influenced by the environmental conditions including pH, and the presence of sugars and bile salts. Minerals such as calcium are known to increase adhesion of some lactobacilli strains to Caco-2 cell lines (Chauviere et al., 1992) by providing a supportive ionic bridge between surfaces of bacterial and epithelial cells (Kleeman & Klaenhammer, 1982). Since dairy products are rich in calcium (Neville et al., 1994) dairy based carrier food matrices may be more likely to facilitate intestinal adhesion of probiotics than carrier materials with less calcium content. Lactic acid bacteria have previously demonstrated improved adhesion levels to human and pig small intestine cells in the presence of milk (Conway et al., 1987). In contrast, dairy based food matrices were demonstrated by Ouwehand et al (2001) to exert negative effects on the adhesive ability of *L. reuteri* and *L. brevis in vitro*.

Clearly, acid-bile tolerance and adhesive ability are essential considerations in evaluating the efficacy probiotic organisms, and as such there have been many *in vitro* studies of these functional properties in potential probiotics prior to incorporation into carrier foods (Schillinger et al., 2005). However, *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, which are known to exhibit poor survival when challenged with gastric acidity *in vitro*, have shown high survival rates in the intestine of Gottingen minipigs when fed with yogurt (Lick et al., 2001). Questions therefore arise regarding the relationship of these functional properties to the carrier food matrix in probiotic foods, and hence the relevance of using "non-food" models when attempting to evaluate the gastro-intestinal tolerance of probiotic organisms. As suggested by Schillinger et al (2005) it would seem to make more sense to

study the tolerance to gastrointestinal conditions with strains incorporated into the final food product. These functional properties of probiotics may vary due to various factors associated with the carrier foods including ingredients used, manufacturing procedures, physico-chemical properties and storage conditions. To date however, there has been little study on the effect of different food carriers on the gastrointestinal tolerance and adhesion ability of probiotic bacteria (Ouwehand et al., 2001; Rivera-Espinoza & Gallardo-Navarro, 2010; Saxelin et al., 2010). To this end, this study aimed to assess *in vitro* the rates of survival and adhesion properties of probiotic strains in actual carrier food matrices.

6.1.1 Experimental design and research hypotheses

The objectives of this study were to evaluate the *in vitro* gastrointestinal tolerance and adhesion properties of *P. jensenii* 702, *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 in the presence of different carrier foods including plain yogurt, stirred fruit yogurts, and ice cream, all made from goat's milk. To facilitate this evaluation, goat's milk ice cream (stored at -20^{o}C for 24 weeks, Chapter 5), and freshly prepared plain and 10% stirred fruit yogurts (Chapter 4), were chosen for this study. These probiotic goat's milk yogurts and ice cream all maintained acceptable characteristics with respect to probiotic viability, physico-chemical and sensory properties, but differed significantly from one another in terms of their composition, preparation methods and storage conditions. As such, these products represent a useful model for examining the variable effects of different food matrices on the chosen functional aspects of probiotic performance.

As explained in Chapter 3, food normally remains in the stomach for 2-4 hours. However the physical nature of food can affect transit time through the stomach, with liquids generally transiting more quickly than solids. For example, liquid foods only take about 20 minutes to pass through the stomach while solid foods may take much longer (Huang & Adams, 2004). In this study the viability of these probiotics in the respective food products was tested prior to addition to simulated gastric juice (0 minute) at three different pH levels (pH 2.0, 3.0 and 4.0) and then at 1, 30, 60 and 180 minutes after exposure. Tolerance to simulated small intestinal juice (both with and without 0.3% bile) was examined in a

similar manner, with viable cell counts taken at 1, 120 and 240 minutes intervals after exposure.

The pH values of goat's milk ice cream plain and stirred fruit yogurts used for this study were 6.6, 4.4 and 4.3 respectively. These differences in pH may possibly cause variations in acid tolerance due to their variable capacity to buffer the simulated gastric juice. Fat contents, which may affect the bile tolerance of probiotics (Stanton et al., 1998; Valerio et al., 2006), were ~10% for goat's milk ice cream and ~5% for both types of yogurts. Probiotic lactobacilli and bifidobacteria can enter a viable-but-nonculturable state (dormant) when exposed to harsh condition and during long storage (Lahtinen et al., 2005; Lahtinen et al., 2006; Possemiers et al., 2010). Certain bacteria can also lose their ability to divide after freezing and thawing. Freezing can cause damage to the cell membrane of bacteria and change its morphology (Thunell et al., 1984). Since specific cellular surface components such as external appendages and adhesion promoting proteins (Servin & Coconnier, 2003) are associated with the bacterial adhesion to intestinal epithelial cells, freezing and thawing may affect their adhesion and colonization in the gut. Since ice cream is a frozen product, probiotics in ice cream may lose their adhesion ability compared to their presence in unfrozen yogurts. *In vitro* assessment of adhesion is considered an important selection criteria for probiotics (Ouwehand et al., 2001) and may provide a reasonable representation of *in vivo* conditions. Therefore, a Caco-2 cell model which was previously described in Chapter 3 was used in this study to assess the adhesion properties. Within the context of the broader research objectives outlined above, this study addressed five specific hypotheses:

1. All three probiotic bacteria would demonstrate their lowest *in vitro* gastric tolerance at pH 2.0 (simulated gastric juice) compared to pH 3.0 and 4.0 in each of the carrier foods.
2. Compared with probiotics in both types of yogurts, probiotics in ice cream would demonstrate higher *in vitro* gastric tolerance at every pH level due to the higher initial pH of ice cream (~6.6) compared to the plain (~4.4) and fruit yogurts (~4.3).

3. In each of the carrier foods, all three probiotic bacteria would demonstrate lower tolerance to simulated small intestinal juice containing 0.3% bile than intestinal juice without bile.
4. Compared with probiotics in both types of yogurts, probiotics in ice cream would demonstrate higher *in vitro* tolerance to 0.3 % bile due to the protective effect of the higher fat content in ice cream.
5. For all three probiotic strains, inclusion in ice cream would result in lower rates of intestinal cell adhesion than inclusion in yogurts, due to the extended frozen storage time of the ice cream.

6.2 Materials and methods

6.2.1 Ice cream and yogurt samples

The food carriers used in this study were the chocolate flavoured goat's milk ice cream stored at -20^{o}C for 24 weeks (refer Chapter 5) and the plain and 10% stirred fruit yogurts (refer Chapter 4) which were prepared within 24 hours prior to analysis and stored at 4°C. All were produced from goat's milk as described in Chapter 2 (section 2.6).

6.2.2 *In vitro* gastrointestinal transit tolerance assay

Simulated gastric and small intestinal juices were prepared as described in Chapter 2 (2.9.1.1). To perform the gastrointestinal transit tolerance assay, 1 g of each dairy food sample containing probiotics was exposed to 9 ml of gastric (pH 2.0, 3.0 and 4.0) and small intestinal juice (pH 8.0) with or without bile salts, as described in Chapter 2 (2.9.1.2).

6.2.3 *In vitro* adhesion assay

One (1) g of each dairy food sample was transferred into each well of the 24-well plate of previously prepared Caco-2 monolayer cells (Chapter 2, section 2.9.2.1). The adhesion assay was performed as described in Chapter 2, (2.9.2.2).

6.2.4 Scanning electron microscopy

Caco-2 monolayer samples with adhered probiotic bacteria were observed using a scanning electron microscope (Philips XL30, Philips, Eindhoven, The Netherlands). Samples for microscopic observations were prepared as described in Chapter 2, section 2.9.2.1 and section 2.9.2.3.

6.2.5 Statistical analysis

Data analyses were performed using SPSS/PASW statistical software version 17 (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) and analysis of variance with repeated measures were used in data analysing with Bonferroni post hoc test for means comparison. A p value <0.05 was considered statistically significant for all analysis.

6.3 Results

The presentation of data in this chapter includes observations based on the influence of carrier foods on *in vitro* probiotic efficacy with special reference to their gastrointestinal tolerance and adhesion ability. It begins with a comparison of the gastric juice tolerance of the probiotics *P. jensenii* 702, *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 in the three different carrier foods during 180 minutes of exposure to simulated gastric juice at three different pH levels (2.0, 3.0 and 4.0). This is followed by a comparison of tolerance to simulated small intestinal juice (with or without bile salt) during 240 minutes of exposure. Finally, the *in vitro* adhesion ability of these probiotics in different carrier foods is presented. While the specific objectives of this study were addressed by these data, additional qualitative assessments were made by observing probiotic adhesion through scanning electron microscopy.

6.3.1 Gastrointestinal tolerance

The pH of the gastric transit test mixture increased as a result of addition of the carrier foods containing probiotics, with the pH of the original mixtures (2.0, 3.0 and 4.0) raised to 2.6, 3.6 and 4.2 respectively in the presence of plain and fruit yogurts and 2.8, 3.9 and 6.3 respectively in the presence of ice cream, at the end of the simulated gastric transit. The pH of the simulated small intestinal juice also changed as a result of the addition of carrier foods. The initial pH of the mixture (8.0) was reduced to 4.3, 4.5 and 6.1 in the case of intestinal juice without bile and to 4.7, 4.8 and 6.4 in the case of intestinal juice with 0.3% bile for the fruit yogurt, plain yogurt and ice cream respectively.

Comparison of bacterial counts in the three gastric juice preparations clearly indicated that the pH level of the simulated gastric juice had a significant effect on probiotic viability regardless of the carrier food matrix. At pH 2.0 each probiotic demonstrated a progressive reduction in viability during *in vitro* gastric transit compared to pH 3.0 and pH 4.0. In general the viability of all three probiotics remained largely unaffected at the end of *in vitro* gastric transit at pH 3.0 and pH 4.0, in all carrier foods. In contrast significant differences were observed between the viabilities of the probiotics in the three carrier foods across the 180 minutes of exposure to gastric juice at pH 2.0 (Table 6.1-6.3). Although strain dependent variation was apparent, all three probiotics demonstrated low gastric tolerance in fruit yogurt while use of ice cream as a carrier food matrix provided a relatively positive influence on viability retention of each probiotic during gastric transit with simulated gastric juice at pH 2.0. Little variation between food matrices in terms of probiotic survivability was evident for *P. jensenii* 702 and *L. acidophilus* LA-5 up to 30 minutes at pH 2. However after 180 minutes of exposure, *P. jensenii* 702 and *B. animalis* subsp. *lactis* BB-12 showed much greater tolerance in ice cream, while *L. acidophilus* LA-5 showed stronger tolerance in plain yogurt. Overall, *P. jensenii* 702 exhibited lowest gastric juice tolerance at pH 2.0.

With regard to simulated intestinal juice, the inclusion of 0.3 % bile salt had a significant influence on reducing probiotic viability during *in vitro* small intestine transit (Table 6.4-

6.6). With the exception of *B. animalis* subsp. *lactis* BB-12 in ice cream, this effect was clearly evident even 1 minute after exposure to 0.3% bile in all three bacteria regardless of carrier food type. Overall, ice cream demonstrated a significant influence on improving probiotic viability in the presence of 0.3% bile compared to plain and stirred fruit yogurts, with reductions in the viable counts of all three bacteria limited to ≤ 2 orders of magnitude in this product, across the 240 minutes of exposure. In general *P. jensenii* 702 showed lower bile tolerance than *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 in plain and fruit yogurts with no viable counts detected after 120 and 240 minutes respectively, but similar tolerance to *L. acidophilus* LA-5 in ice cream.

Table 6.1 Effect of carrier food: goat's milk ice cream, plain and 10% stirred fruit yogurts on the viability of *L. acidophilus* LA-5 during 180 minutes of exposure to simulated gastric juices at pH 2.0, 3.0 and 4.0 (*n* = 3)

Carrier food	pH of simulated gastric juice	Viable counts (log cfu/g) during simulated gastric transit tolerance				
		0 min	1 min	30 min	60 min	180 min
Ice cream	2.0	7.59±0.03^{a}	6.96±0.02^{Aa}	6.39±0.06^{Aa}	4.62±0.04^{Aa}	4.03±0.14^{Aa}
Plain yogurt	2.0	7.24±0.02^{b}	7.63±0.01^{Ab}	7.77±0.00^{Ab}	6.80±0.09^{Ab}	3.73±0.07^{Ab}
Fruit yogurt	2.0	7.58±0.02^{a}	7.42±0.01^{b}	5.65±0.19^{Ac}	3.38±0.12^{Ac}	<1
Ice cream	3.0	7.59±0.03^{a}	7.53±0.04^{a}	7.67±0.05^{a}	7.49±0.02^{ab}	7.45±0.02^{ab}
Plain yogurt	3.0	7.24±0.02^{b}	7.65±0.07^{Aa}	7.62±0.05^{Aa}	7.69±0.07^{Aa}	7.60±0.02^{Aa}
Fruit yogurt	3.0	7.58±0.02^{a}	7.39±0.04^{a}	7.56±0.05^{a}	7.38±0.05^{b}	7.31±0.04^{b}
Ice cream	4.0	7.59±0.03^{a}	7.60±0.01^{a}	7.59±0.03^{a}	7.58±0.01^{a}	7.65±0.05^{a}
Plain yogurt	4.0	7.24±0.02^{b}	7.68±0.06^{Aa}	7.70±0.03^{Aa}	7.69±0.07^{Aa}	7.62±0.07^{Aa}
Fruit yogurt	4.0	7.58±0.02^{a}	7.56±0.03^{a}	7.48±0.05^{a}	7.53±0.02^{a}	7.53±0.04^{a}

Mean value (±SE)

^{A}In the same row indicates a significant difference of mean viable counts compared to that at 0 minutes ($p<0.05$).

^{a,b}Values in the same column having different superscripts for mean viable counts at each pH level differ significantly ($p<0.05$).

Table 6.2 Effect of carrier food: goat' milk ice cream, plain and 10% stirred fruit yogurts on the viability of *B. animalis* subsp. *lactis* BB-12 during 180 minutes of exposure to simulated gastric juices at pH 2.0, 3.0 and 4.0 (*n* = 3)

Carrier food	pH of simulated gastric juice	Viable counts (log cfu/g)				
		0 min	1 min	30 min	60 min	180 min
Ice cream	2.0	8.02±0.02^{a}	7.07±0.05^{Aa}	6.92±0.02^{Aa}	5.18±0.05^{Aa}	5.20±0.02^{Aa}
Plain yogurt	2.0	7.96±0.05^{a}	7.58±0.02^{Ab}	<1	<1	<1
Fruit yogurt	2.0	7.91±0.07^{a}	7.78±0.05^{c}	2.89±0.11^{Ab}	<1	<1
Ice cream	3.0	8.02±0.02^{a}	8.10±0.02^{a}	7.87±0.04^{a}	8.02±0.03^{a}	7.77±0.09^{a}
Plain yogurt	3.0	7.96±0.05^{a}	7.87±0.02^{b}	7.70±0.07^{ab}	7.66±0.03^{b}	7.70±0.02^{a}
Fruit yogurt	3.0	7.91±0.07^{a}	7.75±0.03^{b}	7.55±0.03^{b}	7.67±0.02^{b}	7.70±0.02^{a}
Ice cream	4.0	8.02±0.02^{a}	8.09±0.02^{a}	7.92±0.02^{a}	8.09± 0.06^{a}	8.10±0.03^{a}
Plain yogurt	4.0	7.96±0.05^{a}	7.88±0.06^{b}	7.73±0.04^{ab}	7.85±0.01^{b}	7.82±0.04^{b}
Fruit yogurt	4.0	7.91±0.07^{a}	7.79±0.06^{b}	7.29±0.34^{Ab}	7.70±0.04^{b}	7.75±0.06^{b}

Mean value (±SE)

^{A}In the same row indicates a significant difference of mean viable counts compared to that at 0 minutes ($p<0.05$).

^{a,b}Values in the same column having different superscripts for mean viable counts at each pH level differ significantly ($p<0.05$).

Table 6.3 Effect of carrier food: goat's milk ice cream, plain and 10% stirred fruit yogurts on the viability of *P. jensenii* 702 during 180 minutes of exposure to simulated gastric juices at pH 2.0, 3.0 and 4.0 (*n* = 3)

Carrier food	pH of simulated gastric juice	Viable counts (log cfu/g) during simulated gastric transit tolerance				
		0 min	1 min	30 min	60 min	180 min
Ice cream	2.0	8.65±0.02^{a}	5.46±0.01^{Aa}	6.22±0.04^{Aa}	4.91±0.04^{Aa}	3.36±0.13^{Aa}
Plain yogurt	2.0	8.61±0.04^{a}	8.42±0.00^{b}	6.85±0.00^{Ab}	<1	<1
Fruit yogurt	2.0	8.55±0.01^{a}	8.04±0.00^{Ab}	5.57±0.11^{Ac}	<1	<1
Ice cream	3.0	8.76±0.02^{a}	8.52±0.03^{a}	8.76±0.03^{a}	8.67±0.05^{a}	8.74±0.06^{a}
Plain yogurt	3.0	8.61±0.04^{a}	8.52±0.02^{a}	8.53±0.03^{a}	8.50±0.04^{a}	8.40±0.02^{a}
Fruit yogurt	3.0	8.55±0.01^{a}	8.40±0.01^{a}	8.39±0.01^{a}	8.44±0.03^{a}	8.36±0.03^{a}
Ice cream	4.0	8.76±0.02^{a}	8.77±0.03^{a}	8.73±0.04^{a}	8.81±0.05^{a}	8.75±0.00^{a}
Plain yogurt	4.0	8.61±0.04^{a}	8.55±0.02^{a}	8.54±0.04^{a}	8.48±0.03^{a}	8.37±0.07^{a}
Fruit yogurt	4.0	8.01±0.01^{b}	8.02±0.05^{b}	8.08±0.03^{b}	7.55±0.02^{Ab}	7.71±0.02^{Ab}

Mean value (±SE)

^{A}In the same row indicates a significant difference of mean viable counts compared to that at 0 minutes ($p<0.05$).

^{a,b,c}Values in the same column having different superscripts for mean viable counts at each pH level differ significantly ($p<0.05$).

Table 6.4 Effect of carrier food: goat's milk ice cream, plain yogurt and 10% stirred fruit yogurt on viability of *L. acidophilus* LA-5 during 240 minutes of exposure to simulated small intestinal juice (*n* = 3)

Carrier food	Bile salt percentage	Viable counts (log cfu/g)			
		0 min	1 min	120 min	240 min
Ice cream	0	7.66±0.04[a]	7.52±0.04[a]	ND	7.75±0.03[a]
Plain yogurt	0	7.24±0.02[b]	7.67±0.08[Aa]	7.60±0.04[Aa]	6.80±0.04[Ab]
Fruit yogurt	0	7.58±0.02[a]	7.52±0.04[a]	7.47±0.00[a]	7.37±0.30[c]
Ice cream	0.3	7.27±0.06[a]	6.62±0.08[Aa]	6.00±0.00[Aa]	5.68±0.06[Aa]
Plain yogurt	0.3	7.24±0.02[a]	6.28±0.05[Ab]	4.15±0.04[Ab]	3.80±0.02[Ab]
Fruit yogurt	0.3	7.58±0.02[b]	7.14±0.06[Ab]	4.11±0.05[Ab]	3.07±0.12[Ac]

Mean value (±SE)

ND = Not detected

[A]In the same row indicates a significant difference of mean viable counts compared to that at 0 minutes ($p<0.05$).

[a,b]Values in the same column having different superscripts for mean viable counts at each bile level differ significantly ($p<0.05$).

Table 6.5 Effect of carrier food: goat's milk ice cream, plain yogurt and 10% stirred fruit yogurt on viability of *B. animalis* subsp. *lactis* BB-12 during 240 minutes of exposure to simulated small intestinal juice (*n* = 3)

Carrier food	Bile salt percentage	Viable counts (log cfu/g)			
		0 min	1 min	120 min	240 min
Ice cream	0	8.02±0.02^{a}	8.08±0.01^{a}	ND	7.73±0.05^{a}
Plain yogurt	0	7.96±0.05^{a}	7.81±0.03^{b}	7.46±0.04^{Aa}	7.81±0.02^{ab}
Fruit yogurt	0	7.91±0.07^{a}	7.86±0.02^{b}	8.19±0.32^{b}	8.03±0.06^{b}
Ice cream	0.3	8.02±0.02^{a}	7.70±0.10^{a}	7.49±0.10^{Aa}	7.18±0.09^{Aa}
Plain yogurt	0.3	7.96±0.05^{a}	4.00±0.00^{Ab}	3.34±0.16^{Ab}	3.17±0.09^{Ab}
Fruit yogurt	0.3	7.91±0.07^{a}	5.32±0.06^{Ac}	3.51±0.00^{Ab}	3.50±0.50^{Ab}

Mean value (±SE)

ND = Not detected

^{A}In the same row indicates a significant difference of mean viable counts compared to that at 0 minutes ($p<0.05$).

^{a,b,c}Values in the same column having different superscripts for mean viable counts at each bile level differ significantly ($p<0.05$).

Table 6.6 Effect of carrier food: goat's milk ice cream, plain yogurt and 10% stirred fruit yogurt on viability of *P. jensenii* 702 during 240 minutes of exposure to small intestinal juice (*n* = 3)

Carrier food	Bile salt percentage	Viable counts (log cfu/g)			
		0 min	1 min	120 min	240 min
Ice cream	0	8.76±0.02[a]	8.71±0.06[a]	ND	8.66±0.01[a]
Plain yogurt	0	8.61±0.04[a]	8.60±0.02[ab]	8.42±0.07[a]	8.43±0.05[ab]
Fruit yogurt	0	8.55±0.01[a]	8.46±0.01[b]	8.39±0.02[a]	8.32±0.02[b]
Ice cream	0.3	8.02±0.00[a]	7.40±0.04[Aa]	5.49±0.04[Aa]	5.81±0.08[Aa]
Plain yogurt	0.3	8.61±0.04[b]	6.88±0.05[Ab]	<1	<1
Fruit yogurt	0.3	8.55±0.01[b]	7.02±0.02[Ab]	3.75±0.03[Ab]	<1

Mean value (±SE)

ND = Not detected

[A]In the same row indicates a significant difference of mean viable counts compared to that at 0 minutes ($p<0.05$).

[a,b]Values in the same column having different superscripts for mean viable counts at each bile level differ significantly ($p<0.05$).

6.3.2 Adhesion ability

Compared to the initial cell counts, the proportion of cells of each probiotic strain that were found to adhere to Caco-2 cell layers during the adhesion assay was relatively low regardless of the carrier food type, although the adhesion rates of all three were significantly higher when incorporated into fruit yogurt compared to plain yogurt and ice cream (Table 6.7). *In vitro* viability levels were widely varied between carrier food types as well as probiotic species. Carrier food matrix demonstrated a significant influence on probiotic adhesion ability, with an almost 100-fold difference in the adhesion rate of *B. animalis* subsp. *lactis* BB-12 when incorporated in plain yogurt as opposed to same probiotic in fruit yogurt was the most obvious example. Adhesion of probiotic bacteria on

to Caco-2 cell line from different carrier food matrix was shown by scanning electron microscopy (Figure 6.1). Similar to the observations made in Chapter 3 bacterial clumping during adhesion was apparent only in the specimens which utilized yogurts as the carrier food matrix, while such clustering was not clearly observed among the probiotics adhered into Caco-2 cells from ice cream.

Table 6.7 Percentage of cell adhered, and viable probiotic cell counts in goat's milk ice cream, plain and 10% stirred fruit yogurts, before and after 2 hours exposure to Caco-2 cells (*n* = 3)

Probiotic	Carrier food	Viable counts (log cfu/g)		%
		Before	After	
L. acidophilus LA-5	Ice cream	7.46 ±0.01	5.19 ±0.01	0.54[a]
	Plain yogurt	7.70 ±0.04	5.34 ±0.02	0.44[a]
	Fruit yogurt	7.67 ±0.06	5.70 ±0.01	1.06[b]
B. animalis subsp. *lactis* BB-12	Ice cream	7.79 ±0.07	5.66 ±0.07	0.73[a]
	Plain yogurt	8.48 ±0.09	5.06 ±0.05	0.04[b]
	Fruit yogurt	8.39 ±0.10	6.39 ±0.03	1.00[a]
P. jensenii 702	Ice cream	8.39 ±0.01	6.17 ±0.02	0.61[a]
	Plain yogurt	8.78 ±0.01	6.37 ±0.02	0.39[b]
	Fruit yogurt	8.63 ±0.03	6.59 ±0.02	0.93[c]

Mean value (±SE)

[A, B, C] Values in the same column having different superscripts for a particular bacteria differ significantly ($p<0.05$)

[a, b] Values in the same row having different superscripts differ significantly ($p<0.05$)

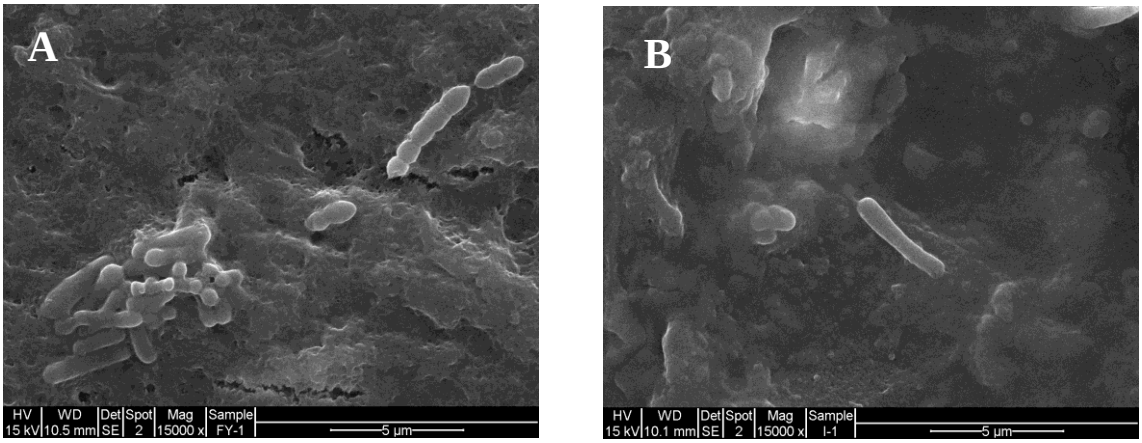


Figure 6.1 Scanning electron micrograph of adhered probiotics into Caco-2 cells from goat's milk stirred fruit yogurt (A) and ice cream (B).

6.3.3 Summary of key findings

Carrier food matrix (goat's milk ice cream, plain and fruit yogurt) had a significant influence on the *in vitro* gastro-intestinal tolerance of all three probiotics when exposed to both highly acidic conditions (pH 2.0) and 0.3 % bile. As hypothesised, exposure to conditions of lower pH (i.e. pH 2.0) resulted in a significant reduction in probiotic viability during simulated gastric transit tolerance compared to pH levels of 3.0 and 4.0. However, ice cream was generally found to improve the acid and bile tolerance of the probiotics compared to plain and stirred fruit yogurts. In a similar manner, the *in vitro* adhesion ability of probiotics was found to be influenced by the carrier food matrix, with fruit yogurt providing the most favourable outcomes, although in all cases a substantial number of viable bacteria (10^5-10^6 cfu/g) were able to attach to the Caco-2 cells.

6.4 Discussion

In relation to the aims of this study, several key findings have emerged with respect to *in vitro* probiotic survivability in simulated gastric juice at different pH levels and in simulated small intestinal juice with or without bile salts. Several lines of evidence among

the findings suggested that carrier food type may have affected the gastrointestinal tolerance and adhesion properties of these probiotics. Most significant among these were the generally higher viable cell numbers in ice cream compared to the yogurts in both simulated gastric and small intestinal juice.

6.4.1 Effect of carrier foods on probiotic viability during simulated gastric and small intestine transit

Both *L. acidophilus* and bifidobacteria have been reported to be moderately resistant to acid and bile, although large differences have nonetheless been shown to exist even between these strains (Charteris et al., 1998a; Liong & Shah, 2005). The results presented in Chapter 3 also confirmed species variations in acid and bile tolerance among lactobacilli, bifidobacteria and propionibacteria. Similarly, different probiotics used in this study have demonstrated different levels of acid and bile tolerance especially at pH 2.0 and in the presence of bile salts. Furthermore, the level of tolerance was observed to be influenced by the type of carrier food matrix. In general ice cream appeared to contribute to an improvement in the viability of probiotics compared to plain or stirred fruit yogurts at pH 2.0 and at 0.3 % bile salts in simulated gastric and small intestinal juice respectively. Similar influences of carrier food matrix on the gastro-intestinal tolerance of probiotics have been observed in a recent clinical study (Saxelin et al., 2010). According to Saxelin et al (2010) yogurt yielded the highest and cheese the lowest faecal quantity of *P. freudenreichii* subsp. *shermanii* JS and *B. animalis* subsp. *lactis* Bb 12 when these probiotics were administered with low fat (0.44% w/w)-low lactose (0.80% w/w) yogurt, low fat (15% w/w and 0% lactose)-semi hard cheese, or in cellulose capsules, although no food matrix effect on gastrointestinal survival of certain strains such as *L. rhamnosus* GG and *L. rhamnosus* LC705 was observed. These findings are nonetheless in general agreement with the results of the present study which has demonstrated strain variation as well as a food matrix effect, on probiotic survival in the presence of simulated gastric and small intestinal juice comprising 0.3% bile.

L. plantarum MF 1298 was also found to survive better during gastrointestinal transit in human subjects when administered with fermented sausage, than by direct administration as a freeze dried form, suggesting a protective role by the sausage (Klingberg & Budde, 2006). Meat may protect bacteria in an acidic environment by acting as a buffer, or by simple physical "encapsulation" within a matrix of sausage meat and fat for example (Tyopponen et al., 2003). Likewise, the higher fat percentage in ice cream (~ 10%) compared to yogurts (~ 5%) may have provided better protection to probiotics by reducing their exposure to acid and bile in the present study. Furthermore, the ice cream contained additional ingredients such as cocoa powder and stabilizers (guar gum and dextrose) not present in the yogurts. These ingredients may also have provided some protection towards probiotic survival during simulated gastric and intestine transit in the present study by acting as a protective cover against gastric and small intestinal juices. Guar gum which is a polysaccharide derived from the seeds of *Cyamopsis tetragonolobus*, has previously used as an efficient protective carrier material for 5-aminosalicylic acid (a colon specific drug in the treatment of inflammatory bowel diseases) in the presence of simulated gastric and small intestinal juices (Krishnaiah et al., 1999). The lipid fraction of cocoa butter has reported to provide a protection for *B. longum* from the surrounding stress factors such as H^+ ions (Lahtinen et al., 2007) and chocolate, of which cocoa is one of the major ingredients has also been reported to enhance *in vitro* gastrointestinal survival of *L. helveticus* and *B. longum* (91% and 80% respectively) compared to milk (20% and 30%) (Possemiers et al., 2010). In addition, these ingredients may possess prebiotic effect (Aragon-Alegro et al., 2007; Edwards & Benjamin, 2003) and may provide additional benefits such as growth promotion during gastrointestinal transit.

Corcoran et al (2005) found that sugars such as glucose enhanced the survival of *L. rhamnosus* GG in the presence of simulated gastric juice at pH 2.0. These authors concluded that glucose provides ATP to F_0F_1-ATPase via glycolysis (a mechanism that gram-positive organisms use for protection against acidic conditions), enabling proton exclusion and thereby enhancing survival during gastric transit. Therefore, it seems possible that higher sugar content in ice cream (12% w/w) compared to the yogurts may have contributed to improved acid tolerance of *P. jensenii* 702 and *B. animalis* subsp. *lactis*

BB-12 at pH 2.0 in the present study. However, in apparent contradiction to this assertion *L. acidophilus* LA-5 in this study demonstrated a satisfactory gastric tolerance at pH 2.0 when incorporated into ice cream as well as plain yogurts which did not add any sugar during manufacturing. This result may reflect differences in the above mentioned mechanism of acid tolerance at the strain/species level. The *in vitro* gastric acid resistance of *L. casei* in flavoured commercial fermented milk has been reported to vary in relation to the presence of flavourings (natural, strawberry, multi-fruits and vanilla), storage time and storage temperature (Vinderola et al., 2011). Likewise, in the present study, differences in the storage temperatures and storage time of the ice cream (-20^{o}C for 24 weeks) and yogurts (4^{o}C, <1 day) may have caused variations in the acid tolerance of the probiotics. The micro-architecture of different food products may also protect probiotics in the harsh gastro-intestinal environment (Lavermicocca, 2006; Lavermicocca et al., 2005; Valerio et al., 2006). Therefore, wide variations in the acid and bile tolerance of probiotics fortified within different food matrices in the present study may have resulted not only from differences in the chemical composition, but also from differences in the physical structure of ice cream, plain and fruit yogurt.

Fermentation pH and duration have also been found to have a significant impact on survivability of *L. rhamnosus* GG under acidic conditions *in vitro*. For example, Ampatzoglou et al (2010) observed that the acid tolerance of *L. rhamnosus* GG cells collected at late exponential phase was considerably higher than the cells collected at mid stationary and late stationary phases of growth. In contrast, Lorca et al. (1998) demonstrated that stationary phase cells of *L. acidophilus* were more resistant to low pH than exponential phase cells. While these examples highlight a lack of consensus within the published literature regarding the acid tolerance of probiotics in different growth stages, it does seem that functional properties such as acid tolerance may vary throughout the life cycle of the culture. In the present study, probiotics in ice cream were fermented for one hour only while yogurts were fermented for approximately 3 ½ hours to obtain the desired consistency and sensory attributes in the final product. Therefore, it is possible that the probiotic cells may have been in different phases of growth in the ice cream and yogurts, resulting in differences in the acid and bile tolerance of the bacteria in the respective

products. Differences in fermentation time may also have contributed to differences in the pH levels of the ice cream and yogurts.

Increases in the pH of the gastric content as a result of addition of a food matrix, has previously been reported to improve the viability of probiotics (Conway et al., 1987; Huang & Adams, 2004; Mainville et al., 2005; Saarela et al., 2006; Wang et al., 2004). According to Salaun et al (2005), one major factor affecting such variations in pH is the buffering capacity of the food product, a physico-chemical characteristic that corresponds to the ability of the product to be acidified or alkalinized. The buffering capacity of dairy products mainly depends on the composition and distribution of small constituents (inorganic phosphate, citrate, organic acid) and milk proteins (casein and whey proteins) between the aqueous and solid phase. Qualitative and quantitative variations in these constituents in different dairy foods due to different manufacturing procedures can therefore cause differences in the buffering capacity of different dairy products. Thus, it is highly possible that the goat's milk ice cream and yogurt produced in this study may vary in their buffering capacity. In the present study, addition of the food matrix into simulated gastric and intestinal juices was observed to change the pH of the food-simulated juice mixture, with ice cream raising the pH of gastric juice to a higher level than the yogurts. Since even small changes in pH can have a large impact on the probiotic survival in low pH environments (Saarela et al., 2006), this factor (change in pH) may have been largely responsible for the observed enhancement in the gastric tolerance of the probiotics in the ice cream compared to the yogurts.

In this study, all three probiotics were able to tolerate higher pH values (3.0 and 4.0) in simulated gastric juice compared to pH 2.0 regardless of the carrier food matrix. These results are comparable to the findings of Huang and Adams (2004) in which 13 dairy propionibacteria strains have been shown to survive well at pH 3.0 and 4.0 compared to pH 2.0. This may be due to the intrinsic resistance of probiotics to higher pH such as pH 3.0 and 4.0 as well as to the fact that optimum activity of the gastric enzyme pepsin, which may also impact on probiotic viability, is closer to pH 1.5-2.0 (Schnaith, 1989; Vinderola et al., 2011).

In probiotic selection tolerance to small intestine conditions is of potentially more importance than gastric survival, because with the development of new delivery systems and use of specific foods probiotic strains can be buffered through the stomach to facilitate their colonization in the gut (Havenaar et al., 1992; Huang & Adams, 2004). Generally there was no significant reduction in viable cell numbers of these probiotics during 240 minutes exposure to the simulated small intestinal juice without bile in the present study, while numbers declined substantially in the presence of bile salts. Many authors have reported a negligible effect of pancreatic enzymes (in simulated small intestinal conditions without bile salts) on the viability of lactic acid bacteria *in vitro* (Champagne & Gardner, 2008; Maragkoudakis et al., 2006; Ruiz-Moyano et al., 2008). This would suggest that by comparison with the effect of bile salts, the impact of pancreatin on the survival of probiotics is relatively low.

In this study, the plain and fruit yogurts contained the starter culture bacteria: *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus,* in addition to probiotic cultures, while the ice cream contained only probiotics. While addition of yogurt starter culture is mandatory in the manufacturing of yogurt to obtain the required product qualities, this is not necessary in manufacturing probiotic ice cream. These differences in the microorganism composition of the different products may affect the functional properties of probiotics due to interactions between different microorganisms. For example, yogurt starter culture bacteria can bind and de-conjugate bile salts (Iyer et al., 2010a; Iyer et al., 2010b; Pigeon et al., 2002; Vinderola & Reinheimer, 2003) and may thus provide additional protection for probiotic species. However, in the present study, probiotic viability remained significantly higher in the presence of 0.3% bile when fortified with ice cream by comparison with the plain and fruit yogurts, probably due to the protective effect of higher fat content of ice cream compared to the yogurt as explained earlier. Therefore, it seems likely that the composition of the carrier food matrix such as fat content is more important than the strain present and their interactions in terms of bile tolerance of probiotics.

Probiotic viability loss is more likely when exposed to stress factors such as acid and bile for longer time periods, and in this study, transit time certainly appeared to influence the bile salt and gastric tolerance (especially at pH 2.0) of probiotics. In accordance with other authors (Huang & Adams, 2004; Mishra & Prasad, 2005; Pan et al., 2009), all the strains showed progressive reduction in viability during 180 minutes of gastric and 240 minutes of small intestine transit in this study, however, strain dependent variations were apparent in the rate of viability loss.

6.4.2 Effect of carrier foods on adhesion ability of probiotics *in vitro*

Both adhesion ability and intestinal transit colonization of probiotic have been found to be influenced by carrier food matrices. Sexelin et al (2010) reported that *B. animalis* subsp. *lactis* Bb 12 was excreted for a longer time when it was consumed in yogurt compared to cheese or in cellulose capsule by healthy humans indicating better colonization of *B. animalis* subsp. *lactis* Bb 12 when consumed in yogurts. Poor adhesive capacity of probiotics to the intestinal lining may result in a shorter excretion time. In this case, improved persistence of *B. animalis* subsp. *lactis* Bb 12 may have been due to extra support provided by yogurts compared to cheese or cellulose capsule for probiotics to adhere strongly into intestinal epithelium. Similarly, *L. acidophilus* LA-5, *B. animalis* subsp. *lactis* BB-12 and *P. jensenii* 702 have shown significant differences in adhesion depending on carrier food type (goat's milk ice cream, plain or fruit yogurt) in the present study (Table 7.7). It seems likely that probiotic adhesion to the intestinal epithelium is carrier food as well as strain specific. Certain probiotics may have strong adhesive capacities due to various physiological and biochemical properties such as the presence of mucus-binding pili on cell wall that facilitate the adhesion process (Kankainen et al., 2009). In general, fruit yogurt improved the adhesion ability of all three probiotics while plain yogurt contributed to a lowering of the adhesion rates of probiotics in the present study. In previous studies, lower adhesion levels have been observed for probiotics when exposed to low pH environments prior to adhesion assays (Marcinakova et al., 2010; Ouwehand et al., 2001). This is somewhat contrary to the results observed here given that the, fruit yogurt, the carrier matrix with the lowest pH, produced the highest adhesion rates. It is possible

that some components of the fruit juice used in manufacturing the fruit yogurts of this study may have strengthened the adhesive capacity of the probiotics, while other physico-chemical properties such as the fat content of the different food carriers may have also influenced their adhesion properties. Milk containing 1.5% fat was previously reported to significantly reduce the adhesion ability of *Lactobacillus* GG and *L. reuteri* ING1 compared to non fat milk (Ouwehand et al., 2001).

The adhesion rates of probiotics may also be affected by the presence of starter cultures in yogurt. Since starter culture bacteria have previously shown their ability to adhere to intestinal epithelium cells (Brigidi et al., 2003; Guglielmotti et al., 2007; Iyer et al., 2010b) these bacteria may potentially compete for available binding sites on the intestinal epithelium and thereby reduce probiotic adhesion. However, evidence of such an effect was not apparent in this case as rates of probiotic adhesion were found to be generally higher in the fruit yogurt than in the ice cream which did not contain starter cultures. As explained in Chapter 3, and as the fermented milk data suggested, interactions between the probiotic strains themselves may also influence intestinal adhesion. Since the yogurt and ice cream products examined here contained all three probiotics, such effects could not be conclusively determined in this case. However, the rates of adhesion were found to be generally similar for all three probiotics, as was the pattern of variation in adhesion rates between the three products. Given that the adhesion rates of the bacteria in the fruit yogurt were significantly higher overall than in the ice cream, the plain yogurt, and indeed the fermented milk (when in triple co-culture - refer Figure 3.3), the results of this study clearly indicate the potential importance of the food matrix as a factor influencing probiotic colonisation of the gut.

Perhaps equally importantly in the case of the ice cream, despite its prolonged frozen shelf life the probiotics were able to demonstrate satisfactory adhesion ability. It therefore seems likely that prolonged storage of probiotics in frozen dairy desserts may not impact negatively on their capacity for adhesion to the intestinal epithelium.

Miyazawa et al (2011) observed changes in cellular morphology of *L. gasseri* TMC0356 based on the culture media that they were grown. *L. gasseri* TMC0356 grown in FG (Food Grade) medium were longer along the longitudinal axis than *L. gasseri* TMC0356 grown in MRS broth. When yeast peptone in the FG medium was replaced with meat extract, the lengths of cultured *L. gasseri* TMC0356 became significantly shorter. A possible explanation for this is significant changes in the composition of the cell wall and other parts of *L. gasseri* TMC0356 when cultured in FG medium, as the amino acid compositions of yeast peptone and meat extract are different. Likewise, differences in the composition/nutrients of yogurts and ice cream may have contributed to the changes in cell morphology of probiotics to varying extent and thereby caused variations in adhesion percentages in the present study. Such changes in cell morphology can also contribute to the clumping of bacteria on Caco-2 cell layers during adhesion. As explained in Chapter 3 cellular components are strongly related to their adhesion ability, bacterial autoaggregation and co-aggregation. Increase in cell length may promote clumping as it may provide more surface area for autoaggregation and co-aggregation (Schillinger et al., 2005; Zareba et al., 1997).

6.5 Conclusions

Different foods are formulated in different and unique ways. These differences in food manufacturing can affect probiotic viability as well as their functional properties. Survivability of probiotics in food during its shelf life alone is not an adequate predictor of strain functionality in adverse conditions such as low pH and the presence of bile. According to the results of this study, acid and bile tolerance of probiotic strains can potentially be improved by choosing an appropriate carrier food matrix. Even though plain and fruit yogurts were similar products to a considerable extent, regarding storage time and conditions, manufacturing procedures and physico-chemical properties such as pH, there were observable differences in acid and bile tolerance and adhesion ability of probiotics when incorporated into these two types of yogurts. All three probiotics have demonstrated significantly better acid and bile tolerance when they were incorporated in ice cream compared to yogurts. Generally, probiotics in fruit yogurt have shown better adhesion to

Caco-2 cells. The findings of this study therefore suggest that the performance of each probiotic strain should be adequately tested in the designated carrier foods, and that simple extrapolation from similar products or strains should not necessarily be accepted as a suitable surrogate by food regulatory authorities.

Chapter 7 : Storage stability, adhesion and rehydration properties of probiotics after spray drying with goat's milk

7.1 Introduction

Spray drying is a well known and widely applied technology in the food industry due to its relative efficiency in allowing high production rates at low operational cost. It is one of the common methods used to prepare food particles which are dry, stable and occupy small volumes (Gardiner et al., 2000; Lian et al., 2002; Potter, 1980), and can be considered the most widely used microencapsulation technique in the food industry (Desai & Hyun-Jin, 2005). Spray drying has been identified as a processing technique which improves the survival of probiotics in food with some additional benefits such as protection of probiotics against subsequent exposure to the harsh conditions of the gastrointestinal tract, because the process encases the bacterial cells in an outer protective coat (Anal & Singh, 2007). Satisfactory viability (10^6-10^7 cfu/g) of *S. thermophilus*, *L. acidophilus* and bifidobacteria in spray dried soy milk during storage was previously reported by Wang et al. (2004), while viability loss of spray dried *L. rhamnosus* GG in skim milk has been shown to be as low as 0.5 log after 5 weeks even at higher storage temperatures such as 25^{o}C and 37^{o}C (Ananta et al., 2005). In a further study *L. acidophilus* La-05 and *B. lactis* Bb-12 which had been microencapsulated by spray drying, were able to maintain significantly higher viability levels in the presence of acid compared to unencapsulated *L. acidophilus* La-05 and *B. lactis* Bb-12 cells (Favaro-Trindade & Grosso, 2002). Furthermore, encapsulated probiotics are protected from bacteriophage and harsh conditions such as freeze storage (Anal & Singh, 2007). Other microencapsulation techniques such as freeze drying (Reid et al., 2005) and different carrier materials such as calcium alginate (Chandramouli et al., 2004) and film-forming protein-carbohydrate-oil emulsion (Crittenden et al., 2006) have also been reported to enhance probiotic viability during gastrointestinal transit.

The research on encapsulation of probiotics has focused mainly on maintaining the viability of the bacterial cells at low pH and higher bile concentrations (Anal & Singh, 2007). It is however, important to investigate the effect of spray drying of probiotics on other functional properties such as adhesion, because of possible detrimental impacts on the cellular integrity of probiotics due to exposure to high temperatures during the spray drying procedure (Golowczyc et al., 2011; Silva et al., 2005; Silva et al., 2002). Such effects may involve a large number of cellular components, including DNA, RNA, cytoplasmic membrane and cell wall (Santivarangkna et al., 2008). As functional properties such as capacity for adhesion and colonization in the gut may be closely related to the structure of the bacterial surface and the other cellular components, spray drying may potentially reduce the functional efficacy of probiotics. Although microencapsulation of novel probiotic *P. jensenii* 702 with various non dairy based carriers, and their acid and bile tolerance following microencapsulation have been examined previously (Kotula, 2008), the adhesion rates of *P. jensenii* 702 after spray drying have not previously been studied.

Although spray drying may have positive effects on probiotic viability and certain functional properties such as gastrointestinal survival, several factors may contribute to a reduction in the survival rate of probiotics during spray drying and subsequent storage, including airflow configuration, dehydration, spray drying temperature conditions, concentration of the spray dried suspension, concentration of the probiotics in the suspension, the carrier materials used in the process, species/strain specific factors, storage temperature and packaging (Ho, 2008). Spray drying has been extensively used in the dairy industry, primarily to maintain starter cultures. Skim milk has been used widely as a spray drying carrier for probiotics due to its protective constituents including protein, carbohydrate and fat (Vega & Roos, 2006). Survival of lactobacilli in simulated gastric and small intestinal juices has been shown to improve when microencapsulated by spray drying with skim milk compared to the various other non dairy carrier materials such as inulin (Ho, 2008). Many authors have utilized cow's milk as a carrier agent in spray drying probiotics (Ananta et al., 2005; Chavez & Ledeboer, 2007; Ho, 2008; Lian et al., 2002). Although spray dried goat's milk powder is available in the market, to the best of this author's knowledge goat's milk has not been utilized as a carrier solution/suspension in

spray drying probiotics. Furthermore, limited information is available in the literature with regard to storage stability of spray dried probiotics at ambient or higher temperatures (Desai & Hyun-Jin, 2005).

Tea and coffee are among the most widely consumed beverages throughout the world (Keenan et al., 2011; Rogers et al., 2008; Sakamoto et al., 2001), and the preparation of tea and coffee with spray dried milk powder is a common practice in some South Asian countries. Thus, use of spray dried probiotic milk powder in hot beverages represents a potential means of delivering probiotics to a large consumer market. However, consideration must be given to the likely negative impact on probiotic viability of the higher brewing temperatures of these hot beverages.

7.1.1 Study design and research questions

This study aimed to evaluate the feasibility of the use of goat's milk as a carrier material in the spray drying of the probiotics *P. jensenii* 702, *L. acidophilus* LA-5, and *B. animalis* subsp. *lactis* BB-12, and to examine the effect of storage temperature on their viability during storage. Their adhesion properties and rehydration viability in maximum recovery diluents (MRD), tea and coffee after spray drying were also evaluated. Spray drying inlet and outlet temperatures were selected based on the manufacturer's recommendations and preliminary studies. During preliminary studies, lower outlet temperatures (<85^{o}C) resulted in products with undesirable properties such as higher moisture content. Therefore, 85^{o}C was selected as the outlet temperature for this study since it helped to produce a powdered product with desirable moisture content. It has also been reported that the stage of growth affects the heat resistance of microorganisms. Essentially, that bacteria in their lag and exponential/log growth phases are more susceptible to heat than bacteria in their stationary phase (Corcoran et al., 2004). For this reason, in the present study probiotic cells were harvested in their stationary phase, and subsequently spray dried. The rehydration temperature for the spray dried probiotics in coffee and tea was selected based on review of the literature regarding brewing and holding temperatures of these beverages (Feria-Morales, 1989; Jayasekera et al., 2011; Keenan et al., 2011). MRD at 25^{o}C was used as the

control. Previous studies have shown that temperature is one of the critical factors for probiotic survival during storage of spray dried powders, and higher survival rates have been obtained at lower storage temperatures (4-8^{o}C) compared to higher storage temperatures (15-30^{o}C) (Gardiner et al., 2000; Teixeira et al., 1995). However, as a dried product, storage at room temperature is desirable for spray dried probiotic powders, especially in commercial applications due to the higher operational costs associated with refrigerated storage, difficulties in transport and distribution (Lorentzen, 1978) as well as limited availability of cold storage facilities in certain areas of the world. In the southern hemisphere, indoor temperatures during summer can be as high as 30^{o}C. Thus, two different storage temperatures (4°C-refrigerated temperature and 30^{o}C-maximum ambient temperature) were used to store the spray dried products in order to determine the most suitable storage temperature.

Primarily, this study was designed to seek answers to the following questions: How does spray drying affect the viability of these probiotics? With respect to the viability retention, is there any effect of storage temperature of spray dried probiotics when goat's milk is used as the carrier material? If so, does it vary between probiotic species? Does spray drying affect the adhesion capacity of different probiotics? Can hot beverages such as tea and coffee be considered viable carriers for the delivery of probiotics to humans?

7.2 Materials and methods

7.2.1 Spray drying process, microbiological analyses and the moisture content

Probiotic *L. acidophilus* LA-5, *B. animalis* subsp. *lactis* BB-12 and *P. jensenii* 702 were resuspended in reconstituted (20% w/v) goat's milk and spray dried in a mini spray dryer (Buchi B-290, Flawil, Switzerland, inlet temperature = 195^{o}C and outlet temperature = 85^{o}C) as described in Chapter 2 (2.6.4). The spray dried powder was stored in air tight glass jars at 4^{o}C and 30^{o}C for 24 weeks. Spray dried probiotic samples from storage were used to enumerate probiotics as described in Chapter 2 (2.7). Moisture content of the spray dried

goat's milk powder was determined at the time of production and 24 weeks after production as described in Chapter 2 (2.8.3).

7.2.2 *In vitro* adhesion assay and scanning electron microscopy

One (1) g of spray dried samples was rehydrated in 9 ml of PBS buffer and 1 ml of probiotic suspension was aliquoted into each well of the 24-well plate of previously prepared Caco-2 monolayer cells (Chapter 2, section, 2.9.2.1) for the adhesion assay as described in Chapter 2 (2.9.2.2).

Samples of spray dried powder were spread thinly onto a double-sided carbon adhesive disc, anchored to the electron microscopy stub, coated with a 20nm layer of gold particles and then examined under a scanning electron microscope (Philips XL30, Philips, Eindhoven, The Netherlands).

7.2.3 Rehydration viability in tea and coffee

One (1) g of spray dried samples was rehydrated in 9 ml of MRD at 25^{o}C, black tea (1.5 g tea bag in 50 ml of tap water for 2 minutes, Dilma, Sri Lanka) and filtered coffee (2 g of powdered coffee in 50 ml of tap water for 2 minutes, Nestle, Australia) which were heated to 85^{o}C. Rehydration solutions were added to the glass test tubes containing the probiotic powders, mixed thoroughly and serial dilution technique was used to evaluate rehydration viability of probiotics as described in Chapter 2 (2.7).

7.2.4 Statistical Analysis

Data analyses were performed using SPSS/PASW statistical software version 17 (SPSS Inc., Chicago, IL, USA). One way ANOVA was used to analyse data with the Bonferroni post hoc test applied. Where appropriate, T-tests were also performed to compare means. A p value <0.05 was considered statistically significant for all analyses.

7.3 Results

The data presented in this chapter includes the effect of storage temperature on viability of spray dried probiotic bacteria *P. jensenii* 702, *L. acidophilus* LA-5, and *B. animalis* subsp. *lactis* BB-12 and their survivability after spray drying. Moisture content of the spray dried product, adhesion ability of probiotics in spray dried product, rehydration viability of probiotics in MRD, tea and coffee, and scanning electron microscopy of spray dried product are also presented.

7.3.1 Viability after spray drying

Both *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 required 24 hours of anaerobic incubation at 37^{o}C in MRS broth and RC medium respectively to enter their stationary phases while *P. jensenii* 702 required 72 hours of anaerobic incubation at 30^{o}C in SL broth. The maximum population was found to be ~ 4.2 x 10^{8} cfu/ml for both *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12, and ~10^{9} cfu/ml for *P. jensenii* 702 at their stationary phases. Spray drying caused a significant viability loss in all three probiotics due to the higher temperatures involved in the process. *B. animalis* subsp. *lactis* BB-12 demonstrated the highest viability loss while *P. jensenii* 702 showed the lowest viability loss. Spray drying resulted in the reduction of viable bacteria, by approximately 1 log cycle in the case of *L. acidophilus* LA-5 and *P. jensenii* 702 and by 2 log cycles in the case of *B. animalis* subsp. *lactis* BB-12. However, all three probiotics were able to maintain high viability levels (>10^{8} cfu/g for *P. jensenii* 702, > 10^{7} cfu/g for *L. acidophilus* LA-5 and > 10^{6} cfu/g for *B. animalis* subsp. *lactis* BB-12) after spray drying, thereby satisfying recommendations regarding the level of viable cells in probiotic foods (Figure 7.1).

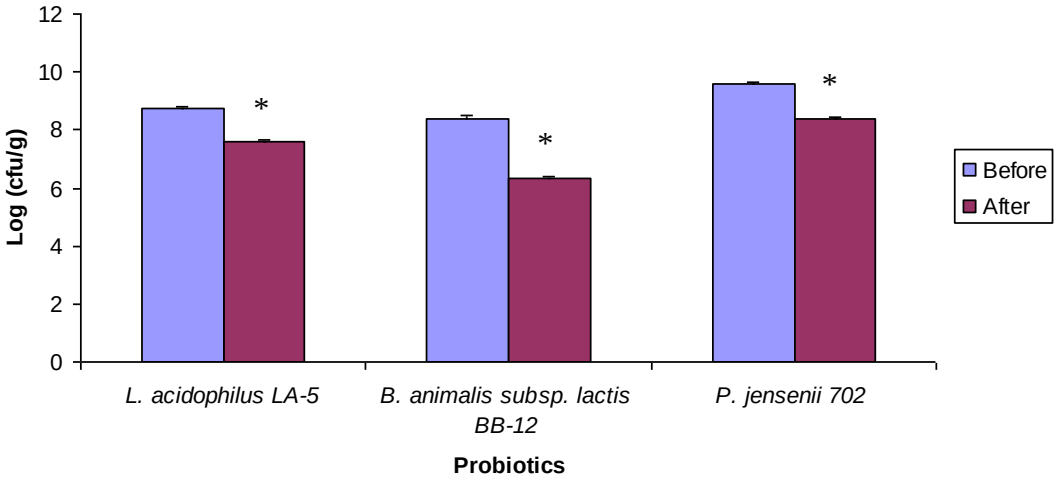
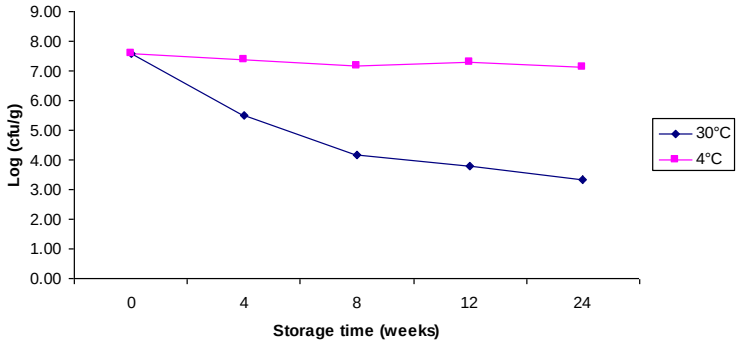


Figure 7.1 Viable probiotic cell counts before and after spray drying. Asterisk (*) indicates a significant difference between corresponding before and after cell counts ($p<0.05$) ($n = 4$).

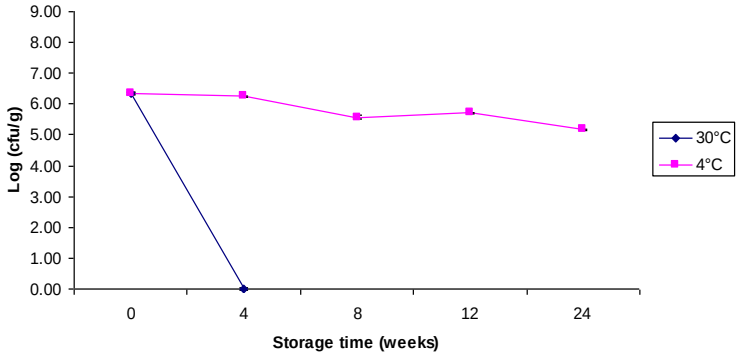
7.3.2 Viability during storage

There was a significant effect of storage temperature in reducing the viability levels of all three probiotics at 30^{o}C. Rapid viability loss was observed in samples stored at 30^{o}C compared to the samples stored at 4^{o}C in all three probiotics. At 30^{o}C, there were no survivors of *B. animalis* subsp. *lactis* BB-12 and *P. jensenii* 702 after 4 weeks and 24 weeks of production respectively (Figure 7.2).

A



B



C

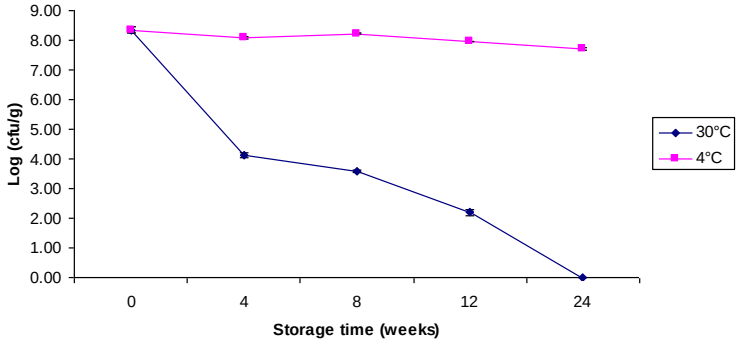


Figure 7.2 Viable counts of spray dried *L. acidophilus* LA-5 (A), *B. animalis* subsp. *lactis* BB-12 (B) and *P. jensenii* 702 (C) during storage at 30°C and 4°C (*n* = 4).

6.3.3 Moisture content of spry dried powder during storage

It is necessary to produce spray dried powders with moisture contents that do not exceed the level required for prolonged powder storage life, quality and stability. The moisture content of the spray dried powder was 2.91 ± 1.49 % at the day of production and fell within the recommended level (~ 4%). Values recorded at week 24 suggested a trend toward increased moisture content after storage at 4°C (3.49±0.05 %) and a decrease when stored at 30°C (2.76±0.12 %), although these apparent shifts in moisture content after storage were not found to be statistically significant (Table 7.1).

Table 7.1 Moisture content (%) of spray dried powder during storage (n = 2)

Storage temperature	**Storage (weeks)**	
	0	**24**
4^oC	2.91 ± 1.49	3.49 ± 0.05
30^oC	2.91 ± 1.49	2.76 ± 0.12

Mean value (±SE)

7.3.3 Adhesion ability

By comparison with *B. animalis* subsp. *lactis* BB-12, for which over 90% of cells were found to have adhered to the Caco-2 cell layer, only a relatively small proportion of *L. acidophilus* LA-5 (25.48%) and *P. jensenii* 702 (1.69%) cells were adherent. It should nonetheless be recognised that in the two latter cases, the viable cell numbers prior to adhesion were substantially greater than those recorded for *B. animalis* subsp. *lactis* BB-12. Importantly however, despite the widely different initial viable counts, approximately similar numbers (~10^5 cfu/g) of each bacterium were ultimately able to adhere to Caco-2 cell layers (Table 7.2).

Table 7.2 Mean viable probiotic counts in spray dried powder before and after adhesion to Caco-2 epithelial cells. (*n* = 6)

Probiotic	Before adhesion (cfu/g)	After adhesion (cfu/g)	Adhesion %
L. acidophilus LA-5	$3.14 \pm 0.27 \times 10^{6a}$	$8.00 \pm 0.26 \times 10^{5b}$	25.48
B. animalis subsp. *lactis* BB-12	$1.75 \pm 0.30 \times 10^{5a}$	$1.60 \pm 0.15 \times 10^{5a}$	91.43
P. jensenii 702	$2.49 \pm 0.22 \times 10^{7a}$	$4.20 \pm 0.23 \times 10^{5b}$	1.69

Mean value (±SE)

[a.b]Values in the same row having different superscripts differ significantly ($p<0.05$).

7.3.4 Rehydration in MRD, coffee and tea

Examination of the rehydration data for each of the probiotics indicated differing outcomes in all three cases. While no significant differences were observed between the viable numbers of *L. acidophilus* LA-5 rehydrated in MRD, tea, or coffee, viability of *B. animalis* subsp. *lactis* BB-12 was found to be significantly reduced after rehydration in tea and coffee by comparison with MRD. A similar result was also observed for *P. jensenii* 702, in that viability was reduced in tea relative to MRD, but was found to be even further reduced in after rehydration in coffee relative to tea.

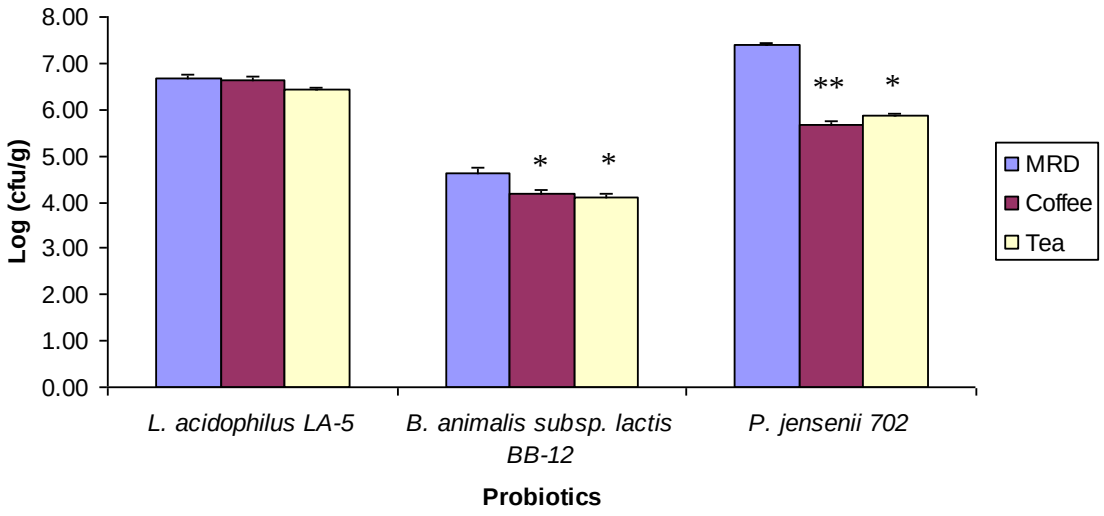
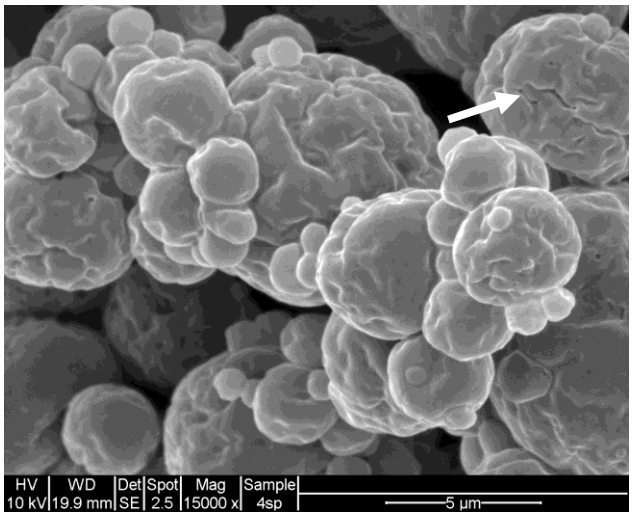


Figure 7.3 Viable probiotic counts in spray dried goat's milk powder rehydrated in MRD (25°C), coffee and tea (85°C). * and ** indicates significant differences with corresponding viability in different rehydration solutions at $p<0.05$ ($n = 6$).

7.3.5 Scanning electron micrograph

The spray dried micro-spheres produced were various in size but small (<15 μm in diameter) and the surface of those microparticles appeared grainy with visible cracks. There was not any evidence of free or non encapsulated bacteria among the spray dried microparticles (Figure 7.4).



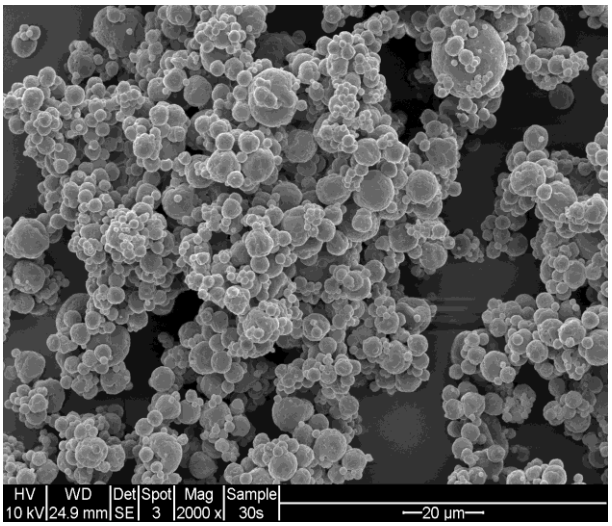


Figure 7.4 Scanning electron micrographs of spray dried microparticles containing probiotics. Cracks in a microparticle are indicated by an arrow.

7.3.6 Summary of key findings

Spray drying probiotics in reconstituted goat's milk resulted in a significant reduction in the viability of all three probiotics. While storage temperature did not appear to have a significant effect on moisture content, the viability of all three strains declined dramatically when stored at 30°C but remained virtually unaffected under storage at 4°C. During the adhesion assay, approximately similar numbers (~10^5 cfu/g) of viable cells were able to adhere to Caco-2 cell layers from each bacterium despite widely varying initial counts. Rehydration of spray dried probiotics in coffee and tea at 85^{o}C lowered the probiotic viability, however, *L. acidophilus* LA-5 was able to maintain promising viability levels (>10^6 cfu/g) even after rehydration at high temperature in tea and coffee.

7.4 Discussion

The primary objective of this study was to attempt to assess the efficacy of spray drying *P. jensenii* 702 together with *B. animalis* subsp. *lactis* BB-12 and *L. acidophilus* LA-5 in goat's milk. In this context, several of the findings have significant implications with regard

to the potential utilisation of probiotics spray dried in goat's milk, especially with respect to viability retention during spray drying and subsequent refrigerated storage, and rehydration in tea and coffee at higher temperatures.

7.4.1 Probiotic viability during spray drying

Generally microencapsulated probiotics have a tendency to survive better in dairy foods compared to free form of the same strain (Adhikari et al., 2003; Adhikari et al., 2000; Anal & Singh, 2007; Shah & Ravula, 2000). Therefore, the use of goat's milk as a carrier material in the spray drying of probiotics may provide several advantages. It can be directly used as a probiotic food or can be used as inoculum for probiotic goat's milk products without any risk of contamination of other carrier materials such as cow's milk, one of the most common carrier materials in producing inoculum.

In the present study the spray drying procedure was found to significantly reduce the viability of probiotics examined (Figure 7.1). Reduction in cell viability after spray drying was most likely due to heat inactivation (To & Etzel, 1997a) and has previously been reported by many authors (Golowczyc et al., 2011; Ho, 2008; Kotula, 2008; Lian et al., 2002). High processing temperatures used in this study may have reduced the viability of probiotic bacteria due to various factors including dehydration of the cell membrane, damage to cellular components and loss of protein from the cell wall (Boza et al., 2004; Gardiner et al., 2000; O'Riordan et al., 2001; Santivarangkna et al., 2008). The reduction in viability associated with the spray drying process was found in this study to be species dependent with *B. animalis* subsp. *lactis* BB-12 experiencing a reduction in viable cell numbers almost 10-fold greater than that observed for either *L. acidophilus* LA-5 or *P. jensenii* 702. Previous studies have demonstrated that different strains of microorganism can vary in their capacity to withstand the elevated temperatures experienced during the spray drying process (Favaro-Trindade & Grosso, 2002; Gardiner et al., 2000; Ho, 2008; Kotula, 2008; Lian et al., 2002). As bifidobacteria are known to be more susceptible to high temperatures than lactobacilli (Doleyres & Lacroix, 2005), the better survival of *L.*

acidophilus LA-5 and *P. jensenii* 702 in the present study may be attributed to a lower sensitivity of these organisms to heat compared to *B. animalis* subsp. *lactis* BB-12.

Although viability losses in the present study were significant, a large number of cells of each probiotic (above the recommended minimum level $>10^6$ cfu/g) were able to survive the spray drying process. This cellular survival during spray drying may be determined by the influence of factors such as cracks in the surface of the spray dried produce (Lian et al., 2002) and the growth stage of probiotic cells at the time of spray drying (Corcoran et al., 2004). The surface of microparticles produced in this study appeared spherical and grainy with visible cracks after spray drying (Figure 7.4) and these observations are in line with the findings of Lian et al. (2002). These cracks may facilitate the escape of heat from inside the particle after drying, resulting in less heat injury to some entrapped probiotics, and this may have been a factor in the satisfactory viability retention of *L. acidophilus* LA-5 and *P. jensenii* 702 and *B. animalis* subsp. *lactis* BB-12 observed. Use of probiotics in their stationary phase may also have contributed to the maintenance of acceptable viability levels (10^6-10^8 cfu/g) for all the probiotics after spray drying.

It has been suggested that the survival of bacterial cells during spray drying is inversely proportional to the outlet temperature, and not directly to the inlet temperature of the dryer (Ananta et al., 2005; Boza et al., 2004; Kotula, 2008; To & Etzel, 1997a). However, high inlet temperatures are necessary in spray drying process, primarily because the specific heat of evaporation must be supplied in a very short time to obtain desirable characteristics in the final product. As a result of this, significant thermal inactivation of probiotic cells may also occur, despite the short residence time (Boza et al., 2004). The moisture content of the spray dried product has also been shown to be inversely proportional to the air outlet temperature (Desmond et al., 2002a). Although improved viability of probiotics can be achieved by reducing the outlet temperature during spray drying, powder quality must also be taken into account when manufacturing spray dried products. A moisture content of ~ 4% in spray dried dairy powders has been reported as a value for this parameter associated with good quality dried-dairy products (Ananta et al., 2005; Gardiner et al., 2000). This low moisture level would minimize the risk of storage related defects such as crystallization of

lactose which may reduce the product quality (Ananta et al., 2005). The spray dried microspheres produced in this study were small (<15 μm in diameter), with low moisture content (<3.5%) (Figure 7.4 and Table 7.1) and fell within the good average microsphere size since particles of this size do not affect the 'mouth feel' properties of most foods (O'Riordan et al., 2001). Large microspheres of size greater than 100 μm may have a negative impact on the mouth feel properties and may create sandiness or grittiness in the texture which is not acceptable in foods such as ice cream and yogurts (Kotula, 2008; Picot & Lacroix, 2003). Scanning electron micrographs of microencapsulated bacterial powder (Figure 7.4) demonstrated that no free, non encapsulated bacteria were present, indicating that the probiotic cells were well encapsulated in the goat's milk powder particles during spray drying in this study. This would suggest a high encapsulation efficacy of probiotics during manufacturing. The findings of this study appear to suggest that spray dried goat's milk probiotic powder may be highly suitable for the manufacturing of dairy products such as ice cream.

7.4.2 Viability of probiotics during storage

Many factors may contribute to a relative decrease in survival of probiotics during storage such as, having high numbers of un-repairable injuries to the cells after spray drying, (Boza et al., 2004; Fu & Etzel, 1995), temperature, humidity and presence of oxygen in the storage environment (Morgan et al., 2006), moisture content of the product, powder composition, exposure to light and storage materials (Meng et al., 2008). Accentuated removal of water during spray drying may also expose cracks and void spaces in the dried particles to air, causing oxidative degeneration of the proteins and cellular compounds (Boza et al., 2004). In this study the viability of probiotics in spray dried powder at two different storage temperatures was measured. The higher storage temperature of 30^{o}C had a significant effect and caused rapid viability loss in all there probiotics compared to lower storage temperature of 4^{o}C. This trend of losing probiotic viability at higher storage temperatures was evident in other studies of spray dried probiotic powders (Ananta et al., 2005; Desmond et al., 2002a; Gardiner et al., 2000; Hsiao et al., 2004; Kotula, 2008; Simpson et al., 2005; Sunny-Roberts & Knorr, 2009). It has been suggested that the storage

of probiotic powders above refrigeration temperatures increases rates of bacterial metabolism which may lead to accumulation of toxic waste and hence reduce their viability (Kotula, 2008).

Although *P. jensenii* 702 had the highest initial viable cell counts their viability loss at 30^{o}C was rapid compared to *L. acidophilus* LA-5. This is contrary to the belief that higher cell densities in the product will improve survival by reducing the exposed area of each cell to the environment (Chavez & Ledeboer, 2007). However, *B. animalis* subsp. *lactis* BB-12 with lowest initial cell counts lost their viability rapidly compared to *P. jensenii* 702 and *L. acidophilus* LA-5 both at 4^{o}C and 30^{o}C. Rapid loss of viability in *B. animalis* subsp. *lactis* BB-12 may also have been attributable to exposure of the cells to oxygen during storage (Jankovic et al., 2010) and more extensive cell damages during spray drying compared to *L. acidophilus* LA-5 and *P. jensenii* 702. Oxygen toxicity is one of the important factors that affects the viability of *Bifidobacterium* (Hsiao et al., 2004). Gardiner et al. (2000) also observed previously the differences in survival of spray dried probiotic species during storage. In that study *L. paracasei* maintained a constant cell concentration for 2 months at 4^{o}C, however *L. salivarius* was found to decline in numbers by an order of magnitude under the same conditions.

The type of packaging material has also been shown to influence the stability of probiotics in spray dried powders. The glass containers which were used in this study may have contributed to retention of cell viability due to their ability to reduce moisture and oxygen permeation (Hsiao et al., 2004; Shah, 2000; Simpson et al., 2005; To & Etzel, 1997b). However, it would seem according to the results of this study that storage temperature may play a more significant role in retaining viability of spray dried probiotics than oxygen tolerance and or packaging materials. Hasiao et al. (2004) stored spray dried bifidobacteria in glass bottles and evaluated the effect of storage temperature on cell viability. Similar to the results of this study they observed a decreased number of viable cells of bifidobacteria as the storage temperature increased. It should also be noted that the effects of light on probiotic viability would have been negligible in the present study, since all the glass

containers were placed and sealed in cardboard cartons for storage at the respective temperatures.

Although refrigerated storage of spray dried powder may be impractical from a commercial point of view (Gardiner et al., 2000), according to the results of present study, it seems likely that, refrigerated storage is necessary for optimal culture viability in spray dried powders over time. However, in order for probiotic powders to be useful, storage at room temperature is desirable. Use of fluidized-bed spray dryers over laboratory scale spray dryers were known to improve the viability of spray dried cultures at room temperature storage (Kotula, 2008; Simpson et al., 2005). Thus use of fluidized-bed spray dryers may be useful in manufacturing probiotic powders on a commercial scale.

7.4.3 Rehydration viability

The rehydration step is often neglected but is an important step in the recovery of microorganisms from dried products. Although an organism can survive various processing steps such as drying and storage, viability losses may occur during rehydration (Jankovic et al., 2010; Wang et al., 2004). The rehydration agent itself (in terms of osmolarity, pH and nutritional energy source) and the rehydration conditions (in terms of temperature and volume) may significantly affect the rate of recovery of probiotics and thus influence the survival rate (Carvalho et al., 2004). High numbers of viable cells have previously been recovered from freeze dried *P. jensenii* 702 when cow's milk and soy milk were used as rehydration agents. Interestingly, the viability of *P. jensenii* 702 was significantly reduced when water and orange juice were used as the rehydration solution (Kotula, 2008). Similarly *P. jensenii* 702 and *B. animalis* subsp. *lactis* BB-12 both demonstrated significant differences in cell recovery in the present study when different rehydration agents were used (Figure 7.3), although there was no significant difference in *L. acidophilus* LA-5 counts in different rehydration media. Small differences (with a maximum of 0.5 log cfu/ml) in the recovery of spray dried lactobacilli were previously observed by Desmond et al (2002b) in various rehydration media including MRD, reconstituted skim milk and sterile water. Therefore, it seems likely that the rehydration viability of encapsulated probiotics in

different media can be species specific. The results of the present study may have been largely influenced by the rehydration temperature, as the temperature of MRD was 25^{o}C while both tea and coffee were at 85^{o}C at the time of rehydration. It was interesting to observe that probiotics in spray dried powder were able to tolerate such a high temperature during rehydration. Improved heat tolerance of probiotic *L. paracasei* during spray drying as a result of exposure to heat (52^{o}C for 15 minutes) before spray drying was previously reported by Desmond et al. (2002b). Likewise, the heat tolerance of the probiotics in the present study may have improved through exposure to high temperatures during spray drying, resulting in a greater ability to tolerate the high rehydration temperature of the tea and coffee.

Due to the numerous applications of spray dried milk powders, not only in manufacturing dairy products, but also in foods such as mayonnaise, confectionary products (Gardiner et al., 2000) and preparing hot beverages, it is possible that the probiotic goat's milk powder could be used in a wide range of functional food applications. Although the laboratory scale experiment conducted in this study provides a promising indication of the performance of these probiotic cultures during spray drying and subsequent storage, further research is needed to evaluate their performance during spray drying on an industrial scale.

7.4.4 Adhesion properties

Different lactobacilli strains were shown by Golowczyc et al (2011) to be different in adhesion capacity *in vitro* after spray drying. For example, *L. kefir* 8348 demonstrated a significant loss of adhesion capacity, while *L. plantarum* 83114 and *L. kefir* 8321 did not lose their capacity to adhere to intestinal cells after spray drying. The adhesion ability of the three probiotics examined in this study was varied even in non encapsulated form in goat's milk (Chapter 3), yogurts and ice cream (Chapter 6). Similarly, these probiotics demonstrated different adhesion levels after spray drying (Table 7.2). Cellular damage during spray drying may vary among different probiotics depending on their ability to withstand heat and may ultimately influence their adhesion ability (Golowczyc et al., 2011). As explained in chapter 3, inter-species competition and morphological and

physiological differences among species may also have had an impact on adhesion ability of these probiotics in the present study. However, regardless of the carrier food type (fermented goat's milk, plain and fruit yogurts, ice cream and spray dried milk powder) and initial cell counts, all three probiotics used in this research study were able to adhere to Caco-2 cell layers in high numbers (~10^5-10^6 cfu/g). Thus, the high temperature employed in spray drying in this study seemed not to have a negative effect on the adhesion ability of these probiotics.

7.5 Conclusions

The results of this study support the use of goat's milk as a carrier material in spray drying *L. acidophilus* LA-5, *B. animalis* subsp. *lactis* BB-12 and the novel probiotic *P. jensenii* 702. Although the spray drying process resulted in a significant viability loss, encapsulated *L. acidophilus* LA-5 and *P. jensenii* 702 were able to maintain a satisfactory viability (~10^7 cfu/g) during 24 weeks of refrigerated storage at 4^{o}C in glass containers. Despite the widely different initial values, approximately similar numbers (~10^5 cfu/g) of viable cells of each bacterium were able to adhere to Caco-2 cell layers during the adhesion assay. Rehydration viability was found to be significantly affected by the specific rehydration agent used in the case of *P. jensenii* 702. Spray dried *L. acidophilus* LA-5 demonstrated remarkable rehydration viability in the presence of tea and coffee at 85^{o}C. Overall, spray dried *P. jensenii* 702 in goat's milk demonstrated promising characteristics in terms of viability retention during spray drying and subsequent refrigerated storage.

Chapter 8 : Overall Conclusions

The primary objective of this thesis was to assess the performance of the novel probiotic *P. jensenii* 702, in combination with established probiotics *B. animalis* subsp. *lactis* BB-12 and *L. acidophilus* LA-5, in several types of goat's milk products. In particular the research aimed to evaluate the effects of the carrier food matrix on probiotic efficacy, with special reference to gastrointestinal tolerance and intestinal cell adhesion rates, as well as the reciprocal influence of the probiotics on the physico-chemical properties and consumer appeal of the products. Functional properties of probiotics such as gastrointestinal tolerance and adhesion to intestinal epithelia can be considered fundamental criteria when selecting potential probiotic microorganisms. In this project, rather than examining the probiotic parameters in isolation of the food matrix, these functional properties were examined by incorporating the probiotic strains into the whole food products, allowing concurrent assessment of the influence of the matrix, packaging materials and storage conditions.

The potential utility of *P. jensenii* 702 in combination with *B. animalis* subsp. *lactis* BB-12 and *L. acidophilus* LA-5 in terms of viability in different goat's milk products were described in Chapters 3, 4, 5 and 7, where these strains were utilized to produce fermented milk, yogurts, ice cream and a spray dried powder respectively. The novel probiotic *P. jensenii* 702 has demonstrated great potential in terms of product manufacture with minimal loss of viability observed over the shelf life of the products analysed. In general, an inoculation level of 10^8 cfu of *P. jensenii* 702 per g or ml of product, resulted in viable cell counts > 10^8 at the end of 3 weeks shelf life at 4^oC in fermented goat's milk, and at the end of 4 weeks (4^oC) and 52 weeks (-20^oC) in yogurt and ice cream respectively, when co cultured with *B. animalis* subsp. *lactis* BB-12 and *L. acidophilus* LA-5. On this basis, *P. jensenii* 702 may be considered a suitable culture to incorporate with *B. animalis* subsp. *lactis* BB-12 and *L. acidophilus* LA-5 in the manufacturing of goat's milk products. Although 3 weeks in the case of fermented milk and 4 weeks in the case of yogurts are relatively short storage times, they were considered reasonable storage periods for

perishable dairy food products which require refrigeration. Nonetheless, *P. jensenii* 702 showed an ability to survive a much longer period in ice cream under frozen storage. Furthermore, *P. jensenii* 702 has also demonstrated considerable viability retention (~10^8 cfu/g) after spray drying and in spray dried powder over 24 weeks at 4^oC, showcasing their potential for diverse application in the food industry.

In Chapter 3, use of *P. jensenii* 702 in combination with *B. animalis* subsp. *lactis* BB-12 and/ or *L. acidophilus* LA-5 was examined with special reference to their contribution to the physico-chemical properties and sensory aspects of the fermented goat's milk, in addition to their viability in the final product. Furthermore, their functional properties such as gastrointestinal tolerance, adhesion properties and stimulation of cytokine production *in vitro* were also assessed. It was found that probiotic viability, product physico-chemical and organoleptic characteristics and probiotic functional properties such as gastrointestinal tolerance, adhesion properties and modulation of cytokine production by intestinal epithelial cells can be affected when combined with other probiotics. Apart from the obvious potential benefits of combining probiotic species with different properties in multi-species products, additional unforseen benefits may also arise from such preparations due to synergistic activities between the species involved. Equally however, inter-species interactions may produce antagonistic effects, thus *in vitro* research is necessary to assess these effects prior to incorporation in commercial products. Obviously research should be targeted toward both finding combinations which show synergistic and symbiotic activities towards each other, in order to maximize the chance of providing more clinically effective probiotic preparations, and to identifying combinations of probiotic strains with mutually inhibitory properties such as production of H_2O_2, bacteriocins or bacteriocin-like substrates. Such inhibitory activities were not apparent in the triplet combination of probiotics applied in these studies.

According to the results described in Chapter 3, it has been identified that *P. jensenii* 702 can be successfully co-cultured with bifidobacteria and lactobacilli in producing fermented goat's milk products. The results further revealed that *P. jensenii* 702 may in fact improve the survival of *B. animalis* subsp. *lactis* BB-12 and *L. acidophilus* LA-5 in fermented goat's

milk. Since lactobacilli and bifidobacteria are natural inhabitants of healthy human gastrointestinal tract, consumption of products containing *P. jensenii* 702 may also improve the gastrointestinal flora of the host. Generally the probiotic products containing lactobacilli and bifidobacteria are well accepted by the public and hundreds of products containing *L. acidophilus* and bifidobateria are available in the market worldwide. Successful application of a new species such as *P. jensenii* 702 into probiotic products together with lactobacilli and bifidobacteria could be a beneficial approach in further expanding the probiotic market.

In this thesis, the importance of use of food additives in masking the unpleasant "goaty" flavour of the fermented goat's milk products has been established. In Chapter 4, all types of yogurts containing fruit juice received overall higher consumer acceptability compared to the plain yogurt without any added fruit juice. In general however, ice cream (Chapter 5) received higher consumer acceptability compared to yogurts (Chapter 4) and fermented milk (Chapter 3). The use of strong flavours such as chocolate/cocoa may also be considered in order to mask the unpleasant flavours and improve the sensory properties of goat's milk products, one of the main constraints faced by the dairy goat industry. However, in general sensory scores for all these products remained low. Further research is needed to explore the ways to improve the sensory properties of these products. Recruitment of trained panellists would be beneficial in this regard.

According to the results of Chapter 5, it can be predicted that, unlike most of the dairy foods stored under normal refrigeration temperatures, probiotic viability of frozen dairy desserts such as ice cream may not be affected by the type of packaging: glass, polyethylene or polypropylene. These findings may be useful in industrial applications of frozen probiotic dairy foods. Another interesting outcome of the experimental work in chapter 5 was the relatively high consumer acceptability for probiotic goat's milk ice cream at weeks 12 of storage compared to the fresh product at week 1 despite the packaging materials. It seems likely that frozen probiotic dairy foods develop desirable organoleptic characteristics over the storage period and these findings may also have potential application in food industry. Many probiotics lose viability through freeze injury during the freezing and agitation process involved in ice cream production. However the ability to

withstand freezing during ice cream manufacture is another important technological characteristic of *P. jensenii* 702 which has been determined in Chapter 5.

Another interesting outcome of this thesis was the satisfactory recovery of certain probiotics in tea and coffee from spray dried powder at high temperatures (Chapter 7). *L. acidophilus* LA-5 was able to sustain > 10^6 cfu/g viable counts in tea and coffee which were heated to 85^{o}C. To the best of the author's knowledge, this research is the first study on rehydration of probiotic powders in black tea and coffee at these temperatures. At this stage, further assessment of the functional and health promoting characteristics of probiotics when subjected to high temperatures is recommended. However, findings of this study have opened many avenues for further research on probiotics and hot beverages.

The ability of probiotic strains to survive passage and to colonise the gastrointestinal tract is considered to be important criteria for providing potential beneficial effects. The findings of chapter 6 supported the underlying hypothesis that the gastrointestinal tolerance and adhesion of probiotics to intestinal epithelia would vary when incorporated into different food matrices. Probiotics in ice cream stored for 24 weeks survived well above the same species in freshly produced yogurts in stressful environments such as low gastric pH and in the presence of bile salts *in vitro*. Thus, this experiment also revealed that the functional properties of probiotics may remain unaffected during the prolonged frozen storage period. However, this phenomenon requires further study, especially under *in vivo* conditions.

The physico-chemical properties of all of the fermented goat's milk products were well within generally acceptable ranges, thus these products may have future commercial potential. For example, goat's milk ice cream (Chapter 5) contained ~10% fat which is a general requirement that should be fulfilled in producing full fat ice cream. The pH values of goat's milk yogurts (Chapter 4, pH 4.40-4.10) also fell within the appropriate pH range for maintaining satisfactory probiotic viability and consumer acceptability. Furthermore, the moisture content of the spray dried powder (Chapter 7) was <4% which is a requirement for proper storage stability. The microsphere size of the spray dried produce also fell within the acceptable range (<15 μm).

According to the results of this thesis the prospect of *P. jensenii* 702 as a novel probiotic in manufacturing probiotic goat's milk products is promising. Furthermore, functional properties of probiotics were shown to be influenced by the carrier food matrices. Therefore, efficacy of probiotics in their carrier food matrices should be taken into account when recommending probiotics for human consumption. Different food matrices have been increasingly tested in the food industry as probiotic carriers due to the allergenicity of cow's milk – (the major probiotic carrier food), desire for novel tastes, and the demand for vegetarian alternatives. However, the findings of this thesis may also be applicable for dairy products from other farm animals including cow's milk products, due to certain similarities in the physico-chemical properties of goat's milk and cow's milk. Furthermore, these findings have opened many avenues for further research, especially the inclusion of an experimental step that determines the effect of carrier food matrix on probiotic efficacy during development of novel functional foods, rather than simple extrapolations from "non-food" models.

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Appendix A: Primer combinations used in qualitative PCR

Probiotic	Primer pair	Sequence
L. acidophilus	IDL04F	5'-AGGGTGAAGTCGTAACAAGTAGCC-3'
	IDL22R	5'-AACTATCGCTTACGCTACCACTTTGC-3'
B. animalis subsp. *lactis*	Lw-A	5'-GCACGGTTTCGGCCGTG -3'
	Lw-B	5'-GGGAAACCGTGTCTCCAC -3'
P. jensenii	PJ	5'-GACGAAGTGCCTATCGGGGTG-3'
	PB2	5'-TGGGGTCGAGTTGCAGACCCCAAT-3'

Primers were obtained from Sigma-Aldrich, Australia.

Appendix B: Sensory studies

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Information Statement for the Research Project:
Sensory Studies of a Novel Fermented Goat's Milk/ Yogurt/ Ice Cream
Document Version []; dated

You are invited to participate in the research project identified as above. This research forms part of Mr Senaka Ranadheera's postgraduate studies at the University of Newcastle, supervised by Dr. Michelle Adams from the School of Environmental and Life Sciences and Dr Surinder Baines from the School of Health Sciences.

Why is the research being done?

Goat's milk is claimed to have many health benefits. Although demand for goat's milk is very high, there are no fermented goat's milk products, such as yogurt/ice cream in the Australian market. The situation is similar in many other countries. The purpose of this research is to produce a novel goats' milk based ice cream and yoghurt which is suitable for the Australian market.

Who can participate in the research?

We are looking for healthy adults between the ages of 18-70. Participants must have a basic understanding of food science and dairy food product development. At the time of participation you must be in a healthy condition. If you have previous experience in sensory evaluation you are most welcome.

What choice do you have?

Participation in this research is entirely your choice. Only those people who give their informed consent will be included in the project. Whether or not you decide to participate, your decision will not disadvantage you.

If you do decide to participate, you may withdraw from the project at any time without giving a reason and have the option of withdrawing any data which identifies you.

What would you be asked to do?

If you agree to participate, you will be asked to do the following things:

You will be provided each sample of ice cream and yogurt/fermented milk numbered with three digit codes and a score card. Instructions on how to fill out the score card and a basic introduction to the testing will be provided before the start of the tasting by the student researcher.

You will be requested to taste each and every sample provided to you (6-8 samples) separately and give your comments (flavour, texture, appearance, colour and overall acceptability) on each and every sample according to the score card. It is not necessary to consume yogurt or ice cream samples in order to evaluate sensory attributes. You will be provided a glass of water at room temperature for mouth rinsing between tasting of each and every sample. At the end of the evaluation score cards will be collected by the student researcher.

Unfortunately we are not in a position to pay any cash reimbursements/payments. Your support and cooperation is most appreciated.

How much time will it take?

It takes only about 30-40 minutes to taste and complete the score card.

What are the risks and benefits of participating?

There is no identified risk associated with the study since all the ingredients used for ice cream and yoghurt production are in accordance with the Australian standards and are safe food materials.

All the yoghurt cultures are safe for human consumption and widely used in dairy food and feed products in Australia.

If you are allergic to cow's milk, goat's milk or other dairy products, please do not participate in this study. However, if you experience any adverse reactions after the study please contact your doctor.

There will be no direct immediate benefits of this study to you as a participant. However if this study is successful the information collected will be used to support the development of a novel goat's milk ice cream/yoghurt/fermented milk to fulfil the consumer demand. These novel food products may provide an alternative dairy product for people who suffer from some allergies and some other digestive problems and provide a market opportunity for Australian dairy goat farmers.

How will your privacy be protected?

The only people who will know that you are a research participant are members of the research team. All records will be stored in a coded form in a locked filing cabinet and/or password protected computer for 5 years as required for any clinical study. When the results of the research are published, no information will be included that would reveal your identity.

How will the information collected be used?

Collected information will form part of the data collection for a PhD thesis being undertaken by the student researcher Mr Senaka Ranadheera. In addition, information will be published in scientific journals and conference/ symposium proceedings. Individual participants, however, will not be identified in any reports, thesis, and conference or journal papers arising from the project.

***What do you need to do to participate*?**

Please read this information statement and be sure you understand its contents before you consent to participate. If there is anything you do not understand, or you have questions, contact the student researcher.

If you would like to participate, please complete and return the attached consent form to the student researcher. Then we will contact you to arrange a time convenient with you for the evaluation.

Feedback

Participants may request a summary of the findings from Dr Michelle Adams (Michelle.Adams@newcastle.edu.au) by contact through email. Results will be presented using figures and tables upon your request.

Further information

If you would like further information please contact Dr Michelle Adams on 0249216431 or Mr Senaka Ranadheera on 0431728163.

Thank you for considering this invitation.

Yours sincerely,

……..	……
Dr. Michelle Adams, PhD	Dr. Surinder Baines,PhD
Senior Lecturer/Microbiology	Senior Lecturer/Nutrition & Dietetics

……
Senaka Ranadheera

PhD student (Food Science)

Complaints about this research

This project has been approved by the University's Human Research Ethics Committee, Approval No. H- 2008-0212

Should you have concerns about your rights as a participant in this research, or you have a complaint about the manner in which the research is conducted, it may be given to the researcher, or, if an independent person is preferred, to the Human Research Ethics Officer, Research Office, The Chancellery, The University of Newcastle, University Drive, Callaghan NSW 2308, Australia, telephone (02) 49216333, email Human-Ethics@newcastle.edu.au.

Poster example for inviting the volunteers for participating in the novel fermented goats' milk/yogurt/ice cream study

Calling
Volunteers for Sensory Testing of a Novel Fermented Goat's Milk, Yogurt and Ice Cream Study

We are looking for volunteers to participate in a sensory evaluation of a novel fermented goat's milk/yogurt/ice cream

Taking part will only take 30-40 minutes and you can taste a few delicious varieties of ice cream and yoghurt/fermented milk

Participants for this study must be healthy adults
between the ages of 18-70
and
should have basic understanding of food/nutrition sciences and dairy product development. If you have previous experience in sensory evaluation you are most welcome

Enquiries
For information on the study, please phone
Senaka on **0431728163 (please leave contact details if not answered)**

This project has been approved by the University of Newcastle Human Research Ethics Committee Approval no. H-2008-0212

Dr Michelle Adams
School of Environmental and Life Sciences
Dr Surinder Baines
School of Health Sciences
Senaka Ranadheera
School of Environmental and Life Sciences
University of Newcastle
Australia

Consent Form for the Research Project:
Sensory Studies of a Novel Fermented Goat's Milk/Yogurt/Ice Cream
Dr. Michelle Adams, Dr. Surinder Baines and Senaka Ranadheera
Document Version []; dated

I agree to participate in the above research project and give my consent freely.

I understand that the project will be conducted as described in the Information Statement, a copy of which I have retained.

I understand I can withdraw from the project at any time and do not have to give any reason for withdrawing.

I consent to complete a sensory evaluation score card and having it recorded.

I understand that my personal information will remain confidential to the researchers.

I have had the opportunity to have questions answered to my satisfaction.

As far as I am aware, I am not allergic to cow or goat's milk products.

By signing this form, I willingly agree to participate in the research as described.

Name: _____

Contacts: _____

Signature: _____

Date: _____

Enquiries regarding this study may be directed to:

Senaka Ranadheera (0431728163)

SENSORY STUDIES OF A NOVEL FERMENTED GOAT'S MILK/ICE CREAM/YOGURT

Panelist Questionnaire

1. Name: ……

Please put a tick next to your answer for the following questions:

2. Sex: Male…… Female……

3. Age: 18-25 years ……

25-35 years ……

35-55 years ……

55-70 years ……

4. Do you regularly consume ice cream?

Yes…… No……

5. If "Yes" which of the following of ice cream do you usually prefer?

Vanilla…… Chocolate……

Strawberry…….. Other (please specify)…….

6. Do you regularly consume yogurt?

Yes…… No……

7. If "Yes" which of the following of yogurt do you usually prefer?

Plain…… Drinking/fermented milk……

Vanilla…… Stirred……..……

Strawberry…….. Other (please specify)…….

SENSORY STUDIES OF A NOVEL FERMENTED GOAT'S MILK

THE SCORE CARD

Name: Prof/Dr/Mr/Ms (Optional) ……
Date:……

Instruction
Please taste the yoghurt samples and indicate how much you like or dislike the sample by scoring each sample against the numerical values given below, ranging from Like extremely (9) to Dislike extremely (1).

Please score one sample at a time. Use water to wash your palate after tasting each sample.

Like extremely	Like very much	Like moderately	Like slightly	Neither like nor Dislike
09	08	07	06	05

Dislike slightly	Dislike moderately	Dislike very much	Dislike extremely
04	03	02	01

Characteristic	**Samples**							**Comments**
	458	227	856	970	150	628	741	
1. Colour & appearance								
2. Aroma								
3. Body & texture								
4. Taste								
5. Overall acceptability								

SENSORY STUDIES OF A NOVEL GOAT'S MILK YOGHURT

THE SCORE CARD

Name: Prof/Dr/Mr/Ms (Optional) ……
Date:……

Instruction
Please taste the yoghurt samples and indicate how much you like or dislike the sample by scoring each sample against the numerical values given below, ranging from Like extremely (9) to Dislike extremely (1).

Please score one sample at a time. Use water to wash your palate after tasting each sample.

Like extremely	Like very much	Like moderately	Like slightly	Neither like nor Dislike
09	08	07	06	05

Dislike slightly	Dislike moderately	Dislike very much	Dislike extremely
04	03	02	01

Characteristic	**Samples**				**Comments**
	175	225	365	487	
1. Colour & appearance					
2. Aroma					
3. Body & texture					
4. Taste					
5. Overall acceptability					

SENSORY STUDIES OF A NOVEL GOAT'S MILK ICE CREAM

THE SCORE CARD

Name: Prof/Dr/Mr/Ms (Optional) ……
Date:……

Instruction
Please taste the ice cream samples and indicate how much you like or dislike the sample by scoring each sample against the numerical values given below, ranging from Like extremely (9) to Dislike extremely (1).

Please score one sample at a time. Use water to wash your palate after tasting each sample.

Like extremely	Like very much	Like moderately	Like slightly	Neither like nor Dislike
09	08	07	06	05

Dislike slightly	Dislike moderately	Dislike very much	Dislike extremely
04	03	02	01

Characteristic	**Samples**			**Comments**
	641	752	923	
1. Colour & appearance				
2. Aroma				
3. Body & texture				
4. Taste				
5. Melting quality				
6. Overall acceptability				

Appendix C: Total solids, fat and ash contents (%) of fermented milk (Chapter 3)

Table 1. Changes in total solids (%) of the fermented goat's milk preparations during 3 weeks of storage at 4^{o}C (*n* = 2)

Storage time (wks)	L	P	B	L+P	L+B	P+B	L+P+B
0	10.43 ±0.03^{Aa}	10.34 ±0.17^{Aa}	10.86 ±0.21^{Aa}	10.57 ±0.01^{Aa}	10.48±0.04^{Aa}	10.53±0.01^{Aa}	10.52 ±0.03^{Aa}
1	10.38 ±0.02^{Aa}	10.54 ±0.01^{Aa}	10.50±0.01^{ABa}	10.38±0.04^{ABa}	10.54 ±0.01^{Aa}	10.62 ±0.03^{Aa}	10.71 ±0.16^{Aa}
2	10.16 ±0.03^{Ba}	10.33±0.03^{Aabc}	10.28 ±0.01^{Bab}	10.18 ±0.08^{Ba}	10.50 ±0.01^{Acd}	10.54±0.02^{Ad}	10.45±0.03^{Abcd}
3	10.20±0.01^{Ba}	10.20 ±0.01^{Aa}	10.20 ±0.02^{Ba}	10.16 ±0.03^{Ba}	10.50 ±0.02^{Abc}	10.58 ±0.01^{Ac}	10.47 ±0.02^{Ab}

Mean value (±SE)

$^{A, B, C}$ Values in the same column having different superscripts differ significantly ($p<0.05$).

$^{a, b, c,d}$ Values in the same row having different superscripts differ significantly ($p<0.05$).

Table 2. Changes in fat content (%) of the fermented goat's milk preparations during 3 weeks of storage at 4°C (*n* = 2)

Storage time (wks)	L	P	B	L+P	L+B	P+B	L+P+B
0	2.65 ±0.15^{Aa}	2.90±0.10^{Aa}	3.00±0.02^{Aa}	3.25±0.05^{Aa}	3.10 ±0.10^{Aa}	3.25 ±0.15^{Aa}	3.00±0.00^{Aa}
1	3.05±0.15^{Aa}	3.00±0.00^{Aa}	3.05±0.05^{Aa}	3.15±0.15^{Aa}	2.95 ±0.05^{Aa}	3.25±0.25^{Aa}	2.95±0.15^{Aa}
2	3.20±0.10^{Aa}	3.05±0.05^{Aa}	3.20±0.10^{Aa}	3.15±0.25^{Aa}	3.15 ±0.15^{Aa}	3.45±0.05^{Aa}	3.00±0.00^{Aa}
3	2.90±0.10^{Aa}	3.05±0.15^{Aa}	3.15±0.05^{Aa}	3.15±0.05^{Aa}	3.25±0.05^{Aa}	3.35 ±0.15^{Aa}	3.10 ±0.10^{Aa}

Mean value (±SE)

$^{A, B}$ Values in the same column having different superscripts differ significantly ($p<0.05$).

$^{a, b}$Values in the same row having different superscripts differ significantly ($p<0.05$).

Table 3. Changes in ash content (% of fresh weight) of the fermented goat's milk preparations during 3 weeks of storage at 4°C (*n* = 2)

Storage time (wks)	L	P	B	L+P	L+B	P+B	L+P+B
0	0.77 ± 0.00^{Aa}	0.81 ± 0.01^{Aa}	0.82 ± 0.01^{Aa}	0.87 ± 0.09^{Aa}	0.78 ± 0.00^{Aa}	0.80 ± 0.00^{Aa}	0.80 ± 0.00^{Aa}
1	0.75 ± 0.00^{Aa}	0.79 ± 0.00^{Abcd}	0.80 ± 0.00^{ABc}	0.78 ± 0.00^{Abd}	0.77 ± 0.00^{Ad}	0.80 ± 0.01^{Abc}	0.78 ± 0.01^{ABd}
2	0.73 ± 0.02^{Aa}	0.79 ± 0.00^{Ab}	0.80 ± 0.00^{ABb}	0.78 ± 0.01^{Aab}	0.78 ± 0.01^{Aab}	0.79 ± 0.00^{Ab}	0.79 ± 0.01^{ABb}
3	0.75 ± 0.00^{Aa}	0.77 ± 0.00^{Ba}	0.74 ± 0.03^{Ba}	0.75 ± 0.01^{Aa}	0.75 ± 0.00^{Ba}	0.77 ± 0.01^{Ba}	0.76 ± 0.01^{Ba}

Mean value (±SE)

$^{A, B, C}$ Values in the same column having different superscripts differ significantly ($p<0.05$).

$^{a, b, c,d}$ Values in the same row having different superscripts differ significantly ($p<0.05$).