

**Comparative Analysis of the Viability and Functional
Performance of Mono- and Multi-Species Probiotic Cultures in
a Non-Dairy Food Matrix**

By

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A thesis submitted for the degree of Doctor of Philosophy

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STATEMENT OF ORIGINALITY

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List of abbreviations

CFU	Colony forming unit
DC	Dendritic cell
EIA	Enzyme immuno-assay
ELISA	Enzyme linked immuno sorbent assay
FAO	Food and agriculture organisation
GIT	Gastro-intestinal tract
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IEC	Intestinal epithelial cells
IgA	Immunoglobulin A
IgM	Immunoglobulin M
IFN- γ	Interferon- γ
IL-1 β	Interleukin-1 β
IL-6	Interleukin-6
IL-8	Interleukin-8
LAB	Lactic acid bacteria
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MRD	Maximum recovery diluent
MRS	deMan, Rogosa Sharp
ng	Nanogram
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
pg	Picogram
ppm	Parts per million
RPMI	Roswell park memorial institute medium
SCFA	Short chain fatty acid
SEM	Scanning electron microscopy
TNF- α	Tumour necrosis factor- α
μ g	Microgram
WHO	World health organisation

Abstract

Probiotics are increasingly being included into food products in order to develop functional foods with health promoting effects, but have to date been exploited mainly in dairy products. Development of non-dairy probiotic foods such as fruit juices may provide consumers with greater choice and be attractive to those who can not eat dairy foods. Orange juice presents as an ideal vehicle for probiotic delivery as it is the most popular fruit beverage worldwide, and like other fruit juices has a short gastro-intestinal transit time which reduces exposure of probiotics to harsh environment of the stomach.

Since probiotic organisms vary in the type and level of their health promoting effects, it is likely that probiotic combinations would offer the consumer more benefit than single strains. Effective design of functional foods containing probiotic combinations, must take into consideration the likely occurrence and impact of potential interactions between individual species within a proposed combination, and between the probiotic and the carrier matrix. The main objectives of the current study were 1) to identify the effect of combining probiotics on their viability and adhesion to intestinal cells and 2) to examine the combined effect of exposure of probiotics to orange juice and low temperatures during refrigerated storage, on their viability and functional properties.

The initial study of long-term (14 days) growth interactions of several lactobacilli and *Bifidobacterium animalis* subsp *lactis* Bb12 (Bb), both alone and in co-culture with *Propionibacterium jensenii* 702 (PJ), revealed that growth patterns of *Lactobacillus* strains were not adversely affected by the presence of PJ, whereas lactobacilli strongly inhibited growth of PJ. In the co-culture of Bb and PJ, a significant enhancement of the growth of both bacteria was observed. The effect of combining probiotics on their adhesion to human intestinal epithelial Caco-2 cells was only evident in the case of *Lb. casei* 01 and *Lb. rhamnosus* GG (LG) which exhibited a decrease in adhesion rate in the presence of PJ.

The viability of LG, *Lb. reuteri* ATCC 55730 (LR), Bb and PJ, both individually and as 2- or 3- multispecies combinations, were then monitored in orange juice (OJ) (with and without 20% pulp) as well as bottled drinking water (BW) over 8 weeks of refrigerated

(4°C) and non-refrigerated storage (only for BW). Lactobacilli remained viable in higher numbers in OJ relative to that observed in BW under refrigeration. In contrast, a better outcome was observed for Bb and PJ in BW. Combining of probiotic species was observed to affect individual strain viability. Presence of pulp did not affect the viability of probiotics in OJ, while storage of BW at room temperature had an adverse effect on viability of all probiotics except of PJ, relative to storage under refrigeration.

Influence of combined exposure to OJ and refrigerated storage of the same probiotic preparations on their *in vitro* gastro-intestinal tolerance, adhesion to intestinal epithelial cells and immunomodulatory effects was then investigated at 10-day intervals during one month of storage. Suspension in OJ did not adversely affect the tolerance of any of the strains examined to simulated gastric juice (SGJ), with the tolerance of LG and PJ considerably enhanced relative to that observed in PBS, but did appear to impair the tolerance of lactobacilli and PJ to simulated intestinal juice (SIJ) at the baseline. High tolerance to SGJ was maintained throughout the storage period. The tolerance of both Bb and PJ to SIJ remained relatively constant during storage. Combining with both Bb and PJ enhanced the tolerance of the lactobacilli to SIJ with little impact on Bb, but adversely affected PJ in all combinations.

The adhesion rate of LG remained relatively constant in all preparations along with the viability during storage. In contrast with LG, adhesion rates and viabilities of other probiotics exhibited variation in relation to strain, presence of other microorganisms, and storage duration. In terms of both viability and adhesion rate, the preparations that provided the best outcomes for all constituents were LG and LR-PJ.

With the exception of LG, all probiotic preparations significantly enhanced non-stimulated interleukin-8 (IL-8) but not interleukin-6 (IL-6) or tumor necrosis factor- α (TNF- α) secretion by Caco-2 cells. Probiotic preparations enhanced *Escherichia coli* lipopolysaccharide (LPS) induced IL-8 release at baseline however this effect was not evident in all preparations at day 10. With the exception of LG, all probiotic preparations enhanced TNF- α induced IL-8 secretion towards day 20 after which it returned to the

control level. In contrast, probiotic preparations significantly reduced IL-1 β induced IL-8 secretion at baseline, with no further effect evident during storage. The relative probiotic effect on IL-1 β and TNF- α induced IL-8 secretion showed an upward and downward trend respectively over the storage period. Probiotic preparations did not affect LPS or IL-1 β induced secretion of IL-6 up to 10 days of storage, while thereafter some of them exhibited variable effects on IL-1 β induced IL-6 secretion. Compared to baseline (day 0), the effect of all four probiotic strains on IL-1 β induced TNF- α production was found to decrease significantly by day 10 of the storage period.

In conclusion, the results provided evidence of variation between individual strains in terms of their viability and intestinal adhesion capacity, and for the same strain when combined with different probiotics. When included in bottled drinking water and orange juice, the viabilities and functional properties of the probiotic preparations were further affected by the duration of their exposure to the carrier matrix and refrigerated storage. Such effects should be considered when formulating probiotic products, and further research is recommended to confirm the observed *in vitro* functional effects *in vivo*.

Chapter I: Introduction

1.1 Overview

Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2001a, 2001b). Although probiotics primarily belong to the genera *Lactobacillus* and *Bifidobacterium* (Prasad *et al.*, 1998), some strains of dairy propionibacteria have also been considered as probiotic due to their reported association with a diverse range of health benefits (Cousin *et al.*, 2011).

Probiotics are increasingly being incorporated into food products in order to develop “functional foods” with additional health promoting effects (Champagne *et al.*, 2005; Stanton *et al.*, 2001), but have to date been exploited primarily in the form of commercial dairy based products such as fermented milk and yoghurt (Mattila-Sandholm *et al.*, 2002; Shah, 2001). Development of dairy-free probiotic foods may, however, suit consumers who have allergy to milk products, are lactose intolerant (Luckow & Delahunty, 2004b) or have no desire to eat dairy foods. As an alternative, fruit juices may be considered ideal delivery vehicles for probiotics due to their short gastro-intestinal transit time which may in turn reduce exposure of probiotics to harsh environments such as low pH in the stomach (Post, 2002). Orange juice, which is the most popular fruit beverage worldwide due largely to its widely appealing flavour and nutritional properties (Rega *et al.*, 2004), is considered a rich source of vitamin C and natural folate. It also contains phytochemicals (*e.g.* polyphenols and carotenoids) and minerals such as potassium, phosphorus, calcium and magnesium (Guarnieri *et al.*, 2007; Ohrvik & Witthoft, 2008; USDA National Nutrient Database for standard Reference, 2009). Therefore, inclusion of probiotics into fruit juices such as orange juice may further enhance the nutritional value of these products and deliver health promoting probiotic microorganisms to a larger consumer population.

Bottled water is also attractive as a potential probiotic delivery vehicle as it is one of the most highly consumed drinks worldwide, representing more than 1/3 of total soft drink consumption (Finlayson, 2005). Thus, as an all-day beverage used by a wide range of consumers, bottled water warrants examination as a potential probiotic carrier.

Since probiotic organisms vary in the type and level of their health promoting effects, it is likely that probiotic combinations may potentially offer even greater benefit to the consumer than single strain preparations. Effective design of functional foods containing

probiotic combinations, must take into consideration the likely occurrence and impact of potential interactions between individual species within a proposed combination, and between probiotic strains and food matrix. However to date, studies addressing the effect of presence of other microorganisms and storage on the viability of probiotics have been limited to dairy products such as yoghurt, although the viability of single strains of probiotics has been studied in fruit juices and fruit drinks during storage (Champagne & Gardner, 2008; Champagne *et al.*, 2008; Sheehan *et al.*, 2007). At this time, to the best of this author's knowledge there have been no studies examining the viability and functional properties of probiotic combinations in fruit juice during storage.

1.2 Research objectives and hypotheses

In this study probiotic strains *Lactobacillus casei* 01 (LC), *Lb. plantarum* HA8 (LP), *Lb. rhamnosus* GG (LG), *Lb. reuteri* ATCC 55730 (LR), *Bifidobacterium animalis* subsp *lactis* Bb12 (Bb) and *Propionibacterium jensenii* 702 (PJ) were used.

The specific objectives of this thesis were:

- To examine long-term growth interactions of five probiotic strains LC, LP, LG, LR and Bb both alone and in combination with PJ in a co-culture system, and to determine their ability to adhere to the human intestinal epithelial cell line Caco-2.
- To monitor viability patterns of probiotics LG, LR, Bb and PJ as mono-cultures and 2- and 3- multispecies combinations over 8 weeks of storage in orange juice and bottled water, in order to examine the influence of the carrier matrix, storage temperature (for water only), and potential species interactions on probiotic viability.
- To determine the effect of duration of combined exposure to orange juice and refrigerated storage on the tolerance of LG, LR, Bb and PJ as mono-cultures and 2- and 3- multispecies combinations to simulated gastric and intestinal juices at 10-day intervals during one month storage.
- To determine the effect of duration of combined exposure of probiotic preparations (LG, LR, Bb and PJ either separately or in 2- and 3- multispecies combinations) to

orange juice and low storage temperature (4°C) on their capability to adhere to intestinal epithelial Caco-2 cells at 10-day intervals during one month storage.

- To determine the impact of duration of combined exposure of probiotic preparations (LG, LR, Bb and PJ as mono-cultures and 2- and 3- multispecies combinations) to orange juice and low storage temperature (4°C) on their *in vitro* modulation of immune responses (secretion of IL-6, IL-8 and TNF- α) of intestinal epithelial cells treated with inflammatory stimuli (*Escherichia coli* LPS, IL-1 β and TNF- α).

In addressing these objectives it was hypothesized that:

- Combining of probiotics would influence the growth/viability and adhesion of constituents of the probiotic combination to the human colon adenocarcinoma cell line Caco-2.
- When included in orange juice or bottled drinking water, viability levels would vary between the different probiotic strains examined, and that these levels would be further influenced by presence of other probiotics, carrier matrix, and the duration and temperature of storage.
- Functional properties of probiotics such as their *in vitro* gastro-intestinal tolerance, adhesion to intestinal epithelial cells and immunomodulatory effects would also vary between the strains examined, and be influenced by the presence of other microorganisms, and the duration of their combined exposure to orange juice and refrigerated storage.

1.3 Thesis Outline

The thesis comprises 8 separate chapters. Immediately following this introductory chapter, which describes the scope, objectives and hypotheses on which the work was based, is a comprehensive review of published literature on probiotic organisms and food products (Chapter 2). Chapters 3-7 include the main studies performed to address the research objectives of this project. The thesis closes with Chapter 8, which includes an overall summary of the findings of this work, conclusions and recommendations for future research.

Chapter II: Literature Review

2.1 Probiotic definition

Probiotics are the subject of evolving definitions as more research is undertaken in this field. The term "probiotic" is derived from the Greek meaning for life, and was first coined by Lilly and Stillwell (1965) to describe substances produced by one microorganism which stimulate the growth of other organisms. Subsequently the definition was expanded to include organisms and substances which improve intestinal microbial balance (Parker, 1974).

Fuller (1989) further developed Parker's definition as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". In 1992 Fuller extended his definition to "live microbial food ingredients that beneficially affect the health of consumers by improving their intestinal microflora balance when ingested live in sufficient numbers" (Fuller, 1992). Fuller's descriptions give emphasis to viability of probiotic and limitation to the intestinal tract.

According to Havenaar and Huis In't Veld (1992) probiotic is "a viable mono- or mixed culture of micro-organisms which, when applied to animals or man, beneficially affects the host by improving the properties of the indigenous microflora".

In 1996 the term probiotic was defined as "microorganisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition" (Schaafsma, 1996). Reuter (1997) described probiotic as "a microbial preparation which contains live and/or dead cells including their metabolites which is intended to improve the microbial or enzymatic balance at mucosal surfaces or to stimulate immune mechanisms". This definition recognised that the preparations do not have to be alive.

An international workshop hosted by the Lactic Acid Bacteria Industrial Platform (LABIP) issued a consensus definition of probiotics: "oral probiotics are living micro-organisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition". According to this definition, probiotics may be administered either as a food ingredient or as a non-food preparation (Guarner & Schaafsma, 1998).

Salminen *et al.* (1999) defined probiotics as "microbial cell preparations or components of microbial cells that have a beneficial effect on health and well being of the host". This definition indicates that it is not necessary need for probiotics to be viable. Moreover, this definition is not limited to beneficial effects concerning the intestinal tract only.

Another definition is that “probiotic is a microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract” (Naidu *et al.*, 1999).

According to Diplock, *et al.* (1999) “a probiotic is a live microbial food ingredient that is beneficial to health”.

Tannock *et al.* (2000) have defined probiotics as “live microbes which transit the gastrointestinal tract and in doing so benefit the health of the consumer”.

Schrezenmeir & de Vrese (2001) proposed a new definition for probiotic as “a preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonisation) in a compartment of the host and by that exert beneficial health effects in this host”.

Regardless of the numerous versions, the most used and widely acknowledged definition by scientific community describes probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2001b).

2.2 Probiotic microorganisms

Probiotics primarily belong to the genera *Lactobacillus* and *Bifidobacterium*, however, other microorganisms including propionibacteria, leuconostoc, pediococci, enterococci and *Escherichia coli* have also been considered as probiotic cultures. A summary of potential probiotic species is provided in Table 2.1. Probiotic preparations may include one or more different microbial strains or species (Champagne & Møllgaard, 2008; Gardiner *et al.*, 2002).

In the following section, physiological and taxonomic characteristics of main probiotic organisms will be discussed.

Table 2.1: Microorganisms used as probiotics

Lactobacilli	Bifidobacteria	Enterococci	Others
<i>Lb. acidophilus</i>	<i>Bif. bifidum</i>	<i>E. faecium</i>	<i>Sacccaromayces boularddi</i>
<i>Lb. plantarum</i>	<i>Bif. infantis</i>	<i>E. faecalis</i>	<i>Lactococcus lactis</i> subsp. <i>cremoris</i>
<i>Lb. casei</i>	<i>Bif. adolescentis</i>		<i>Lactococcus lactis</i> subsp. <i>lactis</i>
<i>Lb. rhamnsus</i>	<i>Bif. longum</i>		<i>Propionibacterium freudenreichii</i>
<i>Lb. debrueckii</i> subsp. <i>bulgaricus</i>	<i>Bif. breve</i>		<i>Pediococcus acidilactici</i>
<i>Lb. fermentum</i>	<i>Bif. lactis</i>		<i>Streptococcus thermophilus</i>
<i>Lb. johnsonii</i>			<i>Leuconostoc mesenteroides</i> subsp. <i>dextranicum</i>
<i>Lb. gasseri</i>			<i>Escherichia coli</i>
<i>Lb. salivarius</i>			<i>Bacillus cereus</i>
<i>Lb. reuteri</i>			<i>Clostridium butyricum</i>
<i>Lb. brevis</i>			
<i>Lb. helveticus</i>			
<i>Lb. lactis</i>			
<i>Lb. paracasei</i>			
<i>Lb. amylovorus</i>			
<i>Lb. crispatus</i>			

Source: adapted from Brunser & Gotteland (2010), Farnworth (2001), Gorbach (2002), Holzapfel *et al.* (1998)

2.2.1 The genus *Lactobacillus*

Lactobacilli are Gram-positive, non-spore forming rods or coccobacilli, catalase-negative, facultative anaerobes, sometimes microaerophilic and often nonmotile. As chemoorganotrophic organisms, they are extremely fastidious and require rich nutritional media to grow. Members of the genus *Lactobacillus* are strictly fermentative and may be either homofermentative, producing mainly lactic acid from glucose, or heterofermentative, producing lactic acid, CO₂, ethanol, and/or acetic acid (Holt, 1994; Prescott *et al.*, 2002). Lactobacilli are taxonomically classified into three groups: obligately homofermentative, facultatively heterofermentative, and obligately heterofermentative (Hammes & Vogel, 1995; Kandler & Weiss, 1986). At present, the genus *Lactobacillus* comprises 100 validly recognised species (Dellaglio & Felis, 2005).

Lactobacillus species are widely found in environments such as animal and vegetable food products, respiratory, gastrointestinal and genital tracts of humans and animals (Dellaglio & Felis, 2005; Holt, 1994).

The ability of lactobacilli to produce lactic acid and other organic acids, as well as flavour compounds, results in the transformation of raw material to a wide variety of new food products, in particular fermented vegetable and dairy products. Furthermore, their ability to lower the pH of the environment and to produce some inhibitory compounds *e.g.* organic acids and bacteriocins causes them to exert an antagonistic action toward harmful microorganisms such as *Escherichia coli*, *Salmonella* spp. and *Helicobacter pylori*. The role of lactobacilli in intestinal ecosystems has received much attention with respect to their beneficial health effect on human and animal, especially in regards to ingestion of lactobacilli as probiotics (Hammes & Hertel, 2006). Some commercial probiotic *Lactobacillus* strains and their clinical benefits are presented in Table 2.2. The health benefits of probiotics are further addressed in section 2.4.

Table 2.2: Examples of recent *in vivo* studies addressing the beneficial health effects of some commercial probiotic *Lactobacillus* strains

Probiotic lactobacillus strain (Owner of the strain)	Health effects	Reference
<i>Lb. rhamnosus</i> GG (Valio Ltd, Finland)	Prevention of atopic eczema/dermatitis, suppression of allergic airway inflammation/asthma, suppression of some symptoms of IBS, healing gastric ulcer, prevention/treatment of rotavirus diarrhoea, prevention of antibiotic associated diarrhoea, treatment of <i>Clostridium difficile</i> - associated diarrhoea	(Blumer <i>et al.</i> , 2007; Feleszko <i>et al.</i> , 2007; Lam <i>et al.</i> , 2007; McFarland, 2006; Nermes <i>et al.</i> , 2011; Pant <i>et al.</i> , 2007; Rose, 2011; Sawada <i>et al.</i> , 2007; Viljanen <i>et al.</i> , 2005)
<i>Lb. reuteri</i> (BioGaia AB, Sweden)	Prevention of enteric colonisation by <i>Candida</i> in preterm newborns; reduction of functional abdominal pain in children; improvement of intestinal comfort in cystic fibrosis patients; improvement of symptoms of rotavirus gastroenteritis ; reduction of symptoms of atopic eczema in children; decreasing antibiotic-associated diarrhoea; control of <i>H. pylori</i> infection; improvement of oral health	(Betta <i>et al.</i> , 2007; Caglar <i>et al.</i> , 2008; Francavilla <i>et al.</i> , 2008; Gromert, 2009; Krasse <i>et al.</i> , 2006; Nikawa <i>et al.</i> , 2004; Romano <i>et al.</i> , 2010; Romeo <i>et al.</i> , 2011; Shornikova <i>et al.</i> , 1997; Twetman <i>et al.</i> , 2009)
<i>Lb. plantarum</i> 299v (Probi AB, Sweden)	Improvement of <i>Clostridium difficile</i> -associated diarrhoea; treatment of IBS; reduction of cardiovascular disease risk factors; inhibition of <i>Escherichia coli</i> -induced intestinal permeability; reduction of pathogenic bacteria in the oropharynx of intubated patients	(Klarin, Molin, <i>et al.</i> , 2008; Klarin, Wullt, <i>et al.</i> , 2008; Mangell <i>et al.</i> , 2002; Naruszewicz <i>et al.</i> , 2002; Niedzielin <i>et al.</i> , 2001; Nobaek <i>et al.</i> , 2000; Waugh <i>et al.</i> , 2009; Wullt <i>et al.</i> , 2007)
<i>Lb. Casei</i> Shirota (Yakult Honsha Co. Ltd., Japan)	Improving stool consistency, constipation and bowel movements, modulating natural killer (NK) cell activity in subjects with low NK cell activity, modulating the immune response in allergic rhinitis, reducing risk of bladder cancer, prevention of colorectal tumors development	(Ishikawa <i>et al.</i> , 2005; Ivory <i>et al.</i> , 2008; Koebnick <i>et al.</i> , 2003; Matsumoto <i>et al.</i> , 2006; Morimoto <i>et al.</i> , 2005; Ohashi <i>et al.</i> , 2002; Sakai <i>et al.</i> , 2011; Takeda & Okumura, 2007; Tamura <i>et al.</i> , 2007)

2.2.2 The genus *Bifidobacterium*

Bifidobacteria were first described by Tissier in 1899. He isolated the bacteria in the faeces of breast-fed infants. Since that time, the taxonomy of bifidobacteria has been changed. In 1974, they were recognised as the genus *Bifidobacterium*, the name initially chosen by Orla-Jensen in 1924 (Gardiner *et al.*, 2002). Within the genus *Bifidobacterium*, 37 species have been described to date (Turroni *et al.*, 2011). Only six species have been considered as probiotics (Table 2.1) (Tamime, 2002). Bifidobacteria are characterised as Gram-positive, catalase negative, polymorphic branched rods, non-motile and non-sporeforming anaerobic heterofermentatives (Dellaglio & Felis, 2005; Tannock, 2002a). The morphology of bifidobacteria depends on the strain/species as well as cultural conditions used (Gardiner *et al.*, 2002). Cell morphology traits only aid a little in identification of a bacterial isolate as *Bifidobacterium*, however, it may be one way to recognize a bifidobacterial species (Biavati & Mattarelli, 2006; Tannock, 2002b). The most reliable and useful non molecular method for identification of bifidobacteria is fructose-6-phosphate phosphoketolase (F6PPK) assay. F6PPK is the key enzyme of bifidobacterial hexose metabolism pathway termed “bifidus shunt”(Biavati & Mattarelli, 2006). Glucose fermentation through this pathway produces acetic and lactic acids in a theoretical ratio of 3:2 (Scardovi, 1986). Differentiation of *Bifidobacterium* species can be achieved using some biochemical tests such as carbohydrate fermentation, cell-wall structure type and electrophoretic tests of cellular proteins, however, molecular techniques (such as 16S rRNA gene sequence analysis) are more valuable tests in the identification of *Bifidobacterium* species (Dellaglio & Felis, 2005; Tannock, 2002b). Examples of the clinical benefits of two commonly available commercial probiotic bifidobacteria are presented in Table 2.3.

Table 2.3: Examples of recent *in vivo* studies addressing the beneficial health effects of some commercial probiotic *Bifidobacterium* strains

Probiotic <i>Bifidobacterium</i> strain (Owner of the strain)	Health effects	Reference
<i>Bifidobacterium animalis</i> subsp <i>lactis</i> BB-12® (Chr. Hansen A/S, Hørsholm, Denmark)	Alleviating symptoms of atopic eczema, decreasing frequency and duration of diarrhea, increasing fecal secretory IgA levels in infants, reducing the incidence of respiratory infections in infants	(Fukushima <i>et al.</i> , 1998; Isolauri <i>et al.</i> , 2000; Taipale <i>et al.</i> , 2011; Weizman <i>et al.</i> , 2005)
<i>Bif. lactis</i> HN019 (marketed as DR10 by Fonterra, New Zealand, and HOWARU Bifido by Danisco, USA)	Decrease in number of iron-deficient preschoolers, confer desired changes in the intestinal microflora of elderly human subjects, enhancement of immunity in the elderly, reduces the severity of <i>Escherichia coli</i> O157: H7 infection, Enhances Resistance to oral <i>Salmonella typhimurium</i> infection, prevention of morbidity in preschoolers, immunomodulatory effect on fetal immune parameters and breast milk	(Ahmed <i>et al.</i> , 2007; Gill, Rutherford, Cross, <i>et al.</i> , 2001; Prescott <i>et al.</i> , 2008; Sazawal <i>et al.</i> , 2010; Shu & Gill, 2001; Shu <i>et al.</i> , 2000)

2.2.3 The genus *Propionibacterium*

The name "*Propionibacterium*" was proposed by Orla-Jensen (1909) for organisms characterised by their ability to produce copious amounts of propionic acid and acetic acid and often small amounts of carbon dioxide during growth. The genus *Propionibacterium* presently comprises 11 recognised species (Stackebrandt *et al.*, 2006). The species of this genus are characterised as irregularly staining Gram-positive, usually catalase positive, diphtheroid, pleomorphic rods that may bifurcate or even branch; they are nonsporeforming, nonmotile and facultative anaerobes, but variable aerotolerant (Holt, 1994; Stackebrandt *et al.*, 2006).

The propionibacteria are comprised of two principal groups: (a) the “classical propionibacteria” that have been mainly isolated from dairy products, especially cheese, and (b) “Cutaneous propionibacteria” or non-dairy strains which are found in spoiled and fermenting fruits, silage and soil, human skin, mouth, the female genital tract, and faeces (Stackebrandt *et al.*, 2006; Tamime, 2002). Some species of the latter such as *Propionibacterium acnes*, *Propionibacterium avidum* and *Propionibacterium granulosum* have been identified to be pathogens and cause diseases including endophthalmitis, brain abscesses, meningitis, arthritis, osteomyelitis, endocarditis and infections of the central nervous system (Funke *et al.*, 1997; Hykin *et al.*, 1994).

Classical propionibacteria or dairy propionibacteria have important roles in food industry especially in cheese making. They produce large gas vacuoles or “eyes” and develop flavour and texture in Swiss cheese varieties (Emmental and Gruyere) during the ripening period. They are also used for the commercial production of propionic acid and vitamin B12 (Stackebrandt *et al.*, 2006; Tamime, 2002). Furthermore, some classical *Propionibacterium* species such as *P. freudenreichii* and *P. jensenii* have been considered as potential probiotic microorganisms (Cousin *et al.*, 2011; Huang & Adams, 2003; Mantere-Alhonen, 1995). Some health benefits of dairy propionibacteria as probiotics are: modulation of short chain fatty acids in human faeces (Jan, Leverrier, *et al.*, 2002), alleviating constipation by improvement of intestinal microbiota (Hojo *et al.*, 2002), anti-inflammatory effect in healthy adults (Kekkonen *et al.*, 2008), synthesis of vitamins such as B12 and folate (Hugenholtz *et al.*, 2002), secretion of antimicrobial compounds (e.g. propionic acid and bacteriocins) (Ouweland, 2004), moderation of lactose intolerance

through production of β -galactosidase (Zarate, Perez Chaia, *et al.*, 2000), modulation of the host's immune system (Perez Chaia, deMacias, *et al.*, 1995), anti-hyperlipemic effect (Perez Chaia, deMacias, *et al.*, 1995), growth stimulation of bifidobacteria (Kaneko, 1999; Kaneko *et al.*, 1994; Kaneko & Noda, 1996; Kouya *et al.*, 2007; Mori *et al.*, 1997; Uchida *et al.*, 2005; Warminska-Radyko *et al.*, 2002), moderation of colonic inflammation by nitrate reduction (Michel *et al.*, 2005) and anticarcinogenic effect (Jan, Belzacq, *et al.*, 2002; Lan *et al.*, 2008; Lan, Lagadic-Gossman, *et al.*, 2007; Perez Chaia *et al.*, 1999).

2.2.4 *Saccharomyces boulardi*

Saccharomyces boulardi is considered a non-pathogenic, mesophilic and non-colonising baker's yeast, which is morphologically and physiologically related to brewer's yeast also known as *S. cerevisiae*. Similarly, resemblance between sequence analysis of *S. boulardi* and *S. cerevisiae* revealed this close relatedness, however it differs from *S. cerevisiae* in some genotypic characteristics. Probably the best designation for this microorganism would be *S. cerevisiae* var. *boulardi* (Hennequin *et al.*, 2001; Kühle & Jespersen, 2003; Mallie *et al.*, 2001).

S. boulardi is known as a unique microorganism that can survive GI tract transit, proliferate in the gut and exert many beneficial effects on humans and animals (McFarland & Bernasconi, 1993). *S. boulardi* has been widely used as a biotherapeutic agent for controlling diarrhoea associated with antibiotic use as well as other types of diarrhoea including diarrhoea caused by *Clostridium difficile* and traveller's diarrhoea in children and adults (Castex *et al.*, 1990; Duman *et al.*, 2005; Elmer *et al.*, 1996; Izadnia *et al.*, 1998; Kollaritsch *et al.*, 1993; Kotowska *et al.*, 2005; Kurugol & Koturoglu, 2005; McFarland, 2007; Szajewska & Mrukowicz, 2005). Also, this organism can protect the intestine against *Cl. difficile* and cholera toxins (Castagliuolo *et al.*, 1999; Dias *et al.*, 1995) and has a positive effect in the treatment of inflammatory bowel disease (IBD) (Dalmasso *et al.*, 2006). It has been reported that *S. boulardi* alleviates Crohn's disease and ulcerative colitis (Guslandi *et al.*, 2003; Guslandi *et al.*, 2000). In addition, it has been demonstrated that *S. boulardi* has an immunostimulatory effect on host through increasing levels of secretory IgA and some cytokines (TNF- α , IL-12 and IFN- γ) (Buts *et al.*, 1990; Rodrigues *et al.*, 2000). Studies have shown that *S. boulardii* inhibits translocation of *Candida albicans* from

the GI tract to liver and kidneys mesenteric lymph nodes (MLN) (Algin *et al.*, 2005; Berg *et al.*, 1993).

2.3 Selection criteria for probiotics

Many criteria have been considered by several researchers as desirable properties for potential probiotic strains (Salminen *et al.*, 1998). Probiotics must fulfil a number of safety, functional and technological properties and characteristics to be used in probiotic food products (Table 2.4).

Table 2.4: Selection criteria of probiotic organisms for human use

Safety	<ul style="list-style-type: none">• Preferably originated from healthy human GI tract• Non-pathogenic and not associated with diseases <i>e.g.</i> infective endocarditis or GI disorders• Non-inflammatory promoting• Not able to deconjugate or dehydroxylate bile salts• Not able to carry transmissible antibiotic resistance genes• Not having clinical side effects
Functional	<ul style="list-style-type: none">• Resistant to low pH, gastric juice, bile acid and pancreatic juice• Adhesion to the intestinal cells and colonisation of the human gut• Modulation of immune system• Antagonistic against pathogens via competition for adhesion sites and production of antimicrobial metabolites• Antimutagenic and antigarcinogenic properties• Potential for the delivery of recombinant proteins and peptides to the human GI tract
Technological	<ul style="list-style-type: none">• Reasonable sensory properties• Phage resistant• Viability during production and storage of the product

Adopted from ([Champagne & Møllgaard, 2008](#); [Collins *et al.*, 1998](#); [Gardiner *et al.*, 2002](#); [Havenaar & Huis In't Veld, 1992](#); [Lee & Salminen, 1995](#); [Saarela *et al.*, 2000](#))

2.4 Health benefits of probiotics

Probiotics have been associated with numerous health benefits (Tables 2.2 and 2.3); however to date only a few of these have been proven by clinical studies. It should be noted that beneficial health effects of probiotics are strain specific ([Sanders, 2008](#); [Shah, 2007](#)). Some of the health benefits claimed include prevention and/or treatment of infections, irritable bowel syndrome (IBS), chronic gut disorder such as inflammatory bowel disease (IBD) and colon cancer, coronary heart disease (CHD), recurrent vaginal thrush, skin problems and food allergy, alleviation of lactose intolerance, treatment of different diarrhoeal diseases, lowering serum cholesterol and triglyceride levels, modulation of the immune system, enhancement of mineral bioavailability, chemopreventative effects,

improvement of constipation, improvement of dermatitis and liver disease. Some of these health promoting effects of probiotics are further discussed below.

2.4.1 Lipid modulation

Hypercholesterolaemia and elevated serum triglyceride levels have been identified as major risk factors for CHD. Studies have shown that reductions in serum cholesterol levels can reduce risk of heart disease (Lourens-Hattingh & Viljoen, 2001). Ingestion of probiotics has been proposed to be an effective way in lowering serum lipid levels including cholesterol and triglycerides (Gardiner *et al.*, 2002; Lovegrove & Jackson, 2004a; Pereira & Gibson, 2002).

Possible mechanisms for hypocholesterolaemic effect of probiotics are as follows:

- 1- Direct cholesterol assimilation by some probiotic bacteria in the presence of bile acids and under anaerobic conditions and thus making it unavailable for absorption into the blood (Gilliland *et al.*, 1985; Noh *et al.*, 1997).
- 2- Enzymatic deconjugation of bile salts by probiotic bile-salt hydrolase (BSH) activity resulting in free (deconjugated) bile salts which are less soluble and may be excreted more likely from the intestinal tract than conjugated bile salts. Faecal loss of bile salts should result in a higher demand for cholesterol as a precursor for the synthesis of new bile salts (in the liver) and therefore may lower serum cholesterol concentrations (DeRodas *et al.*, 1996; Desmet *et al.*, 1994; du Toit *et al.*, 1998).
- 3- Fermentation of food-derived indigestible carbohydrates in the human gut that results in an increased production of short-chain fatty acids (SCFA) which can decrease blood lipid levels either by preventing hepatic cholesterol synthesis, or by redistributing cholesterol from plasma to the liver (Liong & Shah, 2005a, 2005b; Umeki *et al.*, 2004).
- 4- Cholesterol binding to bacterial cell walls (Lovegrove & Jackson, 2004a; Pereira & Gibson, 2002).

2.4.2 Modulation of the immune system

Probiotics appear to be important in the enhancement of immunomodulating activities, *in vivo* and *in vitro*, in human and animals. Possible stimulation of an immune response by probiotic bacteria may explain the potential therapeutic and prophylactic applications of such cultures in the treatment of infections and carcinogenesis (Gardiner *et al.*, 2002). Probiotic cultures have been shown to stimulate both non-specific (innate) and specific (adaptive) immunity (Cross, 2002; Gill *et al.*, 2009). Examples of effects of probiotics on innate and adaptive immunity are presented in Tables 2.5 and 2.6.

It has been documented that administration of probiotics enhanced lymphocyte proliferation (Amrouche *et al.*, 2006; Wagner *et al.*, 2009; Wagner *et al.*, 1997), increased serum levels of IgG and IgM (Wagner *et al.*, 1997), enhanced gut mucosal IgA-secreting cells (Ibnou-Zekri *et al.*, 2003; Park *et al.*, 2002; Perdigon *et al.*, 1995) and stimulated production of different types of interleukin and interferon in immune cells (Amrouche *et al.*, 2006; Kitazawa *et al.*, 1992; Kitazawa *et al.*, 1994; Morita *et al.*, 2002b; Rangavajhyala *et al.*, 1997).

It has been reported that *Lb. acidophilus* and *Lb. gasseri* supernatants possess chemotactic factor(s) (Halper *et al.*, 2003; Kitazawa *et al.*, 2002a, 2002b). Kitazawa *et al.* (2002a, 2002b) first reported that probiotics could produce chemotactic factors, and identified a new chemotactic factor named “Gasserokine” in *Lb. gasseri* supernatant. These researchers proposed that chemotactic activity could be a novel immunological function of probiotic bacteria. Halper *et al.* (2003) showed *Lb. acidophilus* supernatant stimulated the proliferation of macrophages and lymphocytes *in vitro* and the chemotaxis and proliferation inflammatory cells *in vivo*. Roselli *et al.* (2006) indicated that *Bif. animalis* MB5 and *Lb. rhamnosus* GG protect intestinal cells from the inflammation-associated response caused by enterotoxigenic *E. coli* by partly counteracting neutrophil migration.

It has been reported that derived extracts of *Lb. rhamnosus* GG and *Bif. animalis* subsp *lactis* Bb12 prevent lymphocyte proliferation *in vitro* (Saarela *et al.*, 2000). Oral administration of these two probiotic cultures in children with severe atopic eczema resulting from food allergy, showed a considerable improvement in clinical symptoms (Mattila-Sandholm & Saarela, 2000).

Table 2.5: Examples of probiotic effects on innate immunity

Target	Probiotic	Effect	Reference
Phagocytosis activity			
	<i>Lb. acidophilus</i> and <i>Lb. casei</i>	Increased phagocytosis activity of murine peritoneal macrophages	Perdigon <i>et al.</i> (1988)
	<i>Lb. acidophilus</i> La1	Increased phagocytosis activity of human blood leucocytes	Donnet-Hughes <i>et al.</i> (1999), Schiffrin <i>et al.</i> (1997)
	<i>Bif. animalis</i> subsp <i>lactis</i> Bb12	Increased phagocytosis activity of human blood leucocytes	Schiffrin <i>et al.</i> (1997)
	<i>Bif. lactis</i> HN019	Increased phagocytosis activity of peripheral blood mononuclear cells (PBMC)	Arunachalam <i>et al.</i> (2000)
	milk product containing <i>Lb. rhamnosus</i> GG	Up-regulated the expression of phagocytosis receptors like CR1, CR3, FcγRIII and FCαR in neutrophils of healthy individuals	Pelto <i>et al.</i> (1998)
	<i>Lb. johnsonii</i> La1	Increased the respiratory burst of human blood phagocytes	Donnet-Hughes <i>et al.</i> (1999)
	<i>Lb. rhamnosus</i> HN001 and <i>Bif. lactis</i> HN109	Increased <i>ex vivo</i> phagocytosis activity of mononuclear and polymorphonuclear phagocytes	Gill, Rutherford, Cross <i>et al.</i> (2001), Sheih <i>et al.</i> (2001)
Natural Killer (NK)			
	<i>Lb. rhamnosus</i> HN001 and <i>Bif. lactis</i> HN109	Increased the cytotoxic potential of NK cells	Gill, Rutherford, & Cross (2001), Sheih <i>et al.</i> (2001)
	<i>Lb. casei</i> subsp. <i>casei</i> in combination with dextran	Enhanced efficiency of NK cell activity	Ogawa <i>et al.</i> (2006)
	<i>Lb. casei</i> Shirota	Enhanced NK cell activity	Takeda <i>et al.</i> (2006)
	<i>Lb. fermentum</i> CECT5716	Increased proportion of NK cells	Olivares <i>et al.</i> (2007)
Modulation of Cytokine/Chemokine production			
	<i>E. coli</i> Nissle 1017	Induced release of IL-8 in HT-29 cells	Lammers <i>et al.</i> (2002) Otte & Podolsky (2004)
	<i>Lb. plantarum</i> 299v	Increased IL-8 mRNA expression in HT-29 cells stimulated by TNF-α	McCracken <i>et al.</i> (2002)
	<i>Lb. rhamnosus</i> GG	Decreased IL-8 production induced by TNF-α in Caco-2 cells	Zhang <i>et al.</i> (2005)
	<i>Lb. reuteri</i>	Decreased IL-8 production and induced production of nerve growth factor (NGF) in T84 and HT-29 cells	Ma <i>et al.</i> (2004)
	<i>Bif. animalis</i> subsp <i>lactis</i> Bb12	Increased IL-6 production in rats	Ruiz <i>et al.</i> (2005)
	<i>Lb. casei</i> CRL 431 and <i>Lb. helveticus</i> R389	Increased IL-6 in mice	Vinderola <i>et al.</i> (2005)
	<i>Lb. casei</i> , <i>Lb. paracasei</i> and <i>Lb. acidophilus</i>	Induced IL-15 release in Caco-2 cells	Ogawa <i>et al.</i> (2006)
	<i>Lb. casei</i> DN-114	Expression of CXCL-1 and 2, CCL20 in Caco-2 cells	Tien <i>et al.</i> (2006)
	<i>Lb. rhamnosus</i> GG	Induced TNF-α, IL1-α, IFN-γ in IECs	Yan & Polk (2002)

Table 2.6: Examples of probiotic effect on adaptive immunity

Target	probiotic	Effect	Reference
IgA production			
	<i>Bif. bifidum</i> and <i>Lb. acidophilus</i> La1	Increased serum IgA level in subjects who received vaccination against <i>Salmonella typhi</i> Ty21	Link-Amster <i>et al.</i> (1994)
	<i>Lb. rhamnosus</i> GG	Increased number of IgA secreting cells in children (2-5 years old) who received a rotavirus vaccination	Isolauri <i>et al.</i> (1995)
	<i>Lb. rhamnosus</i> GG	Increased IgA seroconversion during the remission phase in children suffering from acute rotavirus-induced diarrhoea	Kaila <i>et al.</i> (1995) H. Majamaa <i>et al.</i> (1995)
	<i>Bif. animalis</i> subsp <i>lactis</i> Bb12	Increased the total amount of IgA in the feces, and more particularly, antipoliiovirus IgA in children receiving polio vaccine	Fukushima <i>et al.</i> (1998)
	<i>Bif. bifidum</i>	Induced IgA production in Peyer's patches and mesenteric lymph nodes in mice	Park <i>et al.</i> (2002)
	<i>Lb. helveticus</i> -fermented milk	Induced local mucosal and systemic IgA immune responses in mice infected with <i>E. coli</i> O157:H7	Leblanc <i>et al.</i> (2004)
	<i>Lb. johnsonii</i> and <i>Lb. paracasei</i>	Induced intestinal IgA production	Ibnou-Zekri <i>et al.</i> (2003)
Dendritic cells and Treg cells			
	<i>Lb. rhamnosus</i>	DCs matured in the presence of <i>Lb. rhamnosus</i> reduced the proliferation of T cells and the secretion of IL-2, IL-4 and IL-10 upon anti-CD3/anti-CD28 stimulation.	Braat <i>et al.</i> (2004)
	irradiated <i>Lb. reuteri</i> , <i>Lb. plantarum</i> Lb20, <i>Lb. casei</i> subsp. <i>alactus</i> , <i>Lb. plantarum</i> 299v and <i>Lb. johnsonii</i> La1	Induced bone marrow-derived murine DCs maturation <i>Lb. casei</i> subsp. <i>alactus</i> has induced pro-inflammatory cytokines (IL-12, IL6, TNF- α) in DCs, <i>Lb. reuteri</i> inhibited the production of IL-12, IL-6 and TNF- α and the expression of B7.2 (CD86) in DCs induced by <i>L. casei</i> subsp. <i>alactus</i> , while maintaining steady DC production of IL-10	Christensen <i>et al.</i> (2002)

Table 2.6: Examples of probiotic effect on adaptive immunity (continued)

Target	probiotic	Effect	Reference
	VSL #3	Increased expression of B7.1 (CD80), B7.2, CD40 and MHC class II molecules; suppressed T cell proliferation and increased IL-10 release in DCs	Drakes <i>et al.</i> (2004)
	VSL #3	Decreased LPS induced production of IL-12 Induced IL-10 release by DCs from blood and intestinal tissue, and inhibited generation of Th1 cells. Of all the probiotics in the VSL #3 preparation, bifidobacteria are the most potent IL-10 inducers. They are also more effective in decreasing surface expression of B7.1 (CD80) in DCs, and they inhibit T cell production of IFN- γ as well	Hart <i>et al.</i> (2004)
	<i>Lb. reuteri</i> and <i>Lb. casei</i>	Induced T cell differentiation into regulatory T cells by binding to the lectin the human monocyte-derived DCs. Regulatory T cells produce large amounts of IL-10.	Smits <i>et al.</i> (2005)
	VSL #3	Induced production of IL-10, and in particular, the generation of greater numbers of Treg cells expressing TGF- β at the surface of the cell membrane of the lamina propria.	Di Giacinto <i>et al.</i> (2005)
	<i>Lb. paracasei</i> NCC2461	Induce development of a CD4+ T cell subset characterised by a low proliferation potential but a marked ability to secrete IL-10 and TGF- β	von der Weid <i>et al.</i> (2001)
	<i>Lb. paracasei</i> NCC2461	Participated in the β -lactoglobulin (BLG) oral tolerance process in mice, attributed to the hydrolysis of BLG into peptides, which stimulate the production of IL-10	Prioult <i>et al.</i> (2003; 2004)
	<i>Lb. gasseri</i> , <i>Lb. johnsonii</i> and <i>Lb. reuteri</i>	Promote DCs to regulate T cell responses toward Th1 pathway by stimulation the secretion of high levels of IL-12 and IL-18, but not IL-10	Mohamadzadeh <i>et al.</i> (2005)
	<i>Lb. casei</i>	Induce high level of IL-12 via macrophages stimulation	Shida <i>et al.</i> (2006)
	<i>Bif. longum</i>	Stimulated colonic DCs in mice to produce IL-10 and IL-12	Rigby <i>et al.</i> (2005)

2.4.3 Prevention/treatment of infections

In spite of medical progress, infectious diseases remain a significant health problem. Many authors have recently shown that probiotics prevent and/or treat some intestinal and urogenital infections and so may be useful as alternatives to antibiotics that have given an increase in the incidence of microbial antibiotic resistance (Bengmark, 1998; Neu, 1992; Reid, 1996; Reid *et al.*, 1998; Zoppi, 1997).

It has been reported that some probiotic bacteria such as *Lb. paracasei* and *Lb. rhamnosus* and *Bif. animalis* subsp *lactis* Bb12 can prevent adhesion of pathogens like *E. coli*, *Listeria monocytogenes*, *Cl. difficile*, *Salmonella enterica* serovar Typhimurium and *Enterobacter sakazakii* *in vitro* (Collado *et al.*, 2008; Collado *et al.*, 2006; Drago *et al.*, 1997; Gibson & Wang, 1994).

Probiotic bacteria can prevent infections by mechanisms which include competition for nutrients, secretion of antimicrobial substances (bacteriocins, hydrogen peroxide, carbon dioxide and diacetyl), reduction of pH, blocking of adhesion sites, attenuation of virulence, blocking of toxin receptor sites, immune stimulation, and suppression of toxin production (Fooks *et al.*, 1999; Mishra & Lambert, 1996).

Diarrhoea can result from many possible causes including infection by a number of bacterial or viral agents, lactose maldigestion, antibiotic therapy, abdominal radiotherapy, surgery and in some cases the etiology is unknown (Gibson *et al.*, 1997).

Lb. rhamnosus GG, has been shown to be effective in the treatment of rotavirus diarrhoea and gastrointestinal disease caused by *Salmonella*, *Shigella* and *E. coli* in human trials (Guarino *et al.*, 1997; Isolauri *et al.*, 1991; Kaila *et al.*, 1995; Kaila *et al.*, 1992; Oberhelman *et al.*, 1999; Pant *et al.*, 1996; Shah, 2007). Other probiotic strains such as *Lb. casei* shirota, *Bif. infantis*, *Bif. breve* and *S. thermophilus* have also been shown to be effective in the prevention/treatment of diarrhoea in children (Saavedra *et al.*, 1994).

Antibiotic-associated diarrhoea (AAD) is also an important clinical problem associated with antibiotic therapy (McFarland, 2006). Pseudomembranous colitis is the most severe form of AAD. *Lb. rhamnosus* GG has been used successfully in the treatment of colitis (Biller *et al.*, 1995; Gorbach *et al.*, 1987). Oral administration of *Lb. rhamnosus* GG was also effective in the prevention of this type of diarrhoea, as were a number of other probiotics including *S. boulardii* (Armuzzi, Cremonini, Bartolozzi, *et al.*, 2001; Armuzzi,

Cremonini, Ojetti, *et al.*, 2001; Kotowska *et al.*, 2005; Mcfarland *et al.*, 1995), *Bif. longum* (Colombel *et al.*, 1987) and *E. faecium* SF68 (Wunderlich *et al.*, 1989).

Some travellers especially those going to and from developing countries may experience specific diarrhoea called Traveller's diarrhoea (Saxelin, 1997), and studies have shown that probiotics have efficacy in the prevention of this form of diarrhoea (Hilton *et al.*, 1997).

Probiotics have also shown a positive effect in the treatment of infections caused by *Helicobacter pylori* which is an important agent in peptic ulcer disease and has also been implicated in some forms of gastric cancer (Bayerdorffer & Morgner, 2000; Palli *et al.*, 2002; Uemura *et al.*, 2001). It has been reported that probiotics such as *Lb. acidophilus*, *Lb. reuteri*, *Lb. casei* shirota and some other LAB can inhibit this pathogen *in vitro* and *in vivo* (Bhatia *et al.*, 1989; Francavilla *et al.*, 2008; Midolo *et al.*, 1995; Sgouras *et al.*, 2004).

2.4.4 Amelioration of lactose maldigestion

Lactose maldigestion is prevalent worldwide and is caused by lactase (β -galactosidase) deficiency in the human gut. Lactase deficiency results in some abdominal discomforts including diarrhoea, bloating, abdominal pain and flatulence after the consumption of milk and some dairy products (Farnworth, 2001; Scrimshaw & Murray, 1988; Shaukat *et al.*, 2010; Wilt *et al.*, 2010). In lactose maldigestors, undigested lactose is fermented by bacteria in colon (Marteau *et al.*, 1997; Wilt *et al.*, 2010). One of the health benefits of probiotics is alleviation of lactose maldigestion symptoms (Guarner & Schaafsma, 1998; Huis in't Veld *et al.*, 1994; Ojetti *et al.*, 2010). It has been reported that oral supplementation with *Lb. reuteri* improved lactose maldigestion symptoms in lactose intolerant patients (Ojetti *et al.*, 2010). Some studies have reported that ingestion of *Lb. acidophilus* can ameliorate the symptoms of lactose maldigestion in humans (Kim & Gilliland, 1983; Mustapha *et al.*, 1997). Jiang *et al.* (1996) found that a *Bif. longum* strain might improve digestion of lactose. However, there are other studies have reported little or no effect of probiotics on lactose malabsorption (Hove *et al.*, 1994; Lin *et al.*, 1991; Saltzman *et al.*, 1999).

2.4.5 Management of allergy

The term “allergy” refers to hypersensitivity responses to particular antigens, known as allergens, resulting in harmful immunologic reactions on subsequent exposures. The incidence of allergy has increased gradually, especially in developed countries. The ‘hygiene hypothesis’ has been proposed to explain the rapid increase in allergy in recent decades. According to this theory, reduced microbial exposure early in life due to increasing sanitary living conditions, increasing consumption of antibiotics and increasing consumption of sterile processed foods has resulted in prevalence of allergic diseases (Bufford & Gern, 2005; Martinez & Holt, 1999; Strachan, 1989; von Mutius *et al.*, 1999).

Studies have shown that stimulation of the immune system by specific microbial preparations may prevent or treat allergic diseases (Feleszko *et al.*, 2006). Probiotic bacteria have been proposed to be effective in the treatment of allergic diseases (Kirjavainen *et al.*, 1999). The possible mechanisms of probiotic therapy include the normalisation of intestinal permeability and improving gut microecology, improvement of the intestine’s immunological barrier functions, especially through intestinal immunoglobulin A (IgA) responses, improvement of intestinal inflammatory responses, and balance control of proinflammatory and anti-inflammatory cytokines (Isolauri *et al.*, 2001). Figure 2.1 shows the treatment targets of probiotics in allergic disease.

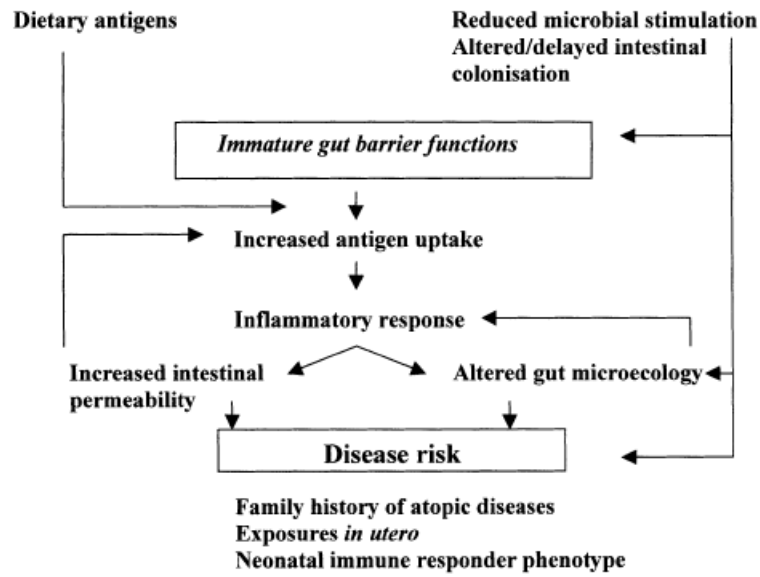


Figure 2.1: The treatment targets of probiotic functional foods in allergic disease (Adopted from (Isolauri, 2001))

Atopic eczema/dermatitis syndrome (AEDS) is a common allergic skin condition which results in dry, itchy, inflamed skin patches. It has been reported that oral administration of an extensively hydrolysed whey formula supplemented with *Bif. animalis* subsp *lactis* Bb12 or *Lb. rhamnosus* GG significantly alleviated the clinical symptoms of atopic dermatitis (Isolauri *et al.*, 2000).

Majamaa & Isolauri, (1997) reported that *Lb. rhamnosus* GG was effective in the treatment of AEDS due to allergy to cow's milk in infants. Another study showed that *Lb. rhamnosus* GG prevented incidence of early atopic eczema in infants at high risk (Kalliomaki *et al.*, 2001). Follow-up studies on the same children demonstrated the persistence of protective effect during the first 4 and 7 years of life (Kalliomaki *et al.*, 2003; Kalliomaki *et al.*, 2007).

Asthma is another common disease, which affects the respiratory system. It has been demonstrated that probiotic organisms have immunomodulatory and anti-inflammatory activity. Recent *in vivo* studies using murine models has shown that probiotics such as *Lb. rhamnosus* GG and *Lb. reuteri* could prevent experimental asthma development and reduced airway hyperresponsiveness in mice (Feleszko *et al.*, 2007; Forsythe *et al.*, 2007; Karimi *et al.*, 2009). However, there is currently insufficient clinical evidence supporting the claim that probiotics can be used effectively in the treatment/prevention of asthma (Feleszko *et al.*, 2006).

2.4.6 Prevention of cancer

It has been proposed that probiotics have anti-cancer effects. There are some potential mechanisms for anti-carcinogenic effect of prbiotics including:

1. Binding, blocking or deactivation of carcinogen/procarcinogen, thereby preventing the induction of DNA damage and genotoxic injury as an early event in the process of carcinogenesis
2. Decreasing levels of certain colonic bacterial enzymes (β -glucuronidase, nitroreductase, azoreductase and dehydroxylase) that produce carcinogens and co-carcinogens (including secondary bile acids) or convert procarcinogens to carcinogens through controlling the growth of fecal bacteria

3. Altering the intestinal bacterial activity and bile acid solubility by lowering the intestinal pH
4. Immuno-stimulating effect
5. Decreasing the colonic transit time, thereby removing faecal carcinogens more rapidly

(Allsopp & Rowland, 2009; Arvanitoyannis & Van Houwelingen-Koukaliasoglou, 2005; Commane *et al.*, 2005; McIntosh, 1996; Swennen *et al.*, 2006)

In an *in vitro* study *Lb. casei* and a blend of *Bif. longum* and *Lb. gasseri* significantly decreased mutagen induced chromosomes (Renner & Munzner, 1991). In another study, several probiotic strains (*Lb. gasseri*, *Lb. confusus*, *Bif. breve*, *Bif. longum*, and *Lb. acidophilus*) inhibited the induction of DNA damage in rat colon cells exposed to 2 carcinogens, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and 1,2-dimethylhydrazine (DMH) (Pool-Zobel *et al.*, 1996). In an *in vitro* study, (Weirich-Schwaiger *et al.*, 1995) it was observed that autolysates of *Lb. gasseri* promoted the repair of damaged DNA.

Some human trials have shown that oral consumption of probiotic strains such as *Lb. acidophilus*, *Lb. rhamnosus* GG and *Bifidobacterium* spp. generally reduced activity levels of glucuronidase and nitroreductase, but there are fewer reports on influence of probiotics on decreasing azoreductase levels (Ayebo *et al.*, 1980; Bouhnik *et al.*, 1996; Goldin & Gorbach, 1984; Goldin *et al.*, 1992; Goldin *et al.*, 1980; Ling *et al.*, 1994; Marteau *et al.*, 1990).

A study with colon cancer patients revealed that consumption of fermented milk containing *Lb. acidophilus* decreased two risk makers for colon cancer including soluble faecal bile acid levels and colonic bacterial enzymes (Lidbeck *et al.*, 1992).

McIntosh *et al.* (1999) studied the effect of oral administration of Lactic Acid Bacteria (LAB) on development of tumours in intestine of rats challenged with a carcinogen, DMH. They found that a commercial probiotic culture, *Lb. acidophilus* LAFTI® L10 was more effective than other LAB. Goldin *et al.* (1996) reported that incidence of intestinal tumors in the rats given a diet containing *Lb. rhamnosus* GG and challenged with DMH, was significantly fewer than rats who did not receive the probiotic culture.

Also, it has been shown that *Lb. casei* prevented the recurrence of superficial bladder cancer in humans (Aso & Akazan, 1992). The results of a study conducted by Tomita *et al.* (1994) indicated that *Lb. casei* treated rats with bladder cancer induced by *N*-butyl-*N* (4-hydroxybutyl) nitrosamine (BBN) had lower tumor volume than control group. It has been shown that consumption of *Lb. casei* Shirota reduced the risk of bladder cancer (Ohashi *et al.*, 2002) and prevented development of colorectal tumours (Ishikawa *et al.*, 2005).

It has been reported that several LAB can inhibit growth of microorganisms which can convert pro-carcinogenic substances to active carcinogens (Gilliland, 1990). Lankaputhra & Shah (1998) reported that live cells of *Lb. acidophilus* and strains of bifidobacteria inhibited or bound some mutagens and promutagens more effectively than killed bacterial cells. Oral administration of *Lb. acidophilus* lowered serum levels of dimethylamine and the carcinogen, nitrosodimethylamine, due to small bowel bacterial overgrowth (SBBO) in hemodialysis patients (Simenhoff *et al.*, 1996). Gourama & Bullerman (1997) found that *Lb. casei* subsp *Pseudopiantarum* inhibited biosynthesis of potential carcinogens, aflatoxins B1 and G1 by *Aspergillus flavus* subsp *parasiticus*.

Also a human trial showed that administration of a fermented dairy product containing *Lb. acidophilus* reduced mutagenic activity in the faeces and urine through absorption of cooked/fried food mutagens (Lidbeck *et al.*, 1992). Renner & Munzner (1991) reported that *Lb. casei* had an anti-mutagenic activity on nitrosated beef extract. Another study showed that administration of *Lb. casei* decreased urine mutagenicity due to consumption of fried ground beef (Hayatsu & Hayatsu, 1993). Hosoda *et al.* (1992) investigated the antimutagenic activity of several lactic acid bacteria including some probiotic strains of lactobacilli and bifidobacteria against MNNG using Ame's test. All cultures indicated antimutagenic effect on the mutagen and *Lb. acidophilus* showed the highest inhibition.

It has been proposed that cell wall fractions of *Bif. infantis* may modulate immune system through activation of immune cells such as neutrophils and macrophages in tumor-bearing animals (Sekine *et al.*, 1995; Sekine *et al.*, 1994).

Mizutani & Mitsuoka (1979, 1980) showed that *Bif. longum* and *Lb. acidophilus* suppressed liver tumorigenesis promoted by some enteric flora such as *E. coli*, *Enterococcus faecalis*, and *Clostridium paraputrificum*.

Biffi *et al.* (1997) reported that fermented milks containing *Bif. infantis*, *Bif. bifidum*, *Bif. animalis*, *Lb. acidophilus* and *Lb. paracasei* inhibit growth of the breast cancer cell line. *Bif. infantis* and *Lb. acidophilus* showed the highest inhibition among the strains.

In vitro and *in vivo* studies have demonstrated the anti-tumour effects of *Lb. casei* LC9018 (Naidu *et al.*, 1999).

Potential probiotic propionibacteria have also been shown to bind a variety of carcinogens including mycotoxins (El-Nezami *et al.*, 2002; Gratz *et al.*, 2005; Gratz *et al.*, 2004; Niderkorn *et al.*, 2006), cyanotoxins (Halttunen *et al.*, 2008), dietary lectins (Zarate & Chaia, 2009) and some heavy metals (Halttunen *et al.*, 2008; Ibrahim *et al.*, 2006). Antimutagenic properties of some dairy propionibacteria also have been reported (Vorobjeva *et al.*, 2001; Vorobjeva *et al.*, 1995). Moreover, it has been demonstrated that *P. freudenreichii* and *P. acidipropionici* induce apoptosis in colorectal carcinoma cells via production of short chain fatty acids including propionate and acetate (Jan, Belzacq, *et al.*, 2002).

2.5 Probiotic products

The many health benefits associated with probiotic bacteria as outlined above, have led to probiotics increasingly being incorporated into food products in order to develop “functional foods” which are defined as “foods claimed to have a positive effect on health” (Champagne *et al.*, 2005; Stanton *et al.*, 2001).

The use of probiotics as functional food ingredients has a long history in both human and animals (Crittenden, 1999). The first products were different types of yogurts but nowadays, a wide range of probiotic products is available in the market including pharmaceuticals, different kinds of dairy products, probiotic drinks, dried fruits, baby foods or confectioneries (Betoret *et al.*, 2003; Mattila-Sandholm *et al.*, 2002).

Probiotic products can be made in three ways:

- Fermented probiotic products: probiotic culture is inoculated into the food product and allowed to ferment the food and provide flavours and organoleptic changes to it.
- Non-fermented probiotic products: probiotics are added to the final product in suitable levels, with no opportunity for culture growth and fermentation.

- Dietary supplements: probiotic cultures are utilised as concentrated and dried cells in the form of powders, capsules, or tablets (Mattila-Sandholm *et al.*, 2002; O'Sullivan *et al.*, 1992; Svensson, 1999).

To be successful in the commercial market, probiotic products should be safe, have good organoleptic characteristics and maintain suitable counts of viable bacteria at the time of consumption (Farnworth, 2001; Mattila-Sandholm & Saarela, 2000).

Factors affecting the quality of the probiotic product include:

1. The ability of the probiotic product in delivering viable probiotic bacteria with desired health benefits at a suitable level to the consumer until the time of consumption
2. Strain selection regarding its reaction to the matrix/components of the targeted food
3. Sensory properties of the product
4. Packaging materials
5. Storage condition of the probiotic food

(Champagne *et al.*, 2005; Hamilton-Miller *et al.*, 1999; Hull *et al.*, 1992; Mattila-Sandholm *et al.*, 2002; Saxelin *et al.*, 1999; Stanton *et al.*, 1998).

In order for the beneficial health effects of probiotics to be realised, regular consumption of high levels of probiotic bacteria is necessary. It has been suggested that minimum cell counts of viable bacteria should be more than 10^6 CFU per gram or millilitre of the probiotic product (Agrawal, 2005; Tamime *et al.*, 1995). Saxelin *et al.* (1991; 1995) showed that the minimum dietary intake of *Lb. rhamnosus* GG (in either freeze-dried powder or gelatine capsules) needed for recovery in the faeces of human subjects was 10^{10} CFU/day. In another study Saxelin *et al.* (1993) reported that when *Lb. rhamnosus* GG was administered in the fermented milk and enterocoated tablets, the lowest dose required for faecal detection was 10^9 CFU/day which is 10-fold lower, showing the significance of the food carrying the culture. Consequently, defining a specific effective number of probiotic microorganisms depends on the type of strain and delivery system used (Champagne *et al.*, 2005; Gardiner *et al.*, 2002; Salminen & Playne, 2001).

2.5.1 Dairy products

During the past few decades, probiotic bacteria have been increasingly exploited in commercial dairy products such as fermented milk and yoghurt. Dairy products are considered to be desirable food systems for the delivery of probiotics to humans. The high buffering capacity of dairy foods protects the probiotic bacteria against high acid levels in the stomach and supports viability of these microorganisms (Salminen & Playne, 2001). In addition, health promoting effects of probiotics are added to the healthful properties (vitamins, minerals and protein) of dairy products and make a healthy functional food (Hekmat & Reid, 2006).

Some examples of the dairy products containing probiotics are: yoghurt, frozen yoghurt, fermented milk, cheese, ice cream, dessert, non-fermented milk with cultures added (Hekmat & McMahon, 1992; Lourens-Hattingh & Viljoen, 2001; Ross *et al.*, 2002).

2.5.1.1 Probiotic yoghurt

Yoghurt has been considered as a healthy product with various desirable effects for consumers. In recent years, the production and marketing of probiotic yoghurts and other fermented milk products has increased significantly throughout the world. Most of the probiotic dairy products in European market are different types of yoghurts (Young, 1998). The main probiotic bacteria incorporated into yoghurt belong to *Lb. acidophilus* and *Lb. casei* as well as *Bif. bifidum* group (Schillinger *et al.*, 2005). It is recommended that one or both of the conventional yoghurt starter cultures (*Lb. bulgaricus* and *Streptococcus thermophilus*) is used in order to manufacture a probiotic yoghurt with desirable flavour and texture (Marshall & Tamime, 1997). Hekmat & Reid (2006) evaluated the sensory characteristics (appearance, flavour, texture and overall quality) of yoghurt containing *Lb. reuteri* RC-14 and *Lb. rhamnosus* GR-1. They found that the probiotic yoghurt had acceptable sensory properties among consumers.

2.5.1.2 Probiotic ice cream

It has been shown that ice cream could be used as a suitable food vehicle for delivery of probiotics to human diet without any unfavourable effect on sensory properties of the final product (Akin, 2005; Davidson *et al.*, 2000; Hekmat & McMahon, 1992).

Hekmat & McMahon (1992) used *Lb. acidophilus* and *Bif. bifidum* to make a probiotic ice cream. They demonstrated that such an ice cream contains high levels of probiotic bacteria even after 17 weeks of frozen storage. In another study, Hagen & Narvhus (1999) produced a probiotic ice cream using four probiotic strains including *Lb. acidophilus*, *Lb. reuteri*, *Lb. rhamnosus* GG and *Bif. bifidum*. Their results indicated that viable counts of the mentioned probiotic bacteria remained above 10^6 CFU/g over 52 weeks of storage at -20°C . All the ice cream samples obtained high scores in the sensory evaluation. Christiansen *et al.* (1996) described a probiotic ice cream produced by adding up to 50% of commercial fermented milk containing *Lb. acidophilus* and *Bif. bifidum*. They concluded that viable numbers of the cultures were $0.5\text{--}1.0 \times 10^7$ CFU/ml after 16 weeks of frozen storage at -20°C . A study conducted by Haynes & Playne (2002) showed that it is feasible to incorporate commercial frozen concentrates of probiotics (*Lb. acidophilus*, *Lb. paracasei* subsp. *paracasei* and *Bif. lactis*) directly into a low-fat ice cream. The viable levels of probiotic organisms remained above 10^6 CFU/g after 52 weeks frozen storage at -25°C . In another trial, the viability of probiotic strains (*Bif. longum* and *Bif. lactis*) used for manufacturing a probiotic ice cream as well as sensory acceptance of the final product was evaluated during 15 weeks of frozen storage at -18°C . The results indicated high levels of viable counts ($>10^6$ CFU/g) and acceptable organoleptic properties (Favaro-Trindade *et al.*, 2006).

2.5.1.3 Probiotic cheese

Cheese has been considered as an effective food delivery system for probiotic cultures. Cheese has certain advantages over other fermented dairy products (such as fermented milk and yoghurt) as a carrier of probiotics including

- Higher pH
- More stable matrix

- Higher fat content
- Higher buffering capacity

These unique characteristics support the long-term survival of probiotic bacteria and protect them during passage through the GI tract (Gardiner *et al.*, 1998; Stanton *et al.*, 1998). The successful production of probiotic cheeses relies on probiotic organisms remaining viable during ripening and shelf-life without adversely affecting cheese flavour, texture, composition and other sensory properties (Corbo *et al.*, 2001; Gomes *et al.*, 1995; Ross *et al.*, 2002).

A number of studies have examined various types of cheese as carriers of probiotic organisms. In one study, *Bif. bifidum* was incorporated into Cheddar cheese. The viability of this strain remained at 2.0×10^7 CFU/g for up to six months with no adverse effect on the sensory characteristics (Dinakar & Mistry, 1994). Gomes *et al.* (1995) made a probiotic Gouda cheese using bifidobacteria in combination with *Lb. acidophilus* strain Ki. After nine weeks of ripening, cheese flavour was significantly affected by the bifidobacteria possibly because of acetic acid production. It has been reported that *Bif. bifidum*, *Bif. longum* and *Bif. infantis* incorporated into a traditional soft rindless Italian cheese (Crescenza cheese), survived at levels of 10^8 , 10^7 and 10^5 CFU/g respectively for to weeks after cheese making (Gobbetti *et al.*, 1998). Ghoddusi & Robinson (1996) indicated that *Bif. bifidum* remained at 10^6 CFU/g for up to 60 days ripening of white brined cheese. In another study, Roy *et al.* (1995) demonstrated that *Bif. longum* strains could be suitable in probiotic cheese-making, due to high viable cell counts in the presence of mesophilic starters and lactic acid bacteria. O'Riordan & Fitzgerald (1998) studied the survival of different bifidobacteria species (*Bif. longum*, *Bif. breve*, *Bif. catenulatum*, *Bif. bifidum*, *Bif. angulatum*, and *Bif. infantis*) in cottage cheese after two weeks storage at 4°C. Their results revealed that viability of bifidobacteria is strain dependant and *B. bifidum* showed the best survival. Kourkoutas *et al.* (2006) produced a probiotic cheese using immobilized *Lb. casei* on apple and pear pieces. They concluded that fruit pieces can support viability of the probiotic cells during 71 days of ripening at 4 to 6°C. Also, the probiotic cheese had acceptable sensory properties compared with commercial Feta cheese.

2.5.2 Probiotic fruit and vegetable juice

For several years, most of the probiotic products in the market have been in the form of fermented milk and dairy products. In recent years, probiotics have also been added to non-dairy-based products (Mattila-Sandholm *et al.*, 2002; Shah, 2001). It has been suggested that fruit juice is an ideal medium for carrying functional food ingredients such as probiotics. One reason is that the residence time in the stomach of fruit juice is short, so that the bacteria are not exposed for too long to the unfavourable acidic conditions of the stomach. Also, fruit juice is a good source of nutrients such as vitamins, minerals, dietary fibres and phytochemicals (*e.g.* polyphenols and carotenoids). Furthermore fruit juice considered as a healthy and refreshing product that is pleasing to a large percentage of the consumers. Development of dairy-free probiotic foods such as probiotic fruit juices may also suit consumers who have allergy to milk products, are lactose intolerant or have no desire to eat dairy foods. Due to the mentioned advantages, there is a growing popularity in the development of fruit juice based probiotic drinks (Luckow & Delahunty, 2004a, 2004b; Post, 2002; Tuorila & Cardello, 2002). Some examples of the most common commercial probiotic fruit drinks are presented in Table 2.7.

2.5.2.1 Fermented fruit/vegetable juice-based probiotics beverages

A number of studies have been done on fermented probiotic fruit or vegetable juice. Yoon *et al.* (2004) produced a tomato juice fermented by four probiotic cultures (*Lb. acidophilus* LA39, *Lb. plantarum* C3, *Lb. casei* A4, and *Lb. delbrueckii* D7) with viable numbers of the cultures ranged from 10^6 - 10^8 CFU/ml after one month of refrigeration at 4°C. Yoon *et al.* (2005) examined the suitability of red beets as a substrate for producing probiotic beet juice by the above four probiotic strains. The results showed that with the exception of *Lb. acidophilus*, the viability of all other cultures remained at levels greater than 10^6 CFU/mL after 4 weeks of refrigerated storage.

These authors conducted another study on fermented cabbage juice using the same probiotic strains with the exception of *Lb. acidophilus* LA39. They found that both *Lb. plantarum* and *Lb. delbrueckii* could survive in the product during four weeks storage at

4°C, whereas *Lb. casei* completely lost its viability in the cabbage juice with low pH and high acidity after 2 weeks of refrigerated storage (Yoon *et al.*, 2006).

2.5.2.2 Non-fermented fruit/vegetable juice-based probiotic drinks

The main steps in producing the probiotic fruit juice are: pasteurisation of the fruit juice, cooling down to < 6°C and adding the probiotic cultures (10^{10} - 10^{11} CFU per litre of beverage). The juice is then packed in suitable containers and stored at refrigeration temperature. The minimum number of viable probiotic bacteria according to relevant standards determines the shelf life of the product (Post, 2002).

A study by Luckow and Delahunty (2004a) on sensory influence of probiotic *Lb. rhamnosus* GG and prebiotic oligosaccharides on orange juice revealed that on average, consumers could recognize a sensory difference between probiotic orange juices and conventional ones and preferred the organoleptic characteristics of conventional juices. However, 11% of consumers preferred the sensory characteristics of probiotic juices. In another study, Luckow & Delahunty (2004b) evaluated consumer acceptance for the sensory characteristics (appearance, aroma, texture and flavour) of probiotic blackcurrant juices containing *Lb. plantarum* 299v. They concluded that age and gender are important factors in the acceptance of probiotic fruit juice. However, on an overall basis, the consumers did not prefer one juice over another but they preferred the appearance of the probiotic fruit juice significantly. Luckow *et al.* (2006) conducted further research to determine whether the following techniques improve the sensory acceptability of orange juice containing *Lb. paracasei* ssp. *paracasei*:

1. Addition of tropical fruit juices including pineapple, mango and passionfruit to mask “off-flavours” caused by probiotics
2. Repeated exposure of members of sensory panel to the probiotic fruit juice
3. Providing health information about the fruit juice ingredients as well as probiotic cultures

Their results revealed that these three strategies can positively affect the sensory quality of probiotic fruit juices.

Table 2.7: Examples of commercial fruit juice-based probiotic drinks

Producer	Country	Brand	Fruit juice Composition	Juice content %	Probiotic strain	Owner of the strain
Skånemejerier	Sweden	Pro Viva	Strawberry, Blackcurrant, Blueberry, Rosehip Exotic (blend of Banana, grape, Apricot, Lime and Lemon)	≈ 20	<i>Lb. plantarum</i> vv	Probi AB (Sweden)
Skånemejerier	Sweden	Pro Viva Active	Exotic (as mentioned above) enriched with vitamins, minerals and WPC	12	<i>Lb. plantarum</i> vv	Probi AB (Sweden)
Skånemejerier	The UK	SHOT	Raspberry, blackcurrant and grape	?	<i>Lb. plantarum</i> 299v	Probi AB (Sweden)
Valio	Finland/ Sweden	Gefilus/ Gfilac	Whey drink with Apricot and Peach juice	17	<i>Lb. rhamnosus</i> GG	Valio (Sweden/ Finland)
Valio	Finland/ Sweden	Gefilus/ Gfilac	Orange/ Peach juice + prebiotic + Vit. C	60	<i>Lb. rhamnosus</i> GG	Valio (Sweden/ Finland)
Valio	Finland/ Sweden	Gefilus/ Gfilac	Pineapple and Carrot + Ca ⁺⁺ + β-caroten	50 and 10	<i>Lb. rhamnosus</i> GG	Valio (Sweden/ Finland)
Valio	Finland/ Sweden	Gefilus/ Gfilac	Multi fruit (Orange, Grape, Peach, Mango and Passion fruit)	80	<i>Lb. rhamnosus</i> GG	Valio (Sweden/ Finland)
Valio	Finland/ Sweden	Gefilus/ Gfilac	Apple and grape	100	<i>Lb. rhamnosus</i> GG	Valio (Sweden/ Finland)
Tine BA	Norway	Biola	Orange-Mango	> 95	<i>Lb. rhamnosus</i> GG	Valio (Sweden/ Finland)
Tine BA	Norway	Biola	Apple-Pear	> 95	<i>Lb. rhamnosus</i> GG	Valio (Sweden/ Finland)
Ingman	Sweden/ Finland	Rela	Orange	?	<i>Lb. reuteri</i>	Biogaia Biologics (Sweden)
Ingman	Sweden/ Finland	“R”	Orange-Pineapple + Ca ⁺⁺	?	<i>Lb. reuteri</i>	Biogaia Biologics (Sweden)
Ingman	Sweden/ Finland	?	Multi fruit (?) + Ca ⁺⁺	?	<i>Lb. reuteri</i>	Biogaia Biologics (Sweden)
Pete & Johnney	The UK	“Its Alive”	Peach-Banana	?	<i>Bif. lactis</i>	Various
Arla Foods	Sweden	Cultura	Blueberry	?	?	?
Arla Foods	Sweden	Cultura	Rosehip	?	?	?
Suntary	Japan	Bikkle	Fruit (?) + whey mineral + Prebiotics + Dietary fibres	?	<i>Bifidobacterium</i> spp	?
A. Lassonde Inc.,	Canada	Oasis Health Break™	fruit juice concentrates (pineapple, apple, orange, pear and/or grape, passion fruit, lemon), purees (peach, strawberry, mango and kiwi),	?	<i>Lb. rhamnosus</i> R0011	Lallemand
Lidl	Germany	Pianola	Orange juice	?	<i>Lb. casei</i>	?
NextFoods	USA	GoodBelly	Pomegranate-Blackberry, Cranberry-Watermelon, Mango, Blueberry-Acai, Strawberry or Lemon Ginger	100	<i>Lb. plantarum</i> 299v	Probi AB (Sweden)
Kerry Group	Ireland	Dawn	Orange juice	100	<i>Bif. animalis</i> subsp <i>lactis</i> Bb-12	Chr Hansen (Denmark)
Danisco	Denmark	Howaru	?	?	<i>Bif. lactis</i> HN019	Danisco

Adapted from ([Anon], 2005; Post, 2002; Searby, 2005a; Stanton *et al.*, 2001)

2.5.3 Probiotic straw and cap

There are some new delivery methods for probiotic bacteria. BioGaia company has developed a new technology for delivery of probiotics namely probiotic straw and cap. The probiotic straw is a telescopic polypropene drinking straw containing a probiotic (*Lb. reuteri*) oil droplet inside. The minimum number of bacteria is 10^8 CFU/straw. When the consumer drinks through the straw, the probiotic bacteria are released. Each straw is wrapped individually in a thick polyester/aluminium/polyethylene sachet that reduces exposure of probiotic to oxygen and moisture and can be attached to the outside of drink container such as single-serve aseptic carton ([Anon], 2005; BioGaia, 2006; Caglar *et al.*, 2006; Searby, 2005b). Probiotic cap is another new vehicle for delivering probiotics to consumers. In this method, a protective blister is incorporated into the bottle cap and when the top is opened, the bacteria fall into the drink (BioGaia, 2006; Searby, 2005b).

The benefits of these products are as follows:

- Increasing shelf life of the probiotics for several months even at room temperature
- Suitable for any type of drink
- Simple and practical ways for supplementing human diet with probiotic bacteria ([Anon], 2005; BioGaia, 2006; Searby, 2005b)

A study on the effect of drinking water through a probiotic straw (Life top straw, Biogaia) containing *Lb. reuteri* on the levels of salivary mutans streptococci and lactobacilli was undertaken in young adults. The results indicated that daily ingestion of *Lb. reuteri* via straw significantly reduced mutans streptococci levels in saliva (Caglar *et al.*, 2006).

2.5.4 Others

Other products that have been evaluated for their potential as probiotic delivery systems include Soy products (Champagne *et al.*, 2005; Heenan *et al.*, 2004; Shimakawa *et al.*, 2003), table olive (Lavermicocca *et al.*, 2005), infant formula (Fukushima *et al.*, 1998; Langhendries *et al.*, 1995), confectioneries (Mattila-Sandholm *et al.*, 2002), cereal bars (Ouwehand *et al.*, 2004), dried fruits (Betoret *et al.*, 2003), edible table spreads (Charteris *et al.*, 2002), chocolate (Possemiers *et al.*, 2010) and chewing gum (Caglar *et al.*, 2007; Twetman *et al.*, 2009).

2.6 Probiotic survival in food matrixes

The factors that affect the viability of probiotics in a food matrix during processing and storage include pH, oxygen levels, temperature, and presence of competing microorganisms and inhibitors. Since the probiotic food should contain viable probiotic cultures at suitable levels at the time of consumption, using some techniques for improving stability of probiotic strains in food systems is of great importance (Champagne *et al.*, 2005; Dave & Shah, 1997; Mattila-Sandholm *et al.*, 2002). Some of these methods are as follows:

- Stress adaptation
- Microencapsulation
- Inclusion of prebiotics
- Modulation of packaging conditions

(Champagne *et al.*, 2005; Mattila-Sandholm *et al.*, 2002)

2.6.1 Stress adaptation

Probiotic organisms are exposed to various stressful conditions (heating, cooling, oxidative stress, low pH, osmotic conditions, bile salts, starvation, etc.) in their natural habitats and during industrial processes, storage and passage through gastro-intestinal tract (Jan *et al.*, 2000; Kosin & Rakshit, 2006; Sanders *et al.*, 1999). An alternative method for improving probiotic viability in such harsh conditions is applying sub-lethal stresses to the cells. Possible stress adaptation may enhance the resistance of the cultures to subsequent stressful conditions (Champagne *et al.*, 2005; Saarela *et al.*, 2004; van de Guchte *et al.*, 2002).

Park *et al.* (1995) reported that acid adaptation (at pH 5.2 for 2 hours) improved survival of the *Bif. breve* in different stressful conditions (pH 2-5, 0.2-1.0 % bile and H₂O₂ 100–1000 ppm). Results of a study conducted by Broadbent *et al.* (1997) revealed that heat shock pre-treatment (50°C), considerably enhanced the ability of exponential phase *Lb. acidophilus* to tolerate subsequent high temperature (63°C). In another study, it has been shown that log phase *Lb. acidophilus* subjected to acid stress (pH 3.8-6.0) is capable of withstanding lower pH values (Lorca *et al.*, 1998). Schmidt & Zink (2000) reported the presence of a heat shock gene for some *Bifidobacterium* spp. (*Bif. longum* strains NCC481, NCC490 and NCC585, *Bif. adolescentis* NCC251, and *Bif. breve*

NCC298.). However it was induced on the transcriptional level only in *Bif. longum* NCC481 and *Bif. adolescentis* NCC251 by rising temperatures. They observed that log phase of *Bif. adolescentis* exposed to a sub-lethal heat stress (45°C and 47°C) or sub-lethal salt stress (1.5 and 2.0% NaCl) showed a considerably risen resistance to lethal temperature of 55°C. Furthermore, pre-treatment of the mentioned strain with 0.1% bile salts led to a noticeable protection against higher bile salts concentrations (0.3% and 0.4%). Lorca & de Valdez (2001) found that *Lb. acidophilus* grown in uncontrolled pH fermentation (final pH 4.5) showed more resistance to acid stress as well as other different stress conditions (including ethanol, hydrogen peroxide, freezing and freeze drying) than the cells grown in controlled pH conditions (pH 6.0). Desmond *et al.* (2002) demonstrated that exposure of probiotic *Lb. paracasei* to sub-lethal temperature (52°C for 15 min.) resulted in 300 and 700 fold protection against lethal temperature of 60°C in MRS medium and skim milk respectively. Sub-lethally heat treated and salt adapted *Lb. paracasei* showed 18 and 16 fold greater survival respectively during spray drying at outlet high temperature (95-105°C) compared to non-treated cells. It has been reported that pre-treatment of *Lb. rhamnosus* with heat (50°C) or salt (0.6 M NaCl) resulted in a marked viability improvement of powdered form of the strain during storage at 30°C (Prasad *et al.*, 2003). Saarela *et al.* (2004) examined the viability improvement of *Lactobacillus* and *Bifidobacterium* strains sub-lethally treated with acid and heat (pH 3.0-4.0 and 47°C for 30 min – 1 h) in subsequent lethal conditions (pH 2.5, 1.5 % bile and 55°C for 1-3 h). They found that stress adaptation enhanced the viability of lactobacillus strains more than that of bifidobacteria at both laboratory and fermentor scale.

2.6.2 Microencapsulation

Microencapsulation is defined as “a technology for packaging solids, liquids, or gaseous materials in miniature, sealed capsules that can release their contents at controlled rates under specific conditions” (Shahidi & Han, 1993). It is a process by which an inner unstable matrix is coated by suitable shell materials for greater stability and protection from the surrounding unfavourable environment (low pH & dissolved oxygen) (Kailasapathy, 2002). Microencapsulation of probiotic organisms is of interest to the probiotic food industry as the best method to maintain the potency of probiotic

microorganisms to be delivered into the gastrointestinal tract (Siuta-Cruce & Goulet, 2001).

Main reasons for using this method for protection of probiotics are as follows:

- Improving viability and stability of probiotic cultures during production, storage and passage through the gastrointestinal tract (Kailasapathy, 2002; Krasaekoopt *et al.*, 2003; Sultana *et al.*, 2000)
- Providing a controlled and efficient release of probiotic bacteria in GIT (Crittenden *et al.*, 2006; Kailasapathy, 2002)
- Easier handling of the cultures (Picot & Lacroix, 2003b)
- Limited effects on sensory properties of the product containing microcapsules (Picot & Lacroix, 2003b)

There are several microencapsulation methods for probiotics including spray drying, freeze drying, fluidised bed drying, extrusion, emulsion, coacervation, phase separation (Kailasapathy, 2002). However, two widely used encapsulation techniques are extrusion and emulsion (Krasaekoopt *et al.*, 2003). A variety of encapsulant materials have been used for the microencapsulation of probiotics including alginate, starch, alginate-starch, cellulose acetate phthalate, κ -carrageenan, κ -carrageenan/locust bean gum, gelatine, xanthan-gellan, chitosan and whey protein (Doleyres & Lacroix, 2005; Krasaekoopt *et al.*, 2003).

2.6.2.1 Methods of microencapsulation

2.6.2.1.1 Spray drying

In this method the core material is dispersed into a polymer solution in order to form an emulsion or dispersion. After homogenisation of the liquid, it is atomised and imploded into the drying chamber. The heat from this chamber evaporates the solvent or aqueous media to form fine particles carrying the microcapsules (Jackson & Lee, 1991). This method is easily scaled up and low cost. Furthermore, it uses apparatus available in the food industry and can be operated continuously (Gibbs *et al.*, 1999; Kailasapathy, 2002). By contrast, cell damage due to relatively high temperature used in this process is considered a disadvantage (Kailasapathy, 2002).

2.6.2.1.2 Extrusion

Extrusion involves preparing a probiotic cell suspension by adding probiotic microorganisms to a suitable hydrocolloid solution (most commonly sodium alginate), then extruding the suspension through a syringe needle or nozzle to form droplets that fall into a hardening solution (calcium chloride) (Figure 2.2). This leads to the formation of beads or microcapsules which their size, shape and sphericity is controlled by the diameter of the needle, the viscosity of the hydrocolloid solution and the distance between of the needle and the hardening solution (Krasaekoopt *et al.*, 2003; Smidsrod & Skjakbraek, 1990). Extrusion is the most common technique to produce microencapsules with hydrocolloids because it is simple, economical and biocompatible. Besides, it provides a gentle condition that ensures high cell viability. However this technique is difficult to scale up due to low rate of bead formation (Krasaekoopt *et al.*, 2003).

2.6.2.1.3 Emulsion

This technique involves adding probiotic cells to a hydrocolloid solution to form a cell-polymer suspension (aqueous or discontinuous phase), then adding the suspension to an edible vegetable oil (organic or continuous phase) followed by homogenisation of the mixture to produce a water-in-oil emulsion. In this step, emulsifiers such as Tween 80 may be added to form smaller microcapsules. Then, water-soluble hydrocolloid must be in-solubilised by cooling, cross-linking, or a chemical reaction to form microcapsules within the oil phase (Figure 2.2). The beads are then collected by filtration. The size of the microcapsules depends on the agitation speed and emulsifier type. The positive features of this method are producing smaller beads (25 µm to 2 mm) compared with those formed by extrusion technique (2-5 mm) and being easy to scale up. The disadvantages are its high operating cost and residual oil in beads that is not desirable for low fat product formulations (Kailasapathy, 2002; Krasaekoopt *et al.*, 2003). Some studies on encapsulation of probiotic microorganisms by different methods have been summarised in Table 2.8.

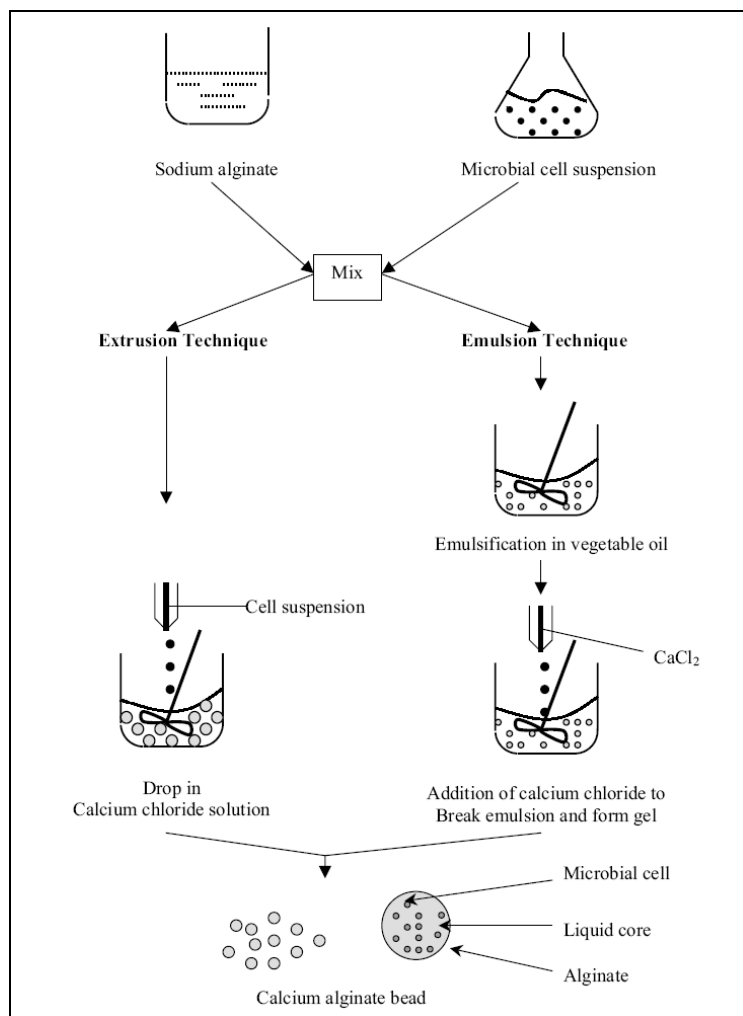


Figure 2.2: Flow diagram of encapsulation of bacteria by the extrusion and emulsion techniques. Adapted from Krasaekoopt *et al.*, (2003)

Table 2.8: Encapsulation of probiotic microorganisms by different methods

Microorganism	Method	Support material	Application	Reference
<i>Bif. infantis</i>	Emulsion/spray drying	Caseinate and prebiotic FOS (RaftiloseP95) plus either dried glucose syrup (DGS) or microfluidized resistant starch	-	(Crittenden <i>et al.</i> , 2006)
<i>Lb. reuteri</i>	Emulsion or extrusion	Alginate	Dry fermented sausage	(Muthukumarasamy & Holley, 2006)
<i>Lb. reuteri</i>	Phase separation (emulsion) or extrusion	alginate, alginate plus starch, κ -carrageenan with locust bean gum, or xanthan with gellan	-	(Muthukumarasamy <i>et al.</i> , 2006)
<i>Lb. acidophilus</i> and <i>Bif. lactis</i>	Emulsion	Calcium-induced alginate-starch	Yoghurt	(Kailasapathy, 2006)
<i>Lb. acidophilus</i> , <i>Bif. bifidum</i> and <i>Lb. casei</i>	Extrusion	Chitosan coated alginate beads	Stirred yoghurt	(Krasaekoopt <i>et al.</i> , 2006)
<i>Lb. acidophilus</i>	Extrusion	alginate plus prebiotics (Hi-maize starch, Raftiline and Raftilose) coated with different coating materials (chitosan, poly-L-lysine, and Alginate)	Yoghurt	(Iyer & Kailasapathy, 2005)
<i>Bif. longum</i> and <i>Bif. infantis</i>	Spray drying	Gelatin, starch, skim milk and Arabic gum	-	(Hsiao <i>et al.</i> , 2004)
<i>Bif. longum</i>	Spray drying	Whey protein	yoghurt	(Picot & Lacroix, 2004)
<i>Lb. acidophilus</i>	Extrusion	Alginate	-	(Chandramouli <i>et al.</i> , 2004)
<i>Lb. casei</i>	Emulsion (Microporous glass (MPG) membrane emulsification)	Alginate	-	(Song <i>et al.</i> , 2003)
<i>Bif. longum</i> and <i>Bif. infantis</i>	Spray drying	Gelatin, starch, skim milk and Arabic gum	-	(Lian <i>et al.</i> , 2003)

Table 2.8: Encapsulation of probiotic microorganisms by different methods (continued)

Microorganism	Method	Support material	Application	Reference
<i>Bif. longum</i>	Gel beads/emulsion	κ -carrageenan	Stirred yoghurt	(Adhikari <i>et al.</i> , 2003)
<i>Lb. acidophilus</i> and <i>Bif. infantis</i>	Gel beads/emulsion	Alginate/starch	Ice cream	(Godward & Kailasapathy, 2003b)
<i>Lb. acidophilus</i> and <i>Bif. infantis</i>	Gel beads/emulsion	Alginate/starch	Yoghurt	(Godward & Kailasapathy, 2003c)
<i>Lb. acidophilus</i> and <i>Bif. infantis</i>	Gel beads/emulsion	Alginate/starch	Cheddar cheese	(Godward & Kailasapathy, 2003a)
<i>Bif. breve</i> , <i>Bif. longum</i> and <i>Lb. acidophilus</i>	Emulsion/spray drying	Milk fat/whey protein	-	(Picot & Lacroix, 2003a)
<i>Bif. lactis</i> and <i>Lb. acidophilus</i>	Gel beads/extrusion	Alginate/starch	Yoghurt	(Talwalkar & Kailasapathy, 2003)
<i>Lb. acidophilus</i> and <i>Bif. lactis</i>	Spray drying	Cellulose acetate phthalate	-	(Favaro-Trindale & Grosso, 2002)
<i>Bifidobacteria species</i>	Freeze drying	starch	-	(Mattila-Sandholm <i>et al.</i> , 2002)
<i>Bif. longum</i> , <i>Bif. bifidum</i> , <i>Bif. infantis</i> , <i>Bif. breve</i> and <i>Bif. adolescentis</i>	Gel beads/emulsion	Alginate	Milk	(Hansen <i>et al.</i> , 2002)
<i>Bifidobacterium</i> PL1	Spray drying	Modified waxy maize starch	-	(O'Riordan <i>et al.</i> , 2001)
<i>Lb. casei</i>	Spray drying	Alginate Alginate/chitosan	-	(Koo <i>et al.</i> , 2001)
<i>Bif. longum</i>	Gel beads/emulsion	κ -carrageenan	Set yoghurt	(Adhikari <i>et al.</i> , 2000)
<i>Bif. lactis</i> and <i>Lb. acidophilus</i>	Gel beads/extrusion	Alginate	-	(Trindade & Grosso, 2000)
<i>Bif. longum</i>	Gel beads/extrusion	Alginate	-	(Koenen <i>et al.</i> , 2004)
<i>Lb. acidophilus</i> and <i>Bifidobacterium spp.</i>	Freeze drying	Alginate	Frozen fermented dairy dessert	(Shah & Ravula, 2000)
<i>Lb. acidophilus</i> and <i>Bif. infantis</i>	Gel beads/emulsion	Alginate/starch	Yoghurt	(Sultana <i>et al.</i> , 2000)
<i>Bif. infantis</i>	Gel beads/extrusion	Gellan/xanthan	Yoghurt	(Yamazaki <i>et al.</i> , 2000)
<i>Bif. bifidum</i> and <i>B. infantis</i>	Emulsion	Alginate or κ -carrageenan	Ice milk	(Kebary <i>et al.</i> , 1998)
<i>Bif. bifidum</i> and <i>B. infantis</i>	Emulsion	Alginate	Mayonnaise	(Khalil & Mansour, 1998)

Table 2.8: Encapsulation of probiotic microorganisms by different methods (continued)

Microorganism	Method	Support material	Application	Reference
<i>Bif. longum</i>	Gel beads/emulsion	κ -carrageenan/locust bean gum	-	(Maitrot <i>et al.</i> , 1997)
<i>Lb. acidophilus</i>	Extrusion	Alginate	Biomass production	(Jankowski <i>et al.</i> , 1997)
<i>Bif. bifidum</i>	Freeze drying	κ -carrageenan	Cheddar cheese	(Dinakar & Mistry, 1994)
<i>Lb. casei ssp casei</i>	Emulsion	κ -carrageenan and locust bean gum	Biomass production	(Arnaud <i>et al.</i> , 1992)
<i>Lb. casei</i>	Emulsion	κ -carrageenan and locust bean gum	Yoghurt	(Lacroix <i>et al.</i> , 1990)
<i>Bif. pseudolongum</i>	Emulsion	Cellulose acetate phthalate	—	(Rao <i>et al.</i> , 1989)

2.6.3 Prebiotics

"Prebiotics are nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health" (Gibson, 1999; Gibson & Roberfroid, 1995).

Some possible beneficial health effects of prebiotics are as follows:

- Modulation of the colonic microflora
- Enhancing resistance to pathogens
- Reducing the risk of colon cancer, heart disease, obesity, diabetes and digestive tract disorders (Lovegrove & Jackson, 2004b)
- Enhancing mineral bioavailability and adsorption
- Lipid modulation

(Delzenne & Kok, 2001; Delzenne & Kok, 1999; Gibson, 1999; Gibson & Roberfroid, 1995; Levrat *et al.*, 1994; Lovegrove & Jackson, 2004b; Naidu *et al.*, 1999; Roberfroid, 1998; Slavin, 1999)

Although any undigested food ingredient (like nondigestible carbohydrates, certain lipids as well as some peptides and proteins) that is selectively fermented by the beneficial bacteria of the gut may be a prebiotic candidate, nondigestible carbohydrates, are the most studied (Gibson, 1999). Prebiotics comprise disaccharides (such as lactulose and lactitol), oligosaccharides [such as fructooligosaccharides (FOSs) and transgalactooligosaccharides (TOSs)], soybean oligosaccharides (mainly trisaccharide raffinose and the tetrasaccharide stachyose), lactosucrose, xylooligosaccharides and polysaccharides (such as resistant starch) (Boehm *et al.*, 2004; Crittenden, 1999; Drakoularakou *et al.*, 2004). The most intensive studies have focused on FOSs and TOSs. FOSs such as inulin (*e.g.* Raftiline HP) and oligofructose (like Raftilose P95) are β -linked fructose monomers and can be found in plants (*e.g.* barley, wheat, asparagus, garlic, leek, onion, artichoke, chicory roots, banana, etc.). TOSs are β -linked galactose units synthesized from lactose via enzymatic transgalactosylation using β -galactosidase and are found in fermented products like yoghurts, as the result of bacterial activity on milk sugars (Boehm *et al.*, 2004; Houdijk *et al.*, 2002). Fructooligosaccharides are not degraded or absorbed in the stomach or in the small intestine and reach the colon (largely intact) where they are fermented by the gut bacteria (specially bifidobacteria and lactobacilli), to short-chain fatty acids (SCFA) (mainly acetate) and other

metabolites (*e.g.*, lactate) (Bielecka *et al.*, 2002; Gibson, 1999; Gibson & Roberfroid, 1995; Roberfroid, 1996).

It has been suggested by many studies that consumption of prebiotics combined with probiotic bacteria as synbiotics may enhance the beneficial effect of each of them (Gmeiner *et al.*, 2000; Rowland *et al.*, 1998; Schaafsma *et al.*, 1998).

A synbiotic has been defined as "a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare" (Gibson & Roberfroid, 1995). It has been shown that consumption of synbiotic products can improve:

- The survival of probiotics during storage
- Shelf life of the product
- The number of viable bacteria passing through the GIT
- The growth and implantation of the live bacteria (both exogenous and endogenous) in the colon

(Gibson & Roberfroid, 1995; Mattila-Sandholm *et al.*, 2002; Shin *et al.*, 2000)

Alander *et al.* (2001) studied the effect of galacto-oligosaccharides (GOS)-containing syrup (60% GOS), *Bif. animalis* subsp *lactis* Bb12 or GOS-containing syrup together with *Bif. animalis* subsp *lactis* Bb12 on the human faecal flora (bifidobacteria, *Cl. perfringens*, lactic acid bacteria and coliforms). They found that consumption of GOS + Bb-12 significantly increased faecal bifidobacterial numbers compared with other treatments but it had no significant effect on the numbers of *Cl. perfringens* and other faecal bacteria. Also, their results showed that GOS-containing syrup did not improve the survival of *Bif. animalis* subsp *lactis* Bb12 in the GIT. Shin *et al.* (2000) reported that addition of (5%) FOS, GOS or inulin to skim milk improved the viability of two commercial *Bifidobacterium* strains after 4 weeks of cold storage. FOS and inulin were the most and the least effective in retaining viability of both strains respectively. In another study prebiotic FOS significantly increased the viability of *Bif. animalis* and *Bif. longum* in yoghurt compared with yoghurt with no prebiotic added (Akalın *et al.*, 2004).

2.7 Probiotic combinations

Probiotic organisms vary in the type and level of their health promoting effects. Therefore, using combinations or cocktails of probiotics may be an appropriate strategy to confer a broad range of beneficial health effects on the host. On the other hand, the effectiveness of probiotic combinations may be different from comprised strains when used separately. *In vitro* studies and *in vivo* animal and human based trails have exhibited that probiotic combinations may be more effective in terms of probiotic beneficial effects compared to their component single strains (Tables 2.9 and 2.10).

Effective design of functional foods containing probiotic combinations, must of course take into consideration the likely occurrence and impact of potentially synergistic or antagonistic interaction between individual strains within a proposed combination as well as their interactions with carrier matrices during storage on their functional performance.

Table 2.9: Summary of *in vitro* works on the beneficial effect of probiotic combinations compared with single strains

Probiotic combination	Probiotic strain(s)	single	Target	Effective preparation	Reference
Mixtures of 2, 3 and 4 strains of <i>Lb. rhamnosus</i> GG, <i>Lb. rhamnosus</i> , <i>P. freudenreichii</i> , <i>Bif. breve</i>	<i>Lb. rhamnosus</i> GG, <i>Lb. rhamnosus</i> , <i>P. freudenreichii</i> or <i>Bif. breve</i>		Preventing adhesion of pathogens	combination	Collado <i>et al.</i> (2008; 2006)
Mixtures of 2, 3 and 4 strains of <i>Lb. rhamnosus</i> GG, <i>Lb. rhamnosus</i> , <i>P. freudenreichii</i> , <i>Bif. breve</i>	<i>Lb. rhamnosus</i> GG, <i>Lb. rhamnosus</i> , <i>P. freudenreichii</i> or <i>Bif. breve</i>		Mucus-binding of probiotics	combination	Collado <i>et al.</i> (2007)
Mixture of <i>Lb. rhamnosus</i> GG, <i>Lb. rhamnosus</i> , <i>P. freudenreichii</i> , <i>Bif. breve</i>	<i>Lb. rhamnosus</i> GG, <i>Lb. rhamnosus</i> , <i>P. freudenreichii</i> or <i>Bif. breve</i>		Protection against barrier function decline, increase in IL-8 secretion	ND	Myllyluoma <i>et al.</i> (2008)
Mixture of strains <i>Lb. rhamnosus</i> GG, <i>Bif. animalis</i>	<i>Lb. rhamnosus</i> GG, <i>Bif. animalis</i>		Inflammatory response to Enterotoxigenic <i>E. coli</i>	ND	Roselli <i>et al.</i> (2006)
Paired combinations of <i>Bif. lactis</i> Bb12 and one of the lactobacilli including <i>Lb. rhamnosus</i> GG, <i>Lb. delbrueckii</i> subsp <i>bulgaricus</i> , <i>Lb. acidophilus</i> , <i>Lb. johnsonii</i>	<i>Bif. lactis</i> , <i>Lb. rhamnosus</i> GG, <i>Lb. delbrueckii</i> subsp <i>bulgaricus</i> , <i>Lb. acidophilus</i> , <i>Lb. johnsonii</i>		Mucus-binding of probiotics	Enhanced adhesion of <i>Bif. lactis</i> Bb12 in combination with <i>rhamnosus</i> GG and <i>Lb. delbrueckii</i> subsp <i>bulgaricus</i> , ND for all other cases	Ouwehand <i>et al.</i> (2000)
Mixture of strains <i>Lb. casei</i> , <i>Lb. acidophilus</i>	<i>Lb. casei</i> , <i>Lb. acidophilus</i>		Pathogen (<i>Shigella sonnei</i>) growth inhibition	combination	Apella <i>et al.</i> (1992)
Mixture of strains <i>Lb. paracasei</i> B21060 and B21070, <i>Lb. acidophilus</i> B21190	<i>Lb. paracasei</i> B21060 and B21070, <i>Lb. acidophilus</i> B21190		Pathogen (<i>E. coli</i> and <i>S. enteritidis</i>) growth inhibition	combination	Drago <i>et al.</i> (1997)
“Ecologic 641” a mixture of strains <i>Lb. acidophilus</i> W70, <i>Lb. casei</i> , <i>Lb. salivarius</i> , <i>Lc. lactis</i> , <i>Bif. bifidum</i> , <i>Bif. infantis</i>	<i>Lb. acidophilus</i> W70, <i>Lb. casei</i> , <i>Lb. salivarius</i> , <i>Lc. lactis</i> , <i>Bif. bifidum</i> , <i>Bif. infantis</i>		Growth inhibition of 13 pathogen strains isolated from pancreatic necrosis	combination	Ridwan <i>et al.</i> (2008)

ND: No Difference between combination and single strains

Table 2.10: Summary of *in vivo* works on the effect of probiotic combinations in the management of disorders/health problems compared with single strains

Probiotic combination	Probiotic single strain(s)	disorder	Effective preparation	Reference
<i>Lb. rhamnosus</i> GG, <i>Bif. breve</i> , <i>Lb. rhamnosus</i> and <i>P. freudenreichii</i>	<i>Lb. rhamnosus</i> GG	Children atopic dermatitis	ND	Viljanen <i>et al.</i> (2005)
<i>Sac. boulardii</i> , <i>Lb. acidophilus</i> , <i>Lb. rhamnosus</i> , <i>Bif. longum</i>	<i>S. boulardii</i>	Rotavirus-associated diarrhoea in children	Significant reduction in duration of diarrhoea and fever for single strain but decreased duration of vomiting in combination group	Grandy <i>et al.</i> (2010)
<i>Lb. casei</i> and <i>Lb. acidophilus</i>	<i>Lb. casei</i> or <i>Lb. acidophilus</i>	Bacterial infection in mice	Combination	Perdigon <i>et al.</i> (1990)
- Mixture of <i>Lb. acidophilus</i> and <i>Enterococcus faecium</i>	<i>Lb. acidophilus</i> or <i>E. faecium</i>	Bacterial infection in lambs	Combination	Lema <i>et al.</i> (2001)
- Mixture of <i>Lb. cidophilus</i> , <i>S. faecium</i> , <i>Lb. casei</i> , <i>Lb. fermentum</i> and <i>Lb. plantarum</i>				
Mixture of <i>Bif. bifidum</i> , <i>Bif. infantis</i> , <i>Bif. longum</i> , <i>Lb. casei</i> , <i>Lb. acidophilus</i> , <i>Lb. salivarius</i> , <i>Lb. brevis</i> , <i>Lb. plantarum</i> , <i>Lb. helveticus</i> , <i>Lb. rhamnosus</i> , <i>S. thermophilus</i> , <i>E. faecium</i>	<i>Lb. casei</i> or <i>Lb. rhamnosus</i>	paediatric GI infections	Combination	Lin <i>et al.</i> (2009)

ND: No Difference between combination and single strains

2.8 Effect of fruit juice as carrier matrix on probiotic survival and functional performances

2.8.1 Survival

As stated earlier (p 33) it has been suggested that the minimum level of viable bacteria should be 10^6 CFU per gram or millilitre of the probiotic product or 10^8 CFU per day at the consumption point (Agrawal, 2005; Tamime *et al.*, 1995). It is therefore important that viability and activity of the probiotic remains optimal throughout the anticipated shelf life of the products (Mattila-Sandholm *et al.*, 2002). Previous research has shown that survivability of probiotics in fruit juice/drink is genus, species and strain dependent (Champagne & Gardner, 2008; Sheehan *et al.*, 2007). Furthermore, the type of fruit juice, intrinsic parameters such as pH and the presence of particular compounds (*e.g.* benzoic acid or lactones), as well as extrinsic factors such as storage temperature, storage duration, packaging material, and dissolved oxygen level, have all been considered as decisive factors in determining the survivability of probiotics in fruit juice (Champagne & Gardner, 2008; Champagne *et al.*, 2008; Sheehan *et al.*, 2007).

The viability of single strains of probiotics has been studied in fruit juices and fruit drinks (Champagne & Gardner, 2008; Champagne *et al.*, 2008; Sheehan *et al.*, 2007). Higher viability of 5 strains of *Lactobacillus* and one *Bifidobacterium* was reported in orange juice (pH 3.65) and pineapple juice (pH 3.40) than in cranberry juice (pH 2.50). Loss of viability of the probiotics occurred more slowly in cranberry juices with higher adjusted pH (pH 4.50 and 5.50) than lower pH values (pH 2.50 and 3.50). Moreover, different probiotic strains showed different survival rates in the same fruit juice over the storage time (Sheehan *et al.*, 2007). In another study, storage stability of 9 *Lactobacillus* strains (*Lb. acidophilus* LB2, LB3 and LB45, *Lb. brevis* LB6, *Lb. rhamnosus* LB11 and LB24, *Lb. fermentum* LB32, *Lb. plantarum* LB42 and *Lb. reuteri* LB38) was investigated in a commercial fruit drink (pH 4.2) containing a mixture of fruit juice concentrates, purees and dairy ingredients over a period of 80 days at 4°C. Viability of *Lb. rhamnosus* LB11 and LB24, *Lb. reuteri* LB38, *Lb. plantarum* LB42 and *Lb. acidophilus* LB45 was maintained throughout the entire storage period in the drink, reducing by less than one order of magnitude across the 80 days of storage, whereas viability of *Lb. acidophilus* LB2, LB3 declined more than 5 logarithmic cycles over the same period (Champagne & Gardner, 2008).

2.8.2 Acid and bile tolerance

To be effective in exerting their health promoting benefits for the host, probiotic microorganisms must adequately survive harsh environmental conditions encountered during gastro-intestinal passage, and then persist in the intestine (Saarela *et al.*, 2000). It is therefore necessary that a potential probiotic be examined for its gastric transit tolerance and intestinal persistence (Saarela *et al.*, 2000).

The strong acidic environment of the stomach as well as the proteolytic activity of pepsin act as a natural, highly protective barrier against harmful microorganisms ingested through the consumption of food and drink. Exposure to hostile conditions of stomach also can result in viability losses of probiotics ingested (Muller *et al.*, 2009). While the normal internal pH of the human stomach ranges from 2.5 to 3.5 (Holzapfel *et al.*, 1998), this value can vary depending on the nature and composition of food and drinks ingested. Another important factor is the residence time of food entering the stomach, which depends largely on its physico-chemical properties. For example liquids, which pass through the stomach more rapidly than solids (Rogers, 2011), may take less than 20 minutes to leave the stomach while a mixed meal can remain in the stomach up to 4 hours (GastroNetAustralia, 2010).

Subsequently probiotics confront with bile salts and pancreatin in the intestine which are further challenges to the viability of probiotics (Muller *et al.*, 2009). Primary role of bile in digestion is the emulsification and solubilisation of lipids. This property is mediated through the amphipathic nature of bile salts. In fact, bile salts act as a detergent, lowering the surface tension of dietary fats and breaking them down into tiny droplets, thus increasing the surface area for lipase activity. In the same way, bile salts may lethally damage bacteria via interaction with membrane lipids (Begley *et al.*, 2005; Begley *et al.*, 2006).

Moreover, it has been shown that the food matrix can influence the ability of probiotics to survive the gastro-intestinal environment, and that incorporation into carrier matrices such as milk, fermented milk, cheese, soymilk and meat may enhance the ability of probiotic bacteria to survive gastrointestinal passage (Ganzle *et al.*, 1999; Huang & Adams, 2004; Leverrier *et al.*, 2005; Saarela *et al.*, 2006; Stanton *et al.*, 1998; Zarate, Perez Chaia, *et al.*, 2000). It has also been speculated that due to the short gastro-intestinal transit time of fruit juices, inclusion in such carriers may reduce exposure of

probiotics to the harsh GI environment, and thereby enhance their effectiveness (Post, 2002).

Saarela *et al.* (2006) reported that the acid and bile tolerance of freeze-dried *Bif. animalis* subsp *lactis* E-2010 (Bb12) included in milk was significantly higher than that in a commercial fruit drink (pH 3.7, a blend of orange, grape and passion fruit). When compared to PBS, Bb12 included in the fruit drink was found to be significantly more tolerant to simulated gastric juice at pH 2.5 in the absence of pepsin, but significantly less tolerant at pH 3.0 with pepsin included.

Champagne and Gardner (2008) showed that 35 day refrigerated storage of *Lb. acidophilus* LB3, *Lb. rhamnosus* LB11, *Lb. reuteri* LB38 and *Lb. plantarum* LB42 included separately in commercial fruit beverages (a blend of 10 fruit juices and purees, pH 4.2) impaired their survival when exposed to simulated gastric juice (pH 2.0) compared with the fresh cultures. The same study also revealed that 35 day storage of the probiotics in the fruit juice, did not affect their tolerance to bile salts (0.3%) or pancreatin.

2.8.3 Adhesion

It has been recognised that in order to exert health promoting properties on the host, probiotic micro-organisms need to survive in sufficiently high number and colonise the gastrointestinal tract. A prerequisite for intestinal colonisation is adherence to intestinal epithelial mucosa (Alander *et al.*, 1999; Beachey, 1981; Boyle *et al.*, 2006). Adhesion to intestinal epithelial mucosa is one of the main criteria by which a microorganism can be selected as a probiotic (Salminen *et al.*, 1996). Bacterial adhesion to intestinal epithelial mucosa is a complicated process, mediated through multiple surface biophysical and biochemical properties of both bacteria and epithelial mucosa such as passive forces, electrostatic interactions, hydrophobicity, steric forces and most importantly specific cellular surface components (Servin & Coconnier, 2003).

The ability of potential probiotics to adhere intestinal epithelial mucosa could be evaluated using *in vivo* and *in vitro* assays (Ouwehand & Salminen, 2003; Servin & Coconnier, 2003). Availability and ethical issues however hamper the widespread use of animal models or human/animal intestinal-derived biopsy samples (Saarela *et al.*, 2000). A number of *in vitro* models have been developed to evaluate the bacterial adhesion to

intestinal mucosa (Ouwehand & Salminen, 2003). Even though *in vitro* assays can not mimic the complexities of *in vivo* conditions completely, various well controlled experimental conditions could be applied to demonstrate the adhesion ability of potential probiotics. Moreover a large number of potential probiotics could be screened using *in vitro* models (Ouwehand & Salminen, 2003). Tissue cultures of intestinal epithelial cell lines Caco-2 and HT-29 are most extensively used *in vitro* models of assessment of adhesion ability of microorganisms. Moreover, since the entire intestine is lined by a thin layer of mucus produced by the epithelial cells, the ability of probiotic candidates to adhere to the intestinal mucosa *in vitro* is tested by performing adhesion assay to intestinal mucus (Ouwehand & Salminen, 2003).

Adhesion to intestinal epithelial mucosa by probiotics depends on many factors such as bacterial strain, bacterial concentration, probiotic formulation (combination), composition of bacterial growth medium, cell culture and co-culture medium, pH of co-culture medium, bacterial growth stage, intestinal cell culture growth conditions, incubation time, host specificity, the intestine section, digestion and composition of gut microbiota (Collado *et al.*, 2007; Deepika *et al.*, 2009; Greene & Klaenhammer, 1994; Kankaanpää *et al.*, 2001; Moussavi & Adams, 2010; Ouwehand *et al.*, 2000; Ouwehand & Salminen, 2003; Tallon *et al.*, 2007; Van den Abbeele *et al.*, 2009).

It is also likely that delivery vehicle matrices affect adhesion characteristics of probiotics, however to date, little is known about the effect of food matrices on adhesion ability of probiotics (Ouwehand & Salminen, 2003; Sanders & Marco, 2010). In order to more closely simulate *in vivo* conditions of bacterial adhesion to intestinal mucosa, it has been recommended that microorganisms are exposed to the food matrix before adhesion assay (Ouwehand & Salminen, 2003). Study on the effect of food matrix on the adhesion ability of probiotics, to our knowledge, is only limited to the work of Ouwehand *et al.* (2001), in which pre-treatment of probiotics with milk was shown to significantly decrease the adhesion of probiotics to intestinal mucus glycoproteins compared with the control (HEPES-Hanks' buffer, pH 7.4). However there are quite a few reports on the effects of food components such as carbohydrates/polysaccharides (Lee & Puong, 2002; Parkar *et al.*, 2010; Van den Abbeele *et al.*, 2009), ethanol (Tuomola *et al.*, 2000), fatty acids (Kankaanpää *et al.*, 2001) and minerals such as calcium (Marcinakova *et al.*, 2010) on the adhesion ability of probiotics to intestinal epithelial mucosa.

2.8.4 Immunomodulation

Immunomodulatory effects of probiotics have been discussed earlier in section 2.4.2.

To our knowledge, there have been no reports on the impact of delivery vehicles or food components on immunomodulatory effects of probiotics. Exposure to low pH of acidic fruit juices such as orange juice (pH 3.7) may influence this functional property of probiotic bacteria. Previous research has shown that probiotics subjected to low pH demonstrated altered ability to modulate immune responses of intestinal epithelial cells. A study by Hosoi *et al.* (2003) has shown that pre-treatment of probiotic *Bacillus subtilis* (natto) cells with 1.0 M hydrochloric acid for 3 hours significantly increased IL-6 and IL-8 production in Caco-2 cells compared to non-treated probiotics.

2.9 Thesis research goal

As an alternative to dairy products, fruit juices could be ideal delivery vehicles for probiotics. Inclusion of probiotic combinations in food carrier matrices such as fruit juices may potentially offer even greater health benefits to the consumer than single strain preparations. However identification and understanding of potential interactions that may occur between the organisms within a proposed combination and between microorganisms and carrier matrix is of importance in functional food development. The main goal of this project is to examine the effect of combining probiotics, and potential interactions between the organisms and food matrices during storage on the viability and functional properties of probiotics.

Chapter III: Bacterial Growth Interactions and Intestinal Epithelial Cell Adhesion Characteristics of Probiotic Combinations

Moussavi, M., & Adams, M. C. (2010). An *in vitro* study on bacterial growth interactions and intestinal epithelial cell adhesion characteristics of probiotic combinations. *Current Microbiology*, **60**(5), 327-335.

3.1 Summary

The aims of this study were to examine long-term growth interactions of five probiotic strains (*Lactobacillus casei* 01, *Lactobacillus plantarum* HA8, *Lactobacillus rhamnosus* GG, *Lactobacillus reuteri* ATCC 55730 and *Bifidobacterium animalis* subsp *lactis* Bb12) both alone and in combination with *Propionibacterium jensenii* 702 in a co-culture system, and to determine their ability to adhere to the human colon adenocarcinoma cell line Caco-2. Growth patterns of probiotic *Lactobacillus* strains were not adversely affected by the presence of *P. jensenii* 702, whereas lactobacilli exerted a strong inhibitory action against *P. jensenii* 702. In the co-culture of *Bif. animalis* subsp *lactis* Bb12 and *P. jensenii* 702, a significant enhancement of the growth of both bacteria was observed ($p \leq 0.05$). The results of the adhesion assay showed that when probiotic strains were tested in combination, there was evidence of an associated effect on percentage adherence; however in most cases these differences were not statistically significant. The adhesion percentages of both *Lb. casei* 01 and *Lb. rhamnosus* GG decreased significantly in the presence of *P. jensenii* 702 compared to their adhesion levels when cultivated individually ($p \leq 0.05$). These results showed that the survival and adhesion capacity of some probiotic strains may be influenced by the presence of other strains and this should be considered when formulating products that contain multiple probiotics.

3.2 Introduction

Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2001a, 2001b). Although probiotics primarily belong to the genera *Lactobacillus* and *Bifidobacterium*, some strains of dairy propionibacteria have also been considered as probiotic due to their reported association with a diverse range of health benefits. These include synthesis of vitamins such as B12 and folate (Hugenholtz *et al.*, 2002), secretion of antimicrobial compounds (*e.g.* propionic acid and bacteriocins) (Ouwehand, 2004), moderation of lactose intolerance through production of β -galactosidase (Zarate, Perez Chaia, *et al.*, 2000), modulation of the host’s immune system (Perez Chaia, deMacias, *et al.*, 1995), anti-hyperlipemic effect (Perez Chaia, deMacias, *et al.*, 1995), growth stimulation of bifidobacteria (Kaneko, 1999; Kaneko *et al.*, 1994; Kaneko & Noda, 1996; Kouya *et al.*, 2007; Mori *et al.*, 1997; Uchida *et al.*, 2005; Warminska-Radyko *et al.*, 2002), moderation of colonic inflammation by nitrate reduction (Michel *et al.*, 2005) and anti-carcinogenic effect (Jan, Belzacq, *et al.*, 2002; Lan *et al.*, 2008; Lan, Lagadic-Gossman, *et al.*, 2007; Perez Chaia *et al.*, 1999).

In most cases, however, it is recognised that in order to confer these reported health promoting benefits on the host, the probiotic microorganisms need to survive in sufficiently high number and colonise the gastro-intestinal tract. A prerequisite for intestinal colonisation is adherence to intestinal epithelial mucosa (Alander *et al.*, 1999; Beachey, 1981). Probiotic adhesion to intestinal epithelial cells using single strains of probiotic propionibacteria has been studied both *in vitro* and *in vivo* (Huang & Adams, 2003; Ouwehand *et al.*, 2002; Tuomola *et al.*, 1999; Zarate, Morata De Ambrosini, Chaia, *et al.*, 2002; Zarate, Morata De Ambrosini, Perez Chaia, *et al.*, 2002), however few studies have investigated how multi-strain interactions could affect either individual bacterial viability or rates of adhesion.

Ouwehand *et al.* (2002) have previously demonstrated that primarily adhered *Lb. rhamnosus* GG, *Bif. animalis* subsp *lactis* Bb12 and *Bif. infantis* Bbi significantly enhanced the subsequent adhesion of certain propionic acid bacteria to human intestinal mucus in paired-strain combinations, while primarily adhered propionibacteria did not increase the subsequent adhesion ability of lactobacilli and bifidobacteria to the mucus. Collado *et al.* (2007) further identified positive changes in the human intestinal mucosal adhesion rates of *P. freudenreichii* ssp. *shermanii* JS in 2-, 3- and 4-strain combinations

with *Lb. rhamnosus* GG, *Lb. rhamnosus* LC705 and *Bif. breve* 99. However, in contrast to the findings of Ouwehand *et al.* (2002), the mucosal adherence of lactobacilli was found by Collado *et al.* (2007) to improve in all combinations containing *P. freudenreichii* ssp. *shermanii* JS.

In addition to measurable changes in adhesion rate, it has been observed that some propionibacteria can stimulate the growth of bifidobacteria *in vivo* and *in vitro* through the production of specific growth stimulating factors (Hojo *et al.*, 2002; Kaneko, 1999; Kaneko *et al.*, 1994; Kaneko & Noda, 1996; Kouya *et al.*, 2007; Mitsuyama *et al.*, 2007; Mori *et al.*, 1997; Satomi *et al.*, 1999; Uchida *et al.*, 2005; Warminska-Radyko *et al.*, 2002). Evidence from a further study indicated that bifidobacteria may also stimulate growth of propionibacteria (Gardner & Champagne, 2005). An earlier study had reported that lactobacilli may have differing effects on growth of propionibacteria including inhibition, stimulation, or no effect at all (Parker & Moon, 1982). It has been reported that selected lactobacilli stimulated the growth of propionibacteria through production of lactic acid which could subsequently be utilised as an energy source (Ouwehand, 2004). Other metabolites produced by lactobacilli may also be involved in the growth stimulation of propionibacteria. For example, Piveteau *et al.* (2002) reported that short peptides produced by *Lb. helveticus* DPC 4571 in milk stimulate the growth of *P. freudenreichii* DPC 3801. Most of studies however have shown that lactobacilli have a strong inhibitory effect on the growth of propionibacteria in associative cultures. These reports have identified that rapid decreases in pH due to the activity of lactobacilli is the main inhibitory factor to the growth of propionibacteria (Parker & Moon, 1982; Perez Chaia *et al.*, 1994; Perez Chaia, Strasser de Saad, *et al.*, 1995).

In general, the existing literature indicates both that the growth interactions between propionibacteria and lactobacilli or bifidobacteria in probiotic combinations are species- and strain-dependent, and that the composition of the growth culture medium may influence the outcome of these interactions.

P. jensenii 702 has been introduced as a novel probiotic bacterium originally isolated from raw bovine milk, and has been shown to survive in *in vitro* and *in vivo* gastrointestinal conditions (Huang & Adams, 2004; Huang *et al.*, 2003) and adhere to human intestinal epithelial cells *in vitro* (Huang & Adams, 2003). *In vivo* safety assessments have also shown that administration of *P. jensenii* 702 to wistar rats for 81 days had no adverse effects on the health status of the animals (Huang *et al.*, 2003). Based on studies using *in vivo* models, it has also been claimed that *P. jensenii* 702 is able to produce

vitamin B12, normalise homocysteine levels, and lower serum cholesterol and triglyceride concentrations in rats (Adams & Huang, 2008). Moreover, *P. jensenii* 702 was identified as a potential live oral vaccine vector for tuberculosis in a mouse model (Adams *et al.*, 2005). Studies on human subjects have also shown *P. jensenii* 702 to survive gastro-intestinal conditions, transiently colonise the GI tract, increase populations of endogenous bifidobacteria, and increase production of faecal short chain fatty acids including acetate, propionate and butyrate (Kotula, 2008). The potential probiotic benefits of this organism are therefore considerable and its performance in multi-species preparations is likely to be critical in determining its future utilisation in probiotic products.

In the current study, associative growth patterns and the viability of *P. jensenii* 702 (PJ) paired with five probiotic strains *Lb. casei* 01 (LC), *Lb. plantarum* HA8 (LP), *Lb. rhamnosus* GG (LG), *Lb. reuteri* ATCC 55730 (LR) and *Bif. animalis* subsp *lactis* Bb12 (Bb) were assessed for 14 days in a co-culture system as a simple model of intestinal conditions, and compared with the performance of pure cultures in the same medium. Furthermore, the capacity of the mono and paired cultures for adhesion to the human colorectal epithelial cell line Caco-2 was also examined.

Four commercial probiotic strains including LC, LG, LR and Bb, were selected as they are among the most widely recognised and extensively studied probiotics of human origin. LP, previously isolated in this laboratory from a Vietnamese traditional fermented food, and identified as a potential probiotic strain (Ho, 2008), was also employed in this study.

Based on the available literature, this investigation aimed to address in particular, the following hypotheses:

- That the growth and viability of PJ would be affected when co-cultivated with the lactobacilli, but that the effects would be variable between the 4 *Lactobacillus* strains.
- That decreases in pH of the co-culture medium due to the activity of lactobacilli inhibit the growth of PJ
- That the growth and viability of the lactobacilli would not be significantly affected by the presence of PJ.
- That the growth and viability of both PJ and Bb would be mutually enhanced when co-cultivated.

- That the co-cultivation of PJ with either Bb or the lactobacilli would result in enhanced adhesion of PJ to Caco-2 cells, with no significant change or increase in the adhesion rates of Bb or the lactobacilli.

3.3 Materials and methods

3.3.1 Bacterial strains and growth conditions

Four commercial probiotic strains LG, LR, LC and Bb, and two new potential probiotic strains LP and PJ, isolated in our laboratory from a Vietnamese traditional fermented food and raw cow's milk respectively, were used in this work. LR was kindly provided by BioGaia Biologics Inc. (BioGaia Biologics Inc. Raleigh, USA). Bb and LC were generous gifts from Chr. Hansen Pty. Ltd. Melbourne, Australia. LG was isolated from a CULTURELLE[®] capsule (a gift from Amerifit Brands Inc., Cromwell, USA). Bacterial identifications were confirmed via DNA extraction and PCR amplification of the 16S rRNA gene using species-specific primers. All extractions were conducted with the Wizard[®] Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA) as per the manufacturer's protocol. For longer term survival and higher quantitative retrieval, the cultures were stored at -80 °C using the Microbank[®] Bacterial and Fungal Preservation System (Pro-Lab Diagnostics, Richmond Hill, Canada). When needed, recovery of strains was undertaken by two consecutive sub-cultures in appropriate media prior to use. The four strains of *Lactobacillus* and the Bb were grown overnight at 37 °C, respectively, in de Man, Rogosa, Sharpe (MRS) and Reinforced Clostridial Medium (RCM) broths (Oxoid Australia Pty Ltd, Adelaide, Australia) under anaerobic conditions (Anaerobic jar and AnaeroGen, Oxoid Ltd). PJ was grown anaerobically in yeast extract lactate (YEL) medium (Malik *et al.*, 1968b) at 30 °C for 48 h.

3.3.2 Chemicals and reagents

Except where otherwise specified, all chemicals used in this study were obtained from Sigma-Aldrich (St. Louis, MO, USA).

3.3.3 Co-culture growth interactions

Growth interactions of PJ with other probiotics were examined in a co-culture system. YEL medium supplemented with 2% glucose (GYEL) was used as the co-culture medium, on the basis of preliminary experiments in which good individual growth of all probiotic strains was observed in this medium. The cultures were individually adapted to GYEL medium prior to examining co-culture growth interactions. This adaptation

was performed by sub-culturing in GYEL medium and incubation at 33 °C overnight (*Lactobacillus* strains and Bb) or for 48 h (PJ). This temperature was selected as it is in optimum growth temperature range for all probiotic strains used in this study. Bacterial cells were then harvested from fresh probiotic cultures in their stationary phases (determined by performing bacterial growth curve experiments) by centrifugation at $1811 \times g$ for 10 min and washed three times with Dulbecco's Phosphate-Buffered Saline (PBS, pH 7.0) (Gibco, Invitrogen Corp., Carlsbad, CA, USA). Bacterial pellets were then resuspended in PBS. Fifty millilitres (50 ml) of the medium dispensed in sterile screw-cap polypropylene containers (Sarstedt Australia Pty Ltd, Mawson Lakes, SA, Australia) was inoculated with an aliquot of 500 µl of each bacterial suspension either alone or in combination with PJ. Containers were incubated anaerobically at 33 °C for 2 weeks. Bacterial counts were determined by plating 100 µl aliquots of decimal dilutions of cultures on agar plates at days 0, 1, 4, 7 and 14. The *Lactobacillus* spp. and the Bb were counted, respectively, on *Lactobacillus* Selective (LBS) agar (Rogosa *et al.*, 1951b) and *Bifidobacterium* Iodoacetate (BIM) agar (Munoz & Pares, 1988) after 3 days of incubation at 37 °C under anaerobic conditions. Since growth of PJ is inhibited on BIM agar, all colonies present on the BIM as a result of plating the PJ and Bb co-culture were considered to be Bb. PJ can grow on LBS agar, however its growth rate is very slow and colonies appear after 5–7 days of incubation. Thus, the colonies which appeared on LBS agar following 24–48 h of incubation were considered to be *Lactobacillus* spp. PJ was counted on YEL agar (Malik *et al.*, 1968b) following 7 days of incubation at 30 °C under anaerobic conditions. In the co-culture of *Lactobacillus* strains and PJ, *Lactobacillus* strains can also grow on YEL agar but their colonies could be easily differentiated from PJ. PJ could be differentiated from *Lactobacillus* strains on the basis of its typical colony morphology and colour as well as by its later appearance on the YEL agar. PJ colonies appear after 7 days of incubation as drop-like mustard coloured colonies. For the presentation of results all bacterial counts have been expressed as Log CFU/ml. In all cases the pH of the culture medium was measured (performed in triplicate) using a Cyberscan 510 pH meter (Eutech Instruments Pty Ltd., Singapore), with all measurements taken on the same days as the bacterial counts were performed.

3.3.4 Caco-2 cell line

The Caco-2 cell line ATCC HTB-37 (American Type Culture Collection, Rockville, MD, USA) was kindly provided by Dr. Matthias Ernst (Ludwig Institute for Cancer Research, Melbourne, Australia). The cells were cultured in Nunc™ tissue culture flasks (Thermo Fisher Scientific, Rochester, NY, USA) containing RPMI 1640 medium (Gibco, Invitrogen Corp., Carlsbad, CA, USA) supplemented with 20% heat inactivated foetal bovine serum (Gibco, Invitrogen Corp., Carlsbad, CA, USA), 2% HEPES buffer (Gibco, Invitrogen Corp., Carlsbad, CA, USA), 2% sodium bicarbonate (Gibco, Invitrogen Corp., Carlsbad, CA, USA), 1% L-glutamine (Gibco, Invitrogen Corp., Carlsbad, CA, USA) and 2% penicillin/streptomycin (Gibco, Invitrogen Corp., Carlsbad, CA, USA). The cells were grown in this medium at 37 °C in a 5% CO₂ / 95% air atmosphere using a humidified HERAcell 150 CO₂ incubator (Thermo Fisher Scientific Inc., Waltham, MA, USA). The cell-culture medium was replaced with fresh medium every other day.

3.3.5 *In vitro* bacterial adhesion assay

The Caco-2 cells were seeded at a concentration of approximately 10⁵ cells/well in each well of a Nunc™ 24-well tissue culture plate (Thermo Fisher Scientific, Rochester, NY, USA), and incubated at 37 °C in 5% CO₂ atmosphere in a humidified incubator until a confluent monolayer had formed. The cell-culture medium was changed every other day. At least 1 hour before the adhesion assay, the RPMI medium was replaced with the same medium but without antibiotic. Prior to the adhesion assay, the monolayers of Caco-2 cells were washed three times with PBS.

A 500 µl aliquot of each bacterial suspension (at concentrations of 10⁷–10⁸ CFU/ml) was added to post confluent monolayers of Caco-2 cells in each well of the 24-well micro-plates and incubated at 37 °C in 5% CO₂ / 95% air for 3 h. Afterwards, the cells were washed three times with PBS in order to remove non-adherent bacteria. Caco-2 cells were then detached from the plastic surfaces of wells by addition of 500 µl trypsin/EDTA (Gibco, Invitrogen Corp., Carlsbad, CA, USA) and 500 µl PBS followed by incubation at 37 °C for 2–3 min. An amount of 1 ml of each suspension was added into a tube containing 9 ml sterile Maximum Recovery Diluent (MRD) (Oxoid Australia Pty Ltd, Adelaide, Australia), and serial decimal dilutions were prepared. One hundred (100) µl of each dilution was then plated onto agar plates. Bacterial counting was

performed as described in detail in the “Co-Culture Growth Interactions” section (page 62). Adhesion was recorded as the number of bacteria adhered to Caco-2 cells, and expressed as a percentage of the number of bacteria initially added to the Caco-2 culture wells.

3.3.6 Scanning Electron Microscopy

In order to qualitative examination of adhesion using scanning electron microscopy (SEM), 13 mm coverslips (Sarstedt Inc., Newton, NC, USA) were placed in the bottom of tissue culture plate wells before seeding with Caco-2 cells. Preparation stages were the same as those applied for other wells during the growth phase of the Caco-2 cells (see “Caco-2 Cell Line” section, page 63). After incubating post-confluent monolayers of Caco-2 cells with each probiotic suspension, coverslips were removed from wells and washed three times with 1 ml pre-warmed (37 °C) PBS buffer to remove nonadherent bacteria. Thereafter, cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h at room temperature and the coverslips washed three times with 0.1 M cacodylate buffer (10 min each time). A second fixation step was performed by exposing the cells to 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h, followed by three times washing with cacodylate buffer. The specimens were then dehydrated with a graded series of ethanol solutions (25, 50, 75, 95, and two times 100%, 10 min each session). Coverslips were then air dried at room temperature for 30 min, mounted on stubs and coated with a conductive material (gold particles) using a SPI Sputter Gold Coater (SPI Structure Probe Inc., West Chester, PA, USA). Specimens were then examined with a Philips XL30 scanning electron microscope (Philips, Eindhoven, The Netherlands) equipped with the EDS Link (Isis, Oxford Instruments, Concord, MA, USA).

3.3.7 Statistical analysis

Statistical analyses were performed using SPSS software Ver. 15 (SPSS Inc., Chicago, IL, USA). Results of adhesion and bacterial interaction experiments were expressed as averages obtained from two independent experiments each performed in triplicate. Adhesion and bacterial interaction data were analysed using the two-tailed t test and general linear model (GLM), respectively. A p value ≤ 0.05 was considered statistically significant for all analyses.

3.4 Results

3.4.1 Growth interactions and viability of probiotics in mixed cultures

The growth, patterns of viability, and pH changes of probiotic mono- and co-cultures in GYEL medium over 14 days incubation are presented in Figures 3.1 and 3.2. The growth and viability patterns of each mono-culture of LG, LC and LP was found to be similar overall to that of each in the presence of PJ. In general, after a dramatic increase in the cell numbers of these three lactobacilli over the first day of incubation, either individually or in combination with PJ, viability was observed to decrease gradually over the remainder of the incubation period. The same trend was also observed for LR, however, after day 7, viable counts of LR decreased more rapidly in mono-culture than in combination with PJ. Viability of LR in combination with PJ remained significantly higher than that of LR mono-culture on day 14, ($p \leq 0.05$).

In contrast with the lactobacilli the mono-culture of Bb exhibited a one day lag phase at the beginning of the incubation period, after which the number of cells increased sharply, reached a peak on day 4 and declined steeply over the next 10 days. No viable cells were recovered on day 14. By comparison, the number of viable Bb cells was found to rise rapidly when in combination with PJ, reaching a peak (5.0×10^7 CFU/ ml) on day 1, and remaining relatively unchanged by day 14.

The mono-culture of PJ was observed to follow a pattern of initial growth in the first 4 days of incubation, followed by a steady decline in numbers, although viability remained relatively strong overall, reducing by less than one order of magnitude across the 14 days of incubation (Figure 3.1). By comparison, co-culturing with lactobacilli appeared to have a significant detrimental impact on the viability of PJ. When cultured with LR, PJ could not be recovered after 7 days of incubation while in all other cases PJ had ceased to be viable by day 4. In contrast the viability of PJ was found to be significantly enhanced when cultured in combination with Bb. In this case, numbers increased initially as they did in the PJ mono-culture, but were subsequently maintained at this elevated level with no decline evident over the remainder of the incubation period.

At the end of the incubation period (day 14), the counts of both PJ and Bb in co-culture were the highest among all cultures examined.

The findings were generally consistent with the hypothesized outcomes except that the impact of the various lactobacilli on the viability of PJ was essentially uniform rather than variable between the different strains.

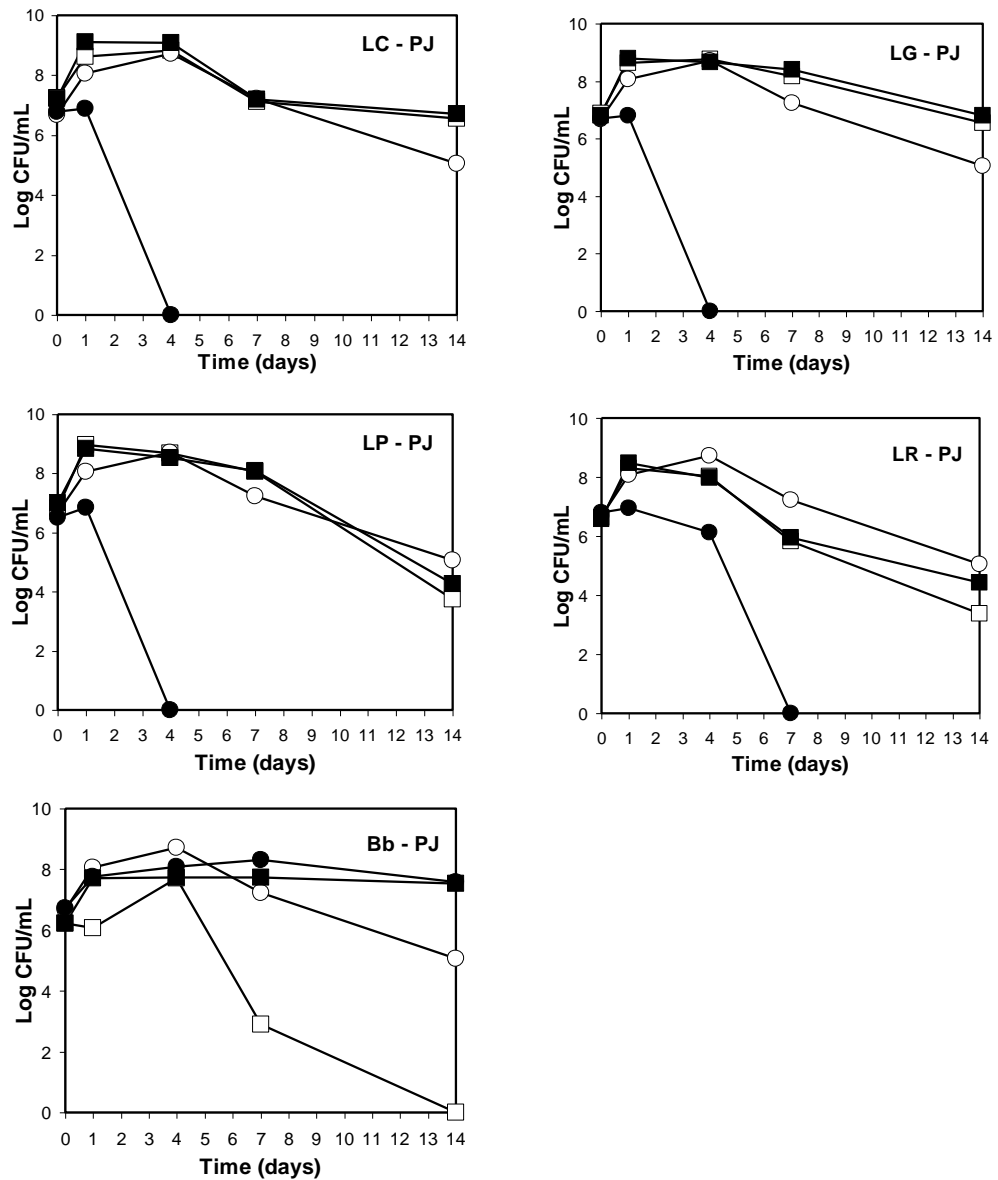


Figure 3.1: Comparative growth and viability patterns during 14 days incubation of mono- and co-cultures of probiotics in GYEL medium at 33 °C. □, viable cell counts of monocultures of *Lactobacillus* strains or Bb; ■, viable cell counts of *Lactobacillus* strains or Bb in combination with PJ; ○, viable cell counts of the PJ mono-culture; ●, viable cell counts of PJ in combination with lactobacilli and Bb.

3.4.2 Patterns of pH change in mono- and co-culture growth media

Changes in the pH of the culture medium of each single *Lactobacillus* strain were the same as those of the medium containing *Lactobacillus* strains in the presence of PJ. In each *Lactobacillus* / PJ co-culture, the pH declined rapidly during the first day of incubation, from an initial value of pH 6.0 to just over pH 4.0, eventually stabilising to a value between pH 3.8 and 4.2 by day 4. The pH value of the culture medium inoculated with PJ alone decreased steadily over the first 4 days of incubation, after which it was observed to stabilise at approximately pH 4.7. At all time points, pH values were lower for lactobacilli either individually or in combination with PJ than that of PJ alone.

Changes in the pH of the culture medium of PJ alone were the same as those of the medium containing the combination of Bb and PJ. The pH values declined by day 4 and remained almost stable for the next 10 days. After 1 day incubation, at all other time points, pH values were lower for PJ either individually or in combination with Bb than those of Bb alone.

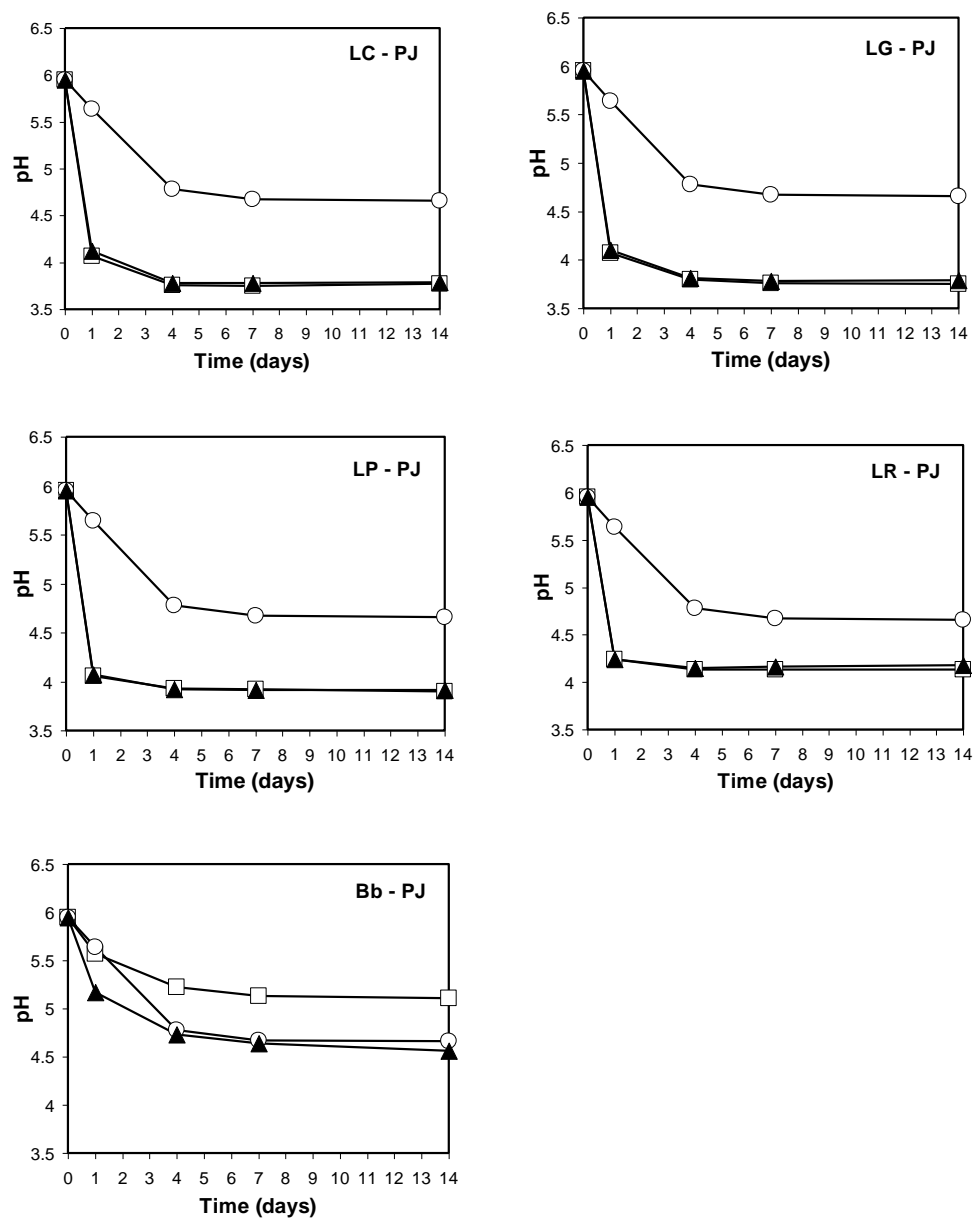


Figure 3.2: Changes in pH during 14 days incubation of mono- and co-cultures of probiotics in GYEL medium at 33 °C. □, pH of mono-cultures of *Lactobacillus* strains and Bb; ○, pH of PJ alone; ▲, pH of co-cultures.

3.4.3 Adhesion of probiotics in mono- and co-culture to the intestinal epithelial cell line Caco-2

All examined probiotic strains, either alone or in combination with PJ, were able to adhere to Caco-2 human intestinal epithelial cells (Figure 3.3). Adhesion rates varied widely however, from 5.07% for Bb in the presence of PJ to 83.15% for LP in mono-culture. LP and LR either alone or in combination with PJ showed a significantly higher adhesion percentage compared with all other cases. There was no significant difference between the adhesion rate of LP either alone or in combination with PJ and that of LR when co-cultured with PJ. When the adhesion of probiotic strains was tested in the presence of PJ, there was evidence of an effect on percentage adherence. The adhesion rates of LC and LG both decreased significantly in the presence of PJ compared to their adhesion levels when alone ($p \leq 0.05$). Variations in adhesion rates between mono- and co-cultures were also observed in relation to the other combinations, however, the differences in these cases were not statistically significant. The percentage adhesion of LR appeared to improve in the presence of PJ, whereas the adhesion rate of LP decreased in combination with PJ. Little difference in the adhesion rate of Bb was apparent whether alone or in co-culture with PJ.

In effect, neither the *Lactobacillus* strains nor Bb appeared to exert any significant effect on the adhesion rate of PJ (Figure 3.4), whereas presence of PJ was shown to adversely affect the adhesion percentages of certain *Lactobacillus* strains (Figure 3.3). The findings were therefore largely unsupportive of the hypothesis that the co-cultivation of PJ with either Bb or the lactobacilli would enhance adhesion of PJ to Caco-2 cells, with either no significant effect or increase in the adhesion rates of Bb or the lactobacilli.

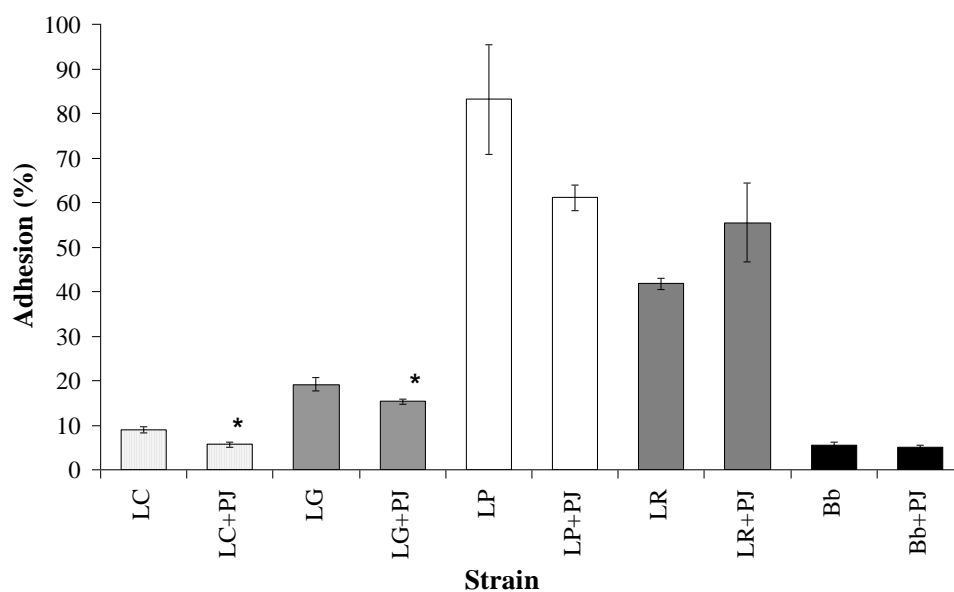


Figure 3.3: Percentage adhesion of LC, LG, LP, LR and Bb, either alone or in combination with PJ to Caco-2 human intestinal epithelial cells. In combinations, the first listed bacterium has been counted. Data represent means \pm standard error of two independent experiments, each performed in triplicate.

* indicates a statistically significant difference between the adhesion rate of each strain when alone and when co-cultured with PJ ($p \leq 0.05$)

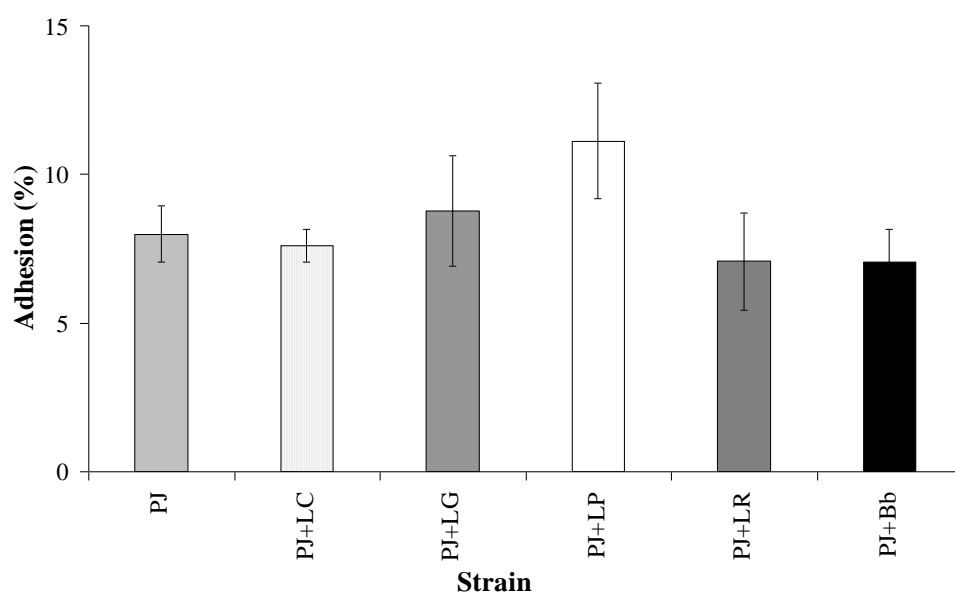


Figure 3.4: Percentage adhesion of PJ either alone or in combination with LC, LG, LP, LR or Bb to Caco-2 human intestinal epithelial cells. Data represent means \pm standard error of two independent experiments, each performed in triplicate.

Scanning electron micrographs of the cell culture preparations taken immediately following the adhesion assay clearly illustrated attachment of the probiotics to Caco-2 cells (Figure. 3.5), but also showed evidence that the distribution of bacterial cells across the tissue monolayer was not uniform, although reasons for this clumping effect were not clear.

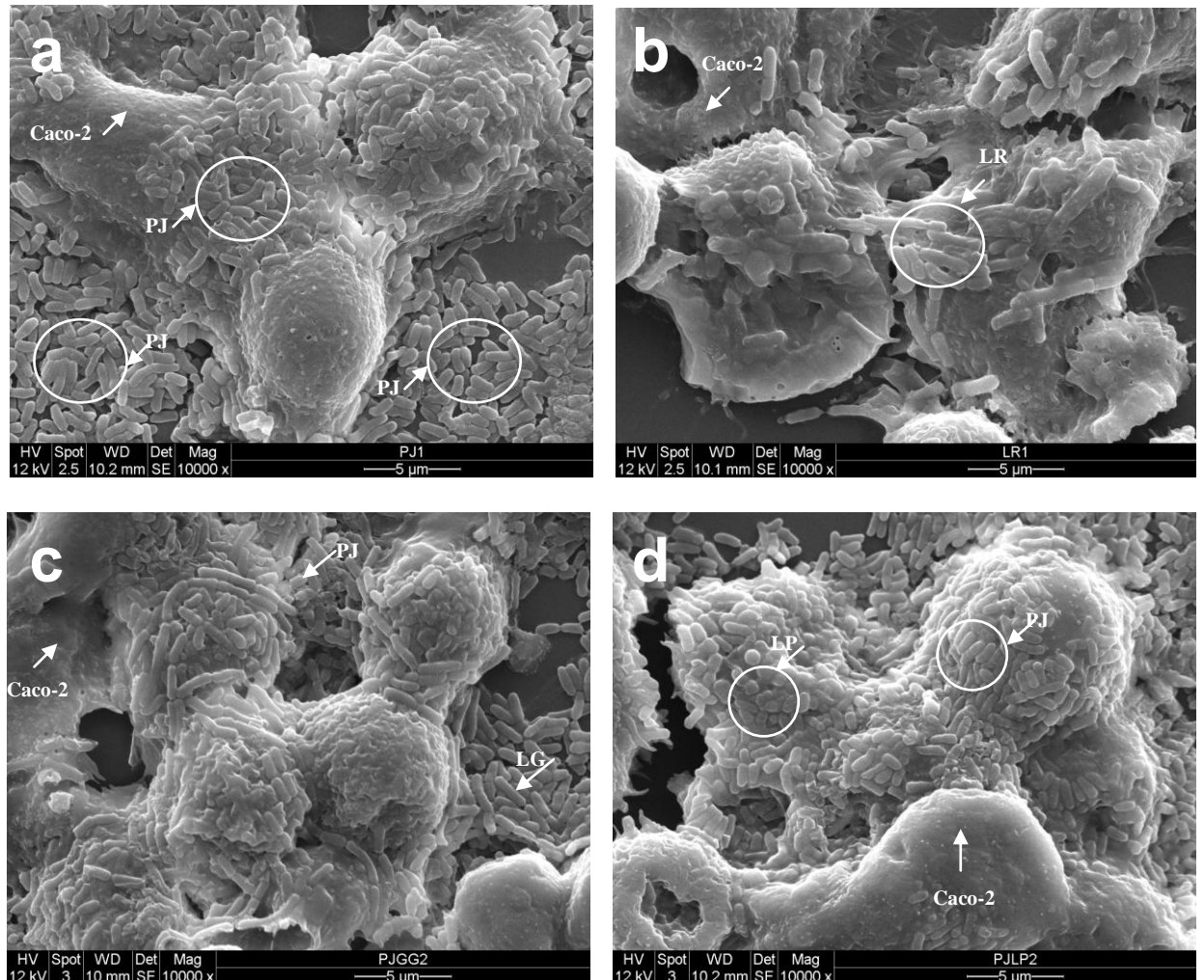


Figure 3.5: Scanning electron micrographs showing adherence of selected probiotic strains to Caco-2 cells. a, PJ; b, LR; c, PJ + LG; d, PJ + LP.

To summarise, growth patterns of probiotic *Lactobacillus* strains were not adversely affected by the presence of PJ, whereas lactobacilli exerted a strong inhibitory action against PJ. The inhibitory effects of LG, LP and LC on the growth of PJ were stronger than that of LR.

Dramatic decline in the pH of co-culture medium to a level below pH 4.5 due to the metabolic activity of lactobacilli was identified as the probable cause of growth inhibition of PJ. In the co-culture of Bb and PJ, a significant enhancement of the growth of both bacteria was observed. The data therefore provides evidence to support the experimental hypotheses with regard to bacterial growth interactions and viability.

The results of the adhesion assay showed that co-cultivation of PJ with either Bb or the lactobacilli did not change adhesion of PJ to Caco-2 cells. Thus the hypothesized enhanced adhesion of PJ to Caco-2 cells was not observed. Out of five bacteria examined, the adhesion percentage of LC and LG both significantly decreased in the presence of PJ compared to their adhesion levels when alone. The results were therefore not completely in support of the hypothesis that no significant change would be observed in the adhesion rates of Bb or the lactobacilli to intestinal cells when co-cultured with PJ.

3.5 Discussion

3.5.1 Growth and viability interactions of probiotics in mixed cultures

The results of co-cultivation of PJ with Bb and several strains of *Lactobacillus* have revealed variable effects on the viability of organisms in the paired co-culture systems. Most significant of the findings was the mutual synergistic viability promoting effect of PJ and Bb when co-cultivated. In such a combination, growth of *Bifidobacterium* might be stimulated by *Propionibacterium* in two ways. Firstly, propionate and acetate produced as end products of fermentation of glucose and lactate by propionibacteria have been shown to enhance the growth of bifidobacteria (Kaneko *et al.*, 1994; Piveteau, 1999). Secondly, some dairy propionibacteria may produce specific growth-stimulating factors for bifidobacteria termed as bifidogenic growth stimulating factors (BGS) such as 2-amino-3-carboxy-1,4-naphthoquinone (ACNQ) and 1,4-dihydroxy-2-naphthoic acid (DHNA) (Hojo *et al.*, 2002; Isawa *et al.*, 2002; Kaneko, 1999; Kaneko *et al.*, 1994; Kaneko & Noda, 1996; Kouya *et al.*, 2007; Mitsuyama *et al.*, 2007; Mori *et al.*, 1997; Satomi *et al.*, 1999; Uchida *et al.*, 2005; Warminska-Radyko *et al.*, 2002). Similarly, the stimulation of PJ by Bb is also consistent with a recent study showing that some strains of bifidobacteria have a growth-promoting effect on propionibacteria, however no particular mechanism was identified (Gardner & Champagne, 2005). Within the context of existing literature, the significance of the finding presented here is that it appears to represent the first report of mutual synergistic growth and maintenance of the viability of *Propionibacterium* and *Bifidobacterium* in an associative co-culture system.

By comparison, co-cultivation of PJ with various lactobacilli proved less successful with an inhibitory effect on the viability of PJ evident in all cases. Aside from evidence of some enhancement in the case of LR, the viability of the lactobacilli remained largely unaffected. Previous reports have shown the effects of lactobacilli on the growth of propionibacteria to be variable, ranging from inhibitory to stimulatory. In two studies, whey from skim milk, pre-fermented by *Lb. acidophilus*, *Lb. delbrueckii* subsp *lactis* and *Lb. helveticus* DPC 4571, was shown to exert a strain dependant stimulation of *Propionibacterium* growth (Piveteau *et al.*, 1995; Piveteau *et al.*, 2002). In the case of *Lb. helveticus* this growth stimulation was found to be associated with the production of specific short chain peptides derived from the proteolysis of casein (Piveteau *et al.*, 2002).

In relation to the inhibition of propionibacteria in co-cultures with lactobacilli, various mechanisms have been identified. For example, sensitivity to formate and acetate, produced by *Lb. casei* from available citrate, was suggested as a possible mechanism for the inhibition of *P. freudenreichii* ssp. *shermanii* in a cheese model (Frohlich-Wyder *et al.*, 2002). Other metabolites produced by lactobacilli have also been implicated. Bacteriocins such as salivacin 140 (Arihara *et al.*, 1996), pentocin TV35b (Okkers *et al.*, 1999), plantaricin S and T (Jimenez-Diaz *et al.*, 1993), plantaricin C (Gonzalez *et al.*, 1994), plantaricin 423 (van Reenen *et al.*, 1998), plantaricin OL15 (Mourad *et al.*, 2005), have all been reported to inhibit growth of propionibacteria. In particular plantaricin, a bacteriocin produced by *Lb. plantarum*, has been reported as an active antimicrobial agent against *Propionibacterium* spp. (Gonzalez *et al.*, 1994; Jimenez-Diaz *et al.*, 1993; Mourad *et al.*, 2005; van Reenen *et al.*, 1998), and several studies have shown *Lb. plantarum* to inhibit growth of *Propionibacterium* spp. (Brink *et al.*, 2006; Warminska-Radyko *et al.*, 2002).

Perhaps the most convincing reports however, are those showing rapid decreases in pH due to the activity of lactobacilli to have a strong inhibitory effect on the growth of propionibacteria in associative cultures (Parker & Moon, 1982; Perez Chaia *et al.*, 1994; Perez Chaia, Strasser de Saad, *et al.*, 1995). In the study of Parker and Moon (1982), this pH associated inhibition of propionibacteria was observed in 15 of 16 experimental paired combinations with various *Lactobacillus* strains.

The results presented here are highly consistent with these findings. In all *Lactobacillus* co-cultures the rapid decline in viability of PJ was coincident with a decline in pH to a level below pH 4.5, with the inhibitory effect most severe where levels fell below pH 4.0. In cases where pH levels remained above pH 4.5 (*i.e.* in mono-culture and in co-culture with Bb), the viability of PJ was maintained throughout the entire incubation period. In all cases the pH levels of the PJ / *Lactobacillus* co-cultures mirrored that of the corresponding *Lactobacillus* mono-culture, suggesting that the effect was driven primarily by the activity of the lactobacilli.

While increasing concentrations of organic acids in the media, especially lactic acid, would appear to be the most likely explanation for the observed reductions in pH, it is interesting to refer again to the study of Parker and Moon (1982). In the only culture pairing in which growth of propionibacteria was found to be enhanced, the authors associated the result with an accumulation of lactate in the medium. Lactic acid has been shown to serve as a suitable energy source for propionibacteria, and is catabolised

to propionic acid by them (Ouwehand, 2004). This would suggest that the mechanisms involved may be rather complex, and further efforts to examine the organic acid composition of the media in co-culture systems may therefore prove insightful.

In this experiment, a simple *in vitro* model of co-culture bacterial interaction was used to investigate growth interactions of PJ and a *Lactobacillus* strain or Bb. Based on the results obtained it might be concluded that due to the inhibition of PJ growth, using PJ in the presence of *Lactobacillus* strains is not advisable, especially in a fermentation process, as the final product may not carry adequate numbers of PJ to ensure efficacy.

In contrast, the results revealed that a combination of PJ and Bb could be successfully used in fermentation processes. Given that the composition of food may influence the probiotic interactions however; this must be tested in other food systems (*e.g.* milk). If the aim is to take advantage of combinations of PJ and the *Lactobacillus* strains, alternative strategies could include incorporating potentially active probiotic combinations into chilled or frozen probiotic food products or in supplement forms such as tablets and capsules. A further issue to consider is the way in which PJ interacts with intestinal microbiota *in vivo*, especially lactobacilli and bifidobacteria, and therefore represents a potential avenue of future research. Existing research on human subjects has shown that consumption of *P. freudenreichii* can result in a significant increase in the population of bifidobacteria in fecal samples (Bougle *et al.*, 1999; Hojo *et al.*, 2002; Satomi *et al.*, 1999).

3.5.2 Adhesion characteristics of probiotics in mono- and co-culture to intestinal epithelial cell Line Caco-2

Adhesion of probiotics to intestinal epithelial mucosa is one of the main criteria which a microorganism should fulfil to be considered as a ‘probiotic’. Adhesion is crucial for intestinal colonisation by probiotics and is therefore necessary to confer their beneficial effects on the host. Bacterial adhesion to intestinal epithelial mucosa is a complicated process, influenced by multiple surface biophysical and biochemical properties of both bacteria and epithelial mucosa such as passive forces, electrostatic interactions, hydrophobicity, steric forces and specific cellular surface components (Servin & Coconnier, 2003).

Since the entire intestine is lined by a thin layer of mucus produced by the epithelial cells, the ability of probiotic candidates to adhere to the intestinal mucosa *in vitro* is

tested by performing assays for adhesion to intestinal cell lines and/or mucus. Previous studies have shown that some dairy propionibacteria have acceptable adhesion ability to both intestinal mucus and epithelial cell lines (Huang & Adams, 2003; Lehto & Salminen, 1997; Ouwehand *et al.*, 2002; Tuomola *et al.*, 1999; Zarate, Morata De Ambrosini, Chaia, *et al.*, 2002; Zarate, Morata De Ambrosini, Perez Chaia, *et al.*, 2002). There are also several recent works on the adhesion of probiotic combinations including dairy propionibacteria spp. to intestinal mucus (Collado *et al.*, 2007; Ouwehand *et al.*, 2002). However, to this author's knowledge, there have been no previous studies examining whether adhesion of probiotics to intestinal epithelial cell lines may be influenced by the presence of other probiotic strains.

In the current study, the initial number of probiotic bacteria inoculated into wells was in all cases far greater than the number adhered to the Caco-2 cells, possibly indicating that available binding sites on the epithelial cells were saturated with probiotic bacteria, although SEM evidence of clumping of bacterial cells on the cell monolayers would appear to suggest otherwise. Our findings also showed that out of five paired probiotic combinations, three combinations did not show any significant adverse effect on the adhesion ability of either strain. This may indicate that the strains used in these combinations have different adhesion sites on the intestinal epithelial cells. Conversely, adhesion of LC and LG to the Caco-2 cells decreased significantly in the presence of PJ (Figure 3.3), suggesting the possibility that these two strains may compete with PJ for the same adhesion sites, although this is yet to be confirmed.

3.6 Conclusion

Previous research has demonstrated that not all probiotic microorganisms are alike, with different genera, species and strains providing differing health promoting effects. Using combinations or cocktails of probiotics may therefore be an appropriate strategy to confer a broad range of beneficial health effects on the host. For the preparation of food products with effective combinations of probiotics however, it is necessary to identify and understand potentially synergistic or antagonistic interactions that may occur between the organisms involved. In this study, reciprocal effects on the viability and adhesion capacity of PJ and other probiotics have been examined in paired *in vitro* co-culture systems. Our findings showed that the survival and percentage adhesion of some strains of probiotic may be influenced by the presence of other strains and this should be considered when formulating the food product. In particular the activity of lactobacilli appeared to induce reductions in pH to levels at which the viability of PJ was adversely affected. A significant reduction in adhesion percentage of LC and LG was observed in the presence of PJ compared to their adhesion levels when alone, while in all other cases variations in adhesion rates between mono- and co-cultures were not statistically significant. PJ and Bb appeared to represent a highly favourable combination, providing mutually enhanced viability with no apparent adverse impact on the adhesion capacity of either organism. The results therefore confirm that it is possible to utilise specific combinations of different probiotic bacteria with differing properties, in order to potentially confer greater health benefits on the host. Further studies are clearly required to elucidate the interaction mechanisms and examine how the probiotic combinations perform *in vivo*. Of particular interest is whether or not strains produce inhibitory or growth-promoting substances that could influence the survival and functionality of the co-administered probiotics in the intestinal tract, and how probiotic combinations interact with gut microbiota.

Chapter IV: Survival of Probiotic Mono-Cultures and Multispecies Combinations During Storage in Orange Juice and Bottled Drinking Water

4.1 Summary

Probiotic bacteria *Lactobacillus rhamnosus* GG (LG), *Lactobacillus reuteri* ATCC 55730 (LR), *Bifidobacterium animalis* subsp *lactis* Bb12 (Bb) and *Propionibacterium jensenii* 702 (PJ), both individually and as 2- or 3-species combinations were incorporated into orange juice (with and without 20% pulp) as well as drinking water. Viability of the probiotic bacteria was monitored at different time points over 8 weeks of refrigerated (4°C) and non-refrigerated (23°C, for drinking water only) storage.

The values for pH and total soluble solid contents (°Brix) of orange juices did not change throughout the storage period indicating minimal metabolic activity of probiotics in juices under refrigeration. Appreciable strain-dependent differences were observed in the survival of the probiotics in carrier drinks during the storage period. With the exception of LR in the presence of Bb, lactobacilli survived in higher number in orange juice than in drinking water under refrigeration. In contrast, a better performance was observed for Bb and PJ in drinking water than in orange juice. Compared to single strains, presence of other probiotics was observed to impact on the viability of probiotics in the experimental carrier drinks. In most cases, presence of pulp was not identified as an influential factor on the viability of probiotics in orange juice. Storage of probiotic drinking water at ambient temperature (23°C) had a detrimental effect on the viability of probiotics with the exception of PJ which exhibited similar survival rates whether under refrigerated or non-refrigerated storage.

These results showed that both chilled orange juice and drinking water could be considered as suitable delivery vehicles for probiotics. Furthermore, the survival of probiotic strains may be influenced by the presence of other strains during storage period and this must be considered when formulating probiotic products.

4.2 Introduction

Probiotics are increasingly being incorporated into food products in order to develop “functional foods” with additional health promoting effects (Champagne *et al.*, 2005; Stanton *et al.*, 2001), but have to date been exploited primarily in the form of commercial dairy based products such as fermented milk and yoghurt (Mattila-Sandholm *et al.*, 2002; Shah, 2001). Development of dairy-free probiotic foods may, however, suit consumers who have allergy to milk products, are lactose intolerant (Luckow & Delahunty, 2004b) or have no desire to eat dairy foods. As an alternative, fruit juices may be considered ideal delivery vehicles for probiotics due to their short gastro-intestinal transit time which may in turn reduce exposure of probiotics to harsh environments such as low pH in the stomach (Post, 2002). Moreover, fruit juices have appealing taste profiles to many consumers and are prominent sources of nutrients such as vitamins, minerals, dietary fibres, phytochemicals and antioxidants (*e.g.* polyphenols and carotenoids). They are therefore considered as healthy and refreshing products, pleasing to a wide range of consumers (Luckow & Delahunty, 2004a, 2004b; Post, 2002; Tuorila & Cardello, 2002). Incorporation of probiotics into fruit juices may further enhance the nutritional value of these products and deliver health promoting probiotic microorganisms to a larger consumer population.

In order for the beneficial health effects of probiotics to be realised, daily consumption of a minimum level of probiotic bacteria is necessary. Defining the specific effective number of probiotic microorganisms depends however on the strain and carrier matrix used (Champagne *et al.*, 2005; Gardiner *et al.*, 2002; Salminen & Playne, 2001). As a general consensus, it has been suggested that the minimum level of viable bacteria should be 10^6 CFU per gram or millilitre of the probiotic product or 10^8 CFU per day at the consumption point (Agrawal, 2005; Boylston *et al.*, 2004; Tamime *et al.*, 1995). It is therefore important that viability of the probiotics remains optimal throughout the anticipated shelf life of the products (Mattila-Sandholm *et al.*, 2002).

The viability of single strains of probiotics has been studied in fruit juices and fruit drinks (Champagne & Gardner, 2008; Champagne *et al.*, 2008; Sheehan *et al.*, 2007). Higher viability of 5 strains of *Lactobacillus* and one *Bifidobacterium* was reported in orange juice (pH 3.65) and pineapple juice (pH 3.40) than in cranberry juice (pH 2.50). Loss of viability of the probiotics occurred more slowly in cranberry juices with higher adjusted pH (pH 4.50 and 5.50) than lower pH values (pH 2.50 and 3.50). Moreover,

different probiotic strains showed different survival rates in the same fruit juice over the storage time (Sheehan *et al.*, 2007). In a further study, storage stability of 9 *Lactobacillus* strains (*Lb. acidophilus* LB2, LB3 and LB45, *Lb. brevis* LB6, *Lb. rhamnosus* LB11 and LB24, *Lb. fermentum* LB32, *Lb. plantarum* LB42 and *Lb. reuteri* LB38) was investigated in a commercial fruit drink (pH 4.2) containing a mixture of fruit juice concentrates, purees and dairy ingredients over a period of 80 days at 4°C (Champagne & Gardner, 2008). The viabilities of *Lb. rhamnosus* LB11 and LB24, *Lb. reuteri* LB38, *Lb. plantarum* LB42 and *Lb. acidophilus* LB45 was maintained throughout the entire storage period in the drink, reducing by less than one order of magnitude across the 80 days of storage, whereas viabilities of *Lb. acidophilus* LB2 and LB3 declined more than 5 logarithmic cycles over the same period (Champagne & Gardner, 2008). Thus these studies have shown that survivability of probiotics in fruit juice/drink is genus, species and strain dependent (Champagne & Gardner, 2008; Sheehan *et al.*, 2007). Furthermore, the type of fruit juice, intrinsic parameters such as pH and the presence of particular compounds (*e.g.* benzoic acid or lactones), as well as extrinsic factors such as storage temperature, storage duration, packaging material, and dissolved oxygen level, have all been considered as decisive factors in determining the survivability of probiotics in these carriers (Champagne & Gardner, 2008; Champagne *et al.*, 2008; Sheehan *et al.*, 2007).

Since probiotic organisms vary in the type and level of their health promoting effects, it is likely that probiotic combinations may potentially offer even greater benefit to the consumer than single strain preparations. Effective design of functional foods containing probiotic combinations, must take into consideration the likely occurrence and impact of potential interactions between individual species within a proposed combination. At this time, to the best of this author's knowledge there are no studies examining the viability of probiotic combinations in fruit juice during storage. Findings in relation to inclusion of mixed cultures in carriers such as fermented dairy products have shown that viability interactions between individual probiotics are evident during storage. A study by Saccaro *et al.* (2009) examined the viability of individual probiotic strains in yoghurt made with starter cultures (*Lb. delbrueckii* subsp. *bulgaricus* LB340 and *Streptococcus thermophilus* TAO) along with paired and triplet probiotic combinations (*Lb. acidophilus* LA5, *Lb. rhamnosus* LBA and *Bif. animalis* subsp. *lactis* BL-04) during 21 days of refrigerated storage. The results of this study showed differences among the survival rate of each probiotic strain in different combinations.

Bif. animalis subsp. *lactis* exhibited a higher viability rate at the end of the storage period when combined with *Lb. acidophilus*, than when combined with *Lb. rhamnosus* or with both lactobacilli in a triplet probiotic combination. *Lb. rhamnosus* showed the highest and lowest viability in the triplet combination and in the presence of *Bif. animalis* subsp. *lactis* respectively.

In the study presented here, probiotics were incorporated into two potential non-dairy carrier products, orange juice (with and without pulp) and non-carbonated bottled drinking water. Orange juice is the most popular fruit beverage worldwide due to its widely appealing flavour and nutritional properties (Rega *et al.*, 2004). According to the Foreign Agricultural Service of United States Department of Agriculture (USDA), total consumption of orange juice in 42 selected countries including members of the EU, USA, China, Brazil and Australia was 2.095, 2.378 and 2.317 million Tonnes at 65 degrees brix in 2007, 2008 and 2009 respectively (USDA, 2010). Orange juice has been considered as a rich source of vitamin C and natural folate. It also contains phytochemicals (*e.g.* polyphenols and carotenoids) and minerals such as potassium, phosphorus, calcium and magnesium (Guarnieri *et al.*, 2007; Ohrvik & Witthoft, 2008; USDA National Nutrient Database for standard Reference, 2009). Orange juice is available in both a 'pulp-included' and 'pulp-free' form. The inclusion of pulp in orange juice may appeal to many consumers as pulp enhances sensory properties such as flavour, aroma, colour and body of the juice (Brat *et al.*, 2003; Rega *et al.*, 2004) in addition to being a good source of dietary fibre (Céspedes *et al.*, 2010). It is also feasible that orange pulp may enhance probiotic viability, as it has recently been reported that inclusion of Açai pulp in a probiotic yoghurt improved viability of probiotics in the product across 4 weeks of cold storage (Santo *et al.*, 2010). Previous to these findings Sendra *et al.* (2008) had reported that citrus fibres enhanced survival of probiotic bacteria in both a MRS medium and fermented milk during storage at 4°C.

Bottled water was also included in the current study because it is one of the most highly consumed drinks worldwide. Classified as a soft drink, global consumption of bottled water doubled over the period 1997-2007 from under 100,000 to over 200,000 million litres per annum, representing more than 1/3 of total soft drink consumption. Moreover, global consumption of bottled water has now overtaken alcoholic beverage consumption (Finlayson, 2005). Thus, as an all-day beverage used by a wide range of consumers, bottled water warrants examination as a potential delivery vehicle for probiotics.

In non-fermented probiotic foods, it is critical to maintain cold conditions (*e.g.* 4°C) throughout the whole supply chain from production to consumption. Refrigeration extends the viability of probiotic microorganisms and inhibits their growth during storage, distribution, and retailing. A break in the cold storage chain may result in increased metabolic activity of microorganisms, and hence unwanted bacterial growth, which may form undesirable flavour in the product. Previous studies on the stability of probiotics in fruit juices have shown that pH values of probiotic fruit juices remained almost unchanged during refrigerated storage indicating little or no metabolic activity of probiotics (Champagne & Gardner, 2008; Champagne *et al.*, 2008; Saarela *et al.*, 2006).

As mentioned before, the viability of probiotics has been studied in single or cocktail fruit drinks (Champagne & Gardner, 2008; Champagne *et al.*, 2008; Saarela *et al.*, 2006; Sheehan *et al.*, 2007), although it has not been compared with that in less complicated carriers such as drinking water. Orange juice has a complex chemical nature characterised by pH values as low as 3.5 and with many constituents including different type of sugars, complex carbohydrates, organic acids, vitamins, flavonoids, and minerals, each of which might differentially affect the viability of included probiotics.

Generally the optimal pH for probiotics varies between 5.5 and 6.5 (Champagne, 2009). Previous research has shown that viability of probiotics was adversely affected during storage in fermented milks with pH values between 4.0 and 5.0 (Champagne *et al.*, 2005). Lower pH values like those found in fruit juices (pH 2.5 - 3.7) make the situation worse (Sheehan *et al.*, 2007). However chemical compounds which could be found in fruit juices such as glucose (Corcoran *et al.*, 2005), ascorbic acid (Shah *et al.*, 2010), hesperidin (the main flavonoid present in orange juice) (McGill *et al.*, 2004; Wilmsen *et al.*, 2005), and minerals such as NaCl (Bucio *et al.*, 2005) might improve the viability of probiotics. Considering combined effects of all these factors, it has been shown that some probiotic bacteria could maintain viability in fruit juices such as orange juice at reasonable levels during storage (Sheehan *et al.*, 2007).

With regard to drinking water, pH is neutral to slightly alkaline (pH 6.5-9.5) and contains some minerals and no or very little organic content (Belitz *et al.*, 2009; Dege, 2005). Little is known about the effect of water on probiotic stability during storage. It could be expected that drinking water with neutral pH may support viability of probiotics better than that with alkaline pH value. However the effect of minerals present in the drinking water and absence of organic chemicals should be considered. It

has been shown that addition of NaCl to distilled water enhanced the viability of *Lb. plantarum* compared with the control (Bucio *et al.*, 2005).

The present study involved incorporation of 4 probiotic strains *Lb. rhamnosus* GG (LG), *Lb. reuteri* ATCC 55730 (LR), *Bif. animalis* subsp *lactis* Bb12 (Bb) and *P. jensenii* 702 (PJ) in various combinations, into both orange juice (OJ) and bottled water (BW). To achieve maximum accuracy, of four *Lactobacillus* strains used in the previous study (Chapter 3), LG and LR were selected to be used in this study as these two strains are among the most extensively studied commercial probiotic lactobacilli with a wide range of health benefits.

The objective was to monitor probiotic viability over 8 weeks of storage in order to examine the influence of the carrier matrix, storage temperature, and potential species interactions on probiotic viability, and thereby assess the suitability of these products as probiotic carriers.

More specifically, the investigation was designed to address the following hypotheses:

- That the viability of probiotic bacteria in orange juice and bottled water would exhibit species/strain dependent variation.
- That the food matrices (orange juice and water) would differentially influence the viability of the probiotics.
- That the inclusion of pulp in orange juice would enhance probiotic viability relative to the pulp-free juice.
- That when incorporated into drinking water, the viability of the probiotics would be compromised by storage at room temperature compared with refrigeration.
- That the viability of individual probiotics in orange juice and bottled water during storage, would be affected by the presence of other probiotic bacteria.
- That the inclusion of probiotics in orange juice would not alter intrinsic properties of the carrier product such as pH and total soluble solids content (Brix).

4.3 Materials and methods

4.3.1 Chemicals and reagents

Unless otherwise specified, chemicals and bacterial culture media used in this study were respectively from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA) and Oxoid Australia (Oxoid Australia Pty Ltd, Adelaide, Australia).

4.3.2 Bacterial strains and growth conditions

Apart from the absence of LC and LP in this study, all other strains (LG, LR, Bb and PJ) and growth conditions were as described in Chapter 3.

4.3.3 Orange juice and drinking water

A commercially available orange juice (OJ) with pulp (The Original Juice Co., a division of Golden Circle Ltd, Mill Park, VIC, Australia) and bottled spring drinking water (Mount Franklin, Coca-Cola Amatil, Pty, Ltd, Sydney, Australia) were used in this study. OJ had been pasteurised at 85°C, for 15 sec by the manufacturer and contained no added preservatives or sugar. The OJ has a 38-day shelf life at 4°C. BW was sterilised at 121 °C, for 20 minutes. Pulp-free OJ was obtained by centrifugation (Eppendorf centrifuge model 5810R, Eppendorf AG, Hamburg, Germany) of the OJ with pulp at 450 ×g for 10 min, with collection of the supernatant as pulp-free juice. Amounts of 50 ml of the OJ (with pulp and pulp-free) and BW were dispensed in sterile screw cap polypropylene containers (Sarstedt Australia Pty Ltd, Mawson Lakes, SA, Australia). All containers were then refrigerated (4 °C) prior to use.

4.3.4 Preparation of probiotic orange juice and drinking water

Bacterial cells were harvested from fresh probiotic cultures in their stationary phases by refrigerated centrifugation (4 °C) at 3220 ×g for 15 minutes and washed three times with Dulbecco's Phosphate-Buffered Saline pH 7.0 (PBS) (Gibco, Invitrogen Corp., Carlsbad, CA, USA). Bacterial pellets were then resuspended in PBS. An aliquot of 500 µL of each bacterial suspension was incorporated into the 50 mL portions of the juice and BW. OJ containers were then stored at 4 °C for 8 weeks. BW containers were stored at 4 °C or 23 °C.

4.3.5 Monitoring the survival of probiotics in drinks

Bacterial counts were determined by plating 100 µL aliquots of decimal dilutions prepared from OJ and BW on selective agar plates at days 0, 1, 4, 7, 14, 21, 28, 42 and 56. Lactobacilli either alone or in combination with PJ were enumerated on LBS agar (Rogosa *et al.*, 1951a) after incubating at 37°C for maximum 72 h under anaerobic condition. In the presence of Bb, lactobacilli were counted on MRS agar supplemented with 1 mg/mL Vancomycin (MRS-V) after anaerobic incubation of the plates at 43°C for 24 hours. Growth of *Bif. animalis* subsp *lactis* Bb12 is inhibited on MRS-V under the incubation conditions described above (Tharmaraj & Shah, 2003). PJ was counted on YEL agar (Malik *et al.*, 1968a) after anaerobic incubation of the plates at 30°C for 7 days. Bb was counted on TOS propionate agar (Yakult Pharmaceutical Ind., Co., Ltd, Tokyo, Japan) following anaerobic incubation of the plates at 37°C for 2 days. The results are expressed as Log CFU/mL of OJ or BW.

4.3.6 Measurement of pH and Brix of drinks

pH of the juice and BW was measured using a Cyberscan 510 pH meter (Eutech Instruments Pte Ltd., Singapore) on the same days as the survival determinations. Total soluble solids or Brix was determined by the refractometer method of the Association of Analytical Chemists (AOAC, 1990) using a refractometer model REF113 (Bacto Laboratories Pty Ltd, Liverpool, Australia).

4.3.7 Scanning electron microscopy

In order to qualitatively evaluate the effect of carrier matrix on the morphology of probiotics included in OJ and BW, scanning electron microscopy was performed. Five (5) mL of well mixed probiotic OJ or BW was taken and centrifuged at 3220 ×g for 10 min. The supernatant was then discarded and the pellet was washed twice with deionised water. The resultant pellet was resuspended in 2 mL of deionised water and well vortex mixed. One (1) mL of the suspension was added to a well of a 24-well micro-plate with a 13 mm cover-slip (Sarstedt Inc., Newton, NC, USA) at the bottom of each well. After 1.5 h incubation at room temperature, cover-slips were removed from wells. Sample fixation and SEM procedure were as described in Chapter 3.

4.3.8 Statistical analyses

Statistical analyses were performed using SPSS software Ver. 18 (SPSS Inc., Chicago, IL, USA). The viability changes of each probiotic strain either alone or in combinations during the storage period and pairwise comparisons at each time point were carried out using a General Linear Model (GLM). A p value ≤ 0.05 was considered statistically significant.

4.4 Results

4.4.1 Changes in the viability of probiotics in the carrier drinks during storage

In order to assess strain dependent variation in probiotic viability and the effect of the carrier matrix, the examination of results in this chapter focuses initially on the mono-culture preparations in the OJ and BW. This is followed by the effect of storage temperature via a comparison of viabilities in BW stored at two different temperatures (4 and 23°C), and finally with an examination of the effect of combining probiotics on individual strain viabilities in these carriers. In general bacterial strain, type of carrier matrix (OJ or BW), presence of other probiotics (in combinations) and storage temperature (for BW), all appeared to impact on the survival of the probiotics examined. In most cases however, there appeared to be little difference between the survival of probiotics included in OJ with and without pulp.

4.4.1.1 Strain dependent variation in probiotic viability

Appreciable differences were observed among mono cultures of the probiotic strains incorporated into juices and BW with respect to their survival during the storage time. In contrast however, with the exception of LR, there was little difference observed between the survival of mono-cultures of probiotics included in OJ with and without pulp. Hence, results relating to strain dependent differences in the viability of probiotics in pulp-free OJ are presented here, and the effect of pulp on probiotics is presented in section 3. In order to standardise the comparisons of viability, the bacterial counts at each time point throughout the storage period have been expressed as a percentage of the initial count for each strain (Log CFU/ml).

In OJ, LG was in general found to be the most stable of the probiotics examined, with viability remaining virtually constant throughout the entire storage period (Figure 4.1). In the mono-cultures of LR and Bb viability was also maintained throughout the entire storage period in OJ with numbers, expressed as Log CFU/mL, reducing by about 10% across the 8 weeks of storage. By comparison, the viability of PJ in OJ remained at almost consistent levels in the first 4 weeks but declined steeply from this point, with no viable cells recovered at the end of storage.

SEM micrographs of PJ in OJ showed that duration of refrigerated storage resulted in extensive cellular shrinkage (Figure 4.2). Morphological changes however were not observed for other probiotics during storage.

In chilled BW, Bb and PJ were the most stable of the probiotics examined with cell counts of both remaining relatively unchanged by the end of the storage period. In contrast, viable cell counts of the two lactobacilli were not maintained in the bottled water. The viability of LG was observed to remain almost unchanged after 2 weeks of storage but then sharply declined, with no viable cells recovered by day 42, while the viability of LR exhibited a continuous decrease throughout storage such that the bacterium could not be recovered at all by day 56.

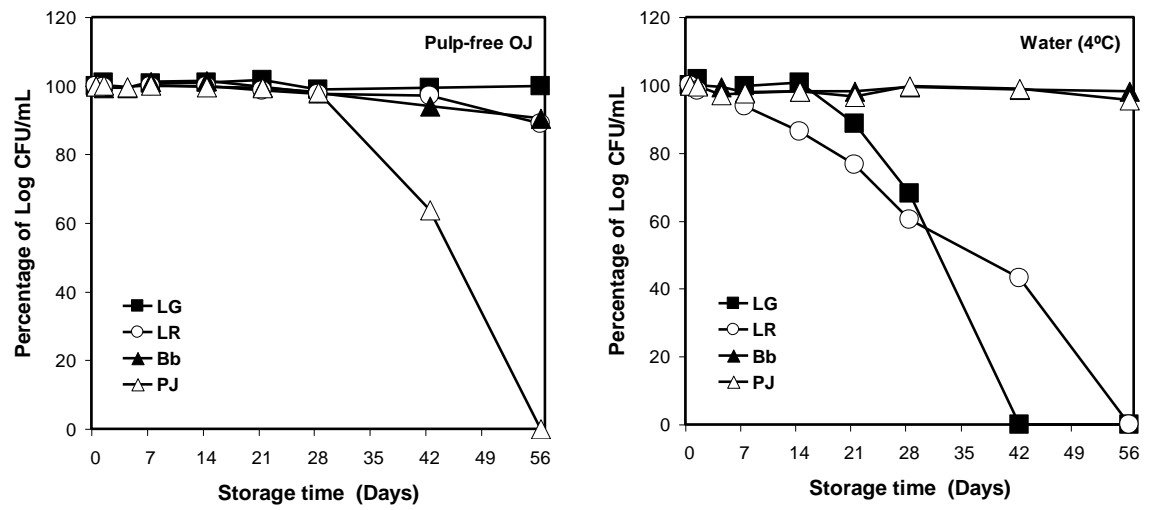


Figure 4.1: Viable cell counts of mono-cultures of each probiotic strain in pulp-free OJ and BW over 8 weeks of storage at 4°C.

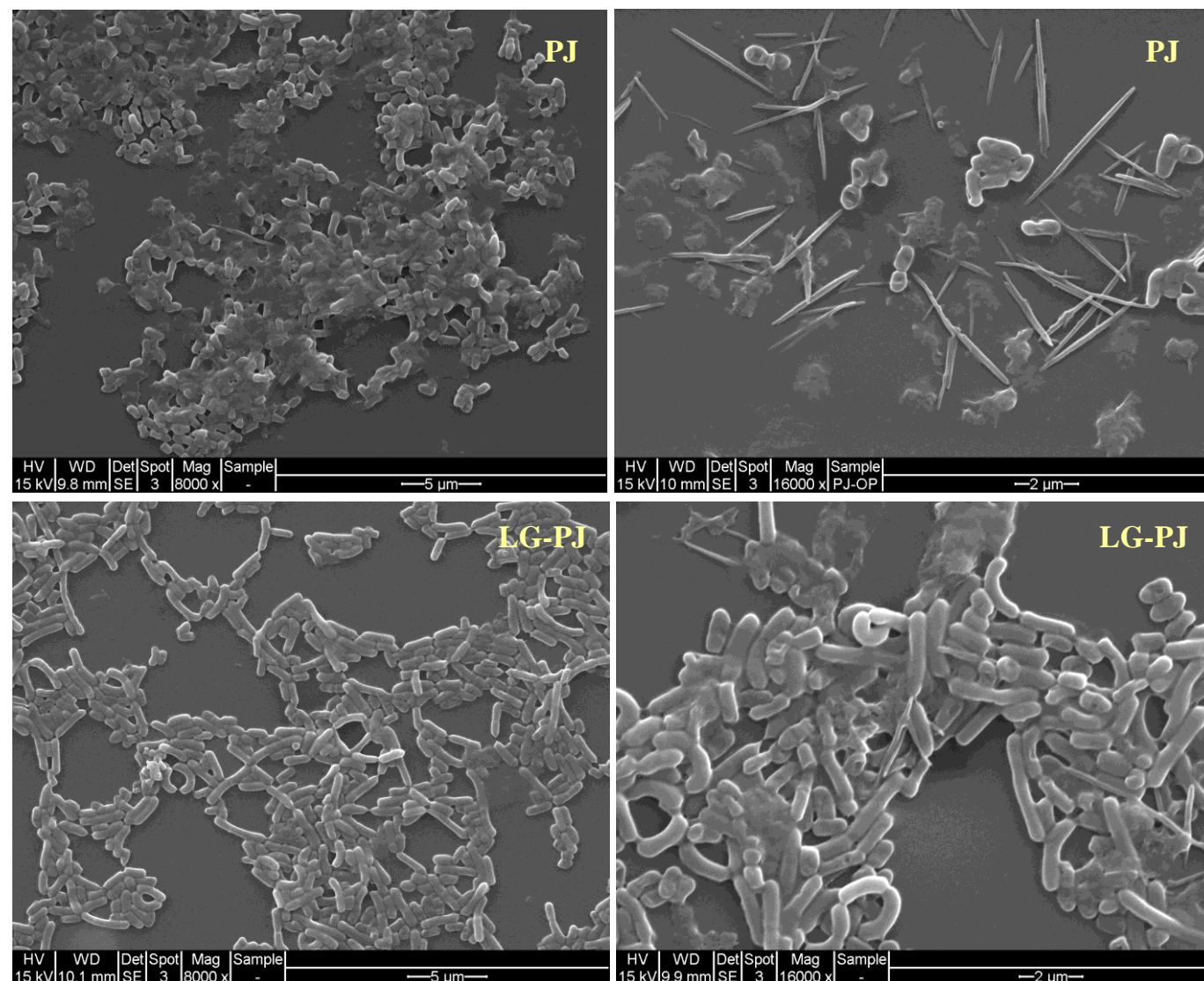


Figure 4.2: Scanning electron micrographs showing differences between the morphology of PJ cells, both in mono-culture (top row) and in combination with LG (bottom) after 3 (left) and 8 weeks (right micrographs) of storage in OJ.

4.4.1.2 Effect of carrier matrix on the viability of probiotics

Examination of Figure 4.3 clearly indicates that the carrier matrices (pulp-free OJ and BW) impacted differently on the viability of the probiotics during refrigerated storage.

The mono-culture of LG remained stable throughout the entire storage period in OJ, whereas in chilled BW, the bacterium was not found to be as stable with viability in the water remaining almost unchanged after 2 weeks of storage after which time cell numbers sharply declined to the point that no viable cells were recovered after week 6. Statistically, all viable cell counts of LG were significantly lower in BW than that of OJ at the same time points after and including day 21 of storage ($p \leq 0.05$).

Viability of LR remained almost unchanged during the first 6 weeks of storage in pulp-free OJ before reducing by less than one order of magnitude across the last 2 weeks of storage. By comparison LR exhibited a continuous decrease during storage such that the viability was lower in BW than that of OJ at the same time points after first week of storage ($p \leq 0.05$).

The viability of the mono culture of Bb remained almost unchanged in OJ by the end of week 4 followed by a steady decrease by less than one order of magnitude over the next 4 weeks while the viability remained at almost consistent levels in the BW throughout the storage period at 4 °C.

Viable cell counts of the mono-culture of PJ in OJ remained at almost consistent levels in the first 4 weeks and then decreased steeply by 2.5 logarithmic cycles between week 4 and 6, and no viable cells were recovered at the end of storage. By comparison, PJ appeared to be more stable in refrigerated BW than OJ and remained relatively strong over the storage time at 4 °C.

In general the lactobacilli performed well in OJ but far less so in the bottled water, while the reverse was true for PJ. Of the four organisms examined only Bb maintained a high level of viability in both products across 8 weeks of storage.

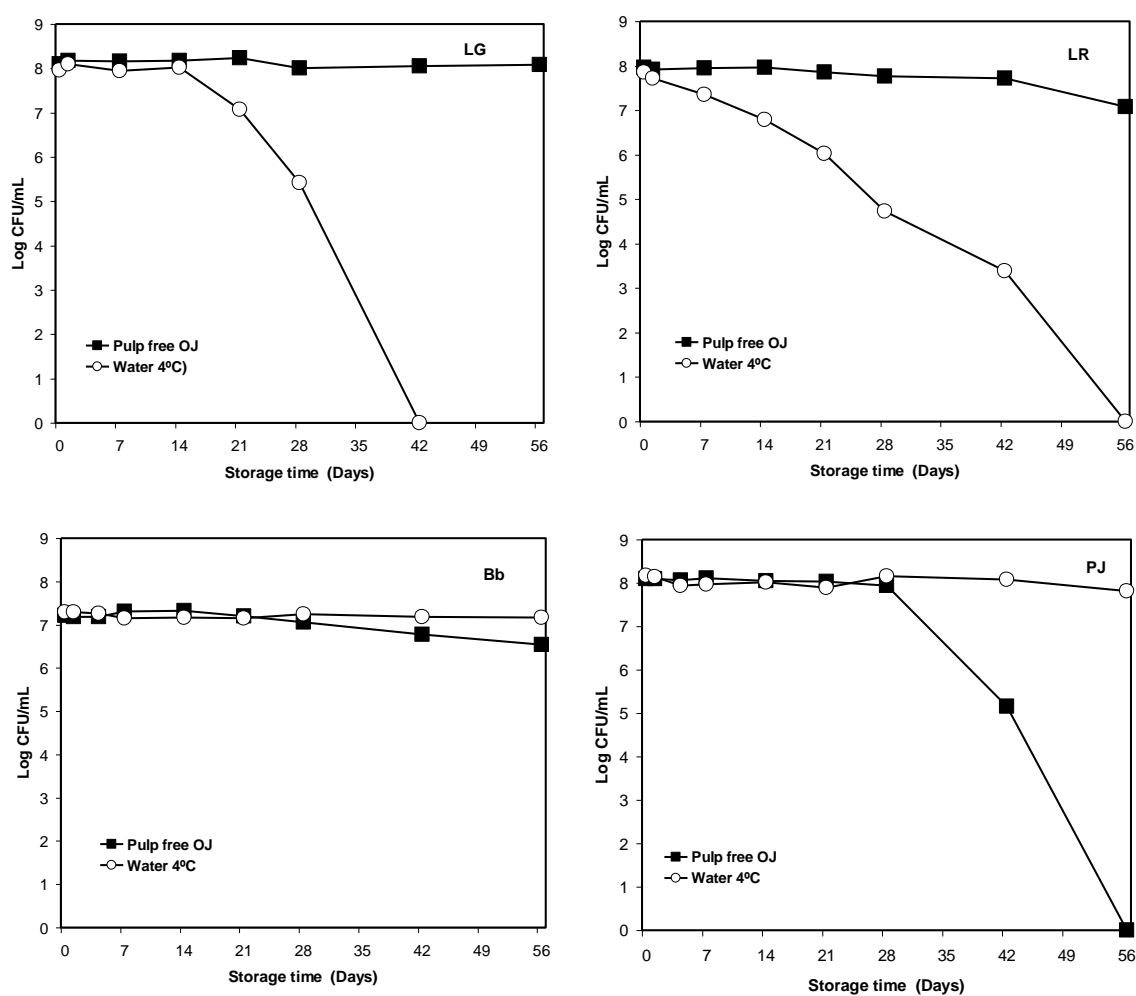


Figure 4.3: Comparison of the viability of mono-cultures of probiotics examined in pulp-free OJ and BW over 8 weeks of storage at 4°C.

4.4.1.3 Impact of pulp on the viability of probiotics in orange juice

Among the mono-culture preparations, significant variation in viability when incorporated into OJ either with or without pulp, was only observed in the case of LR, while among the co-culture preparations significant variation was observed only in the cases of Bb, when combined with either LG or PJ, and PJ when combined with LG. Thus in the majority of cases (16 out of 20), there was little difference between the viability of probiotics in OJ with and without pulp (Data not shown). However, where significant variation was observed, viability was found to decline more rapidly in the OJ containing pulp in all cases (Figure 4.4).

SEM micrographs showed that probiotics were scattered amongst the pulp particles (Figure 4.5), however according to the data, presence of pulp did not protect probiotics and improve their viability.

The figure (4.4) reveals that by comparison with the pulp-free OJ the viability of LR deteriorated in the preparation containing pulp after week 4, such that viable cell counts had declined significantly by day 42, and no viable cells were recovered at the end of storage ($p \leq 0.05$).

Pulp also appeared to adversely affect the viability of Bb when combined with LG or PJ after week 4 and 2 of storage respectively, relative to the pulp-free juice. It should be recognised however that in the combination with PJ, significant declines in the viability of Bb were evident in both carriers across the last 5 weeks of storage, with no viable cells detected by day 56 in either product. Similar declines in the viability of Bb were also evident in both products over the last 2 weeks of storage when co-cultured with LG (Figure 4.4) ($p \leq 0.05$).

The viability of PJ in combination with LG was observed to follow a steady decline in both pulp-free and -included OJ throughout the storage period, reducing by 3 logarithmic cycles by the end of week 6 of storage time. Viability of PJ however reduced by less than one order of magnitude in pulp-free OJ during the last 2 weeks of storage whereas it decreased dramatically by 4 orders of magnitude in pulp-included OJ during the same period and no viable cells were recovered at the end of storage (Figure 4.4).

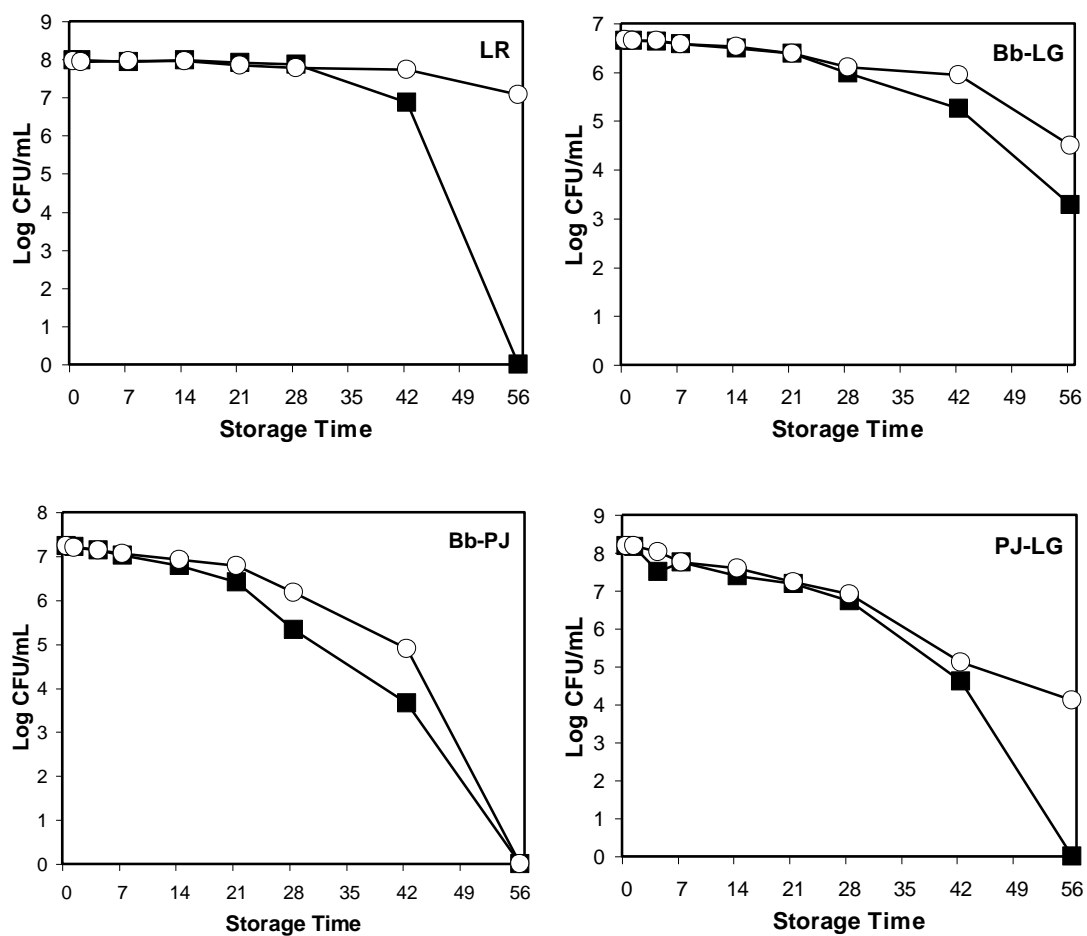


Figure 4.4: Viable cell counts of probiotics in OJ with (■) and without (○) pulp over 8 weeks of storage at 4°C. In combinations, the counts of the first listed bacterium have been reported.

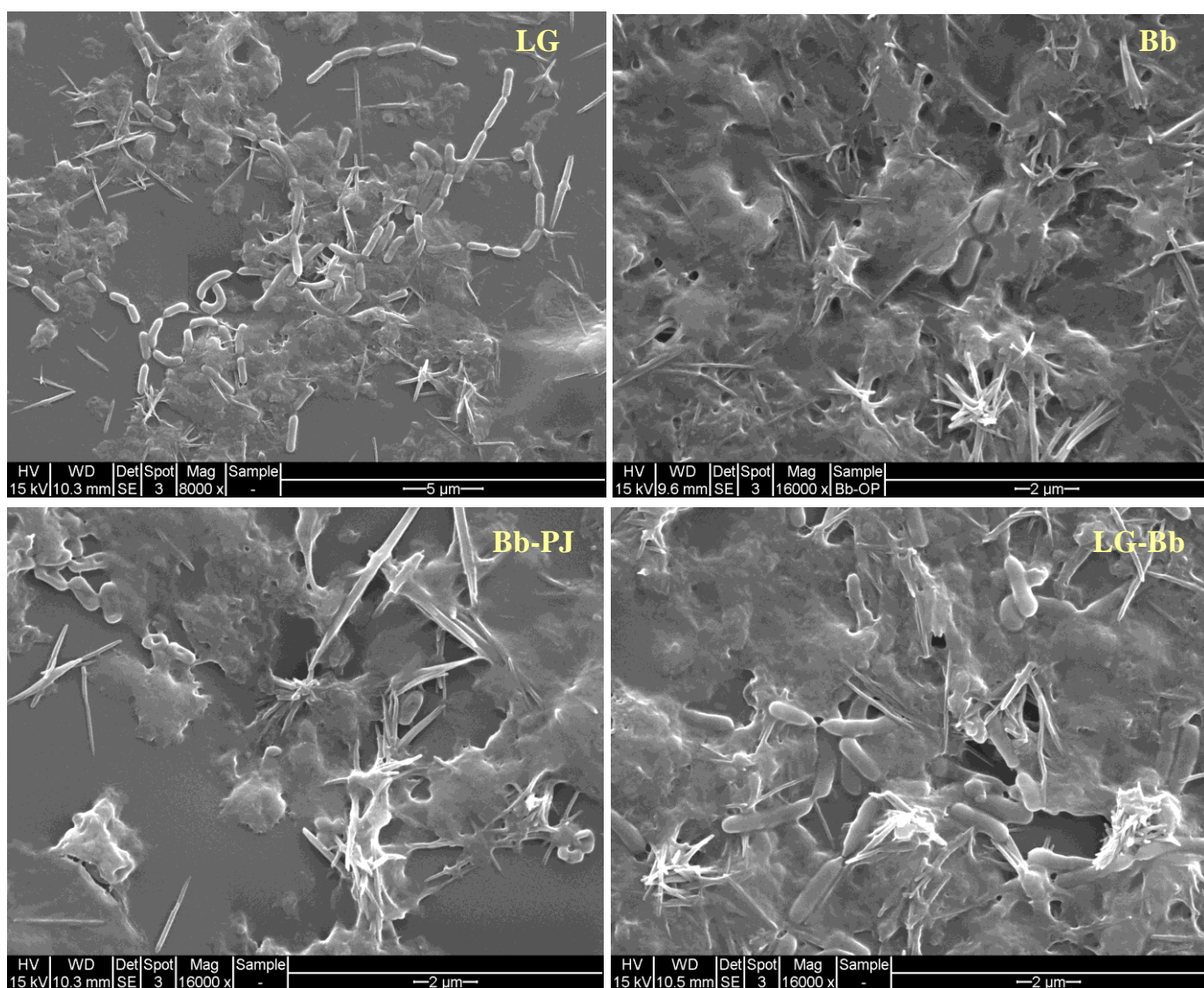


Figure 4.5: SEM micrographs of probiotics scattered among pulp particles in OJ.

4.4.1.4 Effect of storage temperature (4 or 23°C) on the viability of probiotics in drinking water

Examination of the data regarding viability of mono-cultures in bottled water indicated that with the exception of PJ, the probiotics performed poorly in water stored at 23°C, with the effects most dramatic in the case of the lactobacilli. In chilled BW the viability of LG remained almost unchanged for the first 2 weeks of storage but sharply declined below the effective threshold level (10^6 CFU/mL) by the end of week 4, with no viable cells recovered after week 6. However, the decline in viability of LG was found to be even more dramatic when the carrier was stored at room temperature (23 °C), such that the viability reduced rapidly during the first few days of storage and subsequently dropped to zero within the first week (Figure 4.6).

In the case of LR a continuous decrease in viability was observed during storage in chilled water (4°C), such that counts of the bacterium fell below 10^6 CFU/mL after week 3 and viable cells could not be recovered at the end of storage. By comparison, LR had ceased to be viable by the end of the 2nd week of storage at 23 °C.

Bb remained relatively unchanged in the chilled BW over 8 weeks of storage at 4 °C. When compared with the lactobacilli, the viability of Bb in water was found to be substantially more stable, but appeared nonetheless to be adversely affected by unrefrigerated storage. In this case viability was observed to decrease dramatically to a suboptimal level just below 10^6 CFU/mL in the first week of storage, remaining at this level by week 2, but then sharply declining such that no viable cells were recovered after 6 weeks of storage.

In contrast with the other strains examined, PJ was found to maintain a high level of viability throughout the storage period, not only in the chilled water but also in the unrefrigerated product, with viable cell counts remaining $>10^7$ CFU/mL in both cases.

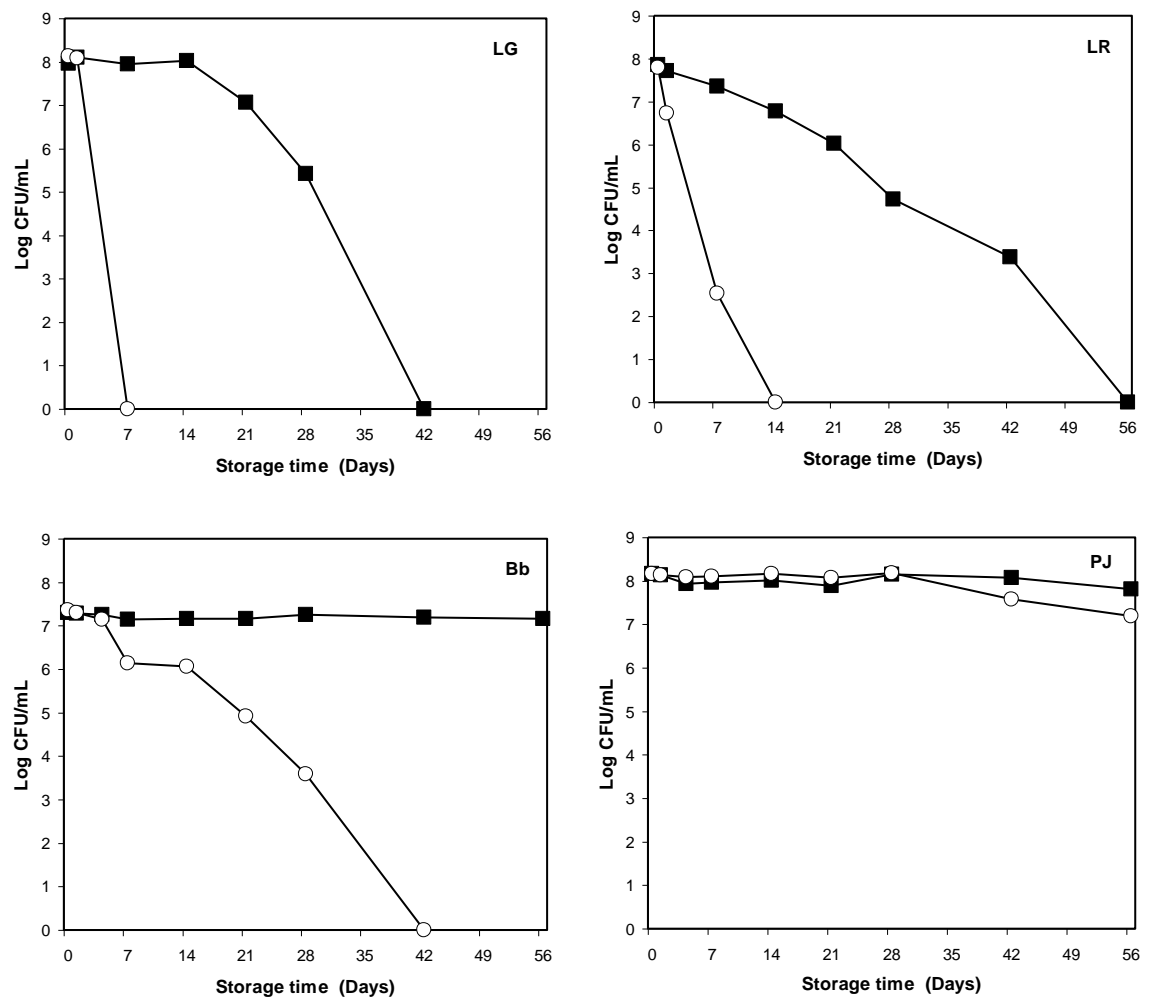


Figure 4.6: Viable cell counts of probiotics in BW over 8 weeks of storage at (■) 4 °C and (○) 23 °C.

4.4.1.5 Probiotic combinations and individual strain viability

In order to assess the effect of combining probiotics on individual strain viability during storage, probiotic combinations were prepared and introduced in 4 different carrier product/storage scenarios - OJ with and without pulp (both stored at 4°C), and bottled water stored at either 4°C or 23°C. The probiotic combinations trialled in each carrier included five paired combinations (LG-Bb, LG-PJ, LR-Bb, LR-PJ, Bb-PJ), and two triplet combinations (LG-Bb-PJ and LR-Bb-PJ), providing a total of 28 separate preparations. In each case the viability of each individual strain was monitored throughout 8 weeks (56 days) of storage, and compared with its viability as a mono-culture in the same carrier. In general, the data revealed little if any difference between the results obtained in OJ with or without pulp. In bottled water poor viability was observed at 23°C for all strains except PJ, for which no discernible difference was apparent in relation to storage temperature. Thus for the purposes of clarity, the presentation of data in this section has been restricted to the results obtained in pulp-free OJ and water stored at 4°C. (*NB: data pertaining to OJ with pulp, and water at 23°C, can be found in appendix A*)

In Figure 4.7, the viability of individual strains in each combination has been plotted along with the respective plot for each mono-culture. The figure reveals that in pulp-free OJ, the observed stability of the LG mono-culture remained completely unaffected by the presence of Bb and/or PJ, while the findings in relation to the other strains were more variable. In particular, the viabilities of both LR and Bb were found to decline significantly when combined together, as was the case for the Bb-PJ combination. In contrast, the combination of all three (LR-Bb-PJ) produced no decline in the viability of either LR or Bb, and a significant enhancement to the viability of PJ, which in this case remained constant throughout the entire 8 week storage.

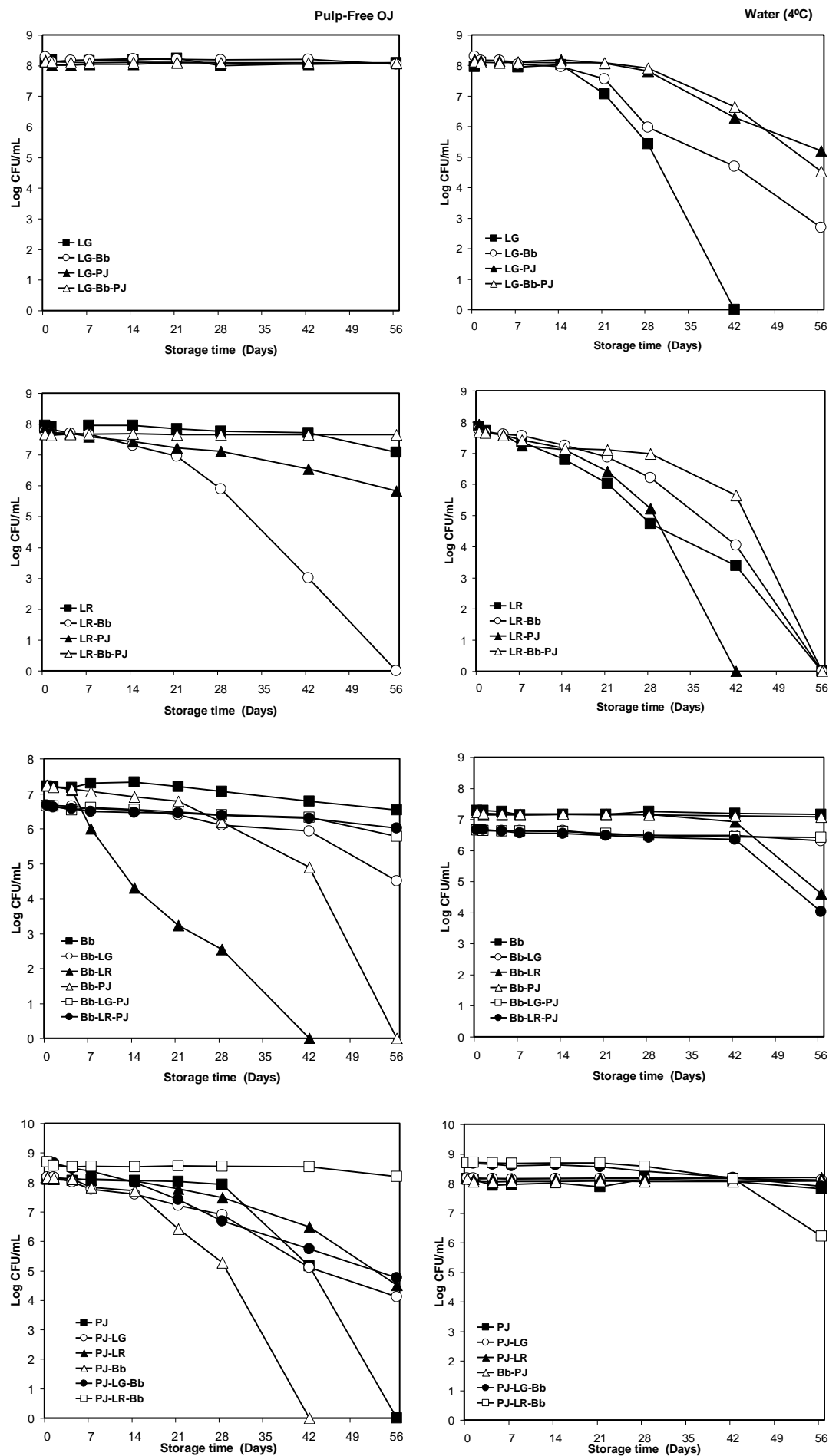


Figure 4.7: Viabilities of the four probiotics, both in mono-culture and in the designated combinations, in pulp-free OJ and BW over 8 weeks of storage at 4°C.

In fact, with exception of the pairing with Bb, the viability of PJ in OJ was found to improve in all combinations by comparison with its viability in mono-culture.

A similar result was observed for LG in bottled water at 4°C, with enhanced viability exhibited in all combinations (Figure 4.7). The viability of LR was also observed to improve in water when combined with either Bb, or Bb and PJ, but was found to decline significantly after day 28 when combined with PJ only. In the bottled water the LR-Bb-PJ combination appeared to be slightly less favourable for both Bb and PJ, although in these cases decreases in viability were only evident beyond day 42. In all other cases, in both OJ and bottled water, the viability of the individual probiotics appeared to be largely unaffected by the presence of other strains.

A more qualitative overview of the effect of probiotic combinations on individual strain viability was obtained by categorising the 32 separate viability plots in Figure 4.7 in terms of the overall trend observed in each, relative to the corresponding mono-culture plot. The results are detailed in Table 4.1. When summarised in this way it can be seen that in 22 cases overall (69%), the viability of the individual strain concerned was either enhanced or unaffected in the presence of other probiotics. When confined exclusively to the outcomes of triplet combinations, this proportion was found to increase to 83% (10 of 12 cases). Of the 10 cases in which a relative decline in viability was observed, only half (5) involved a >2 Log reduction in viable cell numbers across the entire storage period, all of which involved paired combinations. In general, while the hypothesis that ‘combining probiotics in these carriers would not affect viability’ was clearly not supported by the observed variability, significantly impaired viability was only evident in a small minority of cases.

To summarise, LG and PJ appeared to be the strains most amenable to combining with other probiotics in OJ and BW as the carriers, and Bb the least. Probiotic combinations involving the lactobacilli appeared to greatly improve the viability of PJ in OJ, while conversely in water, the viabilities of the lactobacilli were generally enhanced in combinations involving PJ. Finally and perhaps most significantly, triplet combinations were found to produce a greater proportion of improved viability outcomes, and far fewer adverse outcomes, when compared with paired combinations.

Table 4.1: Qualitative summary of individual strain viability in various probiotic combinations, expressed in terms of the viability in combination relative to viability as a mono-culture in the same carrier. (-) = decreased; (+) = increased; (N) = not affected

Combination	Orange Juice				Bottled Water			
	LG	LR	Bb	PJ	LG	LR	Bb	PJ
LG-Bb	N		-		+		N	
LG-PJ	N			+	+			N
LR-Bb		-	-			+	-	
LR-PJ		-		+		-		N
Bb-PJ			-	-			N	N
LG-Bb-PJ	N		N	+	+		N	N
LR-Bb-PJ		N	N	+		+	-	-

4.4.2 pH and Brix changes in orange juices

Both the total soluble solids (sugar) content and pH of the various OJ preparations were monitored throughout the storage period, as a means of detecting potential metabolic activity of the probiotics during refrigerated storage.

Comparison of initial and final pH values in OJ revealed that statistically pH was significantly lower on average at the end of the storage period (Table 4.2). However, pH values were found to remain relatively constant with variations restricted to ≤ 0.1 unit (range = 3.73 – 3.83) across the entire data set. This coupled with the fact that a similar difference between initial and final pH values was also observed in the control preparation (*i.e.* no probiotics present) would suggest that the probiotics had effectively no impact on the pH of the juice.

Brix values were also found to be highly consistent across the entire data set (168 measurements in total), with all recorded values within the range 10.3 – 10.7 and little variation evident either between preparations, or with time (Table 4.3).

Table 4.2: pH values of probiotic OJs (with and without pulp) before and after 56 days of storage at 4°C.

Probiotic(s)	Delivery vehicle					
	Pulp Free OJ			OJ With Pulp		
	^a D0	D56	^b Mean ± SD	D0	D56	Mean ± SD
Control	3.83	3.8*	3.81 ± 0.02	3.81	3.82	3.8 ± 0.02
LG	3.82	3.75*	3.79 ± 0.02	3.83	3.76*	3.79 ± 0.03
LR	3.82	3.8	3.8 ± 0.01	3.83	3.83	3.81 ± 0.02
Bb	3.83	3.82	3.8 ± 0.02	3.815	3.82	3.78 ± 0.03
PJ	3.82	3.84*	3.81 ± 0.03	3.8	3.83*	3.8 ± 0.02
LG-Bb	3.82	3.78*	3.78 ± 0.02	3.82	3.78*	3.79 ± 0.02
LG-PJ	3.81	3.78*	3.79 ± 0.02	3.81	3.78*	3.78 ± 0.04
LR-Bb	3.83	3.79*	3.79 ± 0.02	3.83	3.76*	3.79 ± 0.02
LR-PJ	3.82	3.76*	3.79 ± 0.03	3.81	3.76*	3.79 ± 0.03
Bb-PJ	3.83	3.76*	3.78 ± 0.02	3.82	3.75*	3.78 ± 0.02
LG-Bb-PJ	3.82	3.77*	3.8 ± 0.02	3.83	3.77*	3.79 ± 0.02
LR-Bb-PJ	3.81	3.78*	3.8 ± 0.01	3.81	3.8*	3.8 ± 0.01

^a Data for days 0 and 56 represent means of three pH readings. An asterisk (*) indicates a statistically significant difference ($p \leq 0.05$) between the two means.

^b Data represent means ± standard deviation of pH readings each performed in triplicate obtained from 8 time points during storage.

Table 4.3: Total soluble solids contents (Brix) of probiotic OJs (with and without pulp) before and after 56 days of storage at 4°C.

Probiotic(s)	Delivery vehicle					
	Pulp Free OJ			OJ With Pulp		
	^a D0	D56	^b Mean ± SD	D0	D56	Mean ± SD
Control	10.5	10.6	10.5 ± 0.09	10.5	10.5	10.5 ± 0.05
LG	10.5	10.5	10.5 ± 0.04	10.5	10.5	10.5 ± 0.04
LR	10.6	10.5	10.5 ± 0.09	10.5	10.5	10.5 ± 0.06
Bb	10.4	10.5	10.5 ± 0.05	10.5	10.4	10.5 ± 0.06
PJ	10.5	10.7*	10.5 ± 0.09	10.5	10.5	10.5 ± 0.09
LG-Bb	10.5	10.5	10.5 ± 0.07	10.5	10.5	10.5 ± 0.05
LG-PJ	10.5	10.5	10.5 ± 0.08	10.6	10.6	10.5 ± 0.05
LR-Bb	10.5	10.5	10.5 ± 0.07	10.5	10.4	10.6 ± 0.11
LR-PJ	10.6	10.4*	10.5 ± 0.06	10.5	10.5	10.5
Bb-PJ	10.4	10.5	10.5 ± 0.08	10.5	10.5	10.5 ± 0.05
LG-Bb-PJ	10.5	10.5	10.5 ± 0.08	10.5	10.6	10.6 ± 0.05
LR-Bb-PJ	10.5	10.5	10.5 ± 0.07	10.5	10.6	10.6 ± 0.09

^a Data for days 0 and 56 represent means of three Brix measurements. An asterisk (*) indicates a statistically significant difference ($p \leq 0.05$) between the two means.

^b Data represent means ± standard deviation of Brix measurements each performed in triplicate obtained from 8 time points during storage.

4.4.3 Shelf life of the product

For probiotic foods to provide the desired health benefits, the number of viable probiotic cells present in the food must be maintained at a suitable level throughout the shelf life of the product. A minimum level of 10^6 CFU/ml has been recommended to be present in probiotic foods at the time of consumption.

According to the manufacturer, the OJ used in the current study had a 38-day shelf life at 4°C, therefore probiotic OJs in which the minimum effective level of viable microorganisms was maintained for more than 42 days could be considered acceptable (blue cells in table 4.4). In the case of combinations, the shelf-life was determined as the number of days for which viable cell counts of all probiotic constituents remained above 10^6 CFU /mL. In most cases, pulp did not affect the shelf life of probiotic OJs. OJs containing a mono-culture of either LG, LR or Bb, paired combinations of LG-Bb and LR-PJ, and triplet combinations, all maintained the minimum effective level of viable cell counts of all probiotic constituents beyond week 6. Yellow cells show the probiotic OJs in which the viability of at least one of probiotic constituents dropped below the minimum recommended level between day 28 and 42. These included PJ and LG-PJ. Since the exact day of declining below the effective threshold was unknown in these cases, they may be considered marginal. Probiotic OJs including LR-Bb and Bb-PJ with shelf-life less than 28 days were clearly not acceptable preparations (pink cells).

The same shelf-life grouping as probiotic OJ was used for probiotic BW. Under refrigerated storage both Bb and PJ, and paired and triplet combinations containing both of them, exhibited shelf-lives greater than 6 weeks. At room temperature however only the preparation containing the mono-culture of PJ exhibited an acceptable shelf life.

Table 4.4: Shelf-life (in Days) of the probiotic drinks in terms of cell counts above the minimum effective level of viable microorganisms ($\geq 10^6$ CFU /mL). The colour scheme groups the shelf-lives into 3 categories: blue, >6 wks; yellow, 4-6 wks and pink, 0-4 wks.

Probiotic(s)	Delivery vehicle			
	Pulp Free OJ	OJ With Pulp	BW 4 °C	BW 23 °C
LG	> 56	> 56	21- 28	4 - 7
LR	> 56	42-56	14-21	4-7
Bb	> 56	> 56	> 56	14-21
PJ	28 - 42	28 - 42	> 56	> 56
LG-Bb	42-56	28 - 42	28 - 42	0-4
LG-PJ	28 - 42	28 - 42	42-56	1-4
LR-Bb	7-14	7-14	28 - 42	4-7
LR-PJ	42-56	42-56	21-28	4-7
Bb-PJ	21-28	21-28	> 56	7-14
LG-Bb-PJ	42-56	42-56	42-56	4-7
LR-Bb-PJ	>56	42-56	42-56	4-7

4.4.4 Summary of key findings

1. In OJ, mono-cultures of LG, LR and Bb remained at almost consistent levels throughout the refrigerated storage whereas PJ did so only by the end of week 4 and then decreased sharply over the next 4 weeks.
2. In chilled BW (4 °C), Bb and PJ remained at almost consistent levels during the storage period, whereas LG and LR were adversely affected.
 - These results (findings 1 and 2) therefore provide evidence to support the experimental hypotheses with regard to strain and carrier dependent variation in viability.
3. In OJ, when compared with its viability in mono-culture, viable cell counts of LG remained largely unaffected in the presence of other probiotics. Viabilities of LR and Bb were adversely affected in 2-species combinations, while in 3-species combinations, viabilities were restored to the levels of the bacteria alone. With the exception of pairing with Bb, the viability of PJ was enhanced in all other combinations with the best outcome in the LR-Bb-PJ case, in which viability of PJ remained almost constant throughout the entire storage period.
4. In chilled BW (4 °C), compared with their viability in mono-culture the viabilities of Bb and PJ remained almost unaffected in the presence of other probiotics during the storage period. By comparison with their viability in mono-culture, viability of the lactobacilli improved in all combinations with the exception of LR-PJ.
 - The results (findings 3 and 4) were therefore partly in support of the hypothesis that viability of individual probiotics in OJ and bottled water, would be affected by the presence of other probiotic bacteria during storage.
5. In bottled water poor viability was observed at 23°C for all strains with the exception of PJ which remained at almost consistent levels across 8 weeks of storage.
 - The results (finding 5) were therefore predominantly supportive of the hypothesis that the viability of the probiotics would be compromised by storage at room temperature compared with refrigeration.
6. In a majority of cases (16 out of 20), little if any difference was observed between the probiotic viability obtained in OJ with or without pulp. In the remaining cases, the viability of probiotics was found to decline more rapidly in OJ with pulp.

- Thus the hypothesized enhanced viability of probiotics in OJ with pulp was not supported.
- 7. Little or no change was observed in both pH and °Bx values of the probiotic OJs across the entire storage period.
- The results (finding 7) were therefore in support of the hypothesis that inclusion of probiotics would not alter the pH and °Bx values of the OJs.

4.5 Discussion

This study aimed to examine the impact of the carrier matrix, storage duration and temperature, and potential species interactions, on the viability of single strain and multi-species probiotic cultures incorporated into OJ and BW, over 8 weeks of storage. In general the results provided evidence of variation in viability between the probiotic strains examined; variation between carrier products in terms of the viability of individual strains; and variation in viability under different storage temperatures. Importantly, in a majority of cases, the combining of probiotic species was not observed to adversely affect the individual strain viability to the extent that both OJ and bottled water might be considered useful carrier products for delivery of these organisms.

4.5.1 Strain and carrier dependent variation in the probiotic viability of individual strains

The data presented here has clearly shown that the viability of individual probiotics was strain-dependent and influenced strongly by the carrier. In OJ, mono-cultures of LG and LR remained at almost consistent levels throughout the refrigerated storage, whereas their viability was adversely affected in chilled BW (4 °C). Bb performed well in both carriers. Viability of PJ decreased sharply during the last four weeks of storage in OJ, while it remained at almost unchanged levels in refrigerated BW throughout the storage period.

Several studies have indicated that viability of probiotics in a delivery vehicle is greatly varied in a strain-dependent manner. For example, results of the study by Sheehan *et al.* (2007) revealed several strain dependent differences regarding viability trends of probiotics in orange juice (pH 3.65) during 12 weeks of refrigerated storage. They reported that members of the *Lb. casei* group such as *Lb. rhamnosus* GG, *Lb. casei* DN-114 001 and *Lb. paracasei* NFBC43338 remained viable at levels above 10^7 CFU/mL in orange juice (pH 3.65) for 12 weeks under refrigerated storage, while viable cell counts of *Bif. animalis* subsp *lactis* Bb12 and *Lb. salivarius* strains (UCC118 and UCC500) remained above the critical level of 10^6 CFU/mL for six weeks and just one week respectively. Such a variation in viability between the above probiotic strains was also observed when cultures were included in pineapple juice (pH 3.40) and stored under the same conditions. In a further study, *Lb. rhamnosus* LB11 and LB24, *Lb. reuteri* LB38,

Lb. plantarum LB42 and *Lb. acidophilus* LB45 were identified as being more robust when included in a commercial fruit drink (pH 4.2) in comparison to *Lb. acidophilus* LB2 and LB3 over a period of 80 days storage at 4°C, with the viabilities of the latter cultures declining five orders of magnitude more than the former group over the storage period (Champagne & Gardner, 2008).

The low pH value of fruit juices has been identified as the main determinant of survival of probiotics included in these carriers (Saarela *et al.*, 2006; Sheehan *et al.*, 2007). In the current study LG was identified as the most robust bacterium among the strains examined for its capacity to remain viable in high numbers in OJ throughout the storage period. High survival of LG in OJ is consistent with the findings of a recent study showing that a mono-culture of LG remained at a constant level over 12 weeks of storage at 4 °C in OJ (Sheehan *et al.*, 2007). The study by Champagne and Gardner (2008) has also reported that the viability of other single strain preparations of this species such as *Lb. rhamnosus* LB11 and LB24 remained almost unchanged in a commercial fruit cocktail drink (pH 4.2) over a period of 80 days storage at 4°C.

Such robustness was also observed for LR in the present study, which is also consistent with the findings of Champagne and Gardener (2008) reported that the viability of a single strain preparation of *Lb. reuteri* LB38 remained almost consistent in the fruit drink (pH 4.2) over 80 days of refrigerated storage.

Bb was relatively stable in OJ exhibiting decreases of less than one order of magnitude over 8 weeks of refrigerated storage. Bifidobacteria have been shown to be sensitive to pH values less than 4.6 (Boylston *et al.*, 2004). However *Bif. lactis* strains (Matto *et al.*, 2004) and among them Bb (Crittenden *et al.*, 2001) have been reported to be more resistant to low pH values compared with other species of *Bifidobacterium*. The stability of Bb in OJ is also consistent with findings of Sheehan *et al.* (2007) which indicated that the viability of Bb declined only about 0.7 Log CFU/mL in orange juice (pH 3.65) over 6 weeks of storage at 4°C (Sheehan *et al.*, 2007).

Of the four probiotic strains examined, PJ exhibited considerable instability in OJ, and seemed to be the strain most sensitive to the low pH nature of OJ (pH 3.8). It has been reported that different species/strains of dairy propionibacteria (*P. acidipropionici*, *P. theoni* and *P. jensenii*) exhibited different acid tolerance rates in a broth medium acidified with hydrochloric, lactic or propionic acid to pH values ranging from 3.75 to 6.5 (Rehberger & Glatz, 1998). Heretofore, it was reported that PJ is more sensitive to

low pH than lactobacilli in associative cultures of PJ with a *Lactobacillus* strain (LC, LP, LG or LR) (Moussavi & Adams, 2010).

In addition to pH, the chemical constituents of fruit juices are also considered as important factors influencing the viability of probiotics in fruit juices. They include a range of chemicals which may either enhance or compromise the viability of probiotics. The higher survival rate of lactobacilli in OJ than water is probably due to a protective effect of components such as sugars, vitamin C and minerals present in OJ. Orange juice contains sugars, predominantly sucrose (3.3%), fructose (3.0%) and glucose (2.8%) (Belitz *et al.*, 2009). Previous research has shown that sugars such as glucose enhanced the survival of probiotic *Lactobacillus* strains in acidic conditions (Corcoran *et al.*, 2005). Orange juice is also a prominent source of vitamin C which has been identified as one of the most powerful antioxidants. Ascorbic acid or vitamin C has been reported to contribute 65-100% to the antioxidant capacity of the citrus juices (Gardner *et al.*, 2000). A recent study has shown that addition of vitamin C to a model fruit juice system consisting of trisodium citrate, citric acid powder, saccharose and distilled water (pH 3.8) enhanced the viability of probiotics HOWARU *Lb. rhamnosus* HN001, HOWARU *Bif. lactis* HN001, and *Lb. paracasei* Lpc 37 compared with the control juice and juices with other vitamins such as B2, B3, B6, E during 6 weeks of refrigerated storage (Shah *et al.*, 2010). It is thought that vitamin C scavenges the oxygen present in the juice, thereby generating a more favourable anaerobic environment (Dave & Shah, 1997; Shah *et al.*, 2010).

Another significant antioxidant compound of orange juice is hesperidin, the main flavonoid present in the juice, which can also contribute to the creation of an anaerobic condition more desirable for probiotics (McGill *et al.*, 2004; Wilmsen *et al.*, 2005). Moreover, orange juice has been considered as a source of minerals such as potassium, phosphorus, calcium, magnesium and sodium (Belitz *et al.*, 2009; Guarnieri *et al.*, 2007; Ohrvik & Witthoft, 2008; USDA National Nutrient Database for standard Reference, 2009). Previous research has shown NaCl to slightly enhance the viability of *Lb. plantarum* in distilled water compared with the control (Bucio *et al.*, 2005). In another study, a protective effect has been reported for ions such as Na⁺ against cell death induced under acid stress conditions in *Saccharomyces* spp (Sant'Ana *et al.*, 2009).

Refrigerated BW as a probiotic carrier did not support the survival of the *Lactobacillus* strains but did so for Bb and PJ. With the exception of Bb viability, which remained almost unchanged when included in both refrigerated BW and OJ during storage, other

probiotics exhibited completely different viability trends in these two carriers. This is probably associated with difference in physicochemical properties of the carriers. The pH value of the BW was 6.5 which remained almost constant during the storage period. While this neutral pH value could support the viability of the probiotics, the absence of organic compounds such as sugars and antioxidants such as ascorbic acid and flavonoids in BW, could result in compromised viability of the probiotics. For instance, it has been shown that presence of ascorbic acid in a fruit juice blend protected *Lb. rhamnosus* R0011 during storage period against adverse effect of introducing oxygen in the product (Champagne *et al.*, 2008).

4.5.2 Viability of individual strains in the presence of other probiotics

As shown in section 4.4.1.5 of the results, in probiotic combinations incorporated into OJ and refrigerated BW, effects on the viabilities of individual strains varied between unaffected, enhanced or compromised in the presence of other probiotics. Mechanisms underlying the improved or compromised viability of probiotics in the presence of other probiotics remain unclear.

Probiotics included in OJ and BW were observed to settle as precipitates at the bottom of the containers. Probiotic strains were therefore physically in contact with the same bacterial cells and/or with different strains in combination cases. In the case of enhanced viability of individual strains in the presence of other probiotics, a possible mechanism might be formation of a protective bio-shield by constituents of the probiotic combination, in which they surround and thereby protect each other against unfavourable conditions of the environment such as low pH.

In addition, previous research has shown that probiotic strains may be able to co-aggregate with other probiotic strains through biochemical interactions between the bacterial surface molecules. It has been reported that LG is able to co-aggregate with *Propionibacterium* strains such as *P. freudenrenchii* JS or *Bifidobacterium* strains such as *Bif. breve* 99 (Collado *et al.*, 2007) and Bb (Ouwehand *et al.*, 2000). Bacterial surface compounds and/or substances released by microorganisms may determine the potential mode of action of bacterial interactions on the viability of probiotic strains.

Both bio-physical and bio-chemical interactions might be important determinants influencing the viability of individual probiotic strains in combination cases.

4.5.3 Variation in viability of probiotics in drinking water under different storage temperatures

When compared with refrigerated water, the decline in the viability of LG, LR and Bb was found to be more dramatic when the product was stored at room temperature. Most of the commercial *Lactobacillus* strains used in controlled food fermentations are known to grow well between 25 and 40°C. *Bifidobacterium* spp. also grow at temperatures between 25 and 45°C with optimal growth between 37 and 41°C (Ray, 2004). LG, LR and Bb can also grow at room temperature (23 °C), although it is considered a suboptimal growth temperature for the bacteria (Macedo *et al.*, 2002; Ray, 2004). In spite of the suitability of room temperature for growth of bacteria, absence of carbon energy sources (*i.e.* metabolisable sugars) and other substances necessary for bacterial growth and maintenance in BW could lead to starvation and ultimately bacterial death. Thus the storage of BW containing *Lactobacillus* strains, either alone or in combinations, at room temperature (23 °C) would appear inadvisable.

As presented in Figure 4.6, PJ and Bb were much more stable than lactobacilli in BW at both temperatures. PJ remained at almost consistent levels in BW both at 4°C and 23 °C across 8 weeks of storage. Prolonged survival of another species of this genus (*P. freudenreichii* CIRM-BIA1^T) has been reported when stored at room temperature under carbon starvation (Falentin *et al.*, 2010). Such robustness has also been demonstrated in water for *Mycobacterium* spp. (Whittington *et al.*, 2005) a genus of bacteria which, like *Propionibacterium*, belongs to the class Actinobacteria, and is considered to be closely related (Ventura *et al.*, 2007). A possible underlying mechanism for extended survival of PJ in water at room temperature is that the bacterium may have entered and remained in a dormant phase induced by energy source starvation. Dormancy has been defined as ‘a reversible state of low metabolic activity, in which cells can persist for extended periods without division’ (Kaprelyants *et al.*, 1993). Dormancy has been observed in other Actinobacteria such as *Mycobacterium*, *Koccuria* and *Rhodococcus* spp (Schroeckh & Martin, 2006). Several genes encoding the synthesis and accumulation of polyphosphate (polyP) glycogen, and trehalose and a particular gene encoding a Rpf protein are assumed to be reasons for the long survival of propionibacteria strains under carbon starvation conditions (Falentin *et al.*, 2010). Dormancy also has been indicated as a survival mechanism utilised by bifidobacteria (Lahtinen *et al.*, 2005; Lahtinen *et al.*, 2006). Since the genus *Bifidobacterium* is also classified as a genus of class

Actinobacteria (Biavati & Mattarelli, 2006), similar underlying mechanisms associated with the dormancy of other members of the Actinobacteria are also likely to be involved in prolonged survival of bifidobacteria under carbon starvation.

4.5.4 Impact of pulp on the viability of probiotics

Compared with pulp-free OJ, deterioration in viability was observed in the pulp-included OJ for mono-culture of LR (after week 4), Bb when combined with LG (after week 4) or PJ (after week 2) and PJ in the presence of LG (after week 6). In these cases it would seem that the orange pulp may have had a slight antibacterial effect on these probiotics, although the mechanisms responsible for the death of bacterial cells in the presence of pulp remain unclear. Another recent study has reported an antibacterial effect of orange pulp against Gram negative bacteria such as *E. coli* O157:H7 and *Salmonella typhimurium* (Callaway *et al.*, 2008), however such an effect has not been reported for Gram positive bacteria such as *Lactobacillus* and *Bifidobacterium* spp.

4.5.5 pH and Brix changes in orange juices

Fruit juices need to be stored at temperatures as low as 0 to 8°C (refrigeration), as low temperature decreases the growth and activity rate of microorganism in food matrices (Fellows, 2009). The mechanisms underlying the action of low temperature on microbial growth and activity involves alterations to the structure of the cell membrane, decrease in uptake of substrate through the membrane and decrease in rate of enzymic activities including respiration (Herbert, 1989).

In this study the pH values of probiotic OJs (with and without pulp) were relatively constant during the storage period. Variation in pH between the various preparations was not evident, however changes in pH values during the storage period compared to the initial pH values revealed that although the overall difference was small (less than 0.1 pH value), there were statistically significant changes at some time points during storage. These results are consistent with findings of recent studies in which pH values of commercial fruit beverages such as Oasis Health Break™ (a blend of pineapple, apple, orange, pear and/or grape, passion fruit, lemon, peach, strawberry, mango, kiwi), Health Vision Sante Tradition® (a blend of apple-pear-raspberry) and Valio (a blend of

orange, grape and passion fruit) with probiotic cultures included, remained unchanged or showed little change (within 0.1 pH unit) during refrigerated storage ([Champagne & Gardner, 2008](#); [Champagne *et al.*, 2008](#); [Saarela *et al.*, 2006](#)). Similarly Brix values were found to be highly consistent across the entire data set and little variation was evident either between preparations, or with time. The Brix refers to the total soluble solids content, mainly sugars and fruit acids present in the fruit juice ([Taylor, 2005](#)), however as a general consensus Brix is taken to indicate the percentage sugar content of fruit juices ([Laszlo, 2007](#)). Little or no change in both the pH and °Bx values of the probiotic OJs would suggest very weak or no metabolic activity such as assimilation/fermentation of sugars and production of organic acids by the probiotic bacteria, which might otherwise have reduced pH during storage.

4.6 Conclusion

Appreciable differences were observed among the probiotic strains used in this study in respect to their viability during the storage time. Carrier matrices (OJ and BW) affected the survival of probiotics included. However pulp was not identified as an influential factor on the viability of probiotics.

In OJ, LG and LR remained at almost consistent levels throughout the refrigerated storage, whereas their viability was adversely affected in chilled BW (4 °C). Bb performed well in both carriers. Viability of PJ decreased dramatically during the storage in OJ, while it remained at almost unchanged levels in refrigerated BW throughout the storage period. When compared with BW, OJ was found to be a more suitable carrier for a wider range of probiotics. While the survival of probiotic bacteria could be affected significantly by the presence of other probiotics in most cases the viability of the individual strains was either enhanced or unaffected when combined with other probiotics throughout the refrigerated storage in both carriers.

The findings therefore confirm that it is possible to utilise specific combinations of different probiotic bacteria with differing properties in OJ and BW, in order to potentially confer greater beneficial health effects on the host. However the effect of inclusion of these beneficial bacteria into OJ and BW on sensory characteristics of the end product and functional properties of probiotics needs to be further investigated.

**Chapter V: An *In Vitro* Study on Gastro-Intestinal Tolerance
of Probiotic Combinations Incorporated into Orange Juice**

5.1 Summary

The objective of this study was to determine the effect of duration of combined exposure to orange juice (OJ) and refrigerated storage on the tolerance of probiotics *Lb. rhamnosus* GG (LG), *Lb. reuteri* ATCC 55730 (LR), *Bif. animalis* subsp *lactis* Bb12 (Bb) and *P. jensenii* 702 (PJ), either separately or in 2- or 3-multispecies combinations to simulated gastro-intestinal conditions at 10 day intervals during one month of storage. Data from this study demonstrated that the tolerance of probiotics to simulated gastro-intestinal conditions varied according to the choice of strain, choice of carrier and presence of other probiotic bacteria/strains. Tolerance of LG and PJ to simulated gastric juice (SGJ) was found to be significantly enhanced in OJ compared to PBS at the baseline, while LR and Bb were highly tolerant and of comparable resistance to SGJ in either carrier. In contrast, suspension in OJ appeared to significantly decrease the tolerance of lactobacilli and PJ to simulated intestinal juice (SIJ) at the baseline, with Bb the only strain unaffected. All examined probiotic preparations either alone or in combinations included in OJ showed high tolerance to SGJ which remained virtually unchanged throughout the storage period. Slight variations were also evident in the tolerance of probiotics to SIJ with time during the entire storage period, with changes in viability loss ≤ 0.6 orders of magnitude across the entire data set. The only exception being LR in mono-culture, in which case tolerance to SIJ significantly increased at D10 compared to D0 and remained unchanged by the end of storage period. Different effects including enhancement, suppression, or no effect, were observed with the presence of other probiotic strains on the tolerance to SIJ. Bb (alone and in combinations) included in OJ appeared to be the most robust when confronted with simulated intestinal conditions with little or no viability loss compared to other probiotic strains. The lowest bacterial tolerance to SIJ was observed in LG preparations followed by LR preparations with viability losses of 4-6 and 1-4 orders of magnitude respectively. When combined with Bb and/or PJ significant improvements were observed in the tolerance of lactobacilli to SIJ. In contrast, tolerance of PJ to SIJ was adversely affected in the presence of other probiotics with viability losses ranging from less than 2 (in the presence of only Bb) to 5.5 log reductions in CFU/mL (in combinations containing LG). In conclusion, the results of the present study showed that the gastro-intestinal tolerance of probiotics is strain and carrier dependant and may be influenced by the presence of other strains. These effects should be considered when formulating probiotic products.

5.2 Introduction

To be effective in exerting their health promoting benefits for the host, probiotic microorganisms must adequately survive harsh environmental conditions encountered during gastro-intestinal passage, and then persist in the intestine (Saarela *et al.*, 2000). It is therefore necessary that a potential probiotic be examined for its gastric transit tolerance and intestinal persistence (Saarela *et al.*, 2000).

The strong acidic environment of the stomach acts as a natural, highly protective barrier against harmful microorganisms ingested through the consumption of food and drink. While the normal internal pH of the human stomach ranges from 2.5 to 3.5 (Holzapfel *et al.*, 1998), this value can vary depending on the nature and composition of the food and drinks ingested. Another important factor is the residence time of food entering the stomach, which depends largely on its physico-chemical properties. For example liquids, which pass through the stomach more rapidly than solids (Rogers, 2011), may take <20 minutes to leave the stomach while a mixed meal can remain in the stomach up to 4 hours (GastroNetAustralia, 2010).

In vitro tests for gastrointestinal transit tolerance of potential probiotics commonly include examining their survival at low pH, and in the presence of bile salts and digestive enzymes. More specifically, tolerance to the gastric environment may be examined *in vitro* by exposing the bacteria to conditions of low pH (pH 1-5) for 0-180 minutes in a simulated gastric fluid (Charteris *et al.*, 1998; Huang & Adams, 2004; Zarate, Perez Chaia, *et al.*, 2000), which may include pepsin, a digestive protease secreted by stomach cells (Huang & Adams, 2004).

The intestinal environment may in turn be mimicked by exposure to simulated intestinal juice, a basic solution (pH 8.0) containing bile salts and pancreatin (Holzapfel *et al.*, 1998). The primary role of bile in digestion is the emulsification and solubilisation of lipids. This property is mediated through the amphipathic nature of bile salts. In fact, bile salts act as detergent, lowering the surface tension of dietary fats and breaking them down into tiny droplets, thus increasing the surface area for lipase activity. In the same way, bile salts may lethally damage bacteria via interaction with membrane lipids (Begley *et al.*, 2005; Begley *et al.*, 2006).

Various studies have revealed that simulated gastro-intestinal transit tolerance of probiotic bacteria is both strain and pH-dependent (Huang & Adams, 2004; Lan, Bruneau, *et al.*, 2007; Maragkoudakis *et al.*, 2006; Masco *et al.*, 2007; Zarate,

Gonzalez, *et al.*, 2000; Zarate, Perez Chaia, *et al.*, 2000). Moreover, it has been shown that the food matrix can influence the ability of probiotics to survive the gastro-intestinal environment, and that incorporation into carrier matrices such as milk, fermented milk, cheese, soymilk and meat may enhance the ability of probiotic bacteria to survive gastro-intestinal passage (Ganzle *et al.*, 1999; Huang & Adams, 2004; Leverrier *et al.*, 2005; Saarela *et al.*, 2006; Stanton *et al.*, 1998; Zarate, Perez Chaia, *et al.*, 2000). It has also been speculated that due to the short gastro-intestinal transit time of fruit juices, inclusion in such carriers may reduce exposure of probiotics to the harsh GI environment, and thereby enhance their effectiveness (Post, 2002).

Saarela *et al.* (2006) reported that the gastro-intestinal tolerance of freeze-dried *Bif. animalis* subsp *lactis* E-2010 (Bb12) included in milk was significantly higher than that in a commercial fruit drink (pH 3.7, a blend of orange, grape and passion fruit). When compared to PBS, Bb12 included in the fruit drink was found to be significantly more tolerant to simulated gastric juice at pH 2.5 in the absence of pepsin, but significantly less tolerant at pH 3.0 with pepsin included.

Champagne and Gardner (2008) showed that 35 days refrigerated storage of *Lb. acidophilus* LB3, *Lb. rhamnosus* LB11, *Lb. reuteri* LB38 and *Lb. plantarum* LB42 included separately in a commercial fruit beverage (a blend of 10 fruit juices and purees, pH 4.2) impaired their survival when exposed to simulated gastric juice (SGJ, pH 2.0) compared with the fresh cultures. The same study also revealed that 35 days storage of the probiotics in the fruit juice, did not affect their tolerance to simulated intestinal juice (SIJ) containing bile salts (0.3%) or pancreatin.

It is not yet known whether long term exposure to fruit juice may also impact on the gastro-intestinal tolerance of individual strains in probiotic combinations. This study aimed to examine the tolerance of probiotics, incorporated either separately or in various combinations into orange juice, to SGJ and SIJ during one month of refrigerated storage.

Based on the viability results presented in chapter 4, and the findings of other researchers as detailed above, it was hypothesized that:

- The 4 bacterial species/strains examined would exhibit differential tolerance to simulated gastro-intestinal conditions.
- Incorporation into orange juice would immediately alter the gastro-intestinal tolerance of the bacteria. In particular, it was expected that the gastric tolerance

of Bb12 would be significantly reduced in the orange juice relative to that observed in PBS.

- The ability of the probiotics to survive simulated gastro-intestinal conditions would be further affected by the duration of their combined exposure to the food matrix (orange juice) and low storage temperature (4°C), and more specifically that the gastric juice tolerance of the lactobacilli would decrease significantly with increasing storage time.
- The ability of individual probiotics in orange juice to survive simulated gastro-intestinal conditions would be affected by the presence of other probiotic bacteria.

5.3 Materials and methods

5.3.1 Chemicals and reagents

Unless otherwise specified, chemicals were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA).

5.3.2 Bacterial cultures and growth conditions

As previously described in Chapter 4.

5.3.3 Orange juice

The orange juice used in this study was a commercial, pasteurised (85°C, for 15 sec), pulp-free product containing no added preservatives or sugar (The Original Juice Co., a division of Golden Circle Ltd, Mill Park, VIC, Australia). To ensure the removal of any residual pulp in the product, the orange juice was centrifuged at $3220 \times g$ for 15 min at 4°C - using an Eppendorf centrifuge model 5810 R (Eppendorf AG, Hamburg, Germany) - and the supernatant decanted for use in the experiments. Amounts of 50 ml of the pulp-free orange juice were dispensed into sterile screw cap high density polypropylene (HD-PE) containers (Sarstedt Australia Pty Ltd, Mawson Lakes, SA, Australia). All samples were refrigerated (4 °C) prior to further preparation.

5.3.4 Preparation of probiotic orange juice

Bacterial cells were harvested in their stationary phases by centrifugation ($3220 \times g$, 15 min, 4 °C) and washed 3X with Dulbecco's Phosphate-Buffered Saline (PBS) at pH 7.0 (Gibco, Invitrogen Corp., Carlsbad, CA, USA). Bacterial pellets were then resuspended in PBS. An aliquot of 500 µL of each bacterial suspension was incorporated into the 50 mL portions of the juice. Orange juice containers were then stored at 4 °C for 30 days.

5.3.5 Tolerance to the simulated gastric conditions

Simulated gastric juice was prepared by adding pepsin with an approximate activity of 2500 units/mg (Chem-supply Pty Ltd, Gillman, SA, Australia) to a final concentration of 3.0 gL^{-1} to sterile Dulbecco's Phosphate-Buffered Saline (PBS) (Gibco, Invitrogen

Corp., Carlsbad, CA, USA). The pH value of the solution was then adjusted to pH 2.0 with 0.2N HCl. Tolerance of probiotics incorporated into orange juice to the simulated gastric juice was determined by adding an aliquot of 1.0 mL probiotic orange juice to 9 mL simulated gastric juice followed by vortex mixing for 10 sec and incubation at 37°C for 20 min - the mean residence time of orange juice in stomach (Basit *et al.*, 2001) (Refer Figure 5.1). Immediately after incubation, the suspension was mixed by vortexing briefly, with 1 ml of the suspension then transferred to a tube containing 9 ml sterile Maximum Recovery Diluent (MRD) (Oxoid Australia Pty Ltd, Adelaide, Australia). Serial decimal dilutions were prepared and viable bacterial counts were determined as previously described in Chapter 4.

5.3.6 Tolerance to the simulated intestinal conditions

To determine the tolerance of probiotics to the simulated intestinal conditions, the method of de Palencia *et al.* (2008) was used, with modifications. Briefly, simulated small intestinal juice was prepared by dissolving porcine pancreatin to a final concentration of 1.0 gL⁻¹ in PBS. The mixture was then centrifuged at 2000 ×g for 15 min at 4 °C. Bile Salts No 3 (Oxoid Ltd, Basingstoke, Hampshire, UK) was added to a final concentration of 3.0 gL⁻¹ to the decanted supernatant and the pH adjusted to 8.0 with 1.0N NaOH. Tolerance to the simulated small intestinal conditions was determined by transferring a 2.5 mL aliquot of each probiotic preparation to 7.5 mL of a mixture of simulated gastric juice (45 mL), simulated intestinal juice (60 mL) and 1.0M NaHCO₃ (6.1 mL), as shown in Figure 5.1. The pH value of the mixture without probiotic orange juice was 7.56. The final mixture was vortex mixed for 10 sec and incubated for 180 min at 37°C. The viable bacterial counts were then determined as described in Chapter 4.

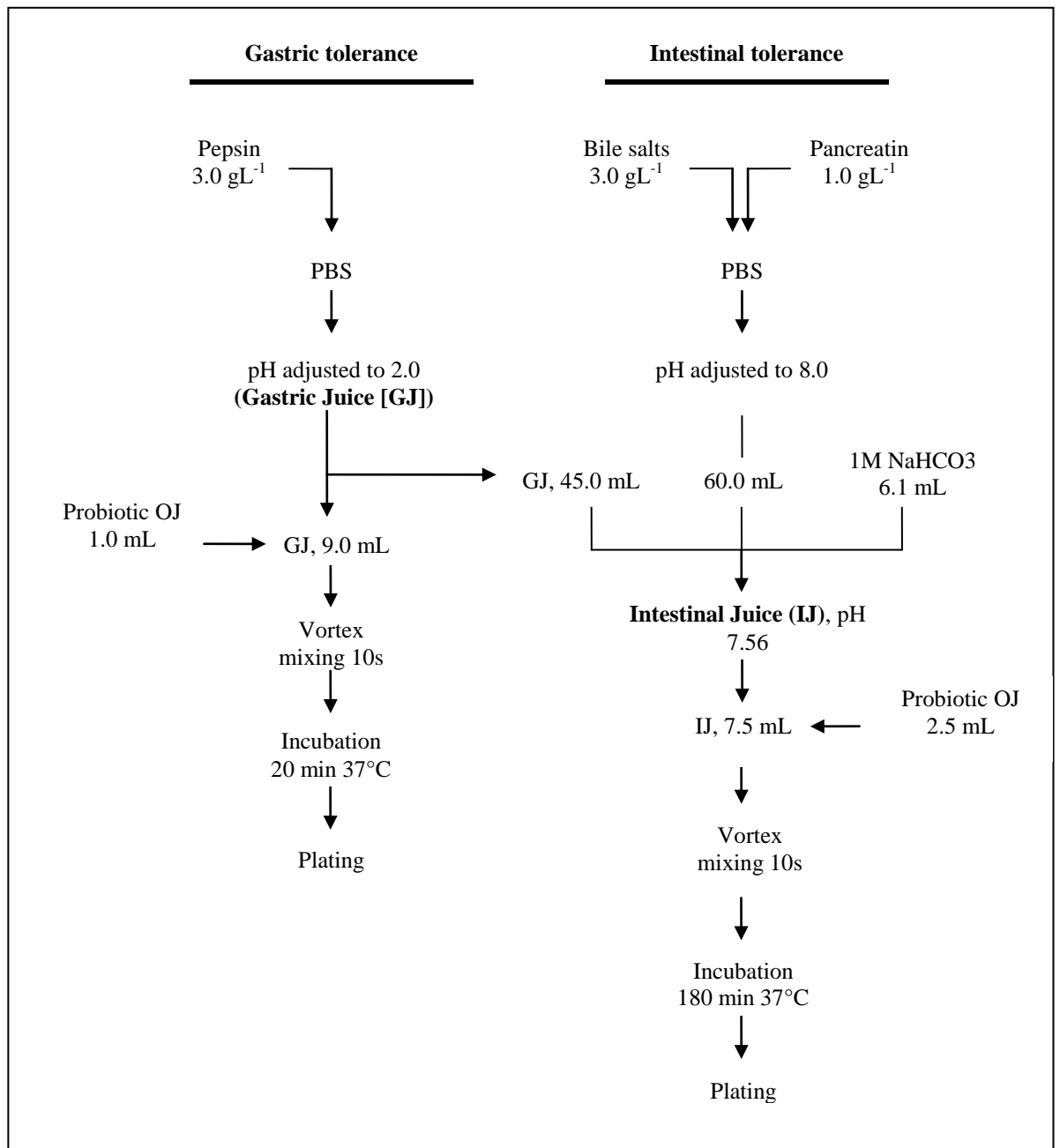


Figure 5.1: Schematic diagram outlining details of the preparation of simulated gastric and intestinal juices, and the *in vitro* gastro-intestinal tolerance assay procedure.

5.3.7 Statistical analyses

Statistical analyses were performed using SPSS software Ver. 18 (SPSS Inc., Chicago, IL, USA). Data were analysed using the linear mixed model procedure to run a general linear model (GLM). In order to give protection against potential false positive (false significant results), the Bonferroni adjustment was used and significance level (α) was set at point $p \leq 0.001$, based on a multiple comparison assessment comprising 50 cases (*i.e.* $0.05/50 = 0.001$). In some cases the number of comparisons was greater, *e.g.* 60, which would strictly stipulate that α be set at 0.00083 (*i.e.* $0.05/60$). However for simplicity, and because the Bonferroni adjustment is conservative, 0.001 was chosen as the acceptable significance level.

5.4 Results

The probiotic preparations employed in this study comprised the same single strain, paired and triplet combinations as those used in the viability analyses of chapter 4. To determine the effect of orange juice as a carrier matrix, tolerance to simulated gastro-intestinal conditions on day 0 was also examined for fresh preparations of the same cultures in PBS. Strain variability was assessed by comparison of the mono-culture preparations, with the effect of storage assessed by examining tolerance to both SGJ and SIJ at 10 day intervals over 30 days of storage. In general the results indicated that bacterial strain, type of carrier matrix (orange juice or PBS), presence of other probiotics (in combinations) and duration of storage, all appeared to impact on probiotic tolerance to simulated gastro-intestinal conditions.

5.4.1 Strain dependent variation and effect of orange juice and refrigerated storage on the gastro-intestinal tolerance of probiotics

When the viabilities of individual strains at baseline (day 0) were compared (Figure 5.2), significant variability in their tolerance to SGJ and SIJ was evident, especially in relation to the carrier matrix. With the exception of Bb, tolerance of the probiotics to SIJ was found to be substantially reduced when suspended in OJ compared with PBS. In contrast, all strains exhibited a strong tolerance to SGJ when suspended in OJ, with substantial losses in viability after exposure to SGJ only evident for LG and PJ in PBS. When suspended in OJ, PJ was found to exhibit a strong tolerance to SGJ and a relatively moderate intolerance to SIJ. Only Bb was observed to maintain full viability in all scenarios.

Importantly, use of OJ as a carrier matrix did not impact adversely on the tolerance of any of the strains examined to SGJ, with the tolerance of LG and PJ considerably enhanced relative to that observed in PBS, but did appear to dramatically impair the tolerance of the lactobacilli to SIJ. With regard to the experimental hypotheses, Figure 5.2 provides clear evidence of strain dependent variability in tolerance to simulated gastro-intestinal conditions. Expectations that incorporation into OJ would significantly influence the gastro-intestinal tolerance of the bacteria were also supported by the data, although the hypothesized reduction to the tolerance of Bb to SGJ was not observed.

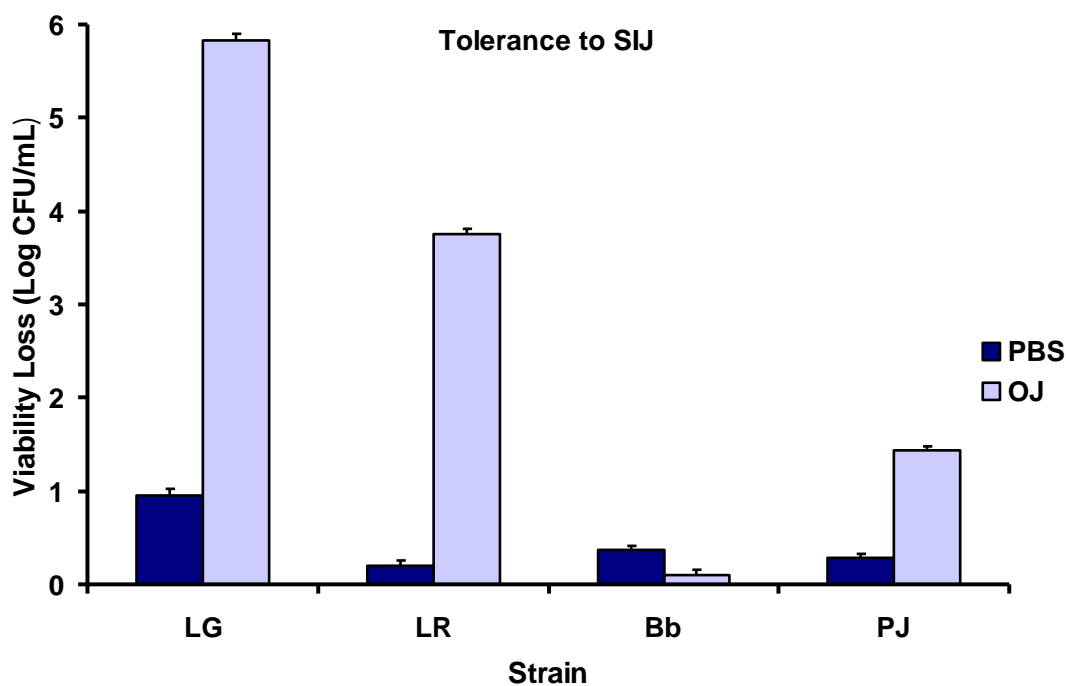
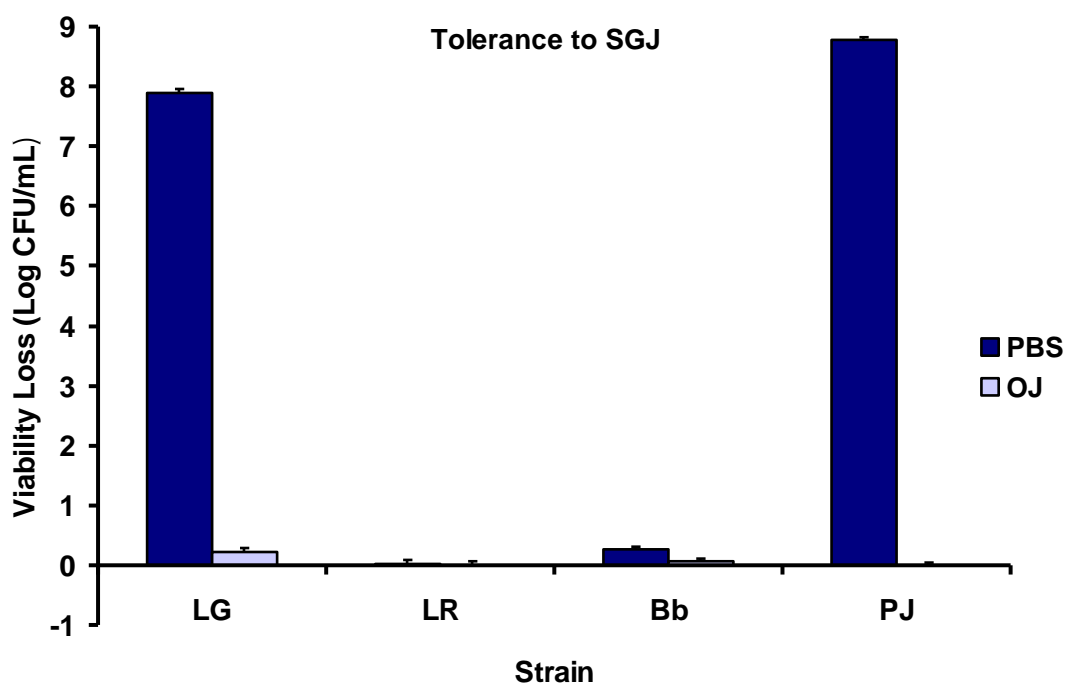


Figure 5.2: Viability losses of individual probiotics incorporated into PBS or orange juice at baseline, after exposure to simulated gastric juice (SGJ) for 20 min at 37°C or simulated intestinal juice (SIJ) for 180 min at 37°C.

Viability losses were determined as the difference between the viable bacterial count of the preparation prior to exposure and the count following exposure (expressed as Log CFU/mL).

In Figure 5.3, the initial (day 0) viability losses of the 4 probiotic mono-cultures have again been plotted, along with the losses measured at 10 day intervals across 30 days of storage. In order to ensure that any apparent variations in gastro-intestinal tolerance were not confounded by general losses in viability due to cold storage, viable cell counts prior to exposure to SGJ and SIJ were determined at each time point. In all cases a high tolerance to SGJ was maintained throughout the storage period. In relation to the SIJ, the tolerance of both Bb and PJ was found to remain relatively constant during storage. By comparison a significant improvement in the tolerance of LR was apparent after 10 days of storage, with the trend continuing for the entire period, while the apparent reduction in the SIJ tolerance of LG between the initial (day 0) and final (day 30) measurements was also found to be statistically significant ($p \leq 0.001$). The data therefore provides limited evidence to support the hypothesis that prolonged exposure to the food matrix (OJ) and low storage temperature would affect probiotic tolerance to simulated GI conditions, but no indication of a specific decrease in the tolerance of the lactobacilli to SGJ.

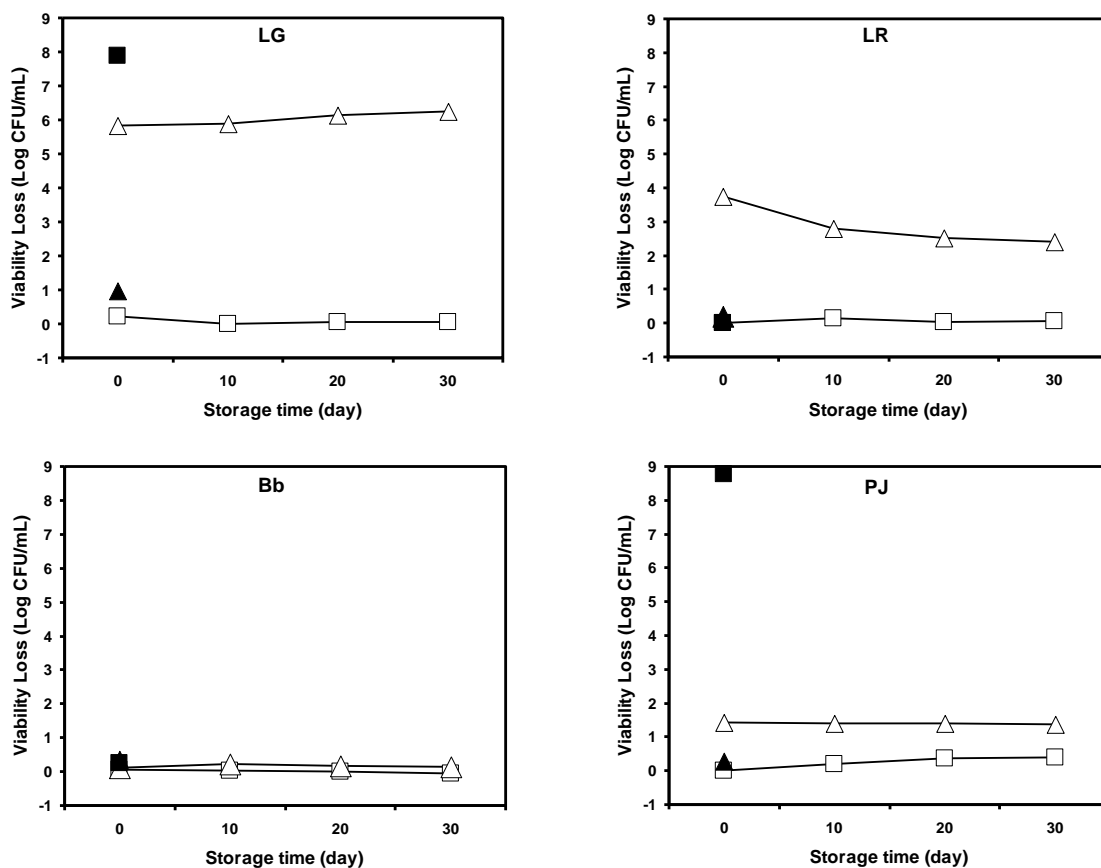


Figure 5.3: Gastro-intestinal tolerance of individual strains of LG, LR, Bb and PJ included in OJ during 30 days of refrigerated storage. The results are presented as reduction in the number of viable probiotic cells after exposure to SGJ and SIJ (expressed as Log CFU/mL). □ probiotic viability loss in OJ after exposure to SGJ; ■ probiotic viability loss in PBS after exposure to SGJ; △ probiotic viability loss in OJ after exposure to SIJ; ▲ probiotic viability loss in PBS after exposure to SIJ.

5.4.2 Effect of probiotic combinations on gastro-intestinal tolerance of individual strains

In order to assess the effect of combining probiotics on the tolerance of individual strains to gastro-intestinal conditions, the probiotic combinations including five paired combinations (LG-Bb, LG-PJ, LR-Bb, LR-PJ, Bb-PJ), and two triplet combinations (LG-Bb-PJ and LR-Bb-PJ) were prepared and introduced in OJ and PBS. In each case, the tolerance of each individual strain was monitored at 10-day intervals during 30 days of storage in OJ and compared with its tolerance as a mono-culture. The results have been presented as 4 sets of figures (5.4 – 5.7), with each set containing separate plots of the viability of one of the 4 probiotics in each of the combined preparations.

Together the figures indicate that while the combining of different strains in a single preparation had little or no impact on the tolerance of any of the individual probiotics to SGJ, a substantial impact on apparent intestinal tolerance was evident in several cases ($p \leq 0.001$). For example, paired and triplet combinations involving LG and PJ appeared to simultaneously result in an approximate 100-fold improvement in the tolerance of LG to SIJ, but a 100000-fold decline in the tolerance of PJ (Figures 5.4 and 5.7). The tolerance of PJ to SIJ also appeared to be adversely affected, although to a lesser extent, when combined with LR. In the case of LR by comparison, significant improvements were apparent when combined with both PJ and Bb (Figure 5.5), although combining with LR appeared to produce a moderate reduction in the tolerance of Bb to SIJ as storage time increased (Figure 5.6). The tolerance of Bb to SIJ was apparently unaffected in other combinations. Variations in intestinal tolerance across the storage period was also evident for individual strains in several of the other combinations, however the magnitude of these variations was in most cases relatively insignificant within the context of initial viability losses ($p \leq 0.001$).

In general, combining with both Bb and PJ appeared to provide a favourable outcome for the lactobacilli with little impact on Bb, while the tolerance of PJ to SIJ was adversely affected to a varying extent in all combinations.

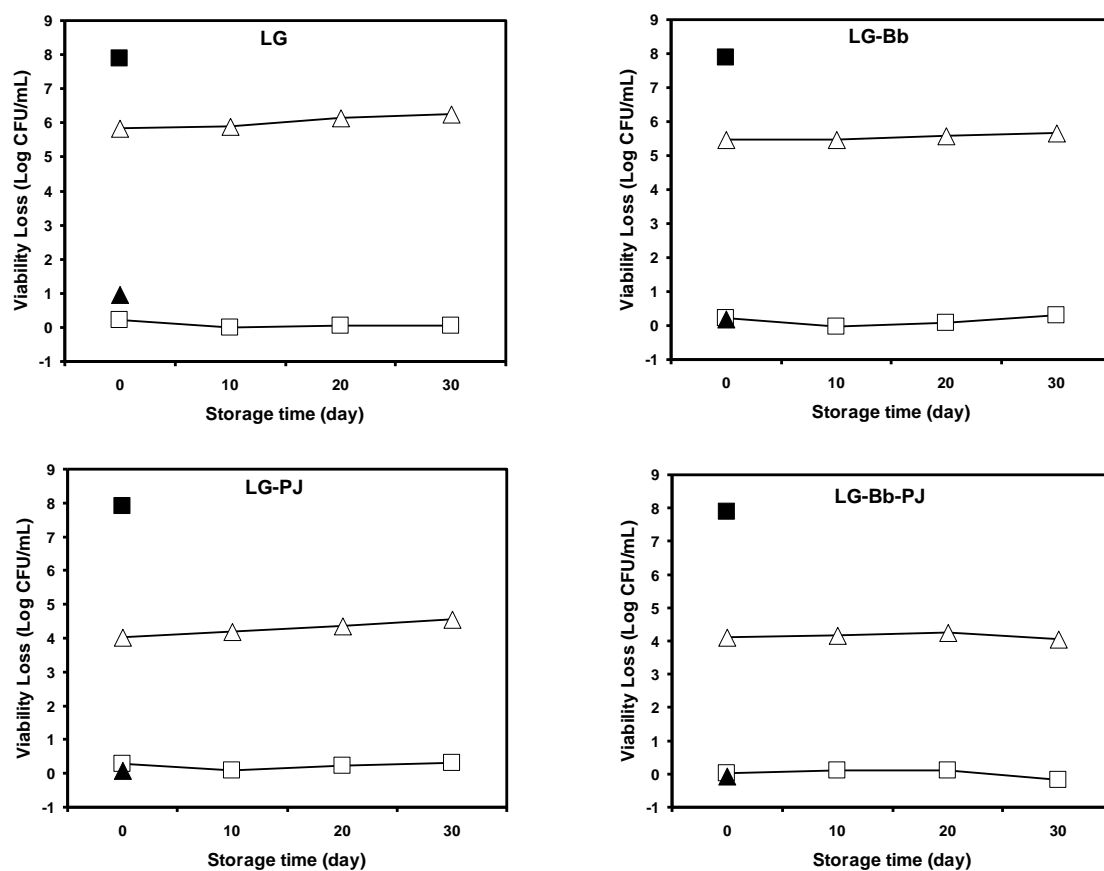


Figure 5.4: Variation in the gastro-intestinal tolerance of LG, in mono-culture and in combination with Bb and/or PJ, during 30 days of refrigerated storage in OJ. The results are presented as reduction in the number of viable probiotic cells after exposure to SGJ and SIJ (expressed as Log CFU/mL). □ probiotic viability loss in OJ after exposure to SGJ; ■ probiotic viability loss in PBS after exposure to SGJ; △ probiotic viability loss in OJ after exposure to SIJ; ▲ probiotic viability loss in PBS after exposure to SIJ.

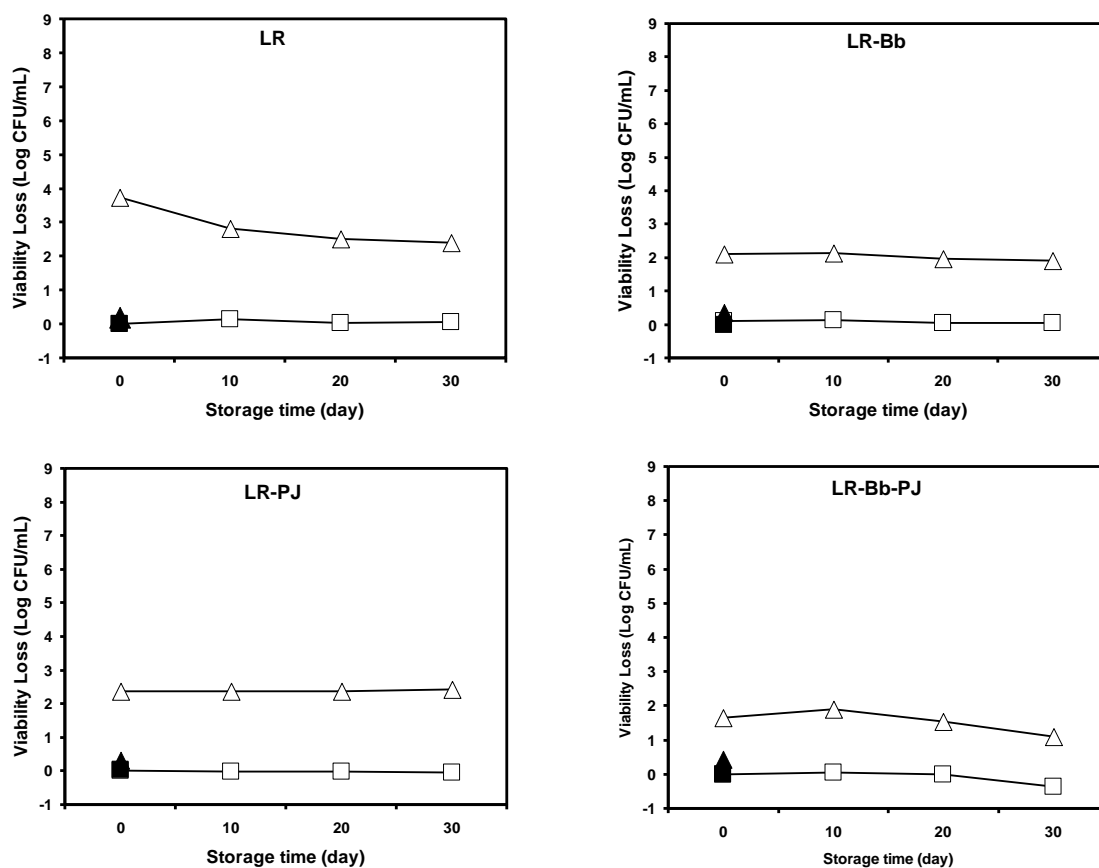


Figure 5.5: Variation in the gastro-intestinal tolerance of LR, in monoculture and in combination with Bb and/or PJ, during 30 days of refrigerated storage in OJ. The results are presented as reduction in the number of viable probiotic cells after exposure to SGJ and SIJ. □ probiotic viability loss in OJ after exposure to SGJ; ■ probiotic viability loss in PBS after exposure SGJ; △ probiotic viability loss in OJ after exposure to SIJ; ▲ probiotic viability loss in PBS after exposure to SIJ.

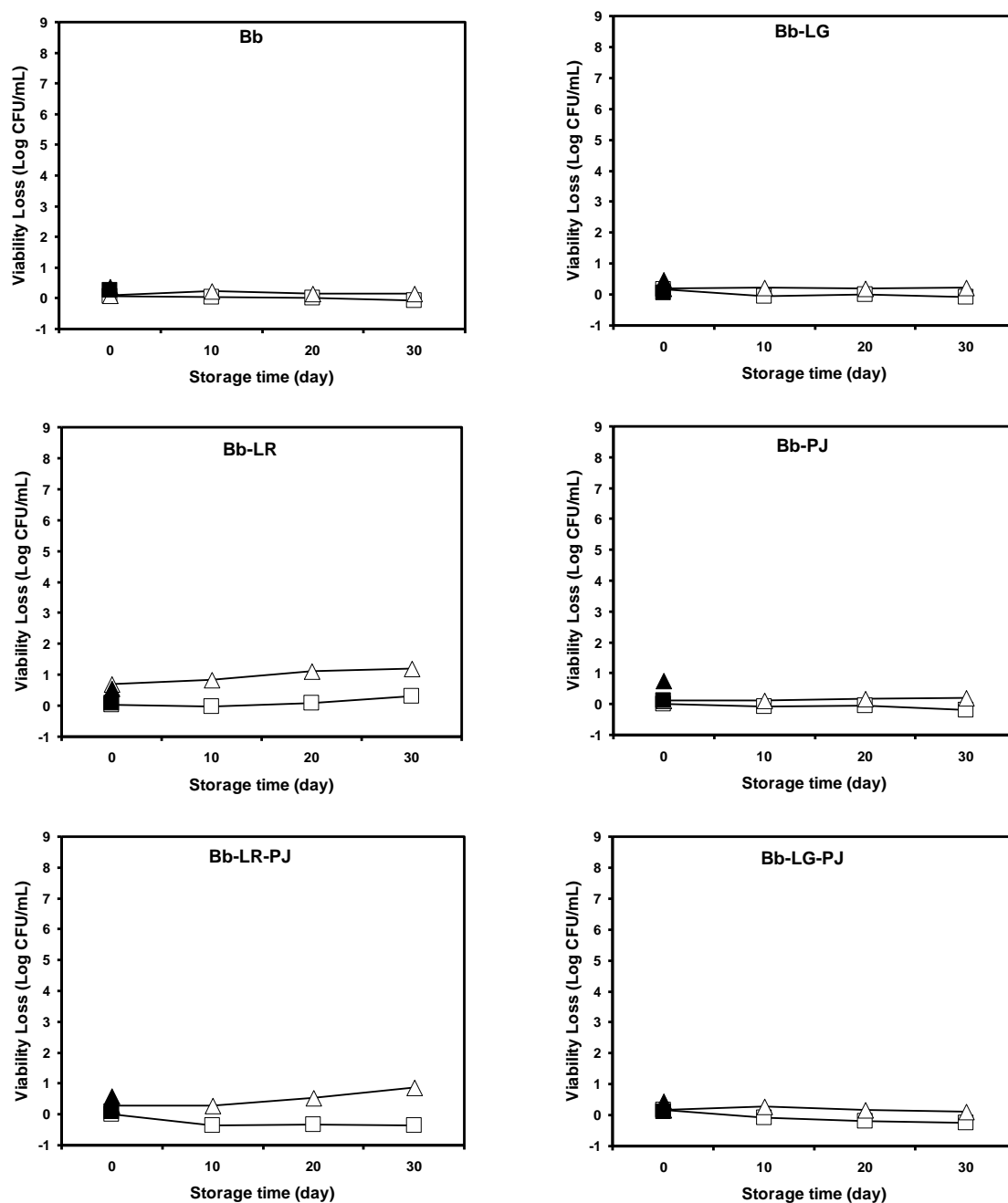


Figure 5.6: Variation in the gastro-intestinal tolerance of Bb, in monoculture and in combination with LG or LR and/or PJ, during 30 days of refrigerated storage in OJ. The results are presented as reduction in the number of viable probiotic cells after exposure to SGJ and SIJ (expressed as Log CFU/mL). □ probiotic viability loss in OJ after exposure to SGJ; ■ probiotic viability loss in PBS after exposure to SGJ; Δ probiotic viability loss in OJ after exposure to SIJ; ▲ probiotic viability loss in PBS after exposure to SIJ.

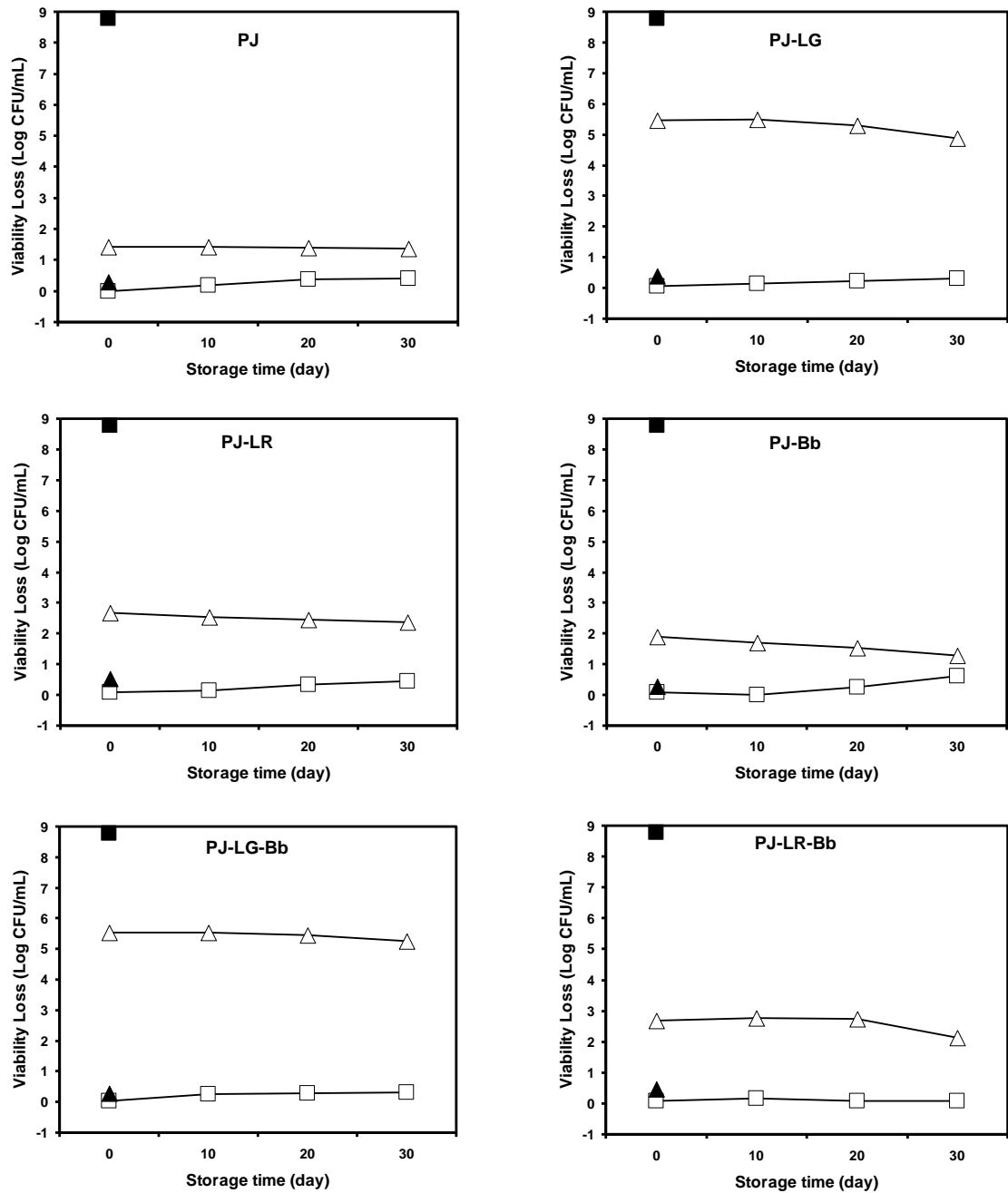


Figure 5.7: Variation in the gastro-intestinal tolerance of *PJ*, in monoculture and in combination with *LG* or *LR* and/or *Bb*, during 30 days of refrigerated storage in OJ. The results are presented as reduction in the number of viable probiotic cells after exposure to SGJ and SIJ (expressed as Log CFU/mL). \square probiotic viability loss in OJ after exposure to SGJ; \blacksquare probiotic viability loss in PBS after exposure to SGJ; \triangle probiotic viability loss in OJ after exposure to SIJ; \blacktriangle probiotic viability loss in PBS after exposure to SIJ.

5.5 Discussion

A general consensus in the selection of a microorganism as a probiotic is that the culture must adequately survive hostile conditions during gastro-intestinal transit so as to exert its beneficial health effects on the host (Corcoran *et al.*, 2008). Data from this study has shown the tolerance of probiotics to simulated gastro-intestinal conditions to vary according to the choice of strain, choice of carrier matrix, and presence of other probiotics.

Specifically, the results revealed the strains, whether included in PBS or OJ, to have differing tolerance to simulated gastro-intestinal conditions. Strain specific tolerance of probiotics to gastro-intestinal conditions is supported by previous research showing that tolerance of probiotic candidates to simulated gastro-intestinal conditions may vary considerably among different genera, different species, or different strains within a species. A study by Masco *et al.* (2007) showed a strain/species dependent variation in the *in vitro* gastro-intestinal tolerance of 66 *Bifidobacterium* strains including 24 strains isolated from probiotic products and 42 human reference strains. *Bif. lactis* has been reported to be the most robust *Bifidobacterium* among bifidobacteria species (Masco *et al.*, 2007; Matsumoto *et al.*, 2004; Matto *et al.*, 2004). Such a variation has also been observed among 29 *Lactobacillus* strains isolated from dairy products (Maragkoudakis *et al.*, 2006), and potential probiotic dairy propionibacteria including 7 reference strains and 6 dairy isolates (Huang & Adams, 2004).

The results of the present study have also revealed that the specific carrier matrix can impact on the gastro-intestinal tolerance of the probiotics. The tolerance of LG and PJ to SGJ was found to be significantly enhanced in OJ compared to PBS at the baseline, while LR and Bb were highly tolerant and of comparable resistance to SGJ in either carrier.

The enhanced tolerance to SGJ of LG and PJ included in OJ, compared to those included in PBS, is assumed to be associated with the physico-chemical properties of OJ. A major component of OJ which may contribute to improved tolerance of the included probiotic to SGJ is sugar - the predominant sugars in OJ being sucrose (3.3%), fructose (3.0%) and glucose (2.8%) (Belitz *et al.*, 2009). The study by Corcoran *et al.* (2005) for example, showed the presence of glucose to enhance the survival of probiotic LG in simulated gastric juice (pH 2.0). This protective effect was thought to have arisen

through provision of ATP to F₀F₁-ATPase via glycolysis, thereby enabling proton (H⁺) exclusion from the cells.

Orange juice is also a prominent source of vitamin C which has been identified as one of the most powerful of known antioxidants. Vitamin C has been reported to contribute 65-100% of the antioxidant capacity of the citrus juices (Gardner *et al.*, 2000). A recent study has shown that addition of vitamin C to a model fruit juice system, consisting of trisodium citrate, citric acid powder, saccharose and distilled water (pH 3.8), enhanced the viability of probiotics *Lb. rhamnosus* HN001, *Bif. lactis* HN001, and *Lb. paracasei* Lpc 37 compared with the control juice and with juices containing other vitamins such as B2, B3, B6 and E (Shah *et al.*, 2010).

Another possible mechanism for improved tolerance of LG and PJ to simulated gastric conditions when included in OJ, is bacterial acid stress adaptation by exposure of the bacteria to milder acidic conditions (OJ, pH 3.8) which may enhance bacterial resistance to subsequent exposure to simulated gastric juice (pH 2.0). Previous research has shown that pre-exposure to sub-lethal or mild acidic pH values may enhance the resistance of the bacteria to subsequent stressful acidic conditions. This phenomenon has been considered as an alternative method for improving probiotic viability in such harsh conditions (Champagne *et al.*, 2005; Van de Castele *et al.*, 2006).

Saarela *et al.* (2004) examined the tolerance of *Lactobacillus* and *Bifidobacterium* strains sub-lethally treated with acid (pH 3.0-4.0) in subsequent lethal conditions (pH 2.5). They observed that stress adaptation enhanced the viability of *Lactobacillus* strains more than that of bifidobacteria. Compared to non-treated bacterium, pre-treatment of *Lb. rhamnosus* E800 with pH 4.0 for 1 h, significantly improved the tolerance of the bacterium to lethal pH 2.5 for 2h in general edible medium (GEM), whereas, its tolerance slightly decreased in PBS with pH 2.5. However pre-treatment with pH 3.5, did not change the tolerance of the bacterium.

Acid adaptation has also been reported in propionibacteria strains such as *Propionibacterium freudenreichii* subsp. *shermanii* in which pre-exposure of the bacterium to a moderate acid stress (30 min at pH 4.5) improved its survival by about 4 orders of magnitude when subsequently exposed to a severe acidic condition (pH 2.0), compared to that of the culture without acid adaptation (Jan *et al.*, 2000).

Saarela *et al.* (2004) has shown that pre-exposure of *Lb. reuteri* E849 cells to pH 3.5 significantly enhanced the tolerance of the bacterium to pH 2.5 in PBS. However the results of their work on *Lb. reuteri* E849 and the current research on *Lb. reuteri* (LR)

could not be compared, as strains and assay procedure (pH value, exposure time and acid adaptation medium) were different.

In contrast to the situation with SGJ tolerance, OJ appeared in the present study to significantly decrease the tolerance of LG, LR and PJ to SIJ, with Bb the only strain unaffected.

This result is in accordance with the findings of Saarela *et al.* (2004), who reported that pre-exposure of *Lb. rhamnosus* E800 to a sub-lethal low pH value (pH 4.0, 1 h) slightly decreased the tolerance of the bacterial strain to subsequent treatment with 1.4 % bile in PBS compared to non-treated bacterium. Pre-treatment at pH 3.5 however, did not change the tolerance of the bacterium to 1.4 % bile. Nor was there any improvement in the bile tolerance (1.4 %) of *Lb. reuteri* E849 cells pre-exposed to pH 3.5 compared to non-treated bacterium, while *Bif. lactis* E2010 (Bb) was reported to tolerate 1.4 % bile in PBS when pre-treated with pH 3.5.

Pretreatment of *P. freudenreichii* with acid (pH 5.0, for 1 h) has also been observed to have an adverse effect on the tolerance of the bacterium to a subsequent bile challenge (1.0 g/L, 60 s) (Leverrier *et al.*, 2003).

The unaffected tolerance of Bb included in OJ and PBS at the baseline to simulated gastro-intestinal conditions is supported by the study of Saarela *et al.* (2006) who reported no significant difference in the acid (2 h at pH 2.5, plus pepsin) or bile (3h at pH 7.2, plus 1.0% bile) tolerance of Bb cells included either in PBS or a commercial fruit drink with pH 3.7.

All examined probiotic strains (either alone or in combinations) included in OJ showed high tolerance to SGJ which remained virtually unchanged over 30 days of refrigerated storage (changes in viability loss ≤ 0.5 orders of magnitude). Such slight variations were also evident in the tolerance of probiotics to SIJ with time during the entire storage period, with changes in viability loss ≤ 0.6 orders of magnitude across the entire data set. The only exception being LR in mono-culture, in which case tolerance to SIJ significantly increased at D10 compared to D0 and remained unchanged by the end of storage period.

In other words, the duration of refrigerated storage of the probiotic strains included in OJ did not have an adverse impact on the bacterial tolerance to SGJ and SIJ over one month of storage. The results were therefore not in support of the hypothesis that the ability of the probiotics to survive simulated gastro-intestinal conditions would be

affected by the duration of their combined exposure to the food matrix (OJ) and low storage temperature (4°C).

These findings are consistent however with the study by Champagne *et al.* (2008), in which refrigerated storage of individual probiotic strains *Lb. rhamnosus* LB11, *Lb. reuteri* LB38, *Lb. acidophilus* LB3 and *Lb. plantarum* LB42 in a fruit juice blend (pH 4.2) for 35 days did not affect their tolerance to bile (0.3%) or pancreatic enzymes. Although in this case, tolerance of the bacteria to acid (pH 2.0 for 2 hours) was significantly impaired by comparison with the fresh cultures.

The tolerance of probiotic strains to SIJ in this study was found to be both strain dependant, and affected by the presence of other probiotic strains. Different effects including enhancement, suppression, or no effect, were observed with the presence of other probiotic strains on the tolerance to SIJ. Bb (alone and in combinations) included in OJ appeared to be the most robust when confronted with simulated intestinal conditions with little or no viability loss compared to other probiotic strains. Previously, milk as a carrier vehicle had been reported to increase susceptibility of monocultures of Bb to both simulated gastric and intestinal juices, after two weeks of refrigerated storage, compared with that of the bacterium at baseline (Saarela *et al.*, 2006). The lowest bacterial tolerance to SIJ was observed in LG preparations with viability loss ranging from 4 to 6 orders of magnitude. The viability loss in LR cases varied from 1 to 4 log reductions in CFU/mL. When combined with Bb and/or PJ significant improvements were observed in the tolerance of LG and LR. On the contrary, tolerance of PJ to SIJ was adversely affected in the presence of other probiotics with viability losses ranging from less than 2 (in the presence of only Bb) to 5.5 log reductions in CFU/mL (in combinations containing LG). Little is known of the possible occurrence of interactions within combinations of probiotics which may impact on the functionality of the individual strains included, with almost all previous studies having focused on the influence of probiotic combinations on the ability of each strain to adhere to the intestinal epithelial mucosa (Collado *et al.*, 2007; Moussavi & Adams, 2010; Ouwehand *et al.*, 2000; Ouwehand *et al.*, 2002). To the authors' knowledge, this is the first report showing that the tolerance to gastro-intestinal conditions of probiotic strains included in a carrier vehicle may be influenced by the presence of other strains. However potential underlying mechanisms are yet to be investigated.

5.6 Conclusion

The results of the present study demonstrated that when included in orange juice, the probiotic strains examined were observed to have a high tolerance to SGJ across one month of refrigerated storage. In terms of their ability to tolerate simulated SIJ, strain specific variation was also clearly evident among the probiotics studied. This ability however, might be further affected by the presence of other probiotics and in some cases could be improved. The results of this study provide a clear indication of the gastro-intestinal tolerance of probiotic cultures included in OJ during refrigerated storage, and the manner in which gastro-intestinal tolerance of probiotics may be manipulated using combinations of different species.

**Chapter VI: Intestinal Epithelial Cell Adhesion
Characteristics of Probiotic Mono-Cultures and
Combinations Incorporated into Orange Juice**

6.1 Summary

The present study aimed to determine the effect of duration of combined exposure of 4 probiotic strains *Lb. rhamnosus* GG (LG), *Lb. reuteri* (LR), *Bif. animalis* subsp *lactis* Bb12 (Bb) and *P. jensenii* 702 (PJ) either alone or in 2-, and 3- multi-species combinations to orange juice and low storage temperature (4°C) on their capability to adhere to intestinal epithelial Caco-2 cells at 10-day intervals during one month storage. While the adhesion rate of LG remained relatively stable in almost all preparations throughout the entire storage period, the data on adhesion ability of other probiotics revealed variations in relation to strain, presence of other microorganisms, and duration of the storage. Adhesion rate of LR either alone or in combination with Bb was found to increase as viability declined during storage, whereas it significantly increased in the presence of PJ and Bb-PJ toward the end of the storage period as viability was maintained throughout the storage period. Adhesion rate of mono-culture of Bb only increased at day 20, while it remained unchanged in combination with LR-PJ during the storage as viability declined for both cases. Adhesion rates of Bb when combined with either of the lactobacilli appeared to decline along with viable cell numbers. In the presence of PJ or LG-PJ significant fluctuations were observed in adhesion rates of Bb during the storage period while viability remained relatively stable. A general trend of increasing adhesion rates was evident for PJ as mono-culture or in combination with LG and/or Bb as viability declined during the storage. A significant increase and decrease in adhesion rate of PJ was observed at day 10 when combined with LR and LR-Bb respectively as viability remained stable in both cases. In terms of both viability and adhesion rate, the preparations that provided the best outcomes were LG in all cases, LR when combined with PJ and Bb-PJ, Bb when combined with PJ and LG-PJ, and PJ either alone or in combination with LR. However when viability and adhesion rates of all constituents included in a preparation were considered, the best outcomes were for LG and LR-PJ. In conclusion, the results of the present study showed that the adhesion ability of probiotics to intestinal epithelial cells is strain/species dependent and is also further affected by the duration of refrigerated storage in orange juice and presence of other probiotics. These effects should be considered when formulating probiotic products.

6.2 Introduction

Adhesion to the intestinal epithelial mucosa is one of the main criteria to be considered in the selection of potential probiotic microorganisms. Adhesion is a prerequisite to the success of bacterial colonisation of the intestinal mucosal surfaces (Salminen *et al.*, 1996), which is thought to be necessary for probiotics to exert their health promoting effects (Boyle *et al.*, 2006). The ability of potential probiotics to adhere to the intestinal epithelial mucosa can be evaluated using *in vivo* and *in vitro* assays (Ouwehand & Salminen, 2003; Servin & Coconnier, 2003). Availability and ethical issues hamper the widespread use of animal models or human/animal intestinal-derived biopsy samples (Saarela *et al.*, 2000), hence a number of *in vitro* models have been developed to evaluate bacterial adhesion to the intestinal mucosa (Ouwehand & Salminen, 2003). Even though *in vitro* assays can not mimic the complexities of *in vivo* conditions completely, various well controlled experimental conditions can be applied to investigate the adhesion ability of potential probiotics. Moreover a large number of microorganisms may be examined using *in vitro* models (Ouwehand & Salminen, 2003). Adhesion to the intestinal epithelial mucosa by probiotics depends on many factors such as bacterial strain, bacterial concentration, probiotic formulation (combination), composition of the bacterial growth medium, the cell culture and co-culture medium, pH of the co-culture medium, bacterial growth stage, bacterial and intestinal cell culture growth conditions, incubation time, host specificity, specific region within the intestine, digestion and composition of gut microbiota (Collado *et al.*, 2007; Deepika *et al.*, 2009; Greene & Klaenhammer, 1994; Kankaanpaa *et al.*, 2001; Moussavi & Adams, 2010; Ouwehand *et al.*, 2000; Ouwehand & Salminen, 2003; Tallon *et al.*, 2007; Van den Abbeele *et al.*, 2009).

It is also likely that the carrier matrix may affect adhesion although the impact of food matrices, or components of them, on the functionality of probiotics has not been extensively researched to date (Sanders & Marco, 2010). To this author's knowledge, study of the effect of food matrices on the adhesion of probiotics, is limited to the work of Ouwehand *et al.* (2001), although there are a number of reports on the effects of food components such as carbohydrates, fatty acids and minerals on the adhesion of probiotics to intestinal epithelial mucosa (Kankaanpaa *et al.*, 2001; Lee & Puong, 2002; Marcinakova *et al.*, 2010; Parkar *et al.*, 2010; Tuomola *et al.*, 2000; Van den Abbeele *et al.*, 2009).

In order to more closely simulate *in vivo* conditions of bacterial adhesion to the intestinal mucosa, it has been recommended that microorganisms be exposed to the food matrix prior to the adhesion assay (Ouwehand & Salminen, 2003).

The study by Ouwehand *et al.* (2001) showed that pre-treatment of 5 probiotic lactobacilli (*Lb. brevis* PEL1, *Lb. reuteri* ING1, *Lb. rhamnosus* E-800, *Lb. rhamnosus* LC705 and *Lb. rhamnosus* GG) with fat-free milk, milk with 1.5% fat or non-homogenised milk (1.6-1.9% fat) for 1 hour in most cases significantly decreased adhesion of the lactobacilli to intestinal mucus glycoproteins relative to the control (HEPES-Hanks' buffer, pH 7.4). Compared with the control, pre-treatment of the lactobacilli with fat-free milk only decreased significantly the adhesion of *Lb. brevis* PEL1 and *Lb. rhamnosus* E-800, while a significant decline was observed in the adhesion percentage of *Lb. brevis* PEL1, *Lb. reuteri* ING1, *Lb. rhamnosus* E-800 and *Lb. rhamnosus* GG when pre-treated with either milk with 1.5% fat or non-homogenised milk (1.6-1.9% fat). The same study also examined the effect of different bacterial growth culture media on the adhesion ability of the lactobacilli. A significant decrease in adhesion percentage was observed for *Lb. brevis* PEL1 and *Lb. rhamnosus* E-800 grown in milk whey medium or the same medium supplemented with 1% glucose compared with MRS medium. Milk whey medium with 1% glucose also decreased adhesion of *Lb. reuteri* ING1 compared with MRS medium. No significant change was observed in all other preparations.

It has also been reported that the presence of fatty acids (γ -linolenic, arachidonic, α -linolenic or docosahexaenoic acid) at concentrations of 20 or 40 $\mu\text{g/mL}$ in the bacterial growth media could, in most cases, significantly decrease *in vitro* adhesion of *Lb. rhamnosus* GG, *Lb. casei* Shirota and *Lb. bulgaricus* to human intestinal mucus, compared with the fatty acid free medium. At a concentration of 10 $\mu\text{g/mL}$ arachidonic acid and α -linolenic acid significantly increased the adhesion of *Lb. casei* Shirota to intestinal mucus and human intestinal epithelial cells respectively (Kankaanpää *et al.*, 2001).

Of 8 different carbohydrates (N-acetyl-glucosamine, galactose, glucose, fructose, fucose, mannose, methyl- α -D-mannopyranoside and sucrose) added by Lee and Puong (2002) to a bacterial suspension prior to an adhesion assay, only methyl- α -D-mannopyranoside and sucrose significantly increased the adhesion of *Lb. rhamnosus* GG to human enterocyte-like Caco-2 cells relative to the control, whereas N-acetyl-glucosamine decreased the adhesion of *Lb. rhamnosus* GG. N-acetyl-glucosamine,

glucose and fucose significantly reduced the adhesion of *Lb. casei* Shirota. No significant change was observed in all other cases.

It has also been reported that 2 h pre-treatment of 4 probiotic lactobacilli with 70% ethanol resulted in enhanced adhesion of *Lb. acidophilus* LA1 to human intestinal mucus glycoproteins (PBS pH 7.4). No significant change was observed for *Lb. rhamnosus* GG, *Lb. rhamnosus* LC-705 and *Lb. casei* Shirota (Tuomola *et al.*, 2000).

Parkar *et al.* (2010) reported that pre-treatment of human intestinal epithelial Caco-2 cells with pectin extracted from kiwifruit for 1 hour followed by incubation with *Lb. rhamnosus* for a further 2 hours, increased the adhesion percentage of the bacterium to Caco-2 cells compared with the control. This study also revealed that functional polysaccharides such as guar gum, citrus pectin, inulin and pectin extracted from kiwifruit using KH_2PO_4 differentially influenced the adhesion ability of *Lb. rhamnosus* and *Bif. bifidum* to Caco-2 cells. They showed that only Kiwifruit pectin significantly enhanced the adhesion of *Lb. rhamnosus* compared to the control whereas the adhesion percentage of *Bif. bifidum* was significantly increased only by inulin and citrus pectin. No significant difference was observed between the adhesion of both bacteria in the presence of guar gum and the untreated control.

Likewise a recent study has demonstrated that inclusion of 1.5% carbohydrate arabinoxylans (AX) in intestinal water (IW) or phosphate buffer saline (PBS) as adhesion media, significantly decreased the adhesion of lactobacilli to porcine mucin (as a model of intestinal mucus) and in contrast adhesion of bifidobacteria declined only in PBS in the presence of AX compared with the media without AX. This study demonstrated that both the composition of the adhesion medium and the bacterial strains can influence adhesion, and further, that the addition of inulin (1.5%) into the adhesion media decreased only the adhesion of bifidobacteria in PBS significantly. No significant change was observed in adhesion rates of lactobacilli (Van den Abbeele *et al.*, 2009).

A recent study has shown that addition of calcium (200 mmol/L) to adhesion assay wells significantly increased the adhesion percentage of probiotic *Enterococcus faecium* (10 strains) and *Lb. reuteri* 12002 to a porcine jejunum epithelial cell line compared with the control (Marcinakova *et al.*, 2010).

Clearly, the environment to which the bacteria are exposed prior to contact with the intestinal epithelium may be critical in determining their capacity to adhere. However, the extent to which long term exposure to various food matrices may impact on the

adhesion properties of probiotics is yet to be established. Moreover, previous research has shown that the adhesion capacity of probiotics to the intestinal epithelial mucosa might be impacted by the presence of other probiotics (Collado *et al.*, 2007; Moussavi & Adams, 2010; Ouwehand *et al.*, 2000; Ouwehand *et al.*, 2002). However this effect has not yet been examined for microorganisms incorporated into food matrices.

This study examines variations in the *in vitro* adhesion of probiotics incorporated both individually and in various combinations in refrigerated orange juice, to Caco-2 cells during one month of storage. The aim of the experiment, comprising the same probiotic combinations as those described in Chapters 4 and 5, was to evaluate the effect of refrigerated storage in orange juice, and the combining of probiotics, on the rates of adhesion of individual strains to intestinal epithelial cells.

The study was designed to address the following hypotheses:

- That the adhesion ability of probiotic bacteria in orange juice would exhibit species/strain dependent variation.
- That the ability of probiotics to adhere to intestinal epithelial Caco-2 cells would be further affected by the duration of their combined exposure to the food matrix (orange juice) and low storage temperature (4°C)
- That the adhesion ability of individual probiotics would be affected by the presence of other probiotic bacteria.

6.3 Materials and methods

6.3.1 Chemicals and reagents

Unless otherwise specified, chemicals were from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Cell culture medium and relevant ingredients/supplements used in this study were from Gibco[®] (Gibco[®], Invitrogen Corp., Carlsbad, CA, USA).

6.3.2 Bacterial cultures and growth conditions

As previously described in Chapter 4.

6.3.3 Orange juice

As previously described in Chapter 5.

6.3.4 Preparation of probiotic orange juice

As previously described in Chapter 4.

6.3.5 *In Vitro* bacterial adhesion assay

Preparation of the Caco-2 monolayer was as described in Chapter 3. Bacterial adhesion to Caco-2 human intestinal epithelial cells was examined on day 0, 10, 20 and 30 during the storage period. For the initial (day 0) adhesion assay the bacterial cells were taken directly from PBS, and had not been exposed to orange juice at all. The probiotic preparations were vortex mixed and a 1 mL aliquot of the preparation was taken and centrifuged (3220 ×g, 10 min, 4 °C). Supernatants were then discarded and pellets re-suspended in 1 mL cell culture medium without antibiotics. In the case of probiotic orange juice preparations, pellets were washed 3X with PBS prior to re-suspension in cell culture medium. Application of bacterial suspensions, incubation, and subsequent assay for percentage of adherent cells, was also as described in Chapter 3.

6.3.6 Statistical analyses

Statistical analyses were performed using SPSS software Ver. 18 (SPSS Inc., Chicago, IL, USA). Data were analysed using the linear mixed model procedure to run a general linear model (GLM). In order to give protection against potential false positive (false significant results), the Bonferroni adjustment was used and significance level was set at point $p \leq 0.001$.

6.4 Results

With the aim of assessing strain variability, and the effect of storage duration and combining of probiotics on the ability of individual strains to adhere to Caco-2 cells, probiotics in the form of single strain (LG, LR, Bb, PJ), paired (LG-Bb, LG-PJ, LR-Bb, LR-PJ, Bb-PJ) and triplet (LG-Bb-PJ and LR-Bb-PJ) combinations - a total of 11 separate preparations - were introduced into orange juice stored at 4°C. In each case the viability and intestinal epithelial adhesion ability of each individual strain were monitored throughout 30 days of storage. In general, the data revealed discernible differences in relation to strain, presence of other microorganisms, and duration of refrigerated storage in orange juice.

6.4.1 Strain variability in adhesion rates and the effect of cold storage in orange juice

In order to assess variability in adhesion rates between the four probiotics, and the effect of refrigerated storage in orange juice, the analysis was focused initially on the performance of the four mono-cultures. The viability and adhesion percentage of the individual strains has been presented in Figure 6.1 revealing differing trends in adhesion among the various probiotic strains during one month of chilled storage.

At baseline, significant species dependent differences were observed between the adhesion rates of individual probiotics with maximum and minimum adhesion rates of 63.6% and 6.9% observed for Bb and PJ respectively. LG and LR exhibited comparable adhesion rates at baseline (35.43 and 37.8% respectively).

Both the stability and adhesion percentage (varying between 35.43-45.72%) of the mono-culture of LG in OJ remained relatively constant throughout 30 days of storage. In contrast with LG, the adhesion rates of LR were found to rise as viability declined across the storage period, until all viable cells were able to adhere by day 30. A similar trend was observed for PJ with adhesion increasing as viability declined, although in this case the decrease in viable cell numbers was considerably less than that observed for LR, with adhesion rates for PJ reaching a maximum of 39% at day 30. A trend very similar to that of LR was also evident in the case of Bb up to day 20 with the adhesion rate approaching 100% at this time. However, in contrast with the LR scenario, rather than all cells maintaining adhesion capacity as numbers of Bb further declined between

D20 and 30, it appeared that some viable cells were unable to adhere, with the adhesion rates falling again to below 60%.

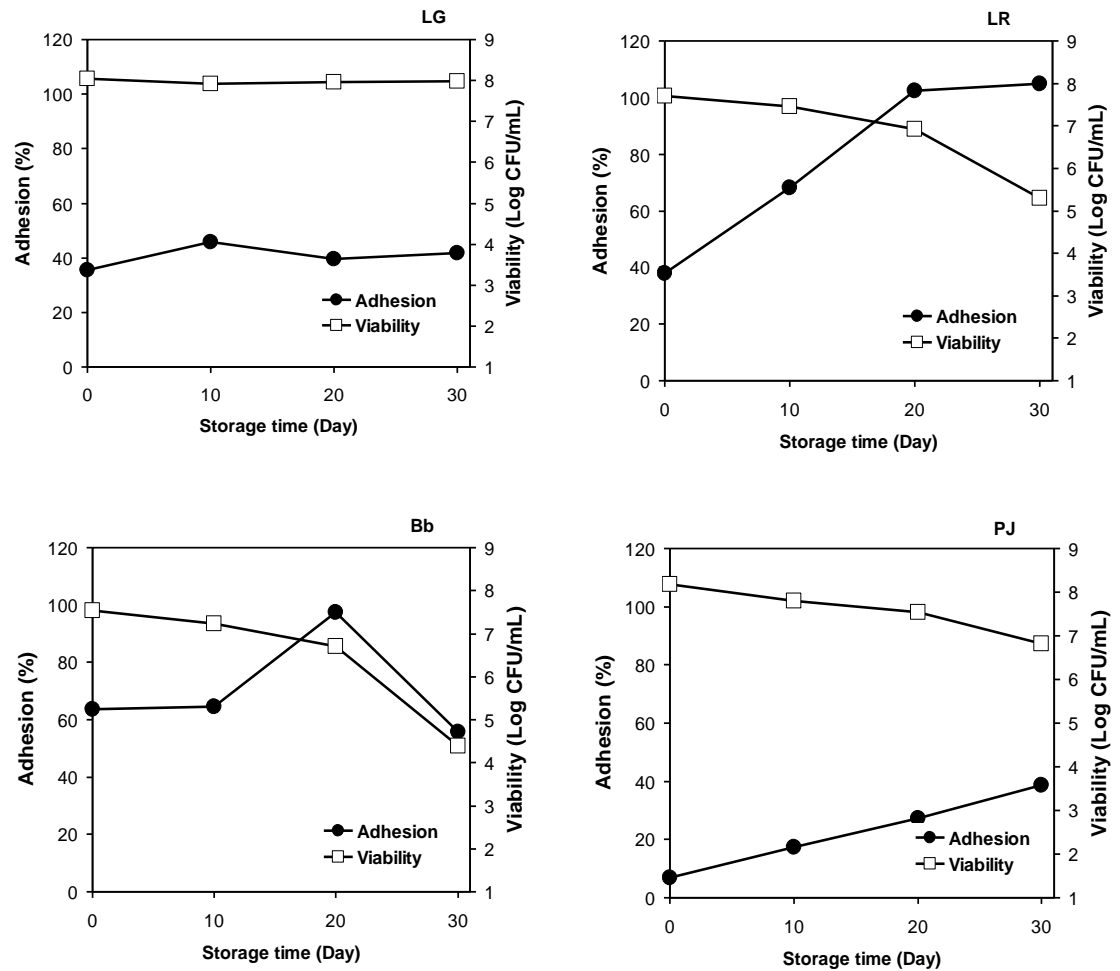


Figure 6.1: Viability (□) and adhesion rate (●) of individual strains of LG, LR, Bb and PJ during exposure to orange juice (OJ) stored at 4°C for 30 days.

6.4.2 Adhesion rates and the effect of combining probiotics

Among the data collected a number of different patterns in adhesion rate relative to viability were evident. In the figures presented (*i.e.* 6.2 to 6.5) the relevant mono-culture plot from figure 6.1 has been reproduced in each case to facilitate visual comparison with the corresponding co-culture results.

Examination of Figure 6.2 indicates that as with the mono-culture, and along with the viability, the adhesion rates of LG remained relatively stable when combined with Bb and/or PJ.

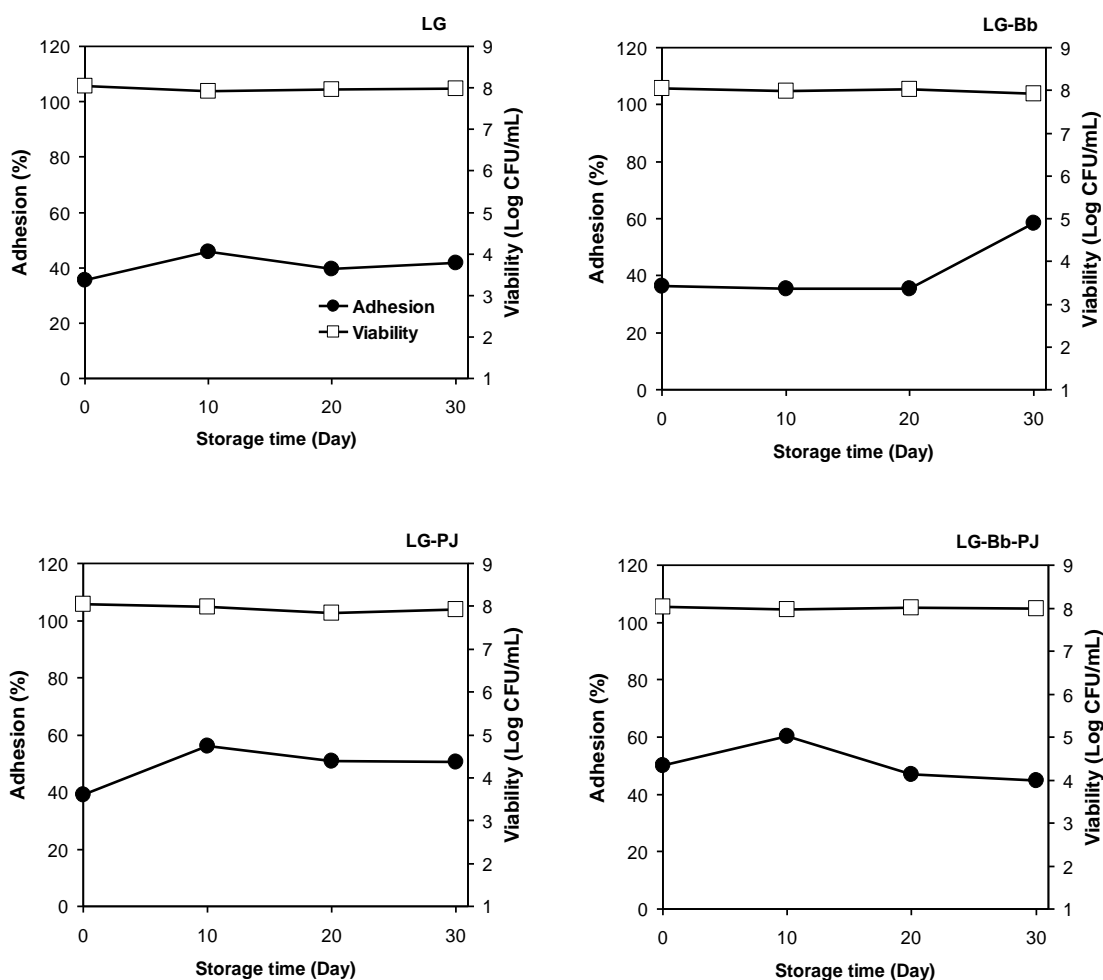


Figure 6.2: Viability (□) and adhesion rate (●) of LG either alone or in combination with Bb and/or PJ during exposure of the probiotics to orange juice (OJ) stored at 4°C for 30 days.

In the case of LR however (Figure 6.3), two distinct patterns in adhesion rate were apparent. The first being that observed for the LR-Bb combination, where the adhesion rate was found to increase as viability declined during storage, resembling that of the LR mono-culture. The second (shared by the LR-PJ and LR-Bb-PJ combinations) was one of sustained viability with a slight upward trend in adhesion rate toward the end of the storage period.

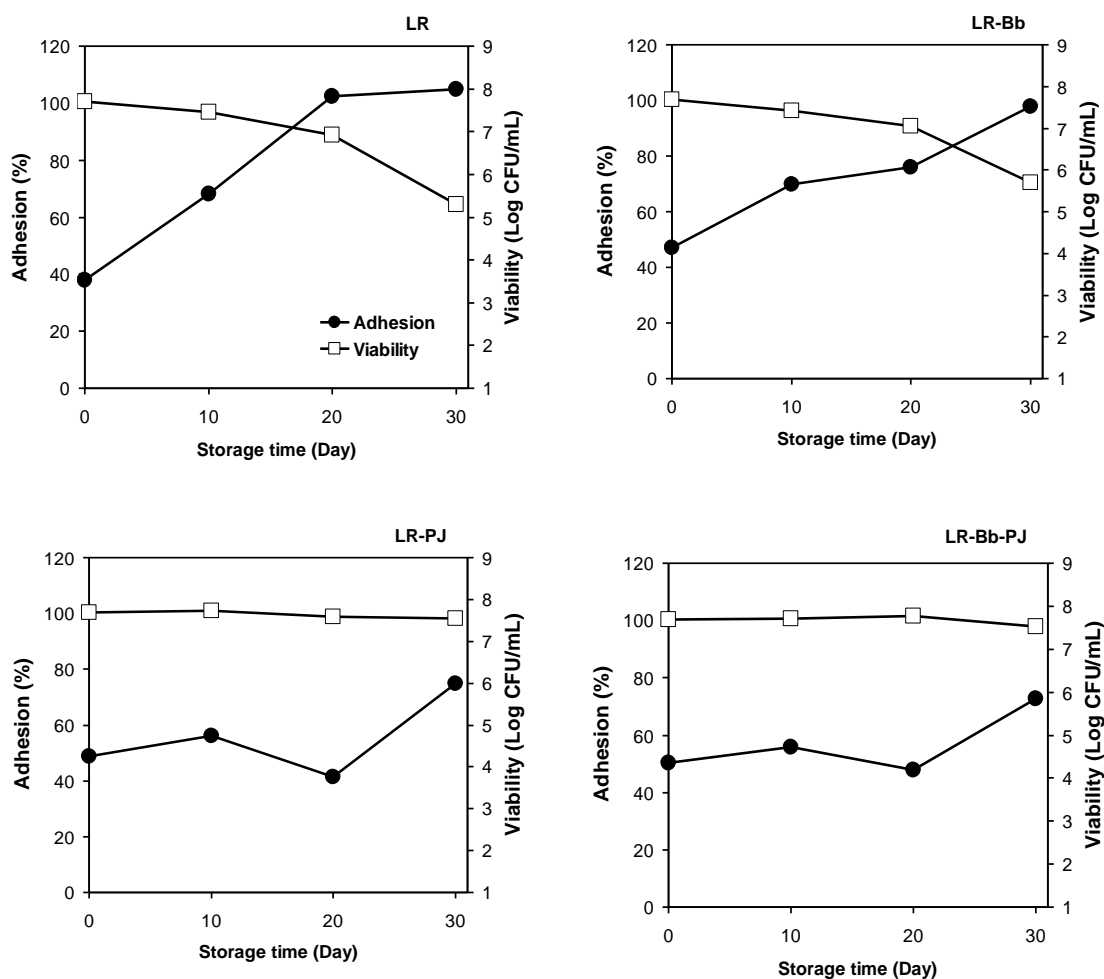


Figure 6.3: Viability (□) and adhesion rate (●) of LR either alone or in combination with Bb and/or PJ during exposure of the probiotics to orange juice (OJ) stored at 4°C for 30 days.

Among the six Bb preparations three distinct patterns were evident (Figure 6.4). Although slightly less exaggerated than in the case of the mono-culture, a similar pattern was also observed for the Bb-LR-PJ preparation. The second pattern was that shared by the Bb-PJ and Bb-LG-PJ preparations in which adhesion rates were respectively found to improve, decline, then improve across the three ten day intervals of the storage period, during which viability remained relatively stable. The third is the pattern observed when combined with either of the lactobacilli. In this case adhesion rates appeared to decline along with viable cell numbers, although in the Bb-LR combination, adhesion percentages were observed to increase somewhat as viable cell numbers plummeted in the final 10 days of storage.

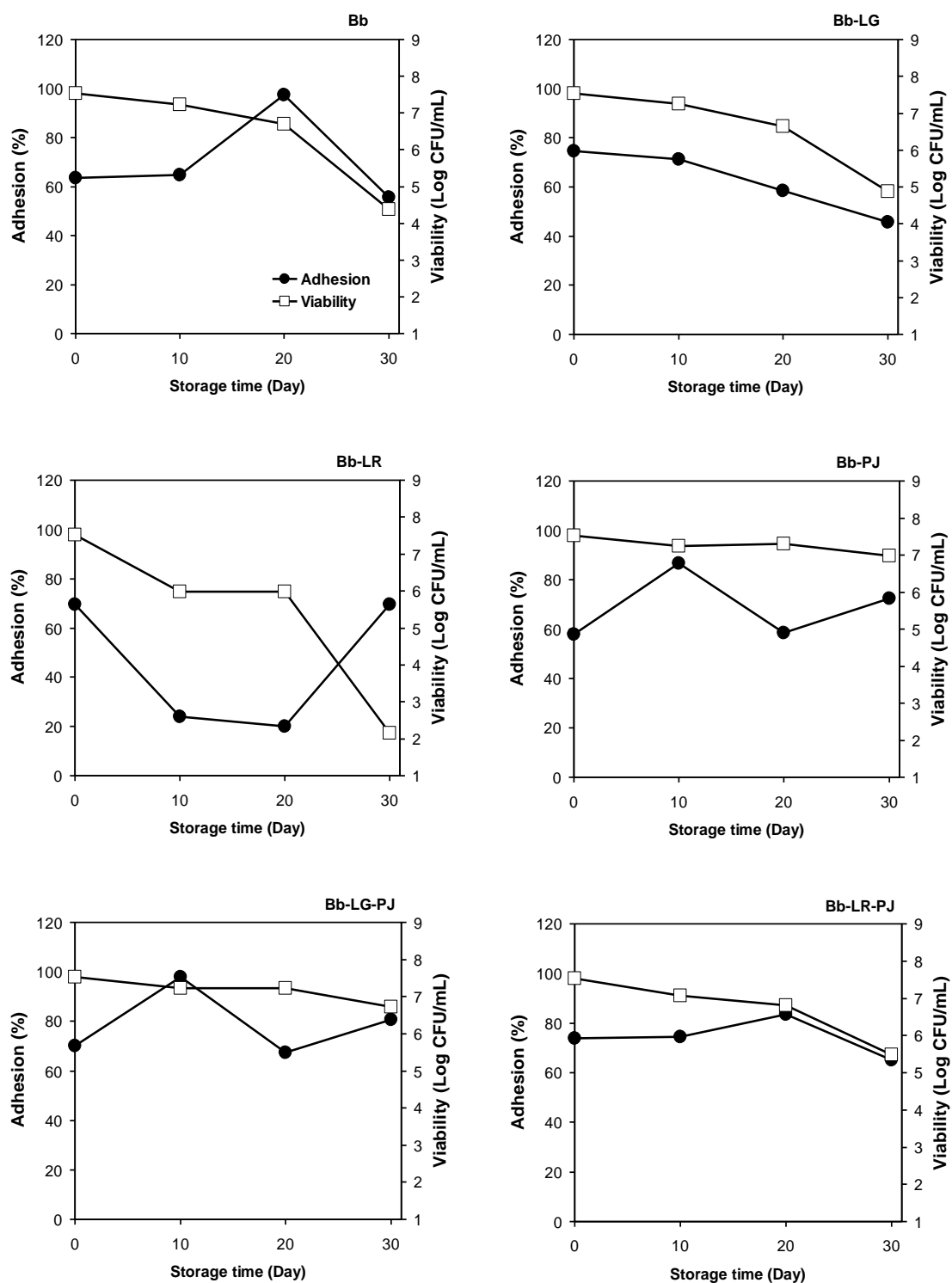


Figure 6.4: Viability (□) and adhesion rate (●) of Bb either alone or in combinations with LG or LR and/or PJ during exposure of the probiotics to orange juice (OJ) stored at 4°C for 30 days

For PJ a general trend of increasing adhesion rates as viable cell numbers declined was evident in four of the six preparations (Figure 6.5). In the PJ-LR preparation by comparison, a 3-fold increase in adhesion was observed over the first 10 days, followed by a decline by day 30 back almost to initial (day 0) levels. In further contrast, the PJ-LR-Bb preparation exhibited an initial adhesion rate almost 3-fold higher than in all other preparations, but declined rapidly thereafter.

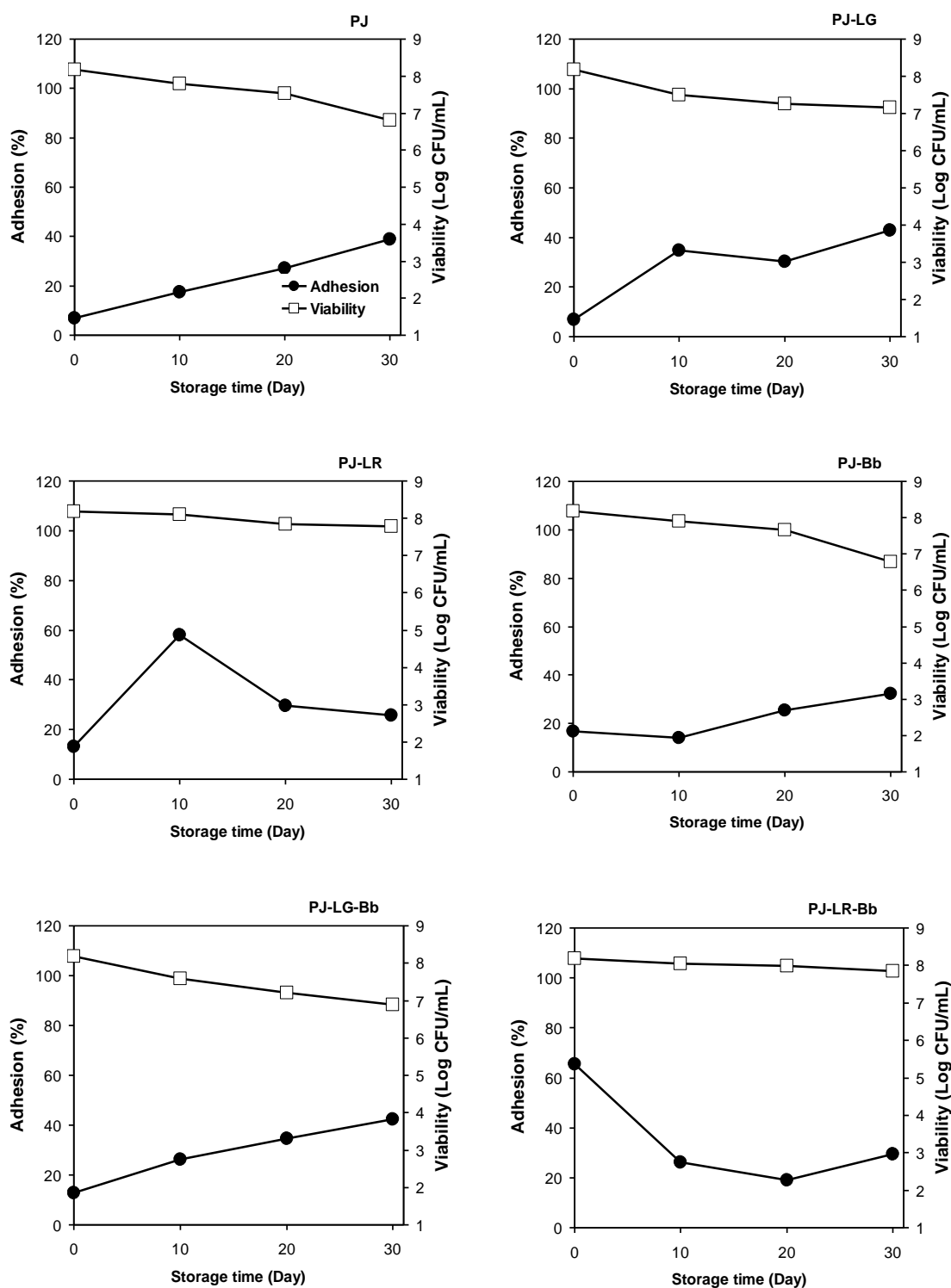


Figure 6.5: Viability (\square) and adhesion rate (\bullet) of PJ either alone or in combinations with LG or LR and/or Bb during exposure of the probiotics to orange juice (OJ) stored at 4°C for 30 days.

With the exception of the adhesion rate of PJ which significantly increased in the presence of LR-Bb compared to that of the mono-culture of PJ, combining probiotics did not significantly affect the adhesion rate of the probiotic constituents. However some effect of the presence of other strains on the adhesion percentage of probiotics in multi-species preparations was evident across the storage period.

In order to simplify the results, they have been summarised in Table 6.1. When summarised in this way it can be seen that there are 60 viability/adhesion sets which could be categorised into two main groups:

- 1- Sets with unchanged viability of the target bacterium compared to the control (68.3%)
- 2- Sets with significantly decreased viability of the target bacterium compared to the control (31.7%)

Each group contains 3 potential subgroups based on changes in the adhesion rate relative to the initial percentage value (day 0): unchanged (N), significantly increased (+) and significantly decreased (-).

Out of 60 possible cases, 25 cases (41.7%) were identified as exhibiting unchanged viability and adhesion (N/N). In 12 cases (20%), viability of the probiotic did not change but adhesion increased significantly compared with the initial value (N/+). Unchanged viability with decreased adhesion percentage (N/-) was only found in the case of PJ when combined with LR and Bb at all time points of probiotic exposure to OJ (4 cases, 6.7%).

In the second group, 4 cases (6.7%) were identified as exhibiting an unchanged adhesion percentage (-/N). In 12 cases (20%), adhesion percentage significantly increased compared to the control (-/+). A significant decrease in adhesion percentage of the four remaining cases (3 cases, 5.0 %) was observed (-/-).

In terms of both viability and adhesion rate, the preparations that provided the best outcomes were LG in all cases, LR when combined with PJ and Bb-PJ, Bb when combined with PJ and LG-PJ, and PJ either alone or in combination with LR (Table 6.1). However when viability and adhesion rates of all constituents included in a preparation were considered, the best outcomes were for LG and LR-PJ (highlighted cells in Table 6.1)

Table 6.1: Qualitative summary of the viability and adhesion percentage of individual strains either alone or in combinations during 30 days of exposure to OJ, expressed in terms of the viability and adhesion percentage relative to those of the control (PBS, day 0). (-) = decreased; (+) = increased; (N) = not affected

Preparations	D10		D20		D30	
	Viability	Adhesion	Viability	Adhesion	Viability	Adhesion
LG	N	N	N	N	N	N
LG-Bb	N	N	N	N	N	+
LG-PJ	N	N	N	N	N	N
LG-Bb-PJ	N	N	N	N	N	N
LR	N	+	-	+	-	+
LR-Bb	N	+	-	+	-	+
LR-PJ	N	N	N	N	N	+
LR-Bb-PJ	N	N	N	N	N	+
Bb	N	N	N	+	-	N
Bb-LG	N	N	N	-	-	-
Bb-LR	-	-	-	-	-	N
Bb-PJ	N	+	N	N	N	N
Bb-LG-PJ	N	+	N	N	N	N
Bb-LR-PJ	N	N	N	N	-	N
PJ	N	N	N	+	-	+
PJ-LG	-	+	-	+	-	+
PJ-LR	N	+	N	+	N	+
PJ-Bb	N	N	-	+	-	+
PJ-LG-Bb	-	N	-	+	-	+
PJ-LR-Bb	N	-	N	-	N	-

6.5 Discussion

Optimal functioning of probiotics depends on their ability to adhere to the intestinal mucosa (Saarela *et al.*, 2000). Delivery vehicle matrices may affect intestinal mucosal adhesion characteristics of probiotics, however to date, little is known about the effect of food matrices on the adhesion of probiotics (Ouwehand & Salminen, 2003; Sanders & Marco, 2010). The objective of the present study was to investigate the influence of combined exposure of probiotics to orange juice and low storage temperature (4°C), on their capacity to adhere to intestinal epithelial cells. The results provided evidence of variation in adhesion rates between individual strains, for the same strain when combined with different probiotics, and in relation to viable cell numbers. Although the experimental design did not specifically allow the possible effects of the carrier matrix and refrigeration to be readily differentiated from one another, it was also found that the ability of the probiotics to adhere to Caco-2 cells was further affected by the duration of their combined exposure to orange juice and refrigerated storage.

As summarised in Table 6.1, six patterns of viability and adhesion could be observed.

- Probiotic preparations with both unchanged viability and adhesion percentage (N/N): Specifically this scenario was evident for LG in almost all cases. As this situation was consistent across the storage period, it could be concluded that the adhesion ability of LG was not influenced by either presence of other probiotics or the duration of its combined exposure to orange juice and refrigerated storage.

Some preparations in the LR, Bb or PJ sets also exhibited this response, providing evidence that the adhesion rate of the target strain can be affected by the presence of other probiotics. This situation however was not observed at all time points during the storage showing the effect of duration of combined exposure of the probiotic preparations to orange juice and refrigerated storage on the adhesion capacity of the target strain.

- Probiotic preparations with unchanged viability but improved or compromised adhesion ability (N/+ or N/-): These situations were again observed for some preparations in a group and for some time points during storage of a preparation. Again it might be concluded that the adhesion ability of target probiotic strain was influenced by the presence of other probiotics and/or duration of its combined exposure to orange juice and refrigerated storage.

Although there have been no previous studies examining the effect of orange juice as a probiotic carrier matrix and/or duration of refrigerated storage of a probiotic food on the adhesion of probiotics to intestinal epithelial cells, several studies have examined the effect of individual food components which could be present in orange juice (*e.g.* carbohydrates, anions/cations and fibers).

Orange juice is a chemically complex medium containing a variety of sugars, organic acids, vitamins, minerals, phytochemicals, flavonoids, volatiles, fibers etc. (Belitz *et al.*, 2009). A distinct characteristic of OJ is its low pH, which varies from 2.9 to 3.9 (Ladaniya, 2008). The main acidifying agents present in the orange juice are citric acid (9.4 g/L) followed by L-malic acid (1.7 g/L) (Belitz *et al.*, 2009). The pH value of the orange juice used in this study was 3.82, and in Chapter 4, it was shown that pH values of probiotic orange juices seemed to be almost constant (changes < 0.1 unit) throughout the storage period at 4°C, suggesting that the incorporated probiotics were, as might be expected, relatively inactive metabolically. The results showed that the adhesion rate of LR either alone or in combination with Bb significantly increased after 10 days of refrigerated storage in OJ (pH 3.8) compared with the control (PBS, pH 7). This observation could be due to the effect of carrier pH on the adhesion rate of LR. If this is the case, the finding would appear consistent with a study by Granato *et al.* (1999) in which significantly enhanced adhesion of probiotic *Lactobacillus* strains such as *Lb. johnsonii* La1 and *Lb. acidophilus* La10 to Caco-2 cells was observed at acidic pH 3 and 3.5 (for La1) and pH 3 (for La10) compared to pH 7. Adhesion at intermediate pH values for both lactobacilli did not differ significantly from that of pH 7.

The results of the present study also showed that the adhesion rate of Bb did not change after 10 days of refrigerated storage in OJ (pH 3.8) compared with the control (PBS, pH 7), whereas significant increases were observed after 20 days storage. Riedel *et al.* (2006) demonstrated strain specific changes for intestinal epithelial adhesion of strains of bifidobacteria when pH of the co-culture medium was shifted from 7.0 to 4.5. In most cases (5 out of 8 strains examined) no significant change was observed between the adhesion of bifidobacteria strains at pH 4.5 and 7.0. *Bif. longum* NCC 2705 however, showed an increase in adhesion percentage at pH 4.5 compared with neutral pH, whereas the adhesion percentage of *Bif. bifidum* strains NCC 189 and S17 decreased significantly at pH 4.5 compared with pH 7.0 (Riedel *et al.*, 2006). In a more recent study pre-exposure of *Bif. bifidum* MIMBb75 to pH values ranging from 3 to 8.5,

changed the adhesion percentage of the bacterium to HT-29 epithelial cells from about 0% at pH 3 to 100% at pH 7.3 (Guglielmetti *et al.*, 2009).

Strain dependent differences have also been reported for adhesion of probiotics to intestinal mucus (Gusils *et al.*, 2003; Ouwehand *et al.*, 2001). The studies by Tuomola *et al.* (2000) and Ouwehand *et al.* (2001) showed that pre-exposure of probiotic lactobacilli, including LG, to low pH values such as pH 2.0 or pH 2.2 did not change the adhesion percentage of the lactobacilli to intestinal mucus glycoproteins compared with the control (HEPES-Hanks' buffer, pH 7.4). Together, this result and the findings presented here regarding unchanged adhesion of LG during exposure to orange juice (pH 3.8) compared with the control (PBS, pH 7), would suggest that the intestinal adhesion properties of this probiotic are not significantly affected under low pH conditions. It should also be noted that acidic pH values have been adjusted in all previous studies using hydrochloric acid (HCl), and it is conceivable that organic acids found in orange juice, such as citric and malic acid, might impact differently on the adhesion properties of probiotics.

Apart from organic acids, sugars such as sucrose (3.3%), fructose (3.0%), and glucose (2.8%) are also major components of natural orange juice (Belitz *et al.*, 2009). Strain dependent differences have also been observed when the effect of sugars on the adhesion of probiotics to intestinal mucosa was examined (Lee & Puong, 2002). It has been reported that inclusion of sucrose, at a concentration of 25 mM, to bacterial suspensions before an adhesion assay, significantly increased the adhesion of *Lb. rhamnosus* GG to human enterocyte-like Caco-2 cells, whereas glucose and fructose did not affect the adhesion ability of the same bacterium (Lee & Puong, 2002). This followed similar findings that inclusion of 0.2 mmol/L glucose, fructose, or sucrose, to a co-culture medium did not change the adhesion percentage of *Lb. fermentum* CRL1015 to chicken intestinal mucus (Gusils *et al.*, 2003). Again these findings might be considered consistent with the results observed in the present study with respect to the adhesion of LG. Guglielmetti *et al.* (2009) have also reported no significant difference between the adhesion percentages of *Bif. bifidum* MIMBb75 to HT-29 epithelial cells when glucose was added at a concentration of 25 mM to the co-culture medium, and that of a control medium without glucose. In turn, the adhesion rates of Bb observed here suggest that the sugars present in the orange juice did not significantly affect its adhesion ability.

Orange juice also contains a wide range of cations such as potassium, sodium, calcium and magnesium, and anions such as phosphate (Belitz *et al.*, 2009; Guarnieri *et al.*, 2007; USDA National Nutrient Database for standard Reference, 2009). Previous research has shown that minerals could influence adhesion of probiotics to intestinal mucosa. Among 4 individual probiotics examined in the present study, the adhesion rate of LR significantly increased after 10 days of exposure to OJ under refrigerated storage. A recent study has shown that addition of calcium (200 mmol/l) to adhesion assay wells significantly increased the adhesion percentage of probiotic *Lb. reuteri* 12002 and *Enterococcus faecium* (10 strains) to a porcine jejunum epithelial cell line (Marcinakova *et al.*, 2010). The enhanced adhesion rate of LR in OJ might therefore be at least partly explained by the effect of cations such as calcium present in OJ. Such an enhanced adhesion rate was observed for *Lb. fermentum* with addition of calcium (0.5 and 1 mM) and magnesium (1 mM) to a co-culture medium, which significantly increased adhesion of the bacterium to chicken intestinal fragments (Gusils *et al.*, 1999). However, a further study reported that addition of calcium chloride to a co-culture medium did not change the adhesion percentage of *Lb. fermentum* CRL1015 to chicken intestinal mucus (Gusils *et al.*, 2003), while the HT-29 cell line study of Guglielmetti *et al.* (2009) reported no significant effect in the adhesion percentages of *Bif. bifidum* MIMBb75 to HT-29 epithelial cells when calcium chloride (CaCl₂, 4 mM) or potassium iodate (KIO₃, 50 Mm) was added to the co-culture medium. Taken within the context of this latter finding, the relatively stable adhesion rates observed for Bb in many of the preparations of this study may indicate that the intestinal cell adhesion capacity of bifidobacteria are largely unaffected by such factors.

It has been reported that orange juice contains pectins (water soluble fibers) at a level of 300 mg/L (Belitz *et al.*, 2009). This orange juice constituent could also impact on the adhesion rate of probiotics included in orange juice, as Parkar *et al.* (2010) reported that pre-treatment of human intestinal epithelial Caco-2 cells with citrus pectin at a concentration of 100 µg/mL for 1 hour followed by incubation with *Lb. rhamnosus* or *Bif. bifidum* for a further 2 hours, only significantly increased the adhesion percentage of the latter compared with the control.

The preceding discussion revealed the effect of some individual components found in orange juice on the adhesion of probiotics to intestinal epithelial mucosa. The combined effects of all components forming a food matrix such as orange juice are however, likely to be different.

The results of this study have shown variation in viability and adhesion rates for individual strains when combined with different probiotics compared to those of the mono-cultures. Variation between viabilities of a strain when combined with other probiotics and that of the mono-culture is clearly associated with the effect of combination. With regard to interpretation of the corresponding adhesion and viability data, differentiation between the effect of combination of probiotics on the viability and adhesion rate of the strain in question is somewhat difficult. However when the same trend in viability as that of the mono-culture was observed for a preparation in a multi-species set, then any difference between the respective adhesion rates could rightfully be associated with the effect of the presence of other probiotics. For example, while the viabilities of LG either alone or in combination with Bb were comparable throughout the storage period, a significant increase in adhesion rate of LG in the presence of Bb at D30 could be due to the synergistic effect Bb on the adhesion ability of LG. The viability trend of Bb in the presence of LG was similar to that of the mono-culture of Bb, but a significant decline in the adhesion rate of Bb in former preparation was observed at D20 compared to that of Bb alone. These findings are consistent with the study by Collado *et al.* (2007) whereby it was reported that while *Bif. breve* 99 significantly enhanced adhesion of LG to intestinal mucus, LG significantly decreased the adhesion rate of *Bif. breve* 99 by comparison with the mono-cultures.

- In the present study, 19 cases were categorised in Table 6.1 as exhibiting significantly reduced viability during storage when compared with initial counts. Of these cases, four showed no significant change in adhesion percentage compared to the control (-/N). Given that the viability decreased significantly compared to the control, no change in the adhesion percentage could indicate that combined exposure of the probiotics to orange juice and refrigerated storage did not affect the ‘proportion’ of total probiotic cells which had capacity to adhere. However this observation would seem to indicate that the total number of bacteria adherent to intestinal epithelial Caco-2 cells was entirely dependent on the number of live bacteria present in the adhesion assay medium in these cases. Such a result is consistent with reports by Greene & Klaenhammer (1994) and Tuomola & Salminen (1998) in which adhesion of probiotic strains to Caco-2 cells was found to be concentration dependent.

Among the cases, 12 belonged to the -/+ group all of which involved preparations comprising LR (4 cases) and PJ (8 cases). A possible explanation for increased adhesion percentage of the bacterium while its viability declined is that the same number of

adhesion sites was available for a lesser number of viable bacteria, resulting in an increased adhesion percentage compared to the control.

Three remaining cases showed reduction in both viability and percentage adhesion (-/-). This situation was only observed for Bb when combined with LG or LR. As each case is compared to the control (PBS), combined exposure to OJ and low storage temperature (4°C) might be considered as the main reason for a decline in both the viability and the adhesion capacity of the bacterium. It is possible that in this scenario, physico-chemical properties of orange juice such as its low pH and/or certain components of it that affected the viability of the bacteria, might also have induced changes in the bacterial surface which led to the reduced adhesion of the bacterium. Exposure to low pH has been shown as a factor decreasing adhesion ability of some strains of *Bifidobacterium* (Guglielmetti *et al.*, 2009; Riedel *et al.*, 2006).

6.6 Conclusion

Adhesion to the intestinal epithelial mucosa is necessary for probiotics to exert their health promoting effects. Little is known about the effect of food carrier matrix and duration of storage on functional properties of probiotics such as their adhesion ability. The results of this study provided evidence of variation in adhesion rates between individual strains and for the same strain in the presence of different probiotics. It was also found that the adhesion ability of the probiotics to IECs would be further affected by the duration of their combined exposure to orange juice and refrigerated storage. In terms of both viability and adhesion rate, the preparations that provided the best outcomes were LG (in all cases), LR when combined with PJ and Bb-PJ, Bb when combined with PJ and LG-PJ, and PJ either alone or in combination with LR. The study has yielded some definitive data on the way in which potential interactions between the organisms and food matrices may affect the adhesion of probiotics to IECs, and demonstrated that the presence of other probiotics may further impact the adhesion ability of the probiotic strains. These findings highlight the importance of such interactions and the necessity that they be considered when formulating probiotic products.

**CHAPTER VII: Impact of Orange Juice as a Probiotic
Carrier Matrix on *in-vitro* Immunomodulatory Effect of
Probiotic Combinations**

7.1 Summary

Lactobacillus rhamnosus GG (LG), *Lactobacillus reuteri* ATCC 55730 (LR), *Bifidobacterium animalis* subsp *lactis* Bb12 (Bb) and *Propionibacterium jensenii* 702 (PJ) either alone or as 2- or 3-multi-species combinations were included into pulp free orange juice and stored at 4°C for 30 days. Effect of the probiotics on the LPS, IL-1 β or TNF- α Induced IL-8, LPS or IL-1 β induced IL-6 and IL-1 β induced TNF- α production in Caco-2 cells was assessed at 10-day intervals during the storage period. Generally at baseline and during the refrigerated storage, with the exception of LG, all probiotic preparations significantly enhanced non-stimulated IL-8 production in Caco-2 cells compared to the control. The probiotic preparations were not observed to affect non-stimulated IL-6 and TNF- α secretion by Caco-2 cells. Exposure to probiotic preparations, significantly enhanced LPS induced IL-8 release at baseline compared to the control, this effect however was not evident for all probiotic preparations at day 10. With the exception of LG, all probiotic preparations enhanced TNF- α induced IL-8 secretion at all time points towards day 20 after which it returned to the control level. In contrast, significant decline in IL-1 β induced IL-8 secretion was observed for all probiotic preparations at baseline, with no further effect evident during storage. The amount of the IL-8 release varied among the probiotic strains/preparations in both stimulation and non-stimulation conditions. However this variation is lower between the preparations in the case of IL-1 β induced IL-8 secretion. The relative probiotic effect on IL-1 β and TNF- α induced IL-8 secretion showed an upward and downward trend respectively over the storage period.

Probiotic preparations did not affect LPS or IL-1 β induced secretion of IL-6 up to 10 days of storage, while thereafter some probiotic preparations exhibited variable effects on IL-1 β induced IL-6 secretion including enhancement and suppression, after 20 and 30 days of storage. PJ at baseline and LG from D10 to the end of the storage period significantly increased IL-1 β induced TNF- α production compared to the control. Compared to baseline (day 0), the effect of all four probiotic strains on IL-1 β induced TNF- α production was found to decrease by D10 and remained subdued until the end of the storage period. In conclusion, the results provided evidence of variation in immunomodulatory effect between the probiotic strains/combinations examined. It was also found that the ability of the probiotics to modulate immune responses of Caco-2 cells induced by different potent pro-inflammatory stimuli would be further affected by

the duration of their combined exposure to orange juice and refrigerated storage. Moreover the data showed that the effect of probiotics on induced cytokines production by Caco-2 cells varied with the particular stimulating agents used.

7.2 Introduction

The intestinal epithelial mucosa plays a crucial role in the host's protection against various invasive pathogens. This intestinal tissue has developed a complicated defence system commonly referred to as the mucosal immune system. The primary cellular barrier of this system are the intestinal epithelial cells (IECs) which along with other immune and non-immune cells such as macrophages, myofibroblasts, and dendritic cells, mediate innate immune responses to antigens at the intestinal mucosal surface through secretion of mucins, expression of Toll-like receptors (TLRs), secretions of antimicrobial peptides (AMPs) and expression/secretion of cytokines and chemokines (Duerkop *et al.*, 2009; Macdonald & Monteleone, 2005). In response to potential invasive and toxigenic pathogens and their components, IECs develop acute inflammation and release pro-inflammatory cytokines (Philpott *et al.*, 2001). There is a growing body of *in vitro* and *in vivo* evidence that probiotics can modulate immune responses to antigens at mucosal surfaces including cytokine production by IECs. The only extensively used and well-established *in vitro* model of investigating the interactions between host and microorganisms involves using co-cultures of immune cells and/or IECs with probiotics, commensals or pathogens (Wohlgemuth *et al.*, 2010). Previous research has shown that IECs are immunologically quiescent, or express low levels of activity in the presence of commensals and probiotics such as lactobacilli and bifidobacteria, but that these bacteria modulate the immune responses of IECs when stimulated by pathogens and their components such as lipopolysaccharide (LPS) or by pro-inflammatory cytokines. This effect however seems to be strain, dose and exposure time dependent (Donkor *et al.*, 2010). The most popular *in vitro* model for investigating the interactions of probiotics with intestinal epithelial cells comprises cells derived from human colon carcinomas such as the Caco-2, HT-29 and T84 cell lines (Vinderola *et al.*, 2005).

7.2.1 CXCL8/IL-8

Interleukin 8 (IL-8) or CXCL8 is a member of the CXC subfamily of structurally-related low molecular weight pro-inflammatory factors known as chemokines (Elgert, 2009). IL-8 has been reported to be synthesised and released by a variety of different cell types including IECs (Mumy & McCormick, 2009) in response to pro-inflammatory stimuli such as IL-1 β , TNF- α , bacterial LPS, and viruses. IL-8 is involved

in a wide variety of physiological and pathological processes including host defence against bacterial infection. IL-8 primarily mediates the activation and chemotaxis of neutrophils from peripheral blood into the sites of inflammation, and damaged or infected tissues (Elgert, 2009). Neutrophils play an important role in combating enteroinvasive pathogens through phagocytosis (Mumy & McCormick, 2009) and production of neutrophil extracellular traps (NETs) (Brinkmann & Zychlinsky, 2007). Moreover, neutrophils generate immune signals to recruit other immune cells such as monocytes and dendritic cells, and help to determine differentiation of macrophage (Nathan, 2006). Neutrophils' activities are therefore necessary to eliminate pathogens in active infection states. In non-infectious states, such as inflammatory bowel disease (IBD), excessive activation of the processes leading to activation, movement and infiltration of neutrophils and unnecessary secretion of antimicrobial products by them, results in chronic inflammation and subsequently substantial intestinal tissue damage (Mumy & McCormick, 2009). In the latter condition, down regulation of IL-8 has been shown to inhibit neutrophil migration and associated tissue injury (Nemeth *et al.*, 2006). Some probiotic strains have been observed to suppress IL-8 production in cultured IECs in an induced inflammatory condition (Table 7.1). A study by Choi *et al.* (2008) has shown that LG suppressed IL-1 β induced IL-8 secretion by Caco-2 cells as a result of declining transcriptional activation of the IL-8 gene, suppression of the NF- κ B signalling pathway, and a decrease in I κ B α degradation.

Reported effects of probiotics on IL-8 secretion by cultured IECs have been controversial. Although most of the work has shown a suppressive effect for probiotics on IL-8 production by cultured IECs when treated with pro-inflammatory stimuli, there are reports that some probiotics enhance release of IL-8 from IECs exposed to pro-inflammatory stimulants. This effect seems to be strain dependent (Delcenserie *et al.*, 2008) (Table 7.1).

Previous research has shown that, IECs are either quiescent (de Palencia, Lopez, *et al.*, 2008; Lammers *et al.*, 2002; Mileti *et al.*, 2009; Morita *et al.*, 2002a; Reilly *et al.*, 2007; Vidal *et al.*, 2002; Vizoso Pinto *et al.*, 2007; Wallace *et al.*, 2003) or produce low levels of IL-8 (Hosoi *et al.*, 2003; Malago, Tooten, *et al.*, 2010; Morita *et al.*, 2002a; Nemeth *et al.*, 2006; Otte & Podolsky, 2004; Vizoso Pinto *et al.*, 2007) when encountering probiotic bacteria, compared with control cells with no probiotic added. It has also been reported that some probiotics significantly reduce IL-8 secretion by IECs in a non-stimulated condition compared with the control (O'Hara *et al.*, 2006; Vizoso Pinto *et al.*,

2007; Wallace *et al.*, 2003). Probiotics can however also modulate responses of IECs in an inflammatory condition. It has been demonstrated that certain probiotics reduce the level of induced IL-8 production in IECs exposed to pro-inflammatory agents such as bacterial LPS or flagellin, cytokines IL-1 β , TNF- α or IFN- γ , or pathogenic bacteria such as *E. coli*, *S. enterica* serovar Enteritidis, *S. enteridis*, *S. Dublin* or *S. typhimurium* (Bai *et al.*, 2004; Candela *et al.*, 2008; Choi *et al.*, 2008; Donato *et al.*, 2010; Grimoud *et al.*, 2010; Liu *et al.*, 2010; Ma *et al.*, 2004; Malago, Nemeth, *et al.*, 2010; Malago, Tooten, *et al.*, 2010; McCracken *et al.*, 2002; O'Hara *et al.*, 2006; Otte & Podolsky, 2004; Stober *et al.*, 2010; Versalovic *et al.*, 2008; Vidal *et al.*, 2002; Wood *et al.*, 2007; Zhang *et al.*, 2005). However a recent study has shown that the probiotics LG and *Lb. plantarum* BFE 1685 significantly enhanced IL-8 production by HT-29 cells in the presence of either TNF- α or *S. enterica* serovar Typhimurium (Vizoso Pinto *et al.*, 2009). Table 7.1 summarises recent findings on the effect of probiotics on non-stimulated and stimulated IL-8 secretion by IECs.

Table 7.1: Summary of published findings on the effect of probiotic strains on non-stimulated and stimulated IL-8 secretion by IECs

IECs	Probiotic strain(s)	Stimulation	Effect	Reference
Caco-2	30 <i>Lactobacillus</i> and <i>Bifidobacterium</i> strains	NS	NF for all except for 8 <i>Bifidobacterium</i> strains ↑	Morita <i>et al.</i> (2002a)
Caco-2	<i>Bacillus subtilis</i> (natto)	NS	↑	Hosoi <i>et al.</i> (2003)
Caco-2	<i>Lb. paracasei</i> and <i>Lb. plantarum</i>	NS	NF	Reilly <i>et al.</i> (2007)
Caco-2	<i>Lb. paracasei</i> subsp <i>paracasei</i> LC-01, <i>Lb. acidophilus</i> LA-5 or <i>Bif. lactis</i> Bb12	NS	NF	de Palencia <i>et al.</i> (2008)
Caco-2	<i>Lb. rhamnosus</i> GG, <i>Lb. paracasei</i> B21060 or <i>Lb. plantarum</i> NCIMB8826	NS	NF	Mileti <i>et al.</i> (2009)
Caco-2	<i>Bif. infantis</i> W52, <i>Lb. casei</i> W56, <i>Lc. lactis</i> W58, <i>Lb. acidophilus</i> W70, <i>Bif. bifidum</i> W23, or <i>Lb. salivarius</i> W24	NS	↑	Malago <i>et al.</i> (2010)
Caco-2	<i>Lb. casei</i> subsp <i>casei</i> 2756, <i>Lb. curvatus</i> 2775, <i>Lb. plantarum</i> 2142	NS	↑	Nemeth <i>et al.</i> (2006)
Caco-2	<i>Lb. rhamnosus</i> GG	TNF- α	↓	Zhang <i>et al.</i> (2005)
Caco-2	<i>Lb. paracasei</i> and <i>Lb. plantarum</i>	IL-1 β	NF	Reilly <i>et al.</i> (2007)
Caco-2	<i>Lb. rhamnosus</i> GG	IL-1 β	↓	Choi <i>et al.</i> (2008)
Caco-2	<i>Lb. rhamnosus</i> GG	IFN- γ (100 ng/mL)/TNF- α (10 ng/mL)	↓	Donato <i>et al.</i> (2010)
Caco-2	<i>Bif. infantis</i> W52, <i>Lb. casei</i> W56 or <i>Lb. lactis</i> W58	<i>Salmonella enterica</i> serovar <i>Enteritidis</i>	↓	Malago <i>et al.</i> (2010)
Caco-2	<i>Lb. casei</i> Shirota or <i>Lb. plantarum</i> 299v	<i>Salmonella enteridis</i> 857	↓ only spent culture supernatant (not whole cells)	Malago <i>et al.</i> (2010)
HT-29	<i>Lactobacillus</i> (<i>Lb. rhamnosus</i> GG, <i>Lb. acidophilus</i> MB443, <i>Lb. casei</i> MB451, <i>Lb. delbrueckii</i> subsp <i>delbrueckii</i> MB 453, <i>Lb. plantarum</i> MB452) and <i>Bifidobacterium</i> strains (<i>Bif. breve</i> Y8, <i>Bif. infantis</i> Y1 and <i>Bif. longum</i> Y10)	NS	NF	Lammers <i>et al.</i> (2002)
HT-29	LTA from <i>Lb. johnsonii</i> La1 and <i>Lb. acidophilus</i> La10	NS	NF	Vidal, <i>et al.</i> (2002)
HT-29	<i>Lb. acidophilus</i> R0052, <i>Lb. delbrueckii</i> subsp <i>lactis</i> R0187, <i>Lb. rhamnosus</i> strains R0011 and R0049, <i>Bif. longum</i> R0175 and IndR0175, <i>Lb. acidophilus</i> ATCC521	NS	NF	Wallace <i>et al.</i> (2003)
HT-29	<i>Lb. rhamnosus</i> strains IndR0011, IndR0049, <i>Lb. rhamnosus</i> GG, <i>Lb. delbrueckii</i> subsp <i>lactis</i> IndR0187, <i>Lb. delbrueckii</i> subsp <i>bulgaricus</i> ATCC 11977, <i>Lb. plantarum</i> ATCC 14917, <i>Lb. acidophilus</i> IndR0052	NS	↓	Wallace <i>et al.</i> (2003)
HT-29	<i>E. coli</i> Nissle 1917	NS	↑	Otte and Podolsky (2004)
HT-29	<i>Bif. infantis</i> 35624, <i>Lb. salivarius</i> UCC118	NS	↓	O'Hara <i>et al.</i> (2006)
HT-29	<i>Lb. plantarum</i> strains BFE 5878 and BFE 1684, <i>Lb. johnsonii</i> strains BFE 6154 and 663, <i>Lb. casei</i> BFE 688	NS	NF	Vizoso Pinto <i>et al.</i> (2007)
HT-29	<i>Lb. plantarum</i> strains BFE 1685 and BFE 5759, <i>Lb. johnsonii</i> BFE 6128	NS	↑	Vizoso Pinto <i>et al.</i> (2007)
HT-29	<i>Lb. rhamnosus</i> GG, <i>Lb. paracasei</i> 675	NS	↓	Vizoso Pinto <i>et al.</i> (2007)
HT-29	<i>Lb. plantarum</i> 299v	TNF- α	↓	McCracken <i>et al.</i> (2002)
HT-29	LTA from <i>Lb. johnsonii</i> La1 and <i>Lb. acidophilus</i> La10	LPS or <i>E. coli</i>	↓	Vidal, <i>et al.</i> (2002)
HT-29	VSL#3™	<i>E. coli</i> , <i>S. dublin</i>	↓	Otte and Podolsky (2004)
HT-29, T84	<i>Lb. reuteri</i>	TNF- α	↓	Ma <i>et al.</i> (2004)
HT-29	<i>Bif. infantis</i> 35624, <i>Lb. salivarius</i> UCC118	<i>S. typhimurium</i> or	↓	O'Hara <i>et al.</i> (2006)

		its flagellin		
HT-29	<i>Lb. rhamnosus</i> strains R0011 and R0049	LPS	↓conditioned media	Wood <i>et al.</i> (2007)
HT-29	<i>Bif. longum</i> or <i>Lb. bulgaricus</i> LB10	TNF- α	↓	Bai <i>et al.</i> (2004)
HT-29	<i>Lb. acidophilus</i> Bar13 and/or <i>Bif. longum</i> Bar33	LPS, TNF- α or IL-1 β	↓	Candela <i>et al.</i> (2008)
HT-29	<i>Lb. coryneformis</i> MM7, <i>Lb. reuteri</i> ATCC PTA 4659, <i>Lb. reuteri</i> ATCC 55730 or <i>Lb. reuteri</i>	LPS	↓ by bacterial conditioned media	Versalovic <i>et al.</i> (2008)
HT-29	<i>Lb. plantarum</i> BFE 1685 or <i>Lb. rhamnosus</i> GG	TNF- α or <i>S. enterica</i> serovar Typhimurium	↑	Vizoso Pinto <i>et al.</i> (2009)
HT-29	13 <i>Lactobacillus</i> and 4 <i>Bifidobacterium</i> strains	<i>E. coli</i> (EHEC) 5 strains	↓	Stober <i>et al.</i> (2010)
HT-29	<i>Bif. bifidum</i> 02, <i>Bif. bifidum</i> 20, <i>Bif. longum</i> R0175, <i>Bif. pseudocatenulatum</i> 14, <i>Lb. farciminis</i> CIP103136, <i>Lb. helveticus</i> R0052, <i>Lb. plantarum</i> R1012, <i>Lb. rhamnosus</i> R1102, <i>Lc. lactis</i> R1058, <i>Ped. acidilactici</i> R1001 and <i>Strep. thermophilus</i> R0083	LPS and IFN- γ	↓	Grimoud <i>et al.</i> (2010)
piglet IPEC-J2	<i>Lb. reuteri</i> strains ATCC PTA4659, ATCC PTA 5289, and ATCC PTA 6475	LPS	↓	Liu <i>et al.</i> (2010)
rat IEC-6	<i>Lb. reuteri</i> ATCC PTA4659	LPS	↓	Liu <i>et al.</i> (2010)

NS: No Stimulation; NF: No Effect

Arrows indicate that either increased (↑) or decreased (↓) secretion of IL-8 was observed

7.2.2 IL-6

Interleukin 6 (IL-6) is a potent pleiotropic pro-inflammatory and immunomodulatory cytokine with a wide variety of biological functions. IL-6 promotes hematopoiesis and regulates the growth and differentiation of various cell types such as hematopoietic stem cells, B- and T-cells (Coico & Sunshine, 2009; Elgert, 2009). IL-6 also plays an important role in immune responses initiated by infection or injury. Along with IL-1 and TNF, IL-6 drives the acute inflammatory response and is a major inducer of synthesis of hepatic acute phase proteins in response to inflammation or tissue injury (Coico & Sunshine, 2009; Elgert, 2009). The role of IL-6 as an anti-inflammatory cytokine is mediated through its inhibitory effects on TNF- α and IL-1 β , and activation of IL-1Ra and IL-10 (Starkie *et al.*, 2003; Steensberg *et al.*, 2003). IL-6 may also influence glucose and lipid metabolism (Glund & Krook, 2008). These multiple actions are integrated within a complex cytokine network, where several cytokines such as IL-1, TNF, and IFNs induce or are induced by IL-6 and the final effects result from either synergistic or antagonistic activities between IL-6 and the other cytokines. IL-6 is expressed by a variety of cells including colonic epithelial cells in response to numerous signals including mitogenic or antigenic stimulation, bacterial LPS, cytokines such as IL-1, IL-2, IFN, TNF and viruses.

A controversy also exists over the effect of probiotics on IL-6 secretion by cultured enterocytes (Table 7.2). Previous reports have shown that some probiotics had no effect on IL-6 production in non-stimulated IECs while others enhanced IL-6 release. Morita *et al.* (2002a) examined the potential of 30 lactic acid bacteria, including *Lactobacillus* and *Bifidobacterium* strains, to induce IL-6 secretion by Caco-2 cells. They observed that co-cultures of *Lactobacillus* strains with Caco-2 cells did not result in induction of IL-6 secretion, while 8 *Bifidobacterium* strains slightly induced cytokine production. A study by Vinderola *et al.* (2005) showed that the effect of exposure to either *Lb. casei* CRL 431 or *Lb. helveticus* R389 on non-stimulated IL-6 secretion by small and large intestinal cells, was bacterial dose dependent. In a study by de Palencia *et al.* (2008), no significant difference was observed between IL-6 concentrations in the supernatants from non-stimulated Caco-2 cells in the presence or absence of either *Lb. paracasei* subsp *paracasei* LC-01, *Lb. acidophilus* LA-5 or *Bif. animalis* subsp *lactis* Bb12. Nor was any significant effect observed by Vizoso Pinto *et al.* (2007) in the secretion of IL-6 by non-stimulated HT-29 cells exposed to different strains of *Lb. plantarum* (4

strains), *Lb. johnsonii* (3 strains) or LG, *Lb. casei* and *Lb. paracasei*, compared with that of untreated HT-29.

It has been shown that *Bif. animalis* subsp *lactis* Bb12 induced IL-6 secretion in the mouse IEC line Mode-K in a dose and time dependent fashion. The highest concentration of IL-6 was detected at a bacterium to epithelial cell ratio of 30 after 6 hours of co-incubation (Ruiz *et al.*, 2005). Potential probiotic *Bacillus subtilis* (natto) has been reported to increase IL-6 release by non-stimulated Caco-2 cells (Hosoi *et al.*, 2003).

It has also been reported that the probiotics *Lb. paracasei* and *Lb. plantarum* enhanced IL-1 β induced IL-6 secretion by human Caco-2 enterocytes, whereas these same probiotics did not affect IL-6 production in the absence of inflammatory stimulus (*i.e.* IL-1 β). Further investigation on *Lb. paracasei* revealed that the effect of the probiotic on modulation of IL-6 production was dose and time dependent (Reilly *et al.*, 2007).

Table 7.2: Summary of published findings on the effect of probiotic strains on non-stimulated and stimulated IL-6 secretion by IECs

IECs	Probiotic strain(s)	Stimulation	Effect	Reference
Caco-2	<i>Lb. paracasei</i> subsp <i>paracasei</i> LC-01, <i>Lb. acidophilus</i> LA-5 or <i>Bif. animalis ssp lactis</i> Bb12	NS	NF	de Palencia <i>et al.</i> (2008)
Caco-2	<i>Lb. paracasei</i> and <i>Lb. plantarum</i>	NS	NF	Reilly <i>et al.</i> (2007)
Caco-2	<i>Lb. paracasei</i> and <i>Lb. plantarum</i>	IL-1 β	↑	Reilly <i>et al.</i> (2007)
HT-29	<i>Lb. plantarum</i> strains BFE 5878, 5759, 1685 and 1684; <i>Lb. johnsonii</i> strains BFE 6154, 6128 and 663; <i>Lb. rhamnosus</i> GG; <i>Lb. casei</i> BFE 688; <i>Lb. paracasei</i> BFE 675	NS	NF	Vizoso Pinto <i>et al.</i> (2007)
Mouse IEC line Mode-K	<i>Bif. animalis ssp lactis</i> Bb12	NS	↑	Ruiz <i>et al.</i> (2005)
Mouse small and large intestinal cells	<i>Lb. casei</i> CRL 431 or <i>Lb. helveticus</i> R389	NS	variable	Vinderola <i>et al.</i> (2005)
Caco-2	<i>Bacillus subtilis</i> (natto)	NS	↑	Hosoi <i>et al.</i> (2003)
Caco-2	30 <i>Lactobacillus</i> and <i>Bifidobacterium</i> strains	NS	NF for all except for 8 <i>Bifidobacterium</i> strains ↑	Morita <i>et al.</i> (2002a)

NS: No Stimulation; NF: No Effect

Arrows indicate that either increased (↑) or decreased (↓) secretion of IL-8 was observed

7.2.3 TNF- α

TNF- α is a primary pro-inflammatory cytokine which mediates inflammation, and is mainly secreted by activated macrophages present at sites of infection (Elgert, 2009). TNF- α has also been reported to be released by IECs (Hosoi *et al.*, 2003; Menard *et al.*, 2004; Vidal *et al.*, 2002; Wallace *et al.*, 2003). In the presence of IL-8, TNF- α increases the toxicity of neutrophils, and along with IL-1 and IL-6, induces the acute inflammatory response (Elgert, 2009).

Hosoi *et al.* (2003) reported that probiotic *Bacillus subtilis* (natto) strains did not induce secretion of TNF- α in non-stimulated Caco-2 cells. Similarly, Visozo Pinto *et al.* (2007) observed no significant difference in the secretion of TNF- α in non-stimulated HT-29 cells treated by different strains of *Lb. plantarum* (4 strains), *Lb. johnsonii* (3 strains) or LG, *Lb. casei* and *Lb. paracasei* compared with that of HT-29 cells in the absence of probiotics.

In contrast however, lipoteichoic acid (a component of the membrane and cell wall of Gram positive bacteria) from *Lb. johnsonii* La1 and *Lb. acidophilus* La10 was shown by Vidal *et al.* (2002) to suppress LPS-induced TNF- α release by HT-29 cells.

A later study by Wallace *et al.* (2003) demonstrated that while probiotic *Lb. rhamnosus* R0049 and *Lb. acidophilus* R0052 had no effect on TNF- α release by non-stimulated human HT-29 cells, *Lb. rhamnosus* R0011, *Bif. longum* R0175 or *Lb. debrueckii* subsp *lactic* R0187 suppressed the secretion of TNF- α in HT-29 IECs. This study also showed that the degree of cytokine suppression was strain and growth condition dependent. It has also been reported that *Bif. breve* conditioned medium significantly decreased TNF- α release by non-stimulated HT29-19A cells, whereas *Strep. thermophilus* conditioned medium had no significant effect on the cytokine release by non-stimulated HT29-19A cells. TNF- α secretion by HT29-19A cells pre-treated with IFN- γ /TNF- α cytokines was significantly reduced by either *Bif. breve* or *Strep. thermophilus* conditioned media compared with that of stimulated cells (Menard *et al.*, 2004).

Reports on the effect of probiotics on TNF- α production by IECs are summarised in Table 7.3.

Table 7.3: Summary of published findings on the effect of probiotic strains on non-stimulated and stimulated TNF- α secretion by IECs

IECs	Probiotic strain(s)	Stimulation	Effect	Reference
Caco-2	<i>Lb. plantarum</i> strains BFE 5878, 5759, 1685 and 1684; <i>Lb. johnsonii</i> strains BFE 6154, 6128 and 663; <i>Lb. rhamnosus</i> GG; <i>Lb. casei</i> BFE 688; <i>Lb. paracasei</i> BFE 675	NS	NF	Vizoso Pinto <i>et al.</i> (2007)
HT29-19A	<i>Bif. breve</i> conditioned media	NS	↓	Menard <i>et al.</i> (2004)
HT29-19A	<i>Bif. breve</i> or <i>Strep. thermophilus</i> conditioned media	IFN- γ /TNF- α	↓	Menard <i>et al.</i> (2004)
HT-29	<i>Lb rhamnosus</i> R0049, <i>Lb acidophilus</i> R0052	NS	NF	Wallace <i>et al.</i> (2003)
HT-29	<i>Lb rhamnosus</i> R0011, <i>Bif. longum</i> R0175, <i>Lb delbrueckii</i> subsp <i>lactis</i> R0187	NS	↓	Wallace <i>et al.</i> (2003)
HT-29	LTA from <i>Lb. johnsonii</i> La1 nad <i>Lb. acidophilus</i> La10	LPS	↓	Vidal <i>et al.</i> (2002)
Caco-2	<i>Bacillus subtilis</i> (natto)	NS	NF	Hosoi <i>et al.</i> (2003)

NS: No Stimulation; NF: No Effect

Arrows indicate that increased (↑) or decreased (↓) secretion of IL-8 was observed

Although previous research and previous chapters of the present study have shown that carrier matrix and storage duration may impact on a variety of probiotic properties such as their survival in food matrices, tolerance to GI conditions and adhesion to IECs, it is not yet known whether these factors may also affect the immunomodulatory properties of probiotics. The goal of this study was to determine the impact of interactions of probiotics included in orange juice with the carrier matrix and duration of refrigerated storage on *in vitro* modulation of immune responses of intestinal epithelial cells treated with inflammatory stimuli. To achieve this goal, we examined the effect of the duration of combined exposure of probiotics (LG, LR, Bb and PJ either alone or in 2- and 3-multi-species combinations) to the food matrix (orange juice) and refrigerated storage on production of cytokines IL-6, IL-8 and TNF- α by cultured intestinal epithelial Caco-2 cell line under inflammatory conditions induced by LPS, IL-1 β and TNF- α .

It was hypothesized that:

- Exposure to probiotics would differentially affect non-stimulated IL-8, IL-6 or TNF- α production by Caco-2 cells.
- Mono-cultures of the probiotic strains would differentially modulate the immune responses of Caco-2 cells when treated with *E. coli* LPS or the pro-inflammatory cytokines TNF- α and IL-1 β .
- The immunomodulatory response of Caco-2 cells would vary under exposure to probiotic combinations (2- or 3- multispecies combinations) compared with that observed when exposed to individual probiotic strains.
- The ability of the probiotics to modulate the immune responses of Caco-2 cells induced by different potent pro-inflammatory stimuli, would be further affected by the duration of their combined exposure to the food matrix (orange juice) and low storage temperature (4°C).
- The effect of probiotics on induced IL-6 and IL-8 production by Caco-2 cells would vary with the particular stimulating agents used.

7.3 Materials and methods

7.3.1 Chemicals and Reagents

As previously described in chapter 4.

7.3.2 Bacterial cultures and growth conditions

As previously described in chapter 4.

7.3.3 Orange juice

As previously described in chapter 5.

7.3.4 Preparation of probiotic orange juice

As previously described in chapter 4.

7.3.5 Intestinal epithelial cell line and growth conditions

As previously described in chapter 3.

7.3.6 Intestinal epithelial cell line responsiveness

Time course responsiveness of Caco-2 cells in the secretion of interleukin 8 (IL-8) was assessed to doses of 0, 5, 10, 25, 50 and 100 ng/mL of Lipopolysaccharide (LPS) from *Escherichia coli* 026:B6 (Sigma-Aldrich, Inc., St Louis, MO, USA) and doses of 0, 5, 10, 25, 50 and 100 ng/mL of recombinant human Tumor Necrosis Factor- α (TNF- α) produced in *Escherichia coli* (Cell Signaling Technology[®], Inc., Danvers, MA, USA).

Time course responsiveness of Caco-2 cells in the secretion of IL-6 was also assessed to doses of 0, 10, 100, 500, 1000 and 10000 ng/mL of LPS, doses of 0, 5, 10, 25, 50 and 100 ng/mL of TNF- α and doses of 0.0, 0.1, 1, 5, 10 and 25 ng/mL of IL-1 β produced in *Escherichia coli* (Cell Signaling Technology[®], Inc., Danvers, MA, USA).

Post-confluent Caco-2 cells in each well of Nunc[™] 24-well tissue culture plates (Thermo Fisher Scientific, Rochester, NY, USA) were washed 3X with PBS. An amount of 1 mL fresh culture medium without antibiotics but with different doses of stimuli were then added to each well and incubated for 0, 3, 6, 12 and 24 h under the

same conditions for growing Caco-2 cells. Cell culture supernatants were then obtained by centrifugation (2000 ×g, 10 min, 4 °C) and analysed for IL-6 and IL-8 concentrations using BD OptEIA™ Human IL-6 and IL-8 ELISA Kits (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions.

7.3.7 Effect of probiotics on pro-inflammatory stimulant induced cytokine production by Caco-2 cells

The Caco-2 cells were seeded at a concentration of 10^5 cells/well in each well of a Nunc™ 24-well tissue culture plate (Thermo Fisher Scientific, Rochester, NY, USA) and incubated at 37°C in 5% CO₂ atmosphere in a humidified incubator until a confluent monolayer had formed. The cell-culture medium was changed every other day. At least 1 hour before the assay, the culture medium was renewed with the same medium without antibiotic. Prior to the assay, the monolayers of Caco-2 cells were washed 3X with PBS. Probiotic orange juice preparations were vortex mixed and an amount of 1 mL of the juice was taken and centrifuged (1811 ×g, 10 min, 4 °C). Supernatants were then discarded and pellets were washed 3X with PBS and finally resuspended in 1 mL cell culture medium without antibiotics, either with or without LPS (100 ng/mL for IL-8 assay and 10000 ng/mL for IL-6 assay), TNF- α (100 ng/mL) or IL-1 β (1 ng/mL). Bacterial suspensions were then added to post confluent monolayers of Caco-2 cells in duplicate into wells of the 24-well micro-plates and incubated at 37°C in 5% CO₂ / 95% air for 24 h. Cell culture supernatants were then obtained by centrifugation (2000 ×g, 10 min, 4 °C) and analysed for IL-6 and IL-8 concentrations using BD OptEIA™ Human IL-6 and IL-8 ELISA Kits (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. TNF- α was also measured using a TNF- α human ELISA kit (Invitrogen, Carlsbad, USA). Due to the resource limitations of the study, the effect of probiotics on LPS induced IL-8 or IL-6 production was examined only for single strains and paired combinations either at the baseline (day 0) or after 10 days of refrigerated storage in OJ. For the same reason the TNF- α assay was conducted only with stimulant IL-1 β for the single probiotic strains (Table 7.4).

Table 7.4: Summary of experimental conditions applied in the measurement of cytokines produced by Caco-2 cells, including the stimulants and probiotic preparations applied, and the storage time over which the relevant measurements were taken.

Measure	Stimulants	Probiotic preparation	Total storage time in OJ (days)
IL-8	LPS	single, paired	10
	TNF- α	single, paired, triple	30
	IL-1 β	single, paired, triple	30
IL-6	LPS	single, paired	10
	IL-1 β	single, paired, triple	30
TNF- α	IL-1 β	single	30

7.3.8 Statistical Analyses

Statistical analyses were performed using SPSS software Ver. 18 (SPSS Inc., Chicago, IL, USA). Data analyses were carried out using the linear mixed model (LMM) procedure to run a general linear model (GLM). A p value ≤ 0.001 was considered statistically significant in all cases.

Ideally it was expected that the amount of cytokines released from the control Caco-2 cells, either stimulated or not stimulated, under the same experimental conditions would remain the same at any time point. However due to the nature of working with tissue cultures this expectation was not fulfilled in all cases. Thus expression of the probiotic effect on cytokine secretion during the refrigerated storage in OJ was standardised using the following formula:

$$PE = [(PS-CS)/(PS-PN)] \times 100$$

Where

PE= Percentage of probiotic effect on cytokine secretion at a nominated time point

PS= Amount of cytokine released by stimulated Caco-2 cells treated with probiotic

CS= Amount of cytokine released by stimulated control Caco-2 cells

PN= Amount of cytokine released by non-stimulated Caco-2 cells treated with probiotic

Values plotted in Figures 7.5, 7.7, 7.12 and 7.14 were derived using the above formula.

7.4 Results

7.4.1 Effect of probiotic preparations on induced IL-8 production by Caco-2 cells

This study aimed to examine not only the initial impact of 4 probiotic strains (either alone or in 2 or 3 multispecies combinations) on LPS, TNF- α or IL-1 β induced IL-8 production by Caco-2 cells, but also the effect of the duration of combined exposure of these probiotic preparations to the food matrix (orange juice) and refrigerated storage on their immune regulation capacity. In order to select the optimum exposure time and dose of stimulants, the time course and dose dependent release of IL-8 production by Caco-2 cells following induction with either LPS or TNF- α (at concentrations ranging from 0-100 ng/mL) was determined over a period of 24 h at 5 different time points (0,3,6,12 and 24 h). LPS and TNF- α were both found to induce IL-8 secretion in Caco-2 cells in a time and dose dependent fashion. In both cases the highest amount of IL-8 was produced by the Caco-2 cells after 24 hours of co-incubation with the stimulant at a concentration of 100 ng/mL (Figure 7.1). Hence for both LPS and TNF- α , a dose of 100 ng/mL and an exposure time of 24 h were selected for subsequent experiments. In the case of IL-1 β , a time course and dose dependent experiment was not undertaken. Instead the method for analysis of IL-1 β induced IL-8 production was carried out at an IL-1 β concentration of 1 ng/mL for an exposure time of 24 h, according to the study of Nanthakumar *et al.* (2000).

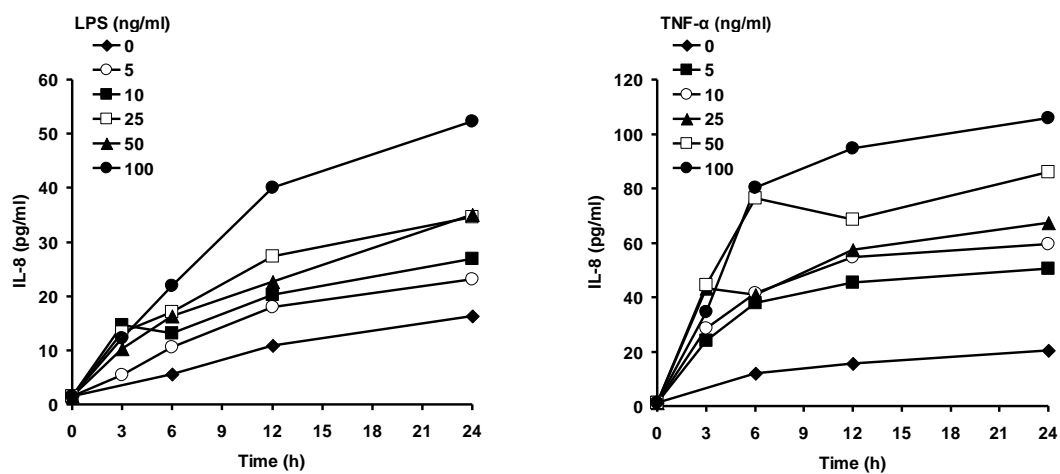


Figure 7.1: Time dependent dose response curves of LPS- (left) and TNF- α - (right) induced IL-8 production in Caco-2 cells

7.4.1.1 Impact of probiotics on LPS induced IL-8 production by Caco-2 cells

Stimulation of the Caco-2 cells with LPS resulted in a significant increase in IL-8 secretion by the cells at baseline (day 0) compared with the levels secreted by unstimulated control cells (Figure 7.2). This figure also illustrates the effect of the various probiotic preparations on the secretion of IL-8 by LPS-treated and non-treated Caco-2 cells. At the baseline, exposure to all probiotic preparations (except LG) significantly enhanced IL-8 production in non LPS treated cells compared to the control. When Caco-2 cells were stimulated with LPS, all probiotic preparations were associated with significantly increased IL-8 release compared to the LPS stimulated control (Figure 7.2). On day 10 (D10) the results were similar except that significantly increased IL-8 release either by LPS induced or non induced Caco-2 cells was not observed when compared to the relevant controls, in the preparations containing LG, PJ and LG-PJ (Figure 7.2).

Comparison of IL-8 secretion by LPS stimulated and non-stimulated Caco-2 cells both treated with the same probiotic preparation indicated that at day 0 (D0), pre-stimulation with LPS resulted in significantly higher levels of IL-8 secretion in the LG and Bb preparations only. On D10 however, a similar effect was observed in relation to four probiotic preparations (LG, LR, PJ and LG-Bb). No significant difference between the responses of unstimulated and LPS stimulated Caco-2 cells was observed for any of the other preparations ($p \leq 0.001$).

The effect of 10 days of refrigerated storage in orange juice on the impact of the probiotics on IL-8 secretion is presented in Figure 7.3. This figure reveals that after 10 days of storage, a significant decrease in IL-8 production was observed in both non-stimulated and stimulated cells treated with LR or PJ compared to that observed at D0. It could be concluded however that in most cases, 10 days of refrigerated storage of the probiotic preparations in OJ had no significant impact on their effect on the IL-8 secretion of Caco-2 cells when compared with the effect associated with the fresh cultures (D0).

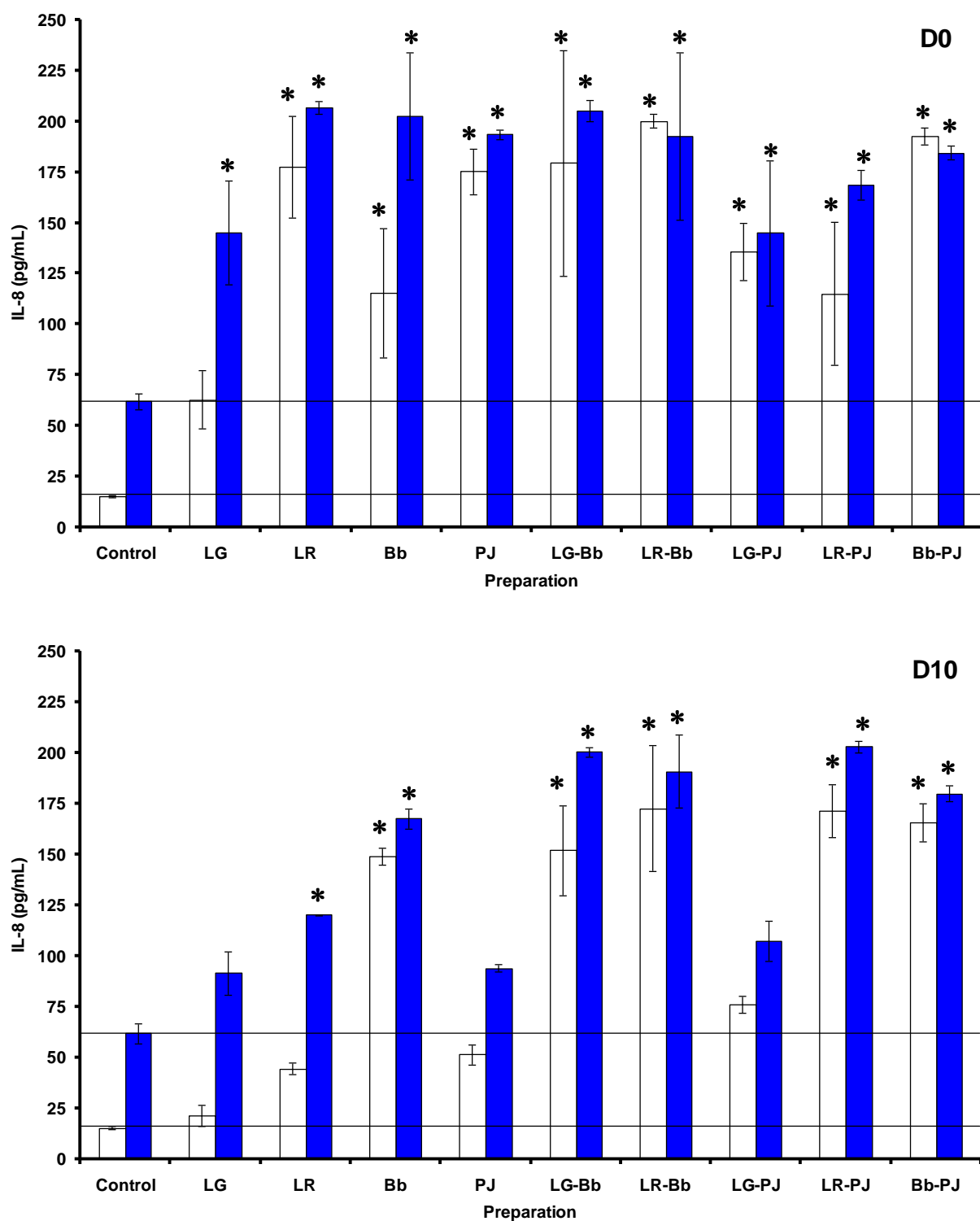


Figure 7.2: Effect of probiotic preparations at baseline (D0) and after 10 days of refrigerated storage in orange juice (D10) on both non-stimulated (white bars) and LPS induced (blue bars) IL-8 secretion by Caco-2 cells. IL-8 production is expressed as mean (pg/mL) \pm SE.

* indicates a statistically significant difference between the level of IL-8 secreted by Caco-2 cells treated with each probiotic preparation and that of the relevant control ($p \leq 0.001$).

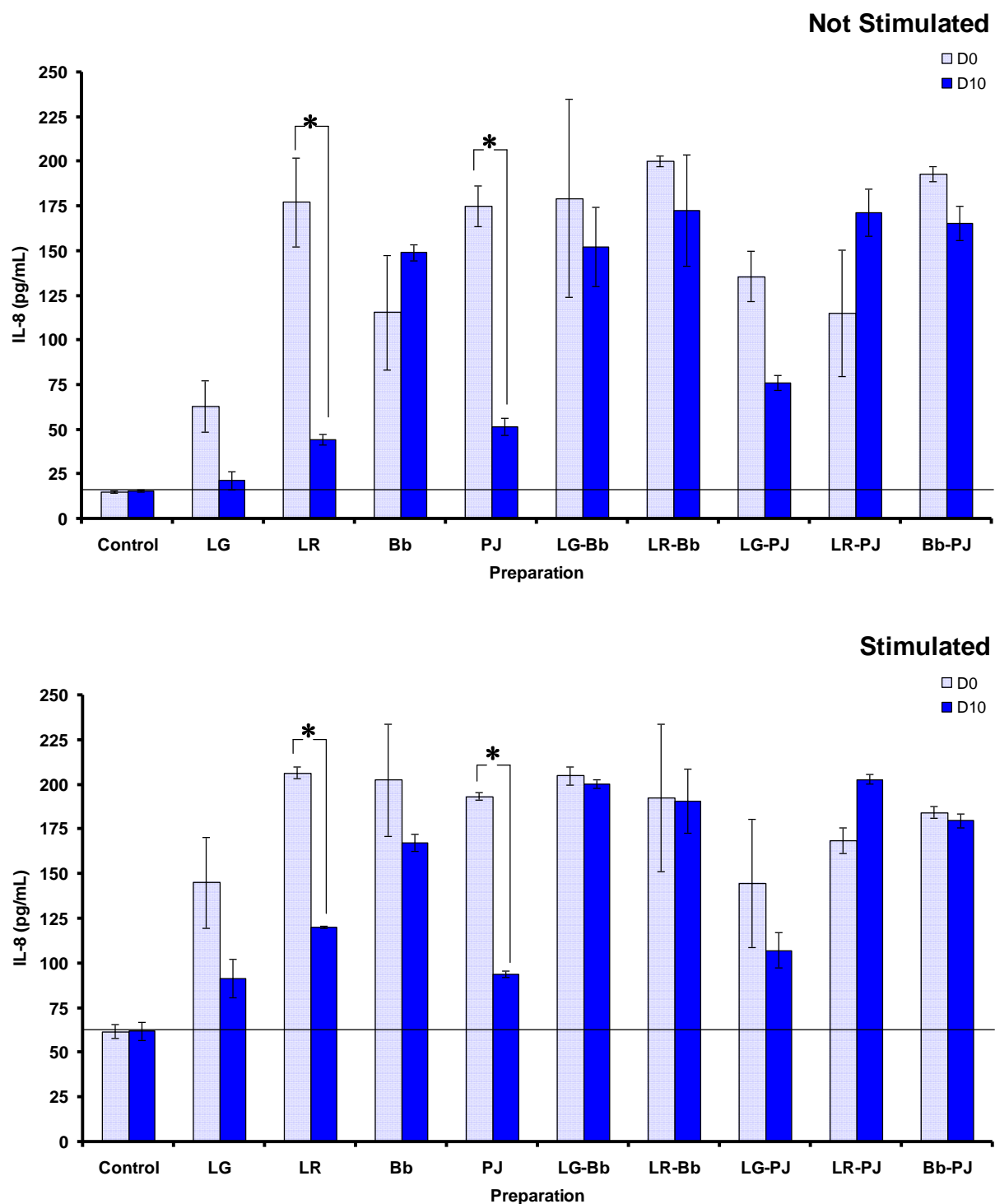


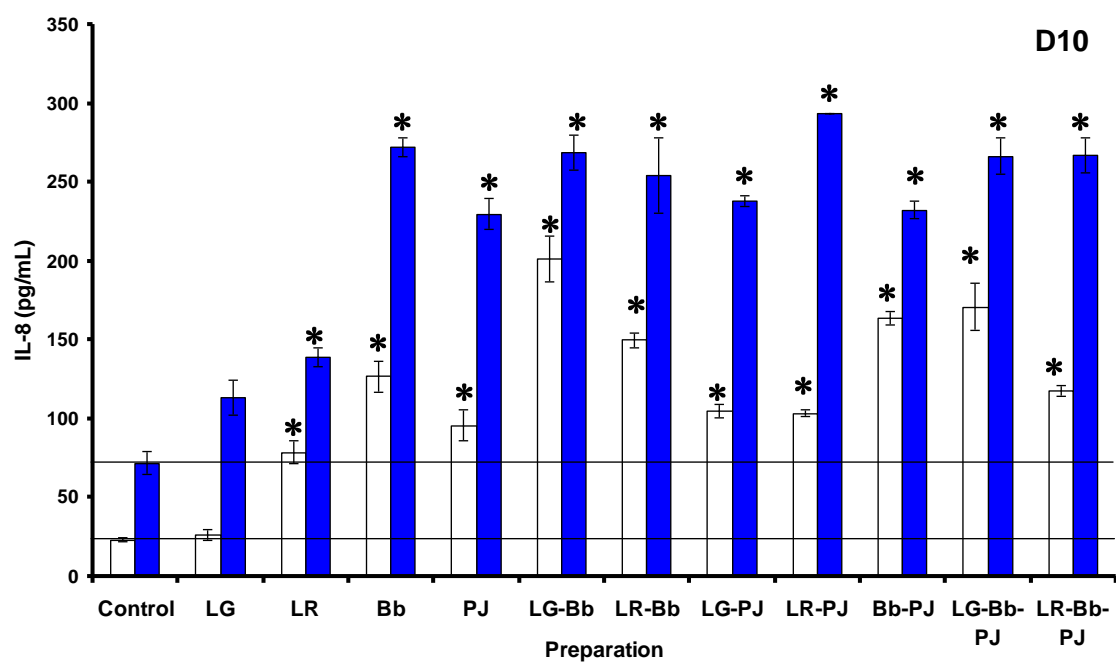
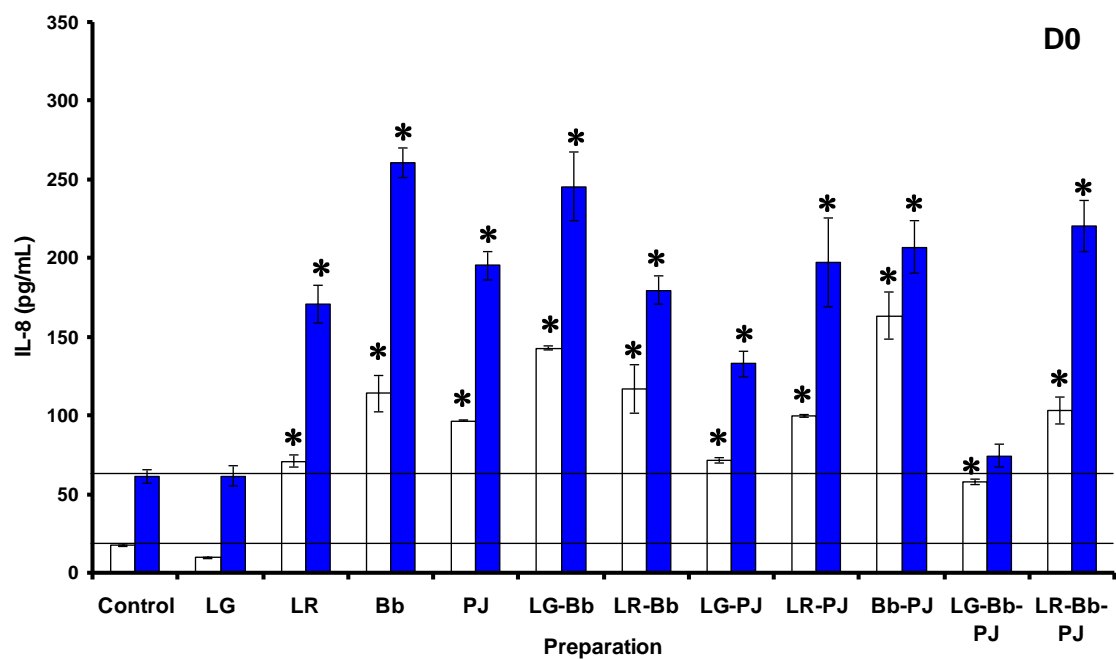
Figure 7.3: Effect of probiotic preparations before and after storage of the probiotics in orange juice at low temperature (4°C) on both non-stimulated and LPS induced IL-8 secretion by Caco-2 cells. IL-8 production is expressed as mean (pg/mL) \pm SE.

* indicates a statistically significant difference between IL-8 secretion by Caco-2 cells treated with the same preparation at baseline and on D10 ($p \leq 0.001$).

7.4.1.2 Effect of probiotics on TNF- α induced IL-8 secretion by Caco-2 cells

With the exception of LG, all probiotic preparations were associated with significantly enhanced IL-8 production in both non-stimulated and TNF- α stimulated Caco-2 cells on days 0, 10 and 20 compared with relevant control cells (Fig 7.4). On day 30, all probiotic treatments were observed to produce the same effect in non-stimulated Caco-2 cells. However, in contrast to measurements recorded prior to day 30, when IL-8 secretion was induced by exposure of Caco-2 cells to TNF- α , no significant difference in secretion levels was observed between the control and any of the probiotics treatments. The only probiotic preparation that did not appear to produce a significant effect at any time point on IL-8 secretion, by either stimulated or non-stimulated Caco-2 cells, was LG.

It seems nonetheless that under the same probiotic treatment, the difference between the amount of IL-8 secreted by unstimulated Caco-2 cells and those stimulated with TNF- α , was decreasing over the storage period. This trend is clearly evident in Figure 7.5 where the relative effect of probiotic exposure on IL-8 production by the Caco-2 cells was observed to decline across the storage period in 8 out of 11 possible cases. Interestingly, the 3 remaining cases (LG, LG-Bb and LG-Bb-PJ) were preparations all having LG in common as one of the constituents. In each of these cases a similar pattern was observed, whereby the ratio increased significantly after 10 days of storage compared with D0, but was followed by a significant decrease at D20 and either no difference (LG alone) or a significant increase again at D30 compared to D20.



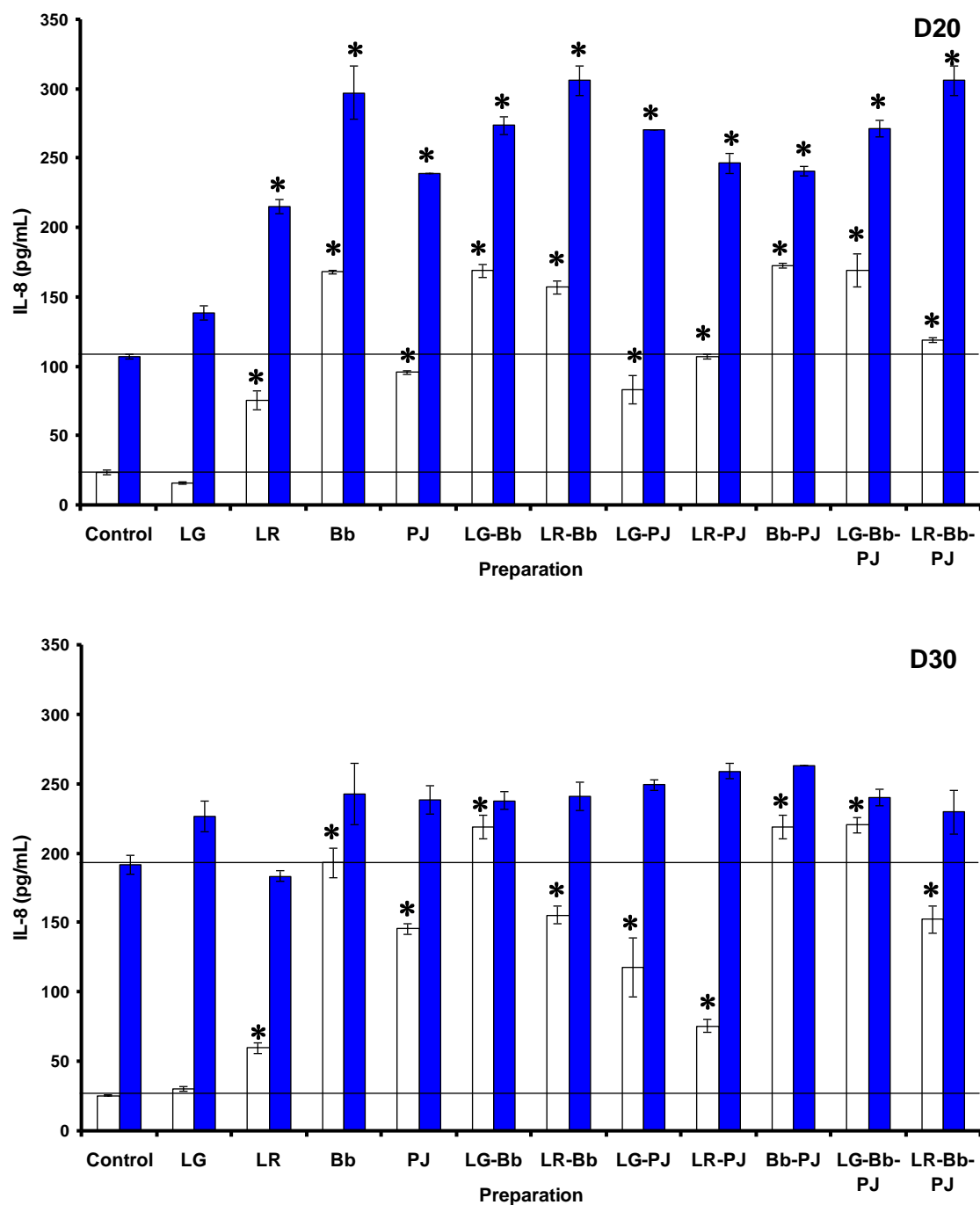


Figure 7.4: The effect of probiotic preparations at baseline (D0), and after 10, 20 and 30 days of refrigerated storage in orange juice, on both unstimulated (white bars) and TNF- α induced (blue bars) IL-8 secretion by Caco-2 cells. IL-8 production is expressed as mean (pg/mL) \pm SE. * indicates a statistically significant difference between the level of IL-8 secreted by Caco-2 cells treated with each preparation and that of the relevant control ($p \leq 0.001$).

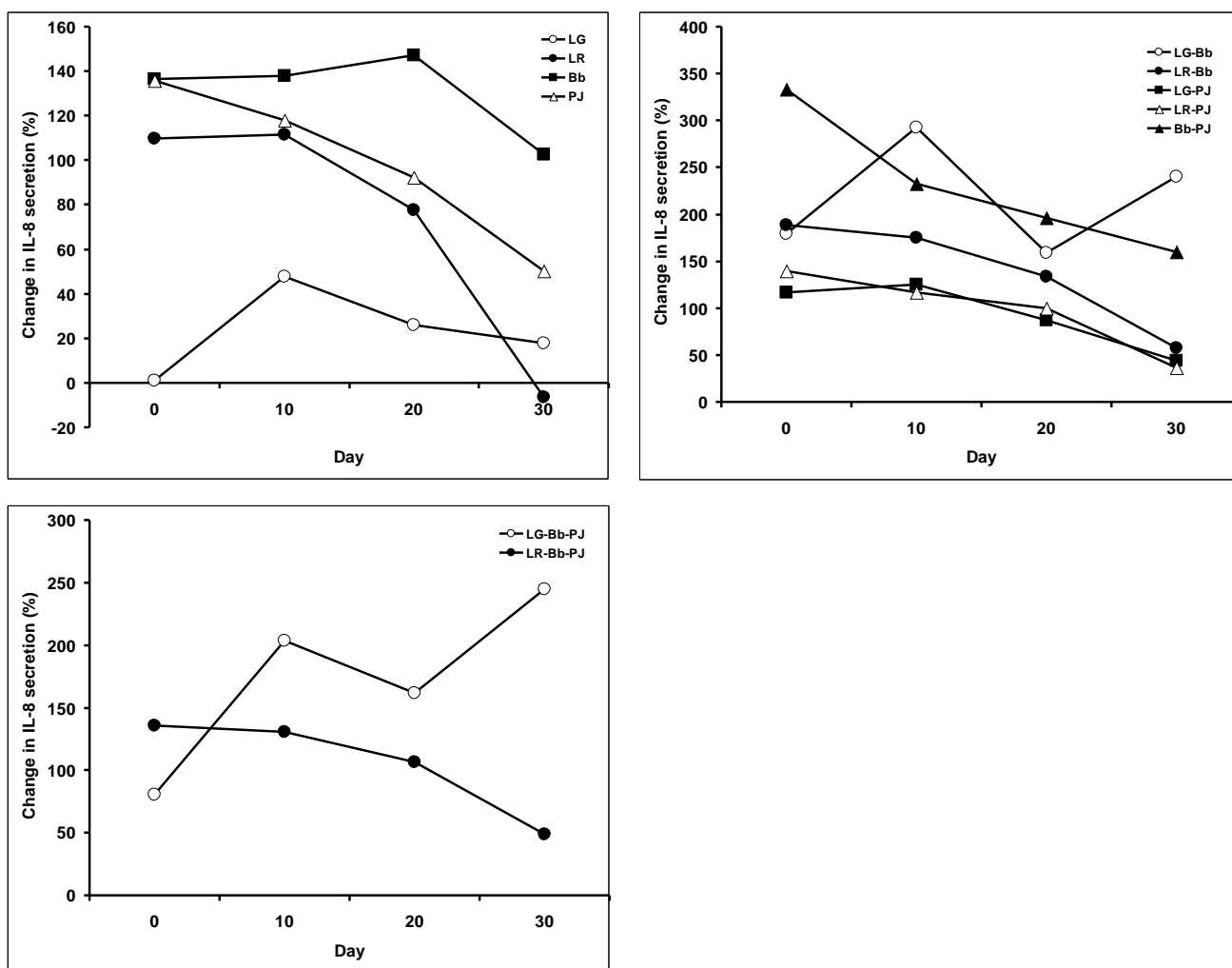
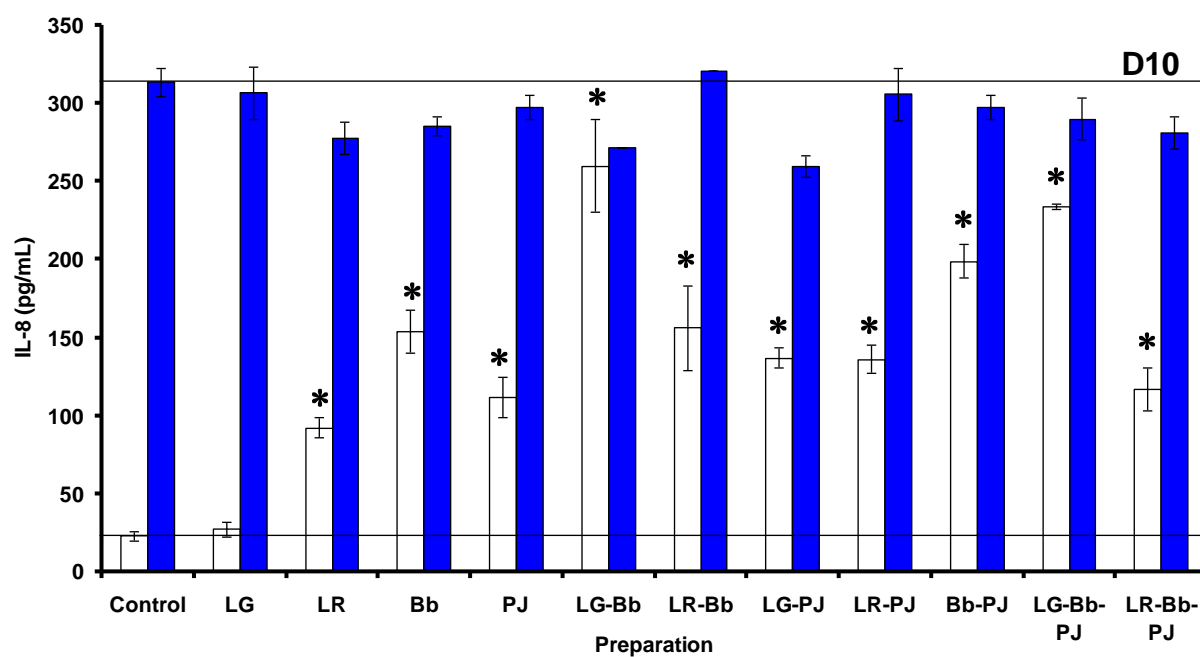
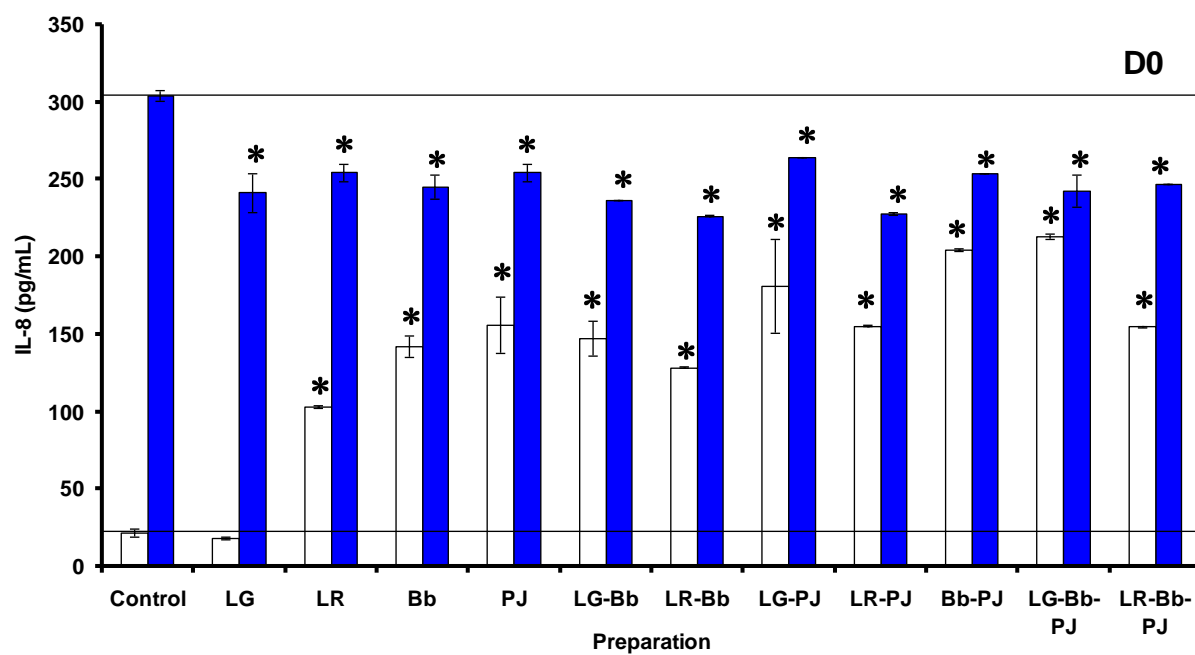


Figure 7.5: Effect of duration of combined exposure of probiotic bacteria to orange juice and low storage temperature (4°C) on TNF- α induced IL-8 secretion by Caco-2 cells

NB: the percentage values plotted in this figure were derived from the concentration data used in Figure 7.4, and calculated as per the formula described on page 195

7.4.1.3 Effect of probiotics on IL-1 β induced IL-8 secretion by Caco-2 cells

As expected, in the absence of stimulation with IL-1 β , effects of probiotic preparations on IL-8 secretion by Caco-2 cells (Figure 7.6) were almost identical to those observed in Figure 7.4. It is noteworthy that when IL-8 production was induced by IL-1 β , initial exposure (D0) of Caco-2 cells to probiotic preparations resulted in a significantly lower level of IL-8 secretion compared to that observed for stimulated control cells. However, thereafter no significant difference was observed between the amount of IL-8 secreted by stimulated control cells and IL-1 β treated Caco-2 cells exposed to probiotic preparations, with the exception of the LG-Bb preparation on day 30. When the effect of stimulation by IL-1 β was examined, with the exception of four cases (LG-Bb D10, Bb-PJ D30 and LG-Bb-PJ D0&D30), there was a significant difference between IL-8 secretion in stimulated and non-stimulated cells both treated with the same probiotic. Figure 7.7 shows that the ratio percentage of probiotic effect on IL-1 β stimulated IL-8 production by Caco-2 cells gradually increased in 10 out of 11 possible cases over the storage time.



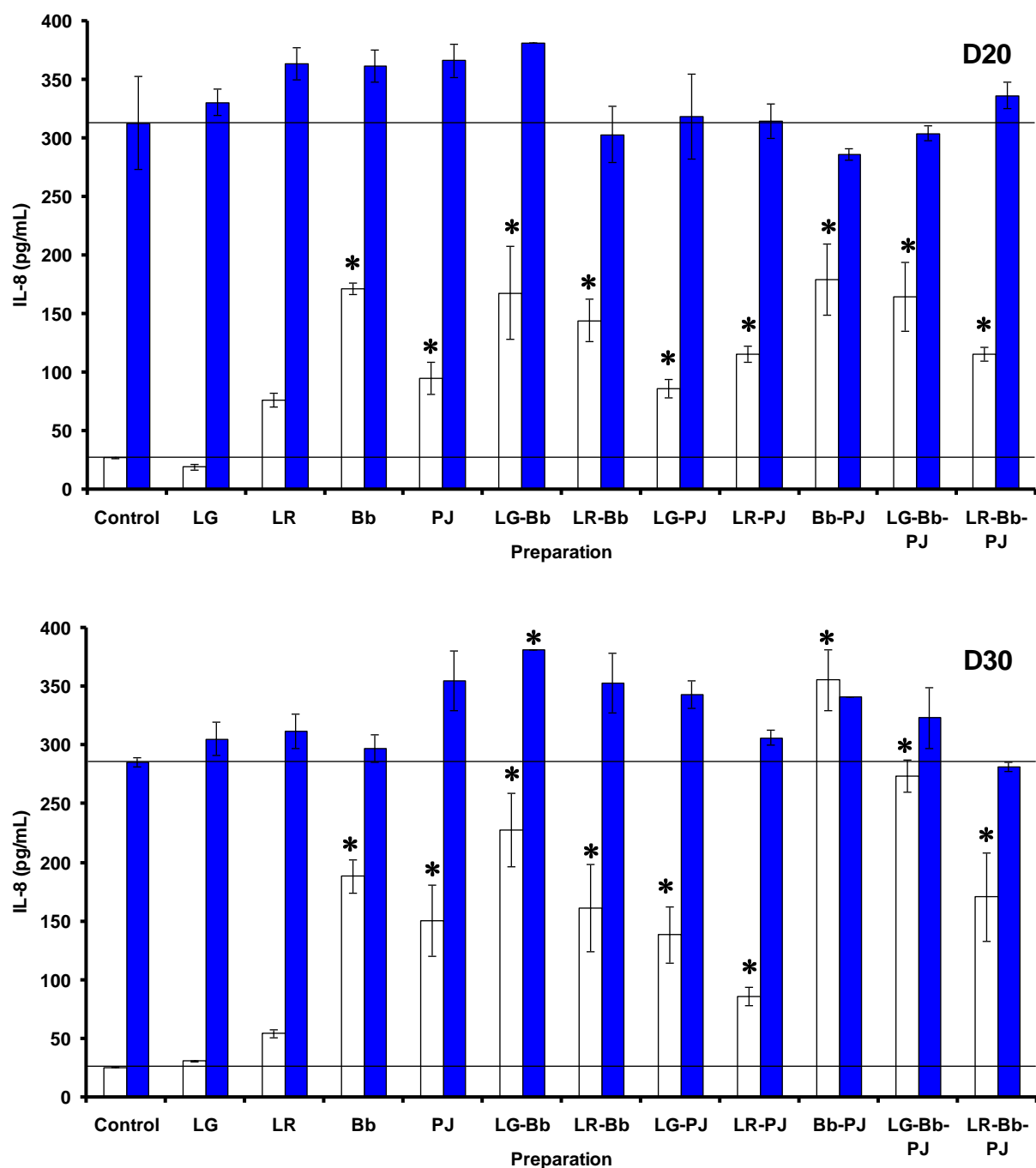


Figure 7.6: Effect of probiotic preparations at baseline (D0), and after 10, 20 and 30 days of refrigerated storage in orange juice on both unstimulated (white bars) and IL-1 β induced (blue bars) IL-8 secretion by Caco-2 cells. IL-8 production is expressed as mean (pg/mL) \pm SE.

* indicates a statistically significant difference between the level of IL-8 secreted by Caco-2 cells treated with each preparation and that of the relevant control ($p \leq 0.001$).

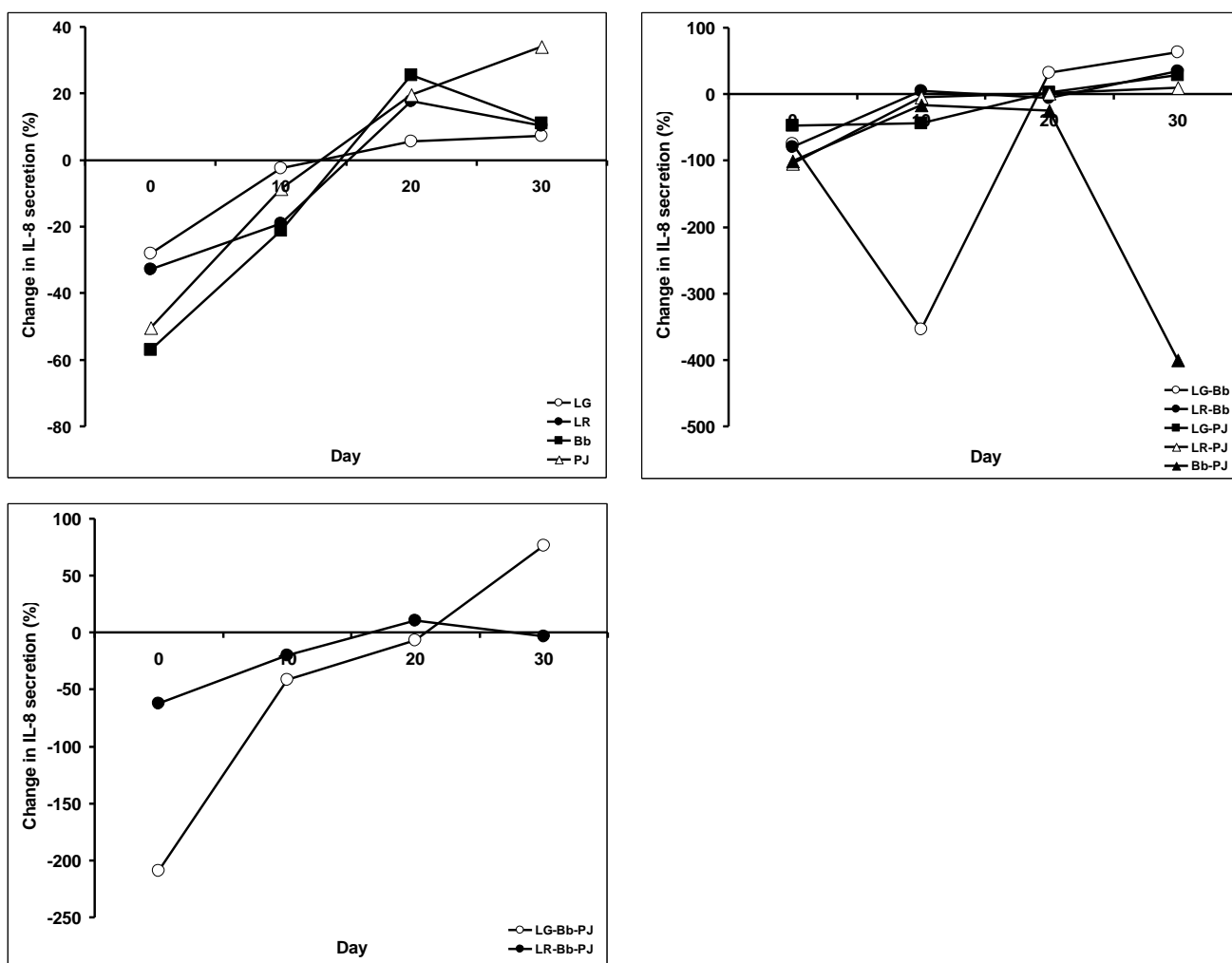


Figure 7.7: Effect of duration of combined exposure of probiotic bacteria to orange juice and low storage temperature (4°C) on IL-1 β induced IL-8 secretion by Caco-2 cells

NB: the percentage values plotted in this figure were derived from the concentration data used in Figure 7.6, and calculated as per the formula described on page 195

7.4.2 Effect of probiotic preparations on induced IL-6 production by Caco-2 cells

The study presented here essentially replicated that presented in section I, except that rather than IL-8, the focus in this case was on secretion of IL-6 by the Caco-2 cells. As with the IL-8 study, in order to choose the optimum exposure time and concentration of stimuli, time course and dose dependant secretion of IL-6 by Caco-2 cells was determined by induction of IL-6 production by LPS (0-10000 ng/mL), TNF- α (0-100 ng/mL) or IL-1 β (0-25 ng/mL) and measurement of IL-6 in cell culture medium after 0, 3, 6, 12 and 24 hours of incubation at 37°C. The time dependent dose response curves of proinflammatory stimuli induced IL-6 secretion by Caco-2 cells is presented in Figure 7.8. The most effective concentration of LPS on IL-6 production was 10000 ng/mL and was selected for further studies; however this level increased IL-6 secretion by only 3.5 pg/mL after 24h. Concentrations of 10 and 25 ng/mL of TNF- α significantly increased IL-6 production compared to other doses after 24 h. Since the effect of TNF- α was comparable to that of LPS in induction of IL-6 by Caco-2 cells (Figure 7.8), TNF- α was not used in further experiments. Doses of 1 and 25 ng/mL IL-1 β significantly increased IL-6 secretion compared to the control and the other doses. Since no significant difference was observed between these two doses in induction of IL-6 release, 1 ng/mL IL-1 β was selected for further studies.

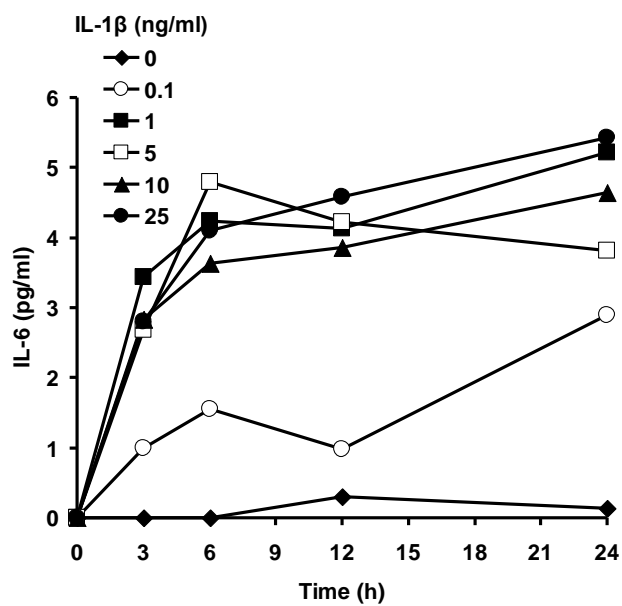
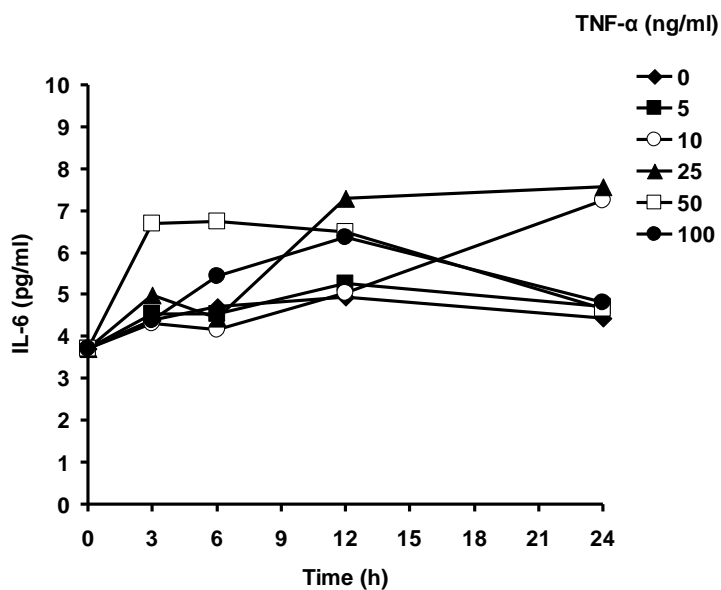
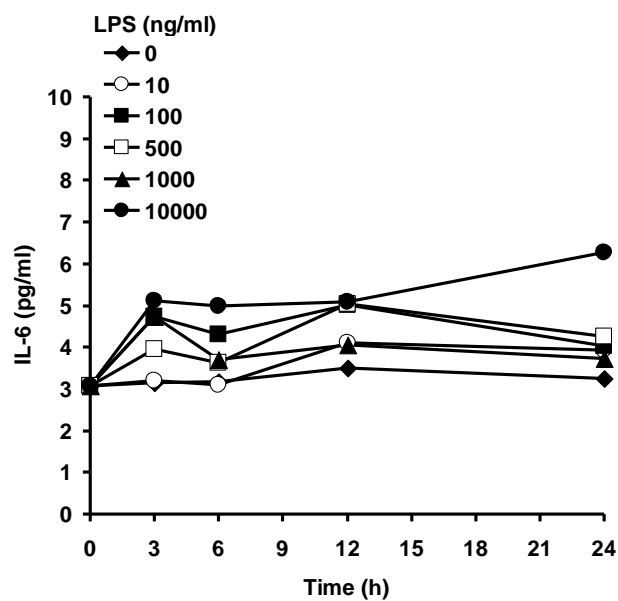


Figure 7.8: Time dependent dose response curves of LPS, TNF- α or IL-1 β induced IL-6 production in Caco-2 cells.

7.4.2.1 Effect of probiotics on LPS induced IL-6 secretion by Caco-2 cells

Probiotic preparations did not significantly affect IL-6 release in the culture medium of LPS-induced or non-induced Caco-2 cells at baseline and after 10 days of storage compared with the relevant controls (Figure 7.9). There was also no significant difference between the level of IL-6 release by LPS induced and non-induced Caco-2 cells treated with the same probiotic preparation at either baseline, or after 10 days of storage. The only exception was observed after 10 days of storage in the LG-Bb set in which LPS induced IL-6 secretion was not only significantly increased compared with that of the non-stimulated cells, but also relative to all other cases (Figure 7.9). Moreover compared to the baseline levels, 10 days of combined exposure of probiotic bacteria to orange juice and refrigerated storage appeared not to influence IL-6 production by Caco-2 cells (Figure 7.10)

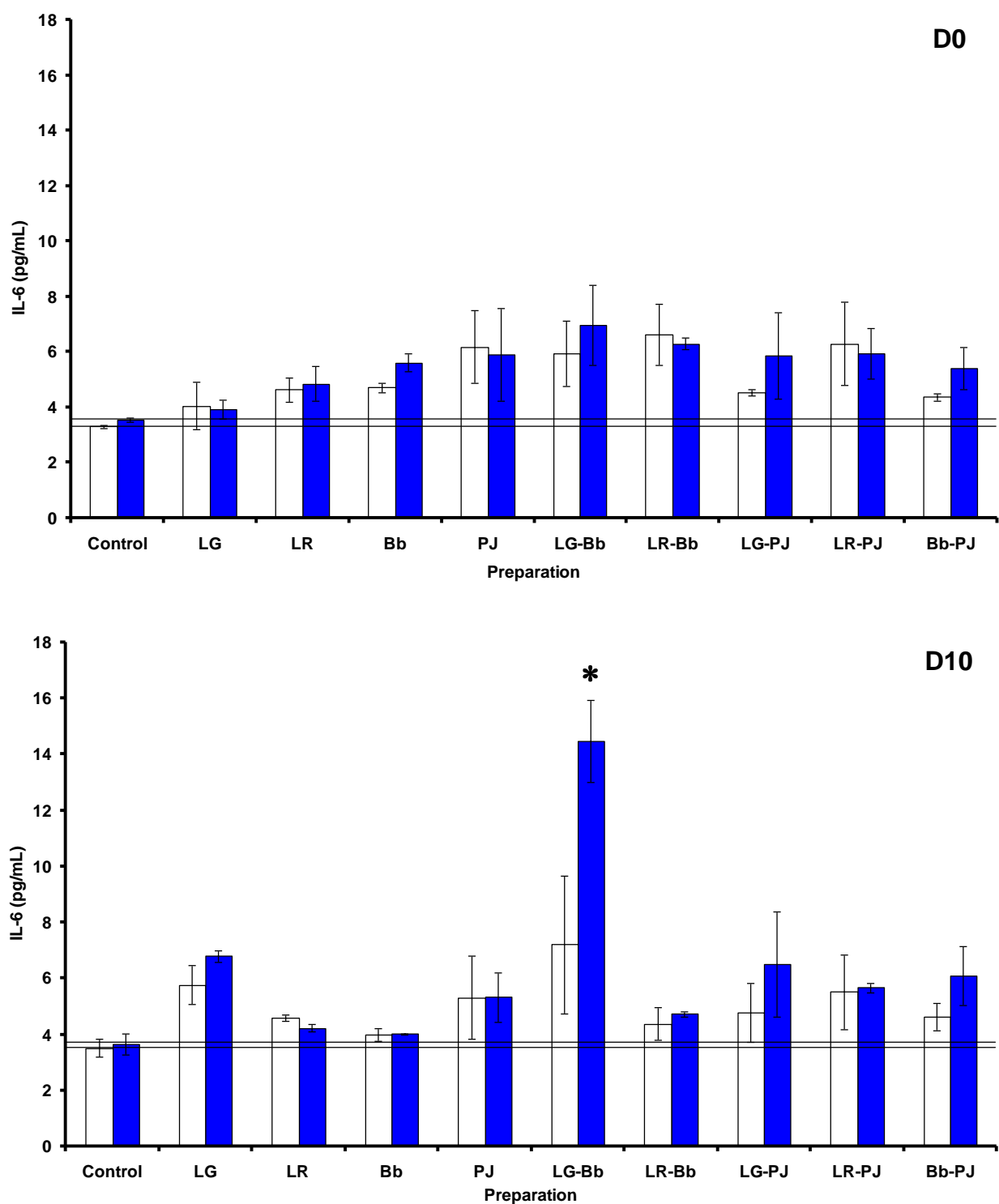


Figure 7.9: Effect of probiotic preparations at baseline (D0) and after 10 days of refrigerated storage in orange juice (D10) on unstimulated (white bars) and LPS induced (blue bars) IL-6 secretion by Caco-2 cells. IL-6 production is expressed as mean (pg/mL) \pm SE.

* indicates a statistically significant difference between the level of IL-6 secreted by Caco-2 cells treated with each preparation and that of the relevant control ($p \leq 0.001$).

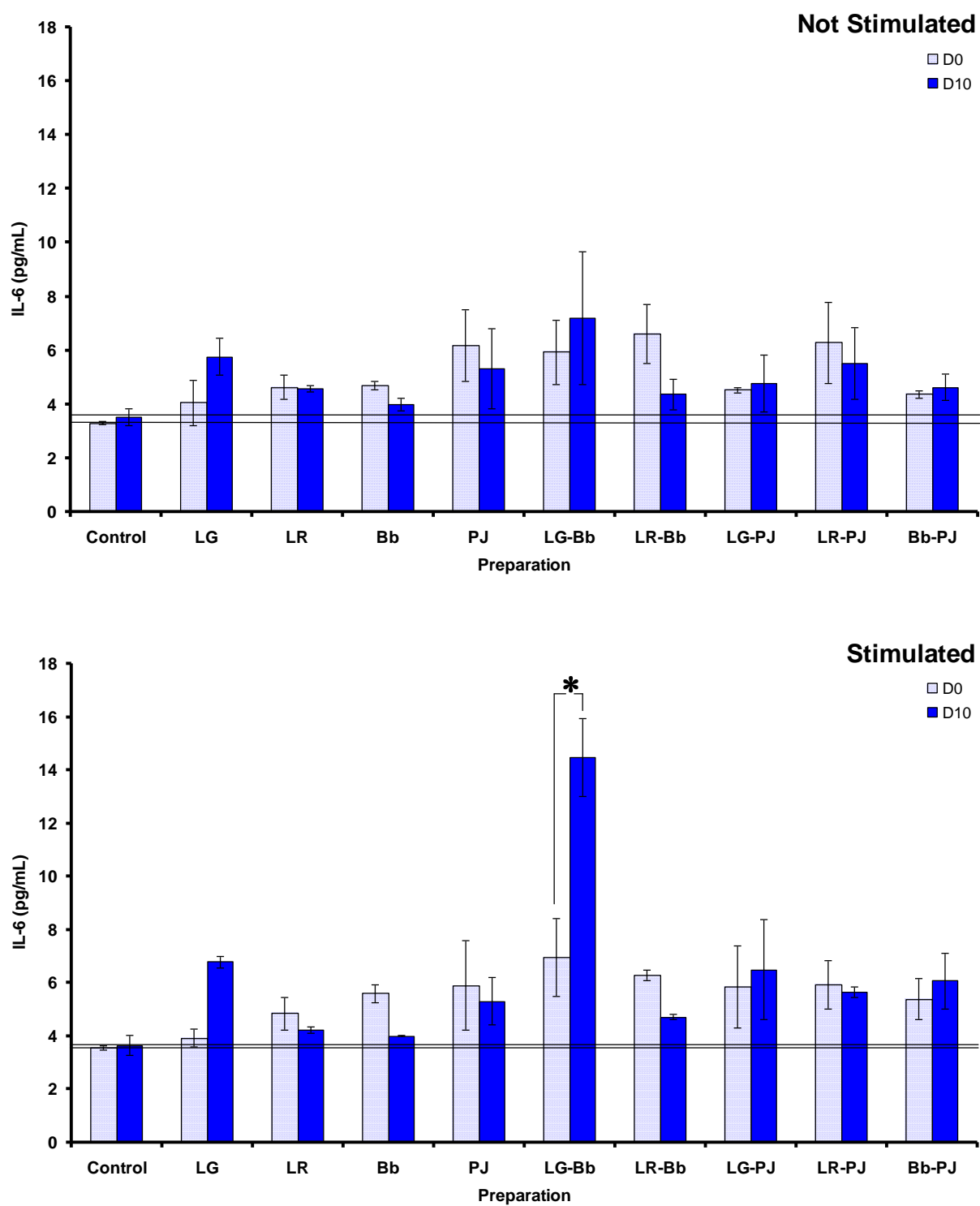
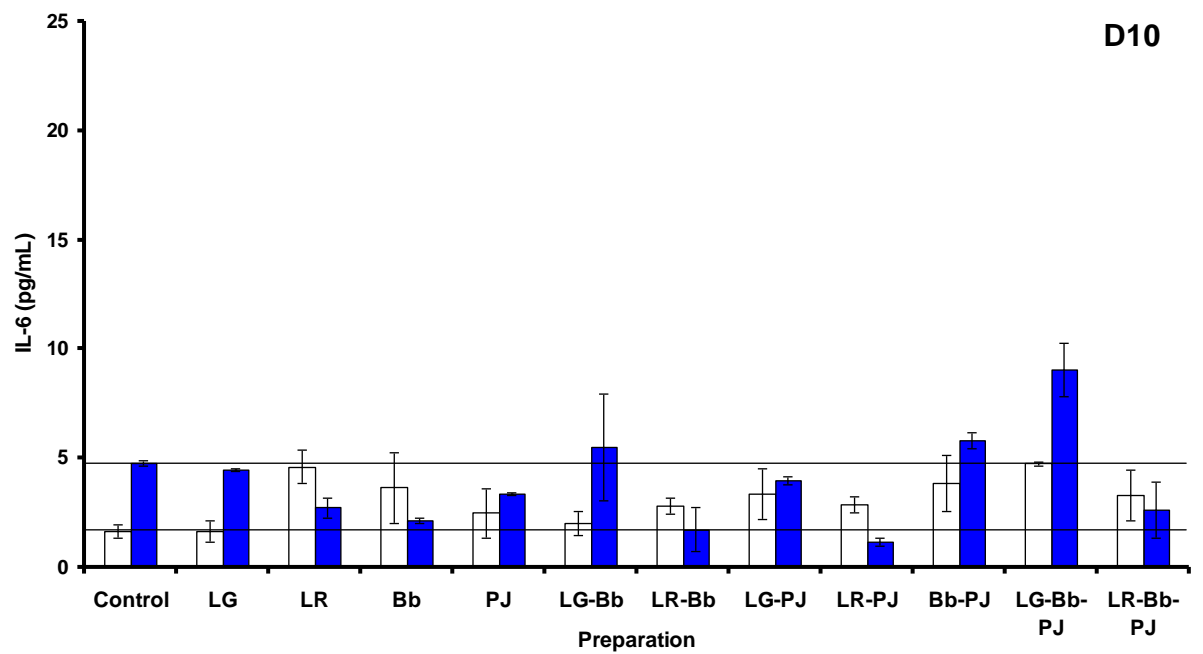
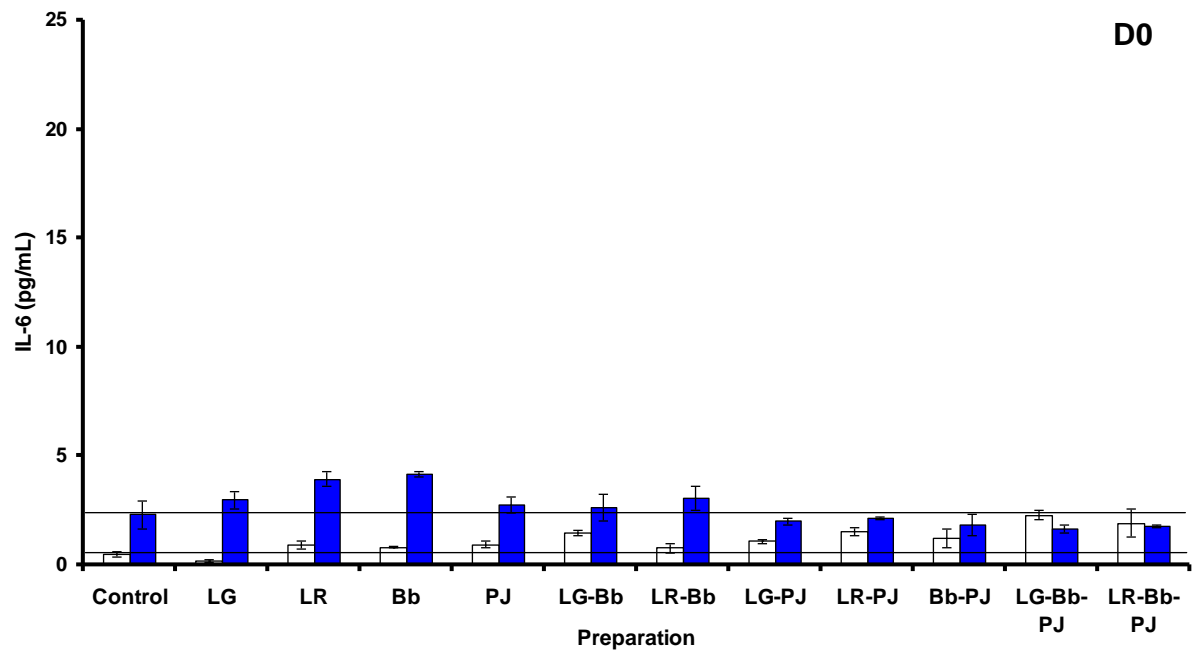


Figure 7.10: Effect of duration of combined exposure of probiotic bacteria to orange juice and low storage temperature (4°C) on unstimulated and LPS induced IL-6 secretion by Caco-2 cells. IL-6 production is expressed as mean (pg/mL) \pm SE.

* indicates a statistically significant difference between IL-6 secretion by Caco-2 cells treated with the same preparation at baseline and on D10 ($p \leq 0.001$).

7.4.2.2 Effect of probiotics on IL-1 β induced IL-6 secretion by Caco-2 cells

The results of this study showed that in general no significant difference was observed among the probiotic preparations and compared with the control in the secretion of IL-6 by non-induced Caco-2 cells (with the exception of Bb on D30) (Figure 7.11). With few exceptions similar relative results were observed for IL-1 β induced IL-6 production over 20 days of storage. On day 30 however the probiotic preparations were respectively associated with a variety of responses including both significant increases and decreases in IL-6 release relative to the control cells. Figure 7.12 shows the effect of probiotics on IL-1 β induced IL-6 production by Caco-2 cells during refrigerated storage in OJ. It is clear from examination of this figure that the responses of the Caco-2 cells under the different probiotic treatments were highly variable. In order to better identify potentially meaningful trends in the data, the results were further summarised. In Table 7.5 the various preparations have been grouped on the basis of the IL-6 secretion level recorded at D10 relative to D0. Of the 11 preparations, a significant increase in the level of IL-6 secretion by the Caco-2 cells at D10 was observed in five, all of which contained either LR and/or Bb. The trend in most of these cases was toward a subsequent decline in IL-6 secretion levels beyond D10. In a further four preparations, all containing PJ, an opposing trend was observed with all exhibiting a significant decrease in IL-6 levels at D10, followed in 3 of these cases by a subsequent increase again at D20. In the two remaining preparations, both of which contained LG, no significant changes in IL-6 concentrations were observed across the entire storage period.



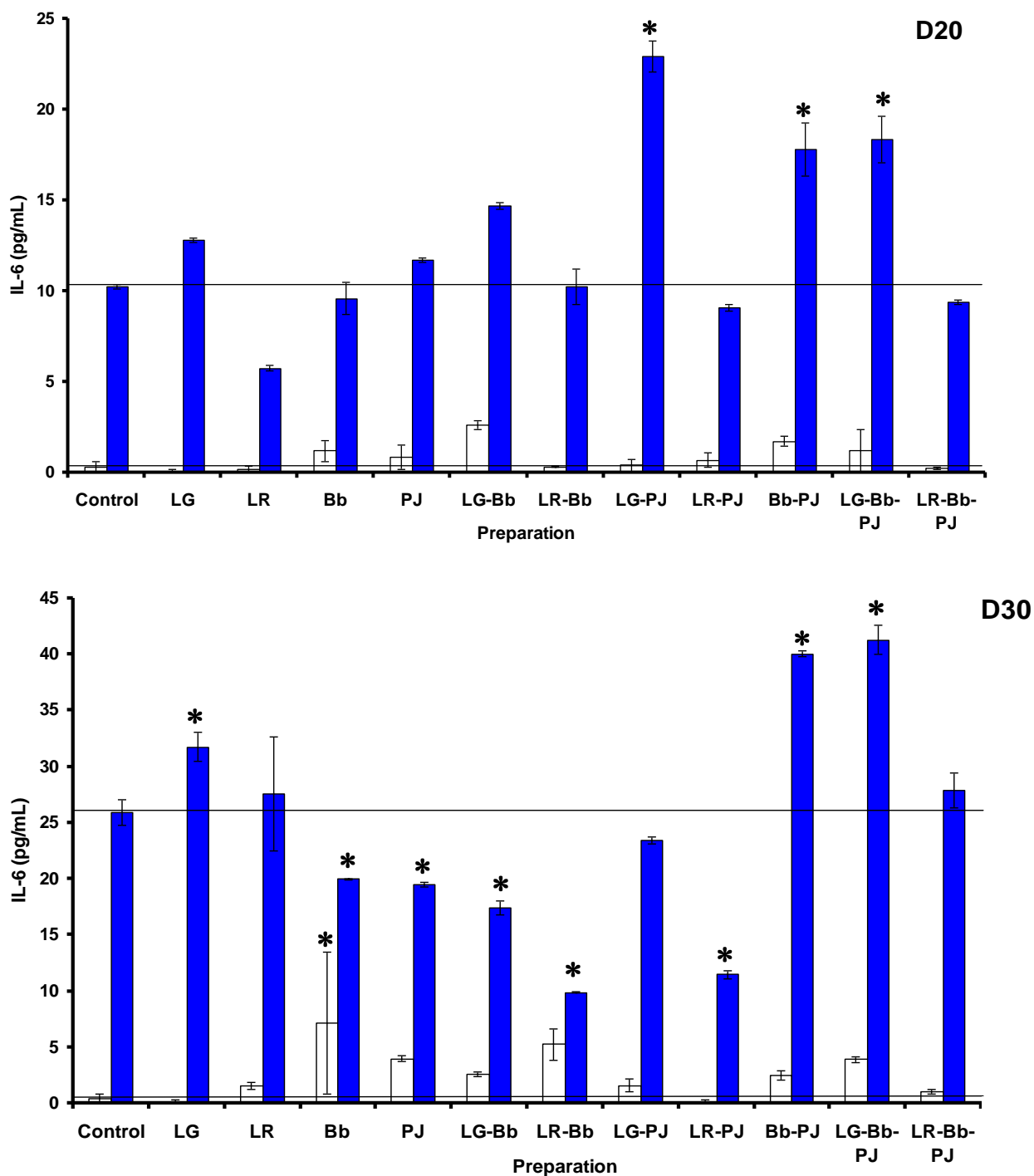


Figure 7.11: Effect of probiotic preparations at baseline (D0) and after 10, 20 and 30 days of refrigerated storage in orange juice on unstimulated (white bars) and IL-1 β induced (blue bars) IL-6 secretion by Caco-2 cells. IL-6 production is expressed as mean (pg/mL) \pm SE.

* indicates a statistically significant difference between the level of IL-6 secreted by Caco-2 cells treated with each preparation and that of the relevant control ($p \leq 0.001$).

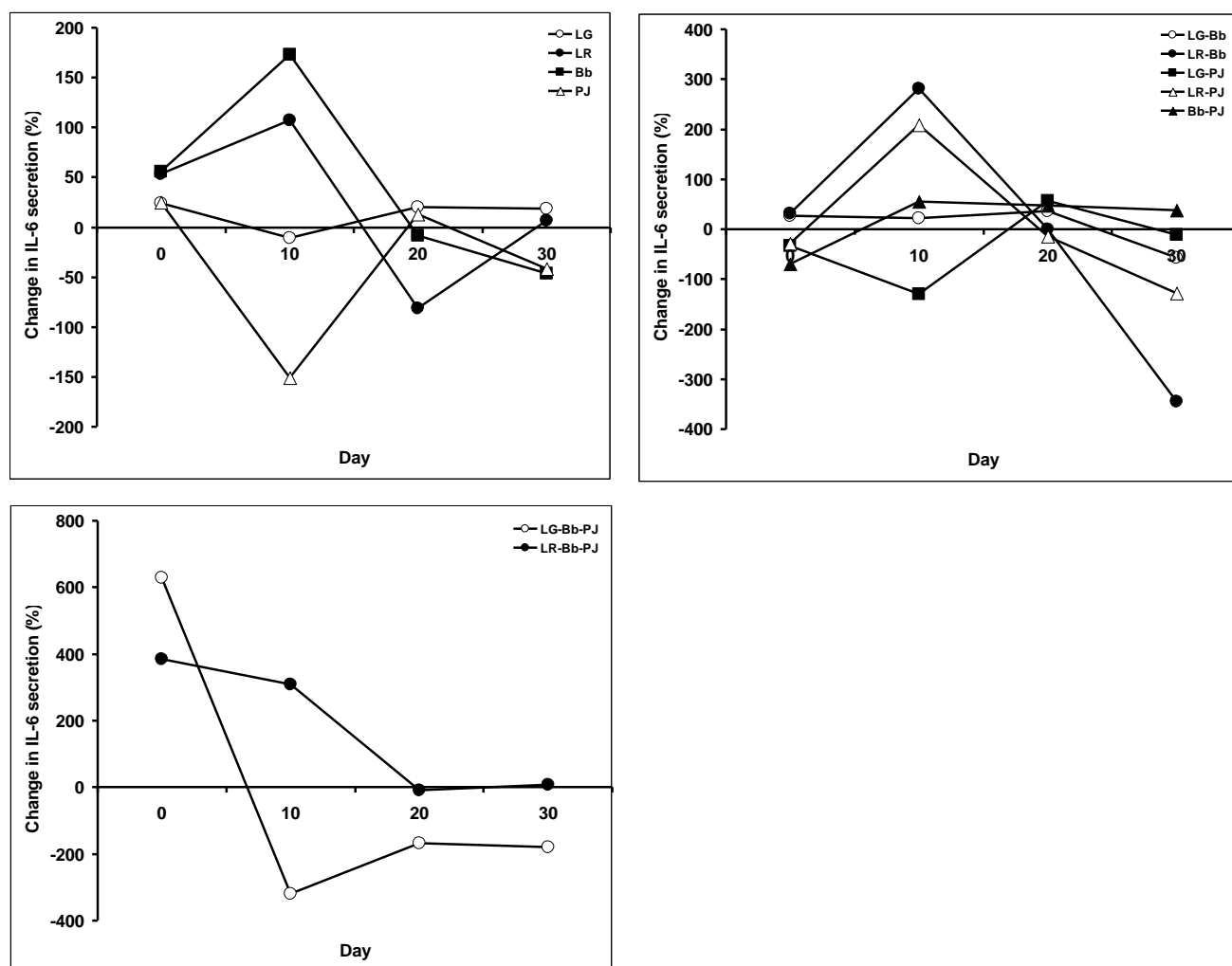


Figure 7.12: Effect of duration of combined exposure of probiotic bacteria to orange juice and low storage temperature (4°C) on IL-1 β induced IL-6 secretion by Caco-2 cells

NB: the percentage values plotted in this figure were derived from the concentration data used in Figure 7.11, and calculated as per the formula described on page 195

Table 7.5: Effect of duration of refrigerated storage on ratio percentage change of probiotic effect on IL-6 production

Probiotic preparation	Effect on IL-6 secretion		
	D10	D20	D30
LR	+	-	+
Bb	+	-	-
LR-Bb	+	-	-
LR-PJ	+	-	-
Bb-PJ	+	N	N
PJ	-	+	-
LG-PJ	-	+	-
LG-Bb-PJ	-	+	N
LR-Bb-PJ	-	-	N
LG	N	N	N
LG-Bb	N	N	N

7.4.3 Effect of probiotics on IL-1 β induced TNF- α secretion by Caco-2 cells

Similar to the previous studies, this final analysis examined IL-1 β induced secretion of TNF- α by Caco-2 cells under exposure to probiotics that were stored in orange juice at 4°C over a period of 30 days. In this case however, the study included only the four single species probiotic preparations (LG, LR, Bb and PJ). Generally at all time points there was no significant difference between TNF- α release in induced or non-induced Caco-2 cells and the relevant control with the exception of LG+IL-1 β on D10, 20 & 30 and PJ+IL-1 β on D0. In all cases stimulation by IL-1 β resulted in significantly increased secretion of TNF- α by Caco-2 cells compared with non-induced cells treated with the same probiotic, except for the fresh cultures of LR and Bb (D0) and LR after 20 days storage. With specific reference to IL-1 β induced TNF- α production by the Caco-2 cells, exposure to the fresh probiotic cultures (D0) was found to result in significantly enhanced levels of secretion (Figure 7.14). However, after 10 days of refrigerated storage of the probiotics in the orange juice the response was found to decline substantially, and remained at these lower levels in subsequent measurements.

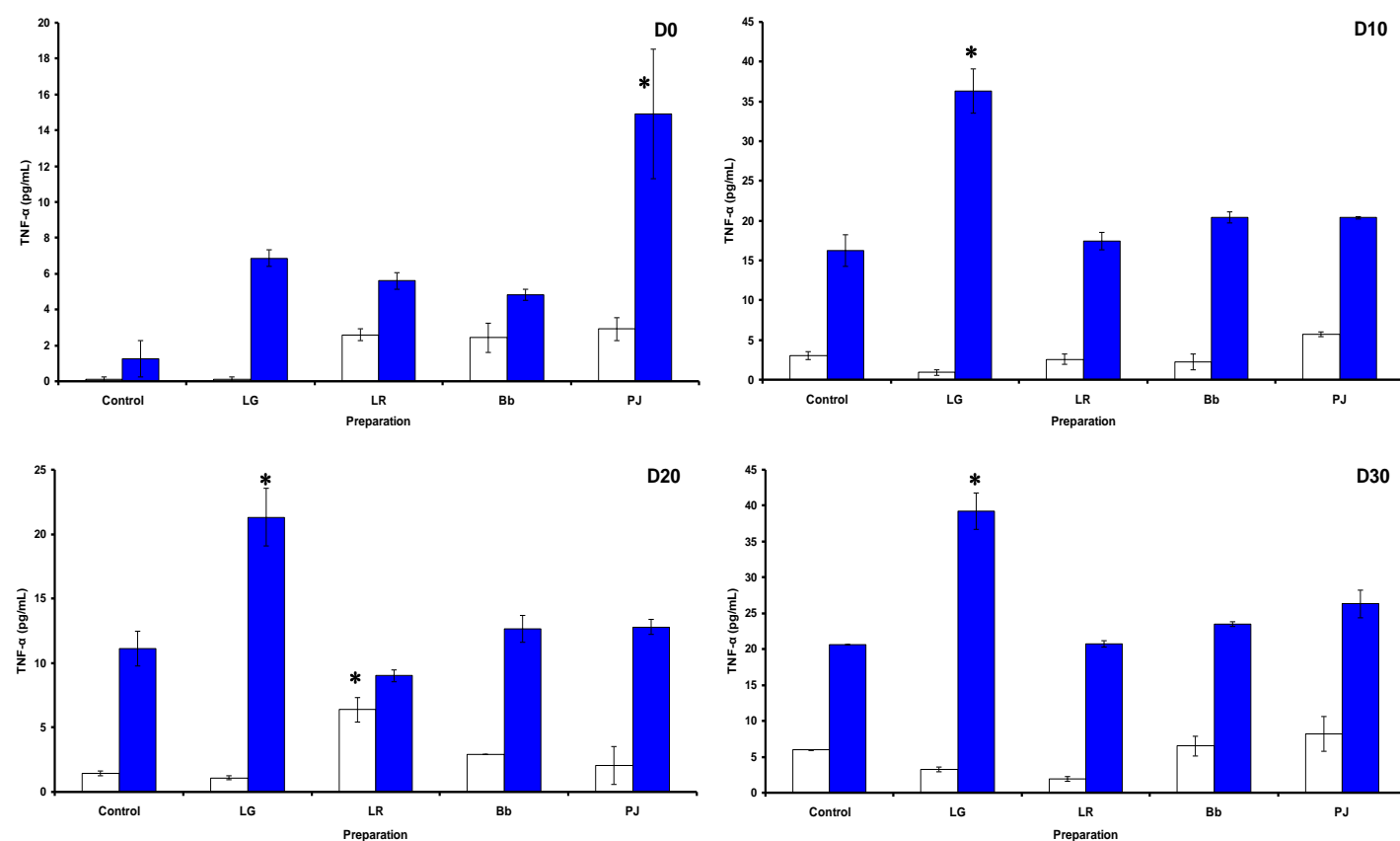


Figure 7.13: Effect of single probiotic strains at baseline (D0) and after 10, 20 and 30 days of refrigerated storage in orange juice, on unstimulated (white bars) and IL-1 β induced (blue bars) TNF- α secretion by Caco-2 cells. TNF- α production is expressed as mean (pg/mL) \pm SE.

* indicates a statistically significant difference between the level of TNF- α secreted by Caco-2 cells treated with each preparation and that of the relevant control ($p \leq 0.001$).

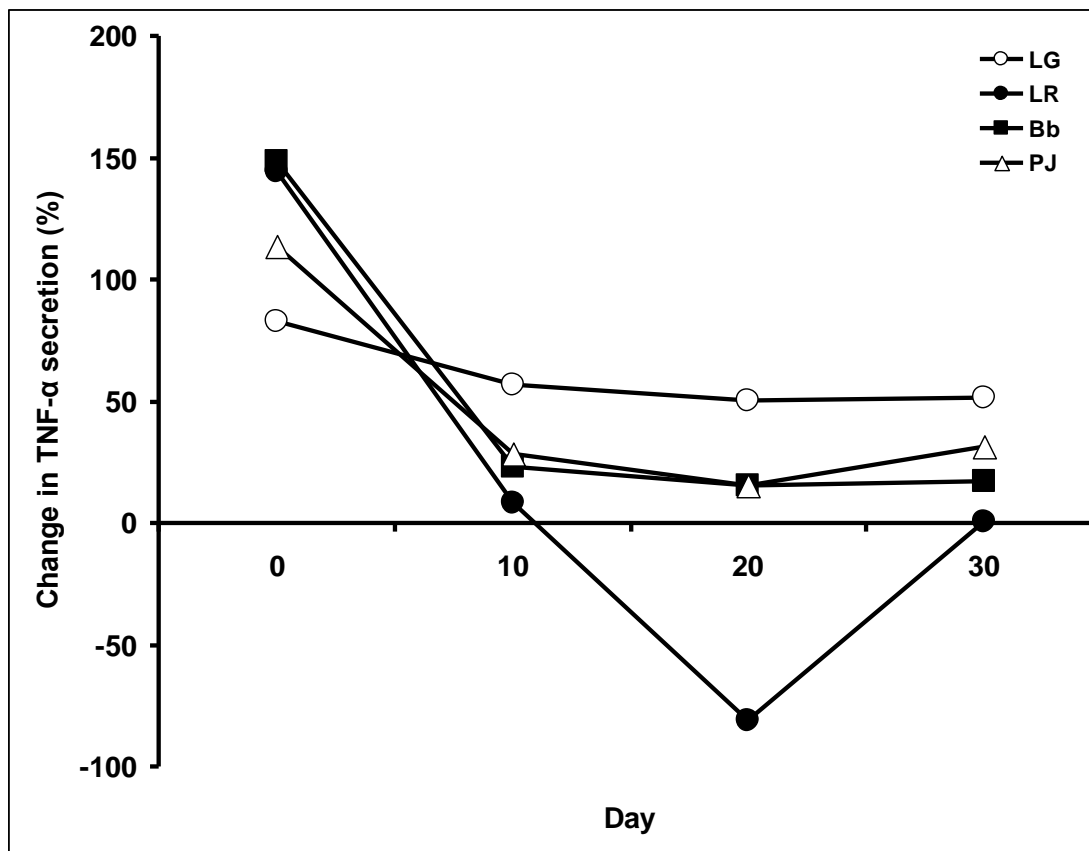


Figure 7.14: Effect of duration of combined exposure of probiotic bacteria to orange juice and low storage temperature (4°C) on IL-1 β induced TNF- α secretion by Caco-2 cells

NB: the percentage values plotted in this figure were derived from the concentration data used in Figure 7.13, and calculated as per the formula described on page 195

7.4.4 Summary of key findings

Major findings/trends are summarised as follows:

Generally, at the baseline and during refrigerated storage, with the exception of LG, exposure to probiotic preparations significantly enhanced non-stimulated IL-8 production in Caco-2 cells compared to the control. Thus, the hypothesized variation among probiotic preparations in non-stimulated production of IL-8 by Caco-2 cells was supported.

While the amount of the IL-8 produced varied among the probiotic strains/preparations in both stimulated and non-stimulated conditions, this variation was lower between the preparations in the IL-1 β induced IL-8 secretion set. These results therefore provide limited evidence of strain and combination dependent variation in the immunomodulatory effect of probiotic preparations on induced IL-8 production.

Exposure to probiotic preparations, significantly enhanced LPS induced IL-8 release at baseline compared to the control. This effect however was not evident for several probiotic preparations at D10. When TNF- α was used as a stimulant, with the exception of LG, all probiotic preparations enhanced IL-8 secretion by D20, while at D30 no effect was observed in IL-8 secretion between probiotics and that of the control. In contrast, a significant decline in IL-1 β induced IL-8 secretion was observed for all probiotic preparations at baseline relative to the control, however no significant effect was evident at subsequent time points during the storage. The percentage of probiotic effect on IL-1 β and TNF- α induced IL-8 secretion showed an upward and downward trend respectively over the storage period. Therefore the ability of probiotics to modulate stimulus-induced IL-8 production by Caco-2 cells varied with particular stimulating agents and further affected by duration of their exposure to the orange juice and refrigerated storage as hypothesized.

The probiotic preparations did not significantly affect non-stimulated IL-6 and TNF- α secretion by Caco-2 cells. These findings were therefore not supportive of the hypothesis that exposure to probiotics would differentially affect non-stimulated IL-6 and TNF- α production by Caco-2 cells.

No difference, either among the different probiotic preparations or between them and the control, was observed in relation to LPS or IL-1 β induced secretion of IL-6 either at baseline or after 10 days of storage. Exposure to some probiotic preparations however appeared to have variable effects on IL-1 β induced IL-6 secretion including

enhancement and suppression, after 20 and 30 days of storage. These findings illustrate an apparently differential effect of prolonged storage on the capability of some probiotic preparations to influence IL-1 β induced IL-6 secretion by Caco-2 cells.

PJ at baseline and LG from D10 to the end of the storage period significantly increased IL-1 β induced TNF- α production compared to the control. Compared to baseline, the effect of all four probiotic strains significantly decreased by D10 and remained subdued until the end of the storage period. This finding demonstrated that the capacity of probiotics to regulate IL-1 β induced TNF- α production was strain dependent and was further affected by their exposure to orange juice and refrigeration.

7.5 Discussion

The objective of this study was to investigate the influence of the duration of combined exposure of probiotics to the food matrix (orange juice) and low storage temperature (4°C) on their capability to regulate immune responses of intestinal epithelial cells in an inflammatory condition. To mimic an infectious and non-infectious inflammatory condition, intestinal epithelial Caco-2 cells were exposed to *E. coli* LPS and pro-inflammatory cytokines (TNF- α or IL-1 β) respectively. Inflammatory responses of Caco-2 cells were then examined by measurement of IL-8, IL-6 or TNF- α in cell culture supernatants. In general the results provided evidence of variation in immunomodulatory effects between the probiotic strains/combinations examined. It was also found that the ability of the probiotics to modulate immune responses of Caco-2 cells to different potent pro-inflammatory stimuli would be further affected by the duration of their combined exposure to orange juice and refrigerated storage. Moreover the data showed that the effect of probiotics on induced cytokines production by Caco-2 cells varied with the particular stimulating agents used.

7.5.1 IL-8

IL-8 release has been considered as a major inflammatory response of IECs upon encounter with invasive pathogens and proinflammatory cytokines such as IL-1 β and TNF- α . IL-8 contributes to neutrophil recruitment and chemoattraction to the sites of inflammation, damaged or infected tissues. While neutrophils play an important role in host defence through phagocytosis and killing of invasive pathogens (Chin & Parkos, 2007; Mumy & McCormick, 2009), unlimited neutrophil activation and migration causes significant tissue damage and destruction, which is seen in non-infectious conditions such as Crohn's disease and ulcerative colitis (Mumy & McCormick, 2009). Therefore it is thought that down regulation of IL-8 production by IECs in non-infectious inflammatory conditions may be useful in helping to suppress neutrophil migration and associated tissue injury.

In the present study, it was shown that almost all probiotic preparations (single strain, 2- or 3-multi-species) at the baseline and during refrigerated storage in OJ significantly enhanced IL-8 production in non-stimulated Caco-2 cells compared to the control cells. However the amount of the cytokine produced varied among the probiotic preparations. These findings are consistent with a number of studies showing that probiotic bacteria

significantly increased IL-8 production by unstimulated intestinal epithelial Caco-2 cells (Hosoi *et al.*, 2003; Malago, Tooten, *et al.*, 2010; Morita *et al.*, 2002a; Nemeth *et al.*, 2006).

However several studies have also demonstrated that some potential probiotic strains have no effect on IL-8 production by Caco-2 cells (de Palencia, Lopez, *et al.*, 2008; Mileti *et al.*, 2009; Morita *et al.*, 2002a; Reilly *et al.*, 2007). These reports have shown that the capability of different probiotic strains to influence IL-8 production in Caco-2 cells is strain dependant (Table 7.1). In this study LG was the only strain which did not significantly affect IL-8 secretion by Caco-2 cells at any time, a finding that concurs with that of a previous study by Mileti *et al.* (2009).

When Caco-2 cells were treated with *E. coli* LPS, at baseline all fresh probiotic cultures significantly increased IL-8 production compared to that of the control cells. There have been no previous reports on the effect of probiotics on LPS-induced IL-8 production by Caco-2 cells, however probiotics have been linked with a significant decrease in LPS-induced IL-8 production by the HT-29 human intestinal epithelial cell line (Candela *et al.*, 2008; Grimoud *et al.*, 2010; Versalovic *et al.*, 2008; Vidal *et al.*, 2002; Wood *et al.*, 2007) and intestinal epithelial cells derived from animals such as piglet or rat (Liu *et al.*, 2010). These contrasting results may reflect differences in the probiotic strains involved, the IECs used, the LPS applied or the assay conditions. For some probiotic preparations (LG, PJ and their paired combination), the enhancement in IL-8 secretion observed at D0, was not evident at D10. This shows that the combined exposure of probiotics to OJ and low storage temperature (4°C) for this duration affected regulation of LPS-induced IL-8 production by Caco-2 cells by the probiotics, in a strain/preparation dependent fashion. The ability of some probiotics to enhance IL-8 production by intestinal epithelial cells may facilitate more rapid elimination of the pathogens in infectious states through increased recruitment of neutrophils.

When IECs were challenged with the pro-inflammatory cytokine TNF- α , all probiotic preparations other than LG significantly enhanced IL-8 production by Caco-2 cells at baseline and after 10 and 20 days of storage. This result is similar to that of a previous investigation using HT-29 cells showing that exposure to probiotic *Lb. plantarum* BFE 1685 and *Lb. rhamnosus* GG also resulted in increased TNF- α -induced IL-8 production (Vizoso Pinto *et al.*, 2009). In contrast several studies have reported that probiotics including *Lb. reuteri* down regulated TNF- α -induced IL-8 production in HT-29 cells (Bai *et al.*, 2004; Candela *et al.*, 2008; Ma *et al.*, 2004; McCracken *et al.*, 2002).

Generally the apparent effect of probiotics enhancing IL-8 production decreased over the storage period such that after 30 days of storage, none of the probiotic preparations showed a significant effect on IL-8 production compared to the control. These observations suggest that the capacity of the probiotics to affect TNF- α -induced IL-8 production by intestinal cells may have been affected during storage either by the orange juice matrix or the low storage temperature. LG was the only probiotic strain not associated with any significant effect on TNF- α -induced IL-8 secretion at any time point, in contrast to a previous study in which exposure to LG was observed to be associated with a significant decrease in TNF- α -induced IL-8 secretion by Caco-2 cells (Zhang *et al.*, 2005). In a further study, induced IL-8 secretion by a mixture of IFN- γ and TNF- α was also found to decline significantly in the presence of LG (Donato *et al.*, 2010). However, a study by Vizoso Pinto *et al.* (2009) demonstrated that LG significantly increased the level of IL-8 production in HT-29 cells pre-treated with TNF- α for 2 h. Therefore it could be concluded that with the exception of LG, other probiotic preparations may not be suitable for patients suffering non-infectious chronic inflammatory conditions such as IBD due to increased IL-8 secretion via an enhanced pro-inflammatory effect of TNF- α .

When IL-8 production was induced by IL-1 β in Caco-2 cells, at baseline all probiotic preparations significantly decreased the level of IL-8 release compared to the control. This finding is similar to that of two previous studies in which probiotic mono-cultures of LG, *Lb. acidophilus* Bar13, and *Bif. longum* Bar33, and a *Lb. acidophilus* Bar13/*Bif. longum* Bar33 co-culture, all appeared to suppress IL-1 β induced IL-8 production by either Caco-2 or HT-29 cells (Candela *et al.*, 2008; Choi *et al.*, 2008). However no significant effect on IL-1 β induced IL-8 production by Caco-2 cells was observed between probiotic preparations and the control over the storage period. Unchanged IL-1 β induced IL-8 release by Caco-2 cells treated with probiotics has been reported previously (Reilly *et al.*, 2007).

Stimulus dependant variations were observed when IL-8 production by probiotic treated Caco-2 cells was provoked by different inflammatory stimuli such as *E.coli* LPS, TNF- α or IL-1 β . The underlying mechanisms by which probiotics differentially influence IL-8 production induced by different stimuli would therefore seem to warrant further investigation.

7.5.2 IL-6

Interleukin 6 (IL-6) is a potent pleiotropic pro-inflammatory and immunomodulatory cytokine with a wide variety of biological functions (Coico & Sunshine, 2009; Elgert, 2009). IL-6 plays an important role in immune responses initiated by infection or injury. (Coico & Sunshine, 2009; Elgert, 2009). Previous research has shown that IL-6 exerts a protective influence on some tissues under inflammation conditions induced by injury (Shanley *et al.*, 1997) or sepsis (Barton & Jackson, 1993). Moreover, IL-6 has been reported to prevent death of Caco-2 cells caused by hyperthermia (Hershko *et al.*, 2003) and improve intestinal integrity (Wang & Hasselgren, 2002). Therefore, the possible ability of probiotics to enhance IL-6 release in IECs could further help to protect intestinal mucosa under inflammatory conditions.

The data from the present study indicated that in general the probiotic preparations did not significantly affect non-stimulated IL-6 production by Caco-2 cells. A similarly neutral impact on IL-6 release by Caco-2 cells has been reported previously for several *Lactobacillus* and *Bifidobacterium* strains including LG and Bb (de Palencia, Lopez, *et al.*, 2008; Morita *et al.*, 2002a; Reilly *et al.*, 2007; Vizoso Pinto *et al.*, 2007).

Regardless of the stimulus used, significant differences, either among the different probiotic preparations or between them and the control, were generally not observed in relation to LPS or IL-1 β induced secretion of IL-6 by Caco-2 cells, either at baseline or after 10 days of storage. In contrast, the study by Reilly *et al.* (2007) showed that fresh cultures of the probiotics *Lb. paracasei* and *Lb. plantarum* enhanced IL-1 β (1ng/mL) induced IL-6 secretion by human Caco-2 enterocytes.

By day 20 however, exposure to three preparations including LG-PJ, Bb-PJ and LG-Bb-PJ, appeared to enhance IL-1 β induced IL-6 secretion, and after 30 days of storage in OJ, variable effects on IL-1 β induced IL-6 release were evident including enhancement and suppression. These findings illustrate an apparently differential effect of prolonged storage on the capability of probiotics to influence IL-6 secretion by Caco-2 cells provoked by IL-1 β . The variable effect of probiotic combinations on IL-1 β induced IL-6 secretion by Caco-2 cells was clearly evident and probiotic preparations with the specific ability to enhance IL-1 β induced IL-6 secretion by Caco-2 cells could be considered as those that may potentially help to protect the intestinal mucosa.

7.5.3 TNF- α

TNF- α has been recognised as a primary pro-inflammatory cytokine, exaggerated release of which is one of the hallmarks of inflammatory responses in the intestinal cells (Hosoi *et al.*, 2003; Menard *et al.*, 2004; Vidal *et al.*, 2002; Wallace *et al.*, 2003). Specifically, increased intestinal levels of TNF- α have been reported in inflammatory bowel disease (IBD) patients. In the presence of IL-8, TNF- α enhances neutrophil toxicity against pathogens. However, as previously explained, excessive activation of neutrophils may cause substantial intestinal epithelial damage (Mumy & McCormick, 2009). Along with IL-1 and IL-6, TNF- α induces the acute inflammatory response (Elgert, 2009). Therefore like IL-8, down regulation of TNF- α may suppress neutrophil activation and associated tissue injury.

In the present study, single probiotic strains were examined for their influence on IL-1 β induced or non-induced TNF- α production by Caco-2 cells. Generally at all time points there was no significant difference between the probiotic preparations and the control in non-induced secretion of TNF- α . This finding is consistent with previous research showing that probiotic bacteria including LG, did not affect production of TNF- α in the absence of inflammatory stimulation (Hosoi *et al.*, 2003; Vizoso Pinto *et al.*, 2007). When Caco-2 cells were treated with IL-1 β , significant differences in TNF- α production were observed between the probiotic preparations and the control in the case of PJ at D0, and LG which was associated with increased IL-1 β induced TNF- α production from D10 to the end of the storage period. These probiotic preparations with the ability to enhance IL-1 β induced TNF- α production (PJ at D0, and LG from D10 to the end of the storage period) may not therefore be advisable for patients with non-infectious intestinal inflammatory conditions.

Two sets of findings in contrast with this were reported in studies that demonstrated significantly decreased TNF- α production by HT-29 cells challenged respectively with IFN- γ / TNF- α or LPS, after exposure to probiotic conditioned media or LTA (Lipoteichoic acid, a component of membrane and cell wall of Gram positive bacteria) (Menard *et al.*, 2004; Vidal *et al.*, 2002). However these two studies did not use probiotic whole live cells and the IEC line was different. Compared to baseline, the effect of all four probiotic strains had significantly decreased by D10 and remained subdued until the end of the storage period (Figure 7.14). This finding also

demonstrated that exposure of probiotics to OJ and refrigeration may have affected their capacity to regulate IL-1 β induced TNF- α production.

7.6 Conclusion

For the preparation of food products with effective probiotic preparations, it is necessary to identify and understand potential interactions that may occur between the organisms and food matrices. Moreover, storage conditions should be considered as another factor which may influence probiotic functionality.

In the present study, the effect of the duration of combined exposure of 4 probiotic strains (either alone or in 2 or 3 multispecies combinations) to orange juice and refrigerated storage on their immune regulation capacity was examined in an *in vitro* model of infection/inflammation induced by *E. coli* LPS or pro-inflammatory cytokines in human intestinal epithelial Caco-2 cells.

The results provided evidence of variation in immunomodulatory effect between the probiotic strains/combinations examined. It was also found that the ability of the probiotics to modulate immune responses of Caco-2 cells induced by different potent pro-inflammatory stimuli would be further affected by the duration of their combined exposure to orange juice and refrigerated storage.

The underlying mechanisms by which the immunomodulatory effect of probiotic preparations is impacted by the duration of their exposure to orange juice under refrigerated storage would seem to warrant further investigation. It would also seem that further research will be required to confirm the observed *in vitro* effects *in vivo*.

Chapter VIII: Overall Conclusions and Future Research Directions

8.1 Overall conclusions

The primary aims of this study were:

- To examine long-term growth interactions of probiotic *Lactobacillus* and *Bifidobacterium* strains either individually or in combination with *Propionibacterium jensenii* 702 in a co-culture system and to determine their adhesion ability to human colon adenocarcinoma cell line Caco-2 (Chapter III)
- To examine the effect of combined exposure of probiotics, in both mono-culture and in co-culture combinations, to orange juice (delivery vehicle) and refrigerated storage on their viability, tolerance to simulated gastrointestinal conditions, adhesion to intestinal epithelial cells, and ability to modulate the immune responses of intestinal epithelial cells (Chapters IV-VII).

Chapter III: Bacterial growth interactions and intestinal epithelial cell adhesion characteristics of probiotic combinations

In this study the growth interactions of each of five probiotic strains *Lb. casei* 01 (LC), *Lb. plantarum* HA8 (LP), *Lb. rhamnosus* GG (LG), *Lb. reuteri* ATCC 55730 (LR) and *Bif. animalis* subsp *lactis* Bb12 (Bb) were examined in a co-culture medium with the novel probiotic *P. jensenii* 702 (PJ), along with their capacity for adhesion to the human colorectal epithelial cell line, Caco-2. The activity of lactobacilli appeared to induce reductions in pH to levels at which the viability of PJ was adversely affected, whereas the growth patterns of probiotic *Lactobacillus* strains were not affected by the presence of PJ. In the Bb / PJ co-culture preparation, a significant enhancement of the growth of both bacteria was observed. A significant reduction in adhesion percentage of LC and LG was observed in the presence of PJ compared to their adhesion levels when alone, while in all other cases variations in adhesion rates between mono- and co-cultures were not statistically significant. One of the key outcomes of this study was that PJ and Bb appeared to represent a highly favourable combination, providing mutually enhanced viability with no apparent adverse impact on the adhesion capacity of either organism. The findings of this study showed that the survival and intestinal cell adhesion of some strains of probiotic may be influenced by the presence of other strains, which should therefore be considered when formulating multi-species probiotic food products.

Chapter IV: Survival of multi-species probiotic combinations in orange juice and drinking water during storage

The probiotic bacteria LG, LR, Bb and PJ, either alone or as 2- or 3-multispecies combinations, were incorporated into orange juice (OJ) (with and without 20% pulp) and bottled drinking water (BW). Viability of the bacteria was monitored at different time points over 8 weeks of refrigerated (4°C) and non-refrigerated (23°C, only for BW) storage.

pH values and total soluble solid contents (°Brix) of orange juices did not change throughout the storage period indicating little or no metabolic activity of probiotics in juices under refrigeration. Appreciable strain-dependent differences were observed in the survival of probiotics in carrier drinks during the storage periods. With the exception of LR in the presence of Bb, lactobacilli survived in higher number in orange juice than in drinking water under refrigeration. In contrast, higher viable cell numbers were observed for Bb and PJ in drinking water than in orange juice. Compared to single strain preparations, the presence of other probiotics was observed to impact on the viability of probiotics in the experimental carrier drinks. In most cases, presence of pulp was not identified as an influential factor on the viability of probiotics in orange juice. Storage of probiotic drinking water at ambient temperature (23°C) had a detrimental effect on the viability of probiotics except for PJ which exhibited similar survival rates as those observed under refrigerated storage.

These results showed that chilled orange juice and drinking water could be effective as delivery vehicles for probiotics. However, consideration should be given to the survival of probiotic strains during long term product storage, as it seems this may be influenced by the presence of other strains in multi-species preparations.

Chapter V: An *in vitro* study on gastro-intestinal tolerance of probiotic combinations incorporated into orange juice

In this chapter the combined effects of exposure to orange juice and refrigerated storage on the tolerance of probiotics LG, LR, Bb and PJ, either separately or in 2- or 3-multispecies combinations, to simulated gastric and intestinal juices was examined. Data from this study demonstrated that the tolerance of probiotics to simulated gastro-intestinal conditions varied according to the choice of strain, choice of carrier matrix, and presence of other probiotic bacteria/strains. Tolerance of LG and PJ to simulated gastric juice was found to be significantly enhanced in orange juice compared to PBS at the baseline, while LR and Bb were highly tolerant, and of comparable resistance, to simulated gastric juice in either carrier. In contrast, orange juice appeared to significantly decrease the tolerance of LG, LR and PJ to simulated intestinal juice, with Bb the only strain unaffected.

In general, the duration of refrigerated storage of the probiotic strains included in orange juice did not have an adverse impact on the bacterial tolerance to simulated gastric (SGJ) and intestinal juice (SIJ) over one month of storage.

The tolerance of probiotic strains to SIJ in this study was found to be both strain dependant, and affected by the presence of other probiotic strains. Different effects including enhancement, suppression, or no effect, were observed with the presence of other probiotic strains on the tolerance to SIJ. Bb (alone and in combinations) included in orange juice appeared to be the most robust when confronted with simulated intestinal conditions with little or no viability loss compared to other probiotic strains.

The lowest bacterial tolerance to SIJ was observed in LG preparations with viability loss ranging from 4 to 6 orders of magnitude. The viability loss in LR cases varied from 1 to 4 log reductions in CFU/mL. When combined with Bb and/or PJ significant improvements were observed in the tolerance of LG and LR. On the contrary, tolerance of PJ to SIJ was adversely affected in the presence of other probiotics.

To the authors' knowledge, this is the first report showing that the gastro-intestinal tolerance of probiotic strains included in a carrier vehicle may be influenced by the presence of other strains.

Chapter VI: Intestinal epithelial cell adhesion characteristics of probiotic combinations incorporated into orange juice

Optimal functioning of probiotics depends on their ability to adhere to the intestinal mucosa. Theoretically, the delivery vehicle and storage conditions may both affect the intestinal mucosal adhesion of probiotics, however to date little is known about the effect of food matrices on this characteristic. As such this study was designed to examine the influence of the duration of exposure to orange juice and refrigerated storage on the adhesion of probiotics (LG, LR, Bb and PJ) to intestinal epithelial Caco-2 cells. In each case the viability and intestinal epithelial adhesion ability of each individual strain were monitored throughout 30 days of storage. In general, the data revealed discernible differences in the adhesion rates of probiotic bacteria in relation to strain, presence of other microorganisms and duration of storage in orange juice.

The viability and adhesion rate of LG, both alone and in combination, were in general found to be the most stable of the probiotics examined, and remained virtually unaffected by the presence of Bb and/or PJ throughout the entire storage period. Adhesion ability of LR was also shown not to be adversely affected by extended refrigerated storage in orange juice. When Bb was alone or in combination with PJ, LG-PJ or LR-PJ, its survival and adhesion rate remained almost unchanged throughout 30 days of storage in orange juice, while the adhesion percentage of PJ was in general found to be significantly higher in OJ than the control in 5 out of 6 preparations.

It could be concluded that the intestinal epithelial cell adhesion characteristics of probiotics were in many cases affected by the duration of their combined exposure to OJ and low storage temperature (4°C). The effect of probiotic combinations on the adhesion ability of component strains was also evident in most cases.

Chapter VII: Impact of orange juice as a probiotic carrier matrix on *in vitro* immunomodulatory effect of probiotic combinations

In this chapter the influence of combined exposure of probiotics (LG, LR, Bb and PJ) to the food matrix (orange juice) and low storage temperature (4°C) on their capability to modulate immune responses of intestinal epithelial cells treated with pro-inflammatory cytokines was examined. Data from this chapter has been summarised in Table 8.1.

Almost all probiotic preparations (single strain, 2- or 3-multi-species) at the baseline and during refrigerated storage in OJ significantly enhanced IL-8 production in non-stimulated Caco-2 cells compared to the control cells. However, the amount of the cytokine produced varied among probiotic preparations. LG was the only strain which did not significantly affect IL-8 secretion by Caco-2 cells compared with the control cells at any time.

Stimulus dependant variations were observed when IL-8 production by probiotic treated Caco-2 cells was provoked by different inflammatory stimuli such as *E.coli* LPS, TNF- α or IL-1 β . However the underlying mechanisms by which probiotics differentially influence IL-8 production induced by different stimuli requires further investigation.

When Caco-2 cells were treated with either *E. coli* LPS or TNF- α , with the exception of LG, virtually all other probiotic preparations significantly enhanced IL-8 production in Caco-2 cells compared with the control cells at baseline and after 10 and 20 days of storage. After 30 days of storage however, none of the probiotic preparations showed a significant effect on IL-8 production compared to the control.

When IL-8 production was induced by IL-1 β in Caco-2 cells, at baseline all probiotic preparations significantly decreased the level of IL-8 release compared to the control.

Probiotics studied in the present work did not show any significant effect on IL-8 production by IL-1 β treated Caco-2 cells over the storage period.

It could be concluded that the effect of probiotics on IL-1 β or TNF- α induced IL-8 release by IECs is influenced by duration of refrigerated storage in OJ.

Table 8.1 shows that apart from a few cases of suppression and enhancement in IL-1 β induced IL-6 secretion by Caco-2 cells after one month of refrigerated storage in orange juice, most probiotic preparations did not significantly affect the production of IL-6 or TNF- α by Caco-2 cells either treated or not treated with LPS or IL-1 β .

Data from this study indicated for the first time that the *in vitro* capability of probiotics, either separately or in various combinations, to regulate the immune responses of IECs,

may be impacted by the duration of their combined exposure to OJ and low storage temperature (4°C). Strain/combination specific variations were also evident among the probiotic preparations. Thus, the data arising from this *in vitro* model suggests that further research may be justified to determine whether similar effects apply *in vivo*.

Table 8.1: Effect of probiotic preparations at baseline (Day 0) and during refrigerated storage in OJ, on cytokine secretion by Caco-2 cells

	Day 0									Day 10									Day 20									Day 30								
	IL-8				IL-6			TNF- α		IL-8				IL-6			TNF- α		IL-8			IL-6			TNF- α			IL-8			IL-6			TNF- α		
Probiotics	NS	LPS	TNF	IL-1 β	NS	LPS	IL-1 β	NS	IL-1 β	NS	LPS	TNF	IL-1 β	NS	LPS	IL-1 β	NS	IL-1 β	NS	TNF	IL-1 β	NS	IL-1 β	NS	TNF	IL-1 β	NS	TNF	IL-1 β	NS	IL-1 β	NS	IL-1 β			
LG	○	●	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	●	○	○	○	○	○	○	●	○	○	○	○	○	○	○	●	○	●	
LR	●	●	●	●	○	○	○	○	○	●	●	●	○	○	○	○	○	○	●	●	○	○	○	○	●	○	●	○	○	○	○	○	○	○	○	
Bb	●	●	●	●	○	○	○	○	○	●	●	●	○	○	○	○	○	○	●	●	○	○	○	○	○	○	●	○	○	○	○	○	○	○	○	
PJ	●	●	●	●	○	○	○	○	●	●	○	●	○	○	○	○	○	○	○	●	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
LG-Bb	●	●	●	●	○	○	○			●	●	●	○	○	●	○			●	●	○	○	○			●	○	○	○	○	○	○	○	○		
LR-Bb	●	●	●	●	○	○	○			●	●	●	○	○	○	○			●	●	○	○	○			●	○	○	○	○	○	○	○	○		
LG-PJ	●	●	●	●	○	○	○			●	○	●	○	○	○	○			●	●	○	○	○			●	○	○	○	○	○	○	○			
LR-PJ	●	●	●	●	○	○	○			●	●	●	○	○	○	○			●	●	○	○	○			●	○	○	○	○	○	○	○			
Bb-PJ	●	●	●	●	○	○	○			●	●	●	○	○	○	○			●	●	○	○	○			●	○	○	○	○	○	○	○			
LG-Bb-PJ	●	●	●	●	○	○	○			●	●	●	○	○	○	○			●	●	○	○	○			●	○	○	○	○	○	○	○			
LR-Bb-PJ	●	●	●	●	○	○	○			●	●	●	○	○	○	○			●	●	○	○	○			●	○	○	○	○	○	○				

○ No significant effect, ● Significant increase and ● Significant decrease in cytokine secretion by Caco-2 cells compared with the relative control

NS, No Stimulation; Cytokine secretion was stimulated by LPS, TNF- α or IL-1 β

Collectively, the results presented in the current study have shown that viability and functional properties of probiotics could be impacted by various factors including bacterial strain, interactions between probiotic strains in a combination and between strains and carrier matrices such as orange juice and drinking water, and duration of refrigerated storage. These results may provide the functional food industry with considerable promise for the future development of non-dairy probiotic foods and should be considered when designing such probiotic foods that contain multiple probiotics.

8.2 Future research directions

- Further studies are clearly required to elucidate the mechanisms of interaction between probiotics in multi-species preparations, and examine how the probiotic combinations perform *in vivo*. Of particular interest is whether or not strains produce inhibitory or growth-promoting substances that could influence the survival and functionality of the co-administered probiotics in the intestinal tract, and how probiotic combinations interact with gut microbiota.
- In order to ascertain the effect of each specific component of a complex natural delivery vehicle such as orange juice on probiotic properties, it may be of value to devise a variety of food models. For example a system containing the same constituents with the same concentrations as in real orange juice with and without sucrose would be an ideal model for examining the effect of sucrose on the viability and functional properties of probiotic.
- A worthwhile future research direction is investigation of the effect of food matrices and/or low temperature storage on the protein expression profiles of probiotics and relationships between the proteins expressed and functional probiotic performance.
- In order to find the most suitable fruit juice based delivery vehicle for probiotics to provide the best outcome in terms of probiotic functional properties, it is worth studying other fruit juices and even fruit cocktail drinks.
- While previous research has found sensory differences between fruit juices including orange juice with and without probiotic mono-cultures added (Luckow & Delahunty, 2004a, 2004b; Luckow *et al.*, 2005), and even between orange juices with different probiotic strains (Luckow *et al.*, 2005), a future research avenue would be evaluation of consumer sensory responses to the functional orange juices containing multi-species probiotic combinations in comparison to their counterparts with probiotic mono-cultures over the refrigerated storage period.

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Appendices

Appendix A

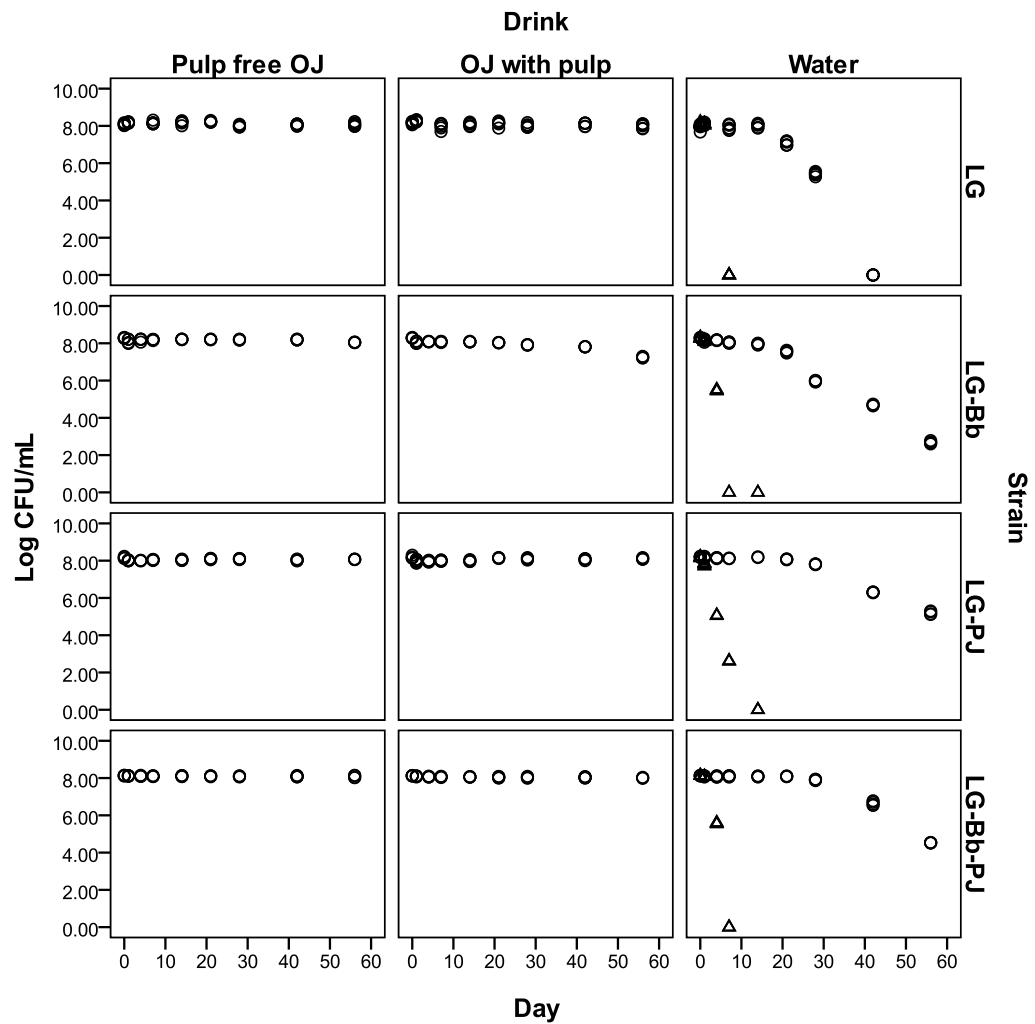


Figure A1: Viability LG either alone or in combination with Bb and/or PJ in orange juice with and without pulp at 4°C (open circle) and in drinking water at 4°C (open circle) or 23°C (open triangle) over 8 weeks of storage.

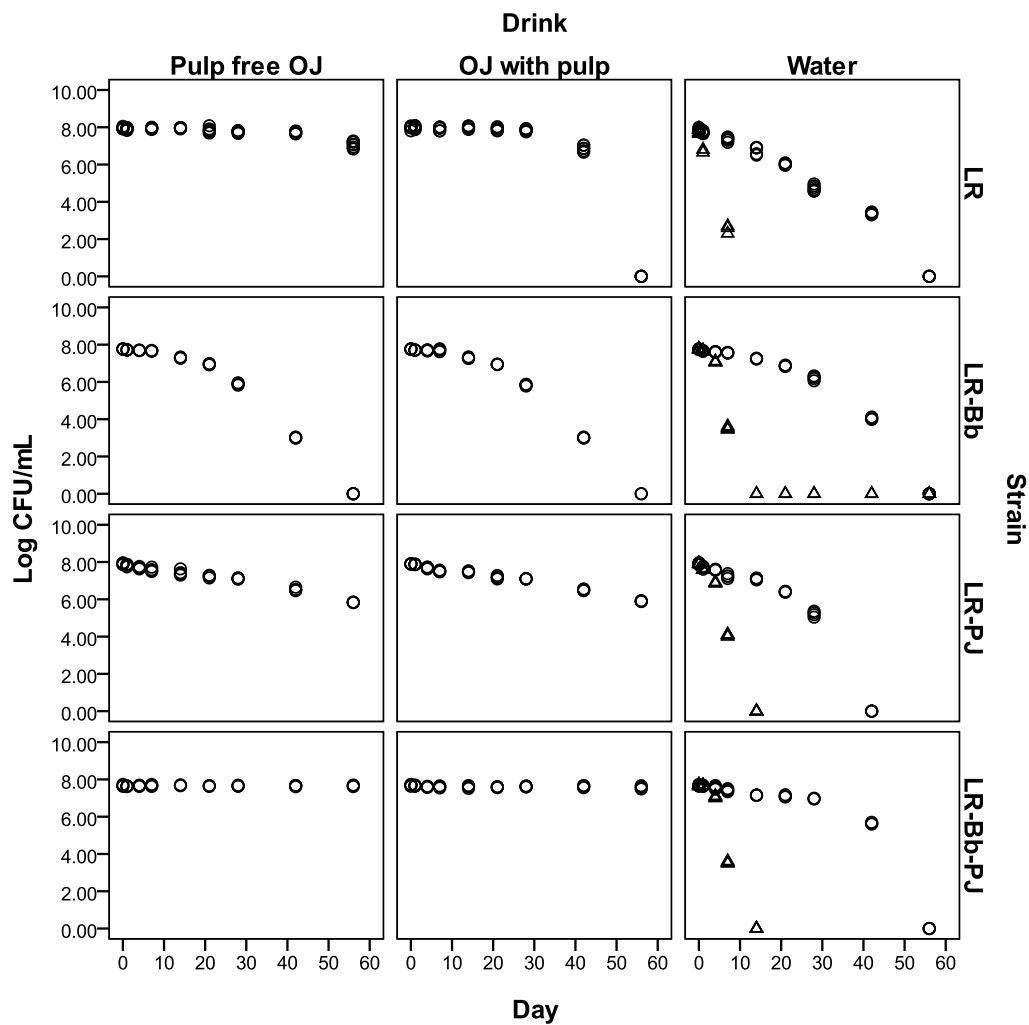


Figure A2: Viability of LR either alone or in combination with Bb and/or PJ in orange juice with and without pulp at 4°C (open circle) and in drinking water at 4°C (open circle) or 23°C (open triangle) over 8 weeks of storage.

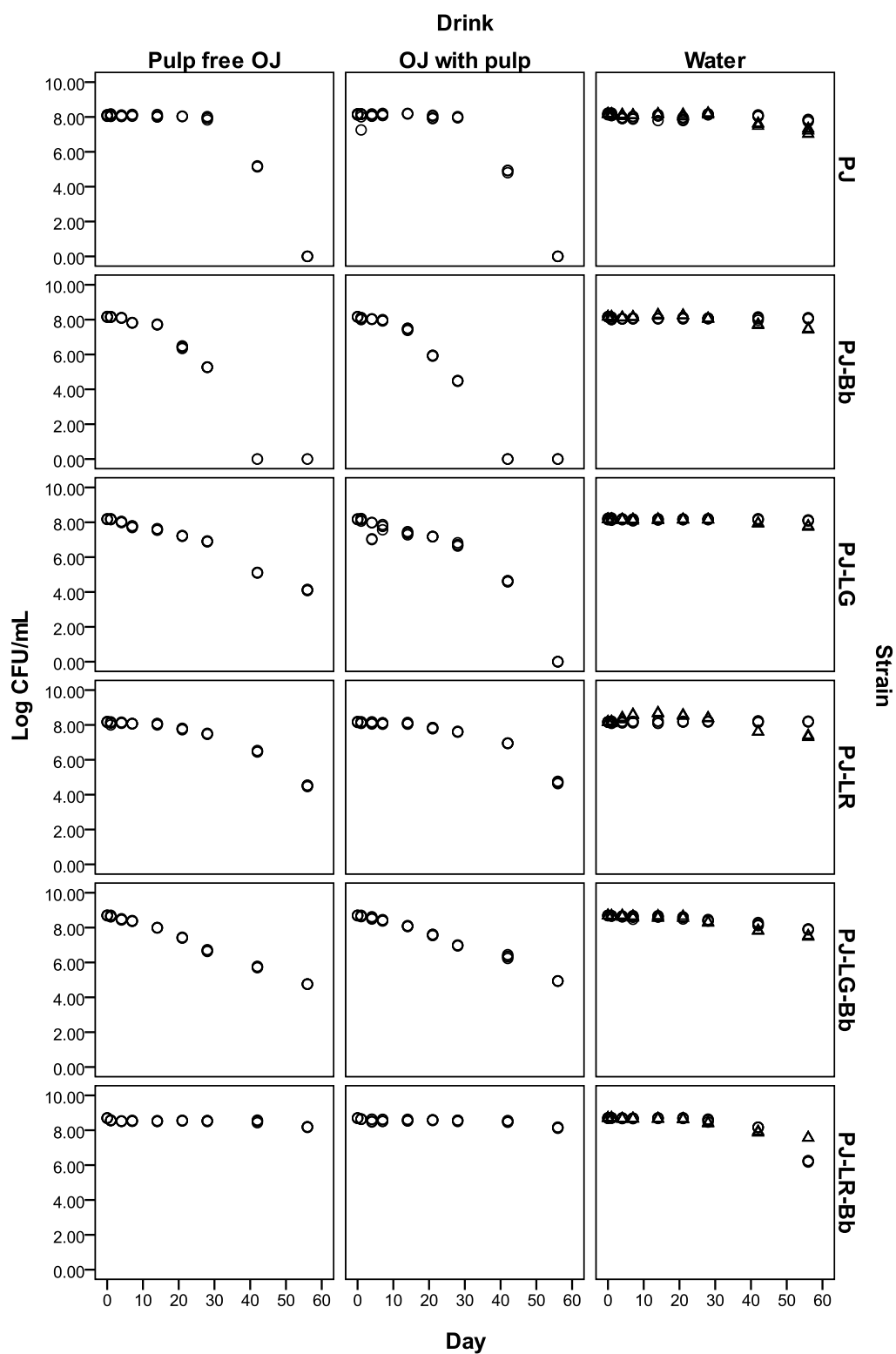


Figure A4: Viability of PJ either alone or in combination with LG or LR and/or Bb in orange juice with and without pulp at 4°C (open circle) and in drinking water at 4°C (open circle) or 23°C (open triangle) over 8 weeks of storage.