USE OF CRYOPROTECTANTS, PREBIOTICS AND MICROENCAPSULATION OF BACTERIAL CELLS IN IMPROVING THE VIABILITY OF PROBIOTIC ORGANISMS IN FREEZE-DRIED YOGHURT

A thesis submitted for the degree of Masters of Science in Food Science by Research

By

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ABSTRACT

The main aim of this study was to improve the viability of probiotic organisms within freeze-dried yoghurt during processing and storage. Initially, probiotic organisms including *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus rhamnosus* and *Bifidobacterium* spp. were incorporated in reconstituted skim milk and freeze-dried in order to select the most robust strains for use in further experiments The viability of *B. infantis* 17930 and *L. rhamnosus* GG was reduced by 0.07 log, while that of *L. casei* 1520 and *B. longum* 1941 was reduced by 0.28 and 0.39 log, respectively. The probiotic organisms selected for further experiments were *L. acidophilus* 33200, *L. casei* 279, *L. rhamnosus* GG and *B. longum* 536.

The effectiveness of adding cryoprotectants and prebiotics in improving the viability of the selected probiotic organisms was investigated. There was a 7% improvement in the viability of *L. casei* 1520 when cryoprotectant 'Unipectine^{TM} RS 150' was added at 2.5% (w/v). The addition of the prebiotic 'Raftilose$^{®}$P95' at 1.5% (w/v) to yoghurt improved the viability of the combined selected probiotic organisms by 1.42 log during four weeks of storage at 4°C.

Microencapsulation of probiotic organisms was assessed to determine whether further improvements in viability were possible. Microencapsulation with calcium-alginate using the emulsion technique was found to improve the viability of the combined selected probiotic organisms by 0.31 log in freeze-dried yoghurt stored at 21°C. Further studies were carried out to compare the extrusion technique with the emulsion technique. It was found that the viability of the probiotic organism was 1.0 $\log_{10}$ CFU/g higher using the emulsion technique after freeze-drying.

Coating with poly-L-lysine or chitosan further improved the survival of the probiotic organisms by 0.03 and 0.07 $\log_{10}$ cfu/g in yoghurt during 4 weeks of storage at 4ºC. The survival of probiotic organisms in yoghurt stored at 4ºC was highest (8.69 $\log_{10}$ cfu/g) in beads coated with chitosan. In freeze-dried yoghurt, coating with poly-L-lysine or chitosan improved survival of probiotic organisms by 0.2 and 0.4 $\log_{10}$ cfu/g in freeze-dried yoghurt during 16 weeks of storage at 21ºC. The survival of probiotic organisms in freeze-dried yoghurt stored at 4ºC was highest (8.47 $\log_{10}$ cfu/g) in beads coated with chitosan.

Although microencapsulation was effective in improving the viability of probiotic organisms in freeze-dried yoghurt, the calcium-alginate beads were too large and influenced the texture of the yoghurt. Consequently, further work was carried out to identify an effective technique for reducing bead-size during microencapsulation of probiotic organisms. The diameters of calcium-alginate beads were reduced by forming emulsions using homogenisation with Ultra-Turrax benchtop homogeniser, Avestin Inc. Piston homogeniser and Silverson mixer. The smallest beads were formed using the Ultra-Turrax benchtop homogeniser with a modal diameter of 56 µm. The counts of *L. casei* 279 after microencapsulation using the Ultra-Turrax benchtop homogeniser at 8000 rpm for 2 min were not adversely affected as the population was 8.8 $\log_{10}$ cfu/g. However, the count of microencapsulated *B. longum* 536 using the Silverson mixer was 6.3 $\log_{10}$ cfu/g. Overall, small beads containing viable probiotic organisms were created when an emulsion formed by homogenisation during microencapsulation.

It is recommended that probiotic organisms present in freeze-dried yoghurt should be microencapsulated to improve their viability. The emulsion technique should

be used and the emulsion. The sodium alginate/probiotic and oil mixture should be homogenised using the Ultra-Turrax Benchtop mixer at 8000 rpm for 2 minutes to effectively reduce bead size. Ideally, calcium-alginate beads should be coated in chitosan to provide maximum protection. Cryoprotectants should also be added to the yoghurt to provide probiotic organisms with additional protection during freeze-drying.

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CHAPTER 1

INTRODUCTION

The health benefits of consuming fermented dairy products containing *Lactobacillus acidophilus* and *Bifidobacterium* spp. are numerous. A high population of probiotic organisms in the colon contributes to good intestinal health. A low population of probiotic organisms in the colon can give rise to the domination of pathogenic organisms causing ailments such as intestinal bloating and diarrhea. Consequently, humans should consume products such as yoghurt containing viable probiotic organisms.

Freeze-dried yoghurt is a nutritional food suitable for delivering viable probiotic organisms to the colon. However, the viability of probiotic organisms in freeze-dried yoghurt is affected during processing and storage. Probiotic organisms in freeze-dried yoghurt are protected by adding prebiotics and cryoprotectants to the yoghurt mix. Microencapsulation of probiotic cells further improves the survival of probiotic organisms during processing in fresh and freeze-dried yoghurt.

1.1 Aim of this study

The aim of this investigation was to enhance survival and improve the viability of probiotic organisms in yoghurt and freeze-dried yoghurt using cryoprotectants, prebiotics and microencapsulation.

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1.2 The specific objectives of the study

1. To select probiotic organisms based on their ability to withstand the stresses associated with freeze-drying in order to incorporate them in yoghurt and freeze-dried yoghurt,

2. To investigate the effect of prebiotics and cryoprotectants on the survival and viability of probiotic organisms in yoghurt and freeze-dried yoghurt,

3. To observe the effect of microencapsulating probiotic organisms using the emulsion and extrusion techniques on bead size and on the viability of probiotic organisms,

4. To investigate the effect of coating on the viability of microencapsulated probiotic organisms in yoghurt and freeze-dried yoghurt during storage,

5. To determine the effect of homogenisation on the uniformity and diameter of calcium alginate beads and to investigate its effects on the counts of selected microorganisms, and

6. To examine the effect of homogenising sodium alginate (containing probiotic organisms) and oil using high speed mixers and high pressure valve homogenisers in order to reduce the size of calcium alginate beads.

Chapter 2 of this thesis contains the literature review. Chapter 3 involves the selection of probiotic organisms for use in further experiments throughout this research project. Chapter 4 investigates the effects of cryoprotectants and prebiotics on the viability of probiotic organisms in yoghurt and freeze-dried yoghurt. Chapter 5 focuses on the improvement in the viability of probiotic organisms in yoghurt and freeze-dried yoghurt using microencapsulation and co-encapsulation. Chapter 6 deals with the effect of forming an emulsion using homogenisation during microencapsulation, and its effect on the size of calcium alginate beads. Chapters 7, 8 and 9 give overall conclusions, future research directions and references.

CHAPTER 2

LITERATURE REVIEW

2.1 Yoghurt as a functional Food

Functional foods include products such as yoghurt containing beneficial cultures, margarine fortified with plant sterols and eggs enriched with omega-3 fatty acids. Food Standards Australia and New Zealand (FSANZ) defines functional food as '... similar in appearance to conventional foods and intended to be consumed as part of a normal diet, but modified to serve physiological roles beyond the provision of simple nutrient requirements'. An example is addition of probiotic organisms to yoghurt; this does not significantly alter the appearance of their conventional counterparts and provides health-promoting benefits. Probiotic yoghurt is aimed at reducing medical conditions such as constipation and diarrhoea by restoring the beneficial microbial population in the colon (Tanaka and Shimosaka, 1982; Ouwehand et al., 2002).

In Australia, the national centre of excellence in functional foods (NCEFF) has been established for the advancement of functional foods. In Australia's food processing industries, functional food products represent an enormous opportunity as experts foresee applications estimated at providing approximately $47.6 billion of value annually for the global market (www.nceff.com.au).

2.1.1 Functional Food- International outlook

The concept of "functional foods" originated in Japan (Ashwell, 2001). The Japanese functional food market is one of the most advanced in the world and is often used as a model for developments in Europe and the United States (http://www.cspinet.org) as shown in Table 2.1. In 1991, the Japanese government instituted an approval system for functional foods (Nutrition Improvement Law Enforcement Regulations; Ministerial Ordinance No. 41, July 1991). Consequently, a system is present which helps promote the manufacture of foods that remedy serious health problems.

Table 2.1. Functional Foods: An International Comparison of Health Claims Regulation

Japan	FOSHU System (Food for Specified Health Use): Health claims permitted for specific products upon approval of application including scientific documentation demonstrating the medical or nutritional basis for a health claim and ingredient safety information.
US	Food, Drug, and Cosmetic Act: 1) Health claims linking a nutrient to a particular disease or health-related condition. Health claims must be pre-approved by the FDA. They may also be based on an authoritative statement from another government agency with scientific expertise so long as FDA does not object within a 120-day period after the company notifies FDA of its intent to make a claim. 2) Structure/function claims linking a substance to an effect on a structure or function of the body,
UK	Food Safety Act: Prohibits medicinal claims - claims to treat a disease or restore, correct or modify physiological functions. Health maintenance claims that do not specifically refer to a disease may be lawful. There is no pre-market approval requirement. Enforcement is carried out by local trading standards officers and self-regulating bodies.
Heller et al., (1999).	

2.1.2 Yoghurt

At the turn of the 20th century, scientist Élie Metchnikoff attributed the long health life of Bulgarian peasants to their consumption of fermented milk products (Bibel, 1988). The word "yoghurt" is derived from Turkish "jugurt", which is used to describe any fermented food with an acidic taste (Younus et al., 2002). Historically, yoghurt was made by fermenting milk with indigenous microorganisms. Live microorganisms have been used to create cultured dairy products for thousands of years with references found in the first book of the Bible (Genesis 18:8). Such yoghurt, referred to as "dahi" (traditional Indian yoghurt), is still made today in developing countries such as India, Pakistan and Sri Lanka. However there is limited quality control during the manufacture of dahi, which affect its sensory attributes due to variations in microorganisms used during fermentation (Masud et al., 1991).

2.1.3 Yoghurt- Current manufacturing process

At present, yoghurt is made using a carefully controlled process involving the fermentation of milk commonly with *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. In Australia, several types of yoghurt are manufactured which include set, stirred and drinking yoghurts. To produce set yoghurt, milk is heated to 80-95ºC for 5-30 min to destroy pathogens and change the physiochemical properties of whey proteins. The milk is cooled to 42-45ºC and *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* are added at 0.5-3.5% (w/w). The cultured milk is incubated for 3-5 h. Fermentation is halted by cooling to 4ºC when the pH is reduced from 6.8 to 4.5-4.6.

2.1.4 Yoghurt starter culture

In 1905, Grigoroff reported three different rod and rod/coccal *Lactobacillus* during his bacteriological study of yoghurt. While others such as Lüerssen and Kühn (1908) reported similar observations, in 1910, Metchnikoff proposed that ingesting *Bulgarian bacillus* found in yoghurt inhibited the growth of putrefactive organisms in the intestine thus prolonging the life of humans. *Bulgarian bacillus,* which was later named *Thermobacterium balgarican,* is currently known as *Lactobacillus delbrueckii* spp. *bulgaricus*. Together with *Streptococcus thermophilus,* these organisms are most widely used for the manufacture of yoghurt. These organisms have a symbiotic association during the fermentation of milk as they produce molecules and compounds that beneficially affect each other.

L. delbrueckii ssp. *bulgaricus* is believed to stimulate the growth of *S. thermophilus* by releasing certain amino acids into the milk during fermentation, while *S. thermophilus* produced a stimulatory factor for the growth of *L. delbrueckii* spp. *bulgaricus* (Pette and Lolkema, 1950). *L. delbrueckii* ssp. *bulgaricus* provides *S. thermophilus* with the essential amino acids required for growth. Consequently, the rate of acid production is greatest when the two organisms are grown together rather than independently as observed by Pette and Lolkema (1950)**.** The substances produced during fermentation not only assist growth but also influence sensory characteristics of the yoghurt such as the aroma, flavour and texture (Tamine and Robinson, 1999).

Yoghurt is believed to assist in curing gastrointestinal disturbances and provides humans beneficial therapeutic properties (Athar, 1986). However, the potential of *L.*

delbrueckii ssp. *bulgaricus* to directly benefit the host is limited due to poor survival during transit to the colon (Rettger et al., 1935).

2.1.5 Australian food standards for the manufacture of yoghurt

In Australia, food manufacturers are required to comply with the food standards as set out by Food Standards Australian New Zealand (FSANZ). Standard 1.6.2 in the Australian New Zealand Food Standards Code (ANZFSC) states that milk during the manufacture of yoghurt must be pasteurised by heating to a temperature of no less than 72°C and retained at such temperature for no less than 15 seconds and immediately shock cooling to a temperature of 4.5°C (www.foodstandards.gov.au -Standard 1.6.2.). This is to ensure that the time and temperature have a lethal effect on the bacteria. The requirements for the manufacture of yoghurt in Australia are shown in Table 2.2.

Table 2.2 Requirements for yoghurt in Australia

Component or parameter	Proportion
Protein (measured as crude protein)	minimum 30 g/kg
pH	maximum 4.5
Microorganisms from the added culture	minimum 1,000,000 cfu/g

Source: Australia New Zealand Food Standards Code

Standard 2.5.3 in the ANZFSC defines and sets compositional requirements for fermented milk, including yoghurt. Fermented milk is defined as a milk product obtained by fermentation of milk and/or products derived from milk, where the fermentation involves the action of microorganisms and results in coagulation and a reduction in pH. Specifically, yoghurt is defined as fermented milk where the fermentation has been carried out with lactic acid producing microorganisms (www.foodstandards.gov.au -Standard- 2.5.3). A product must comply with each of the requirements listed in ANZFSC for it to legally be called yoghurt and to ensure that it is safe for consumption.

2.1.6 Consumption of yoghurt in Australia

During the last decade, the consumption of dairy products such as yoghurt has steadily increased (Fig 2.1). This increase is possibly due to the greater awareness of the health benefits such as 'calcium and probiotic organisms' obtained from their consumption.
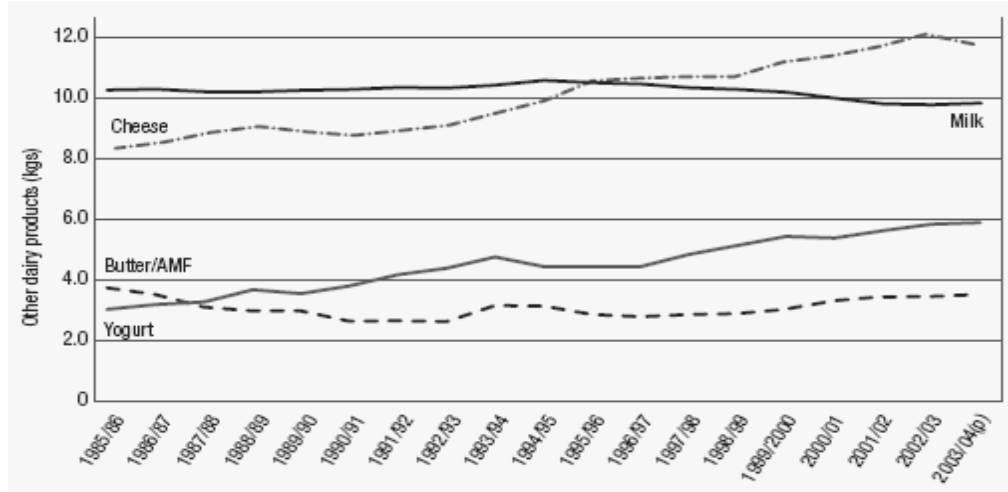
Fig. 2.1 Consumption of major dairy products in Australia
(Source: Dairy Australia: Australian dairy industry in focus, 2004, www.dairyaustralia.com.au)

Table 2.3 Per capita consumption of dairy products in Australia

	Milk (litres)	Cheese (kgs)	Butter/AMF (kgs)	Yoghurt (kgs)
1996/99	102.5	10.7	2.9	5.1
1999/2000	101.5	11.1	3.0	5.4
2000/01	99.6	11.3	3.3	5.3
2001/02	97.7	11.6	3.4	5.6
2002/03	97.4	12.0	3.4	5.8
2003/04	98.0	11.7	3.5	5.9

(Source: Dairy Australia: Australian dairy industry in focus, 2004, www.dairyaustralia.com.au)

Table 2.3 shows the changes in consumption of dairy products including yoghurt during the past decade.

2.1.7 Freeze-dried yoghurt

Freeze-dried yoghurt is a food product made by freezing yoghurt and removing water by sublimation. Water is removed from the yoghurt to increase the shelf-life of the product. Historically, dried yoghurt was used in the preparation of food dishes, soups or consumed as biscuits with tea (Tamine and Robinson, 1999). Yoghurt powder was originally manufactured by shaping concentrated low-fat natural yoghurt into flat rolls followed by drying by sun exposure (Kurmann et al., 1992). Commercially, yoghurt powder is generally manufactured either by freeze-drying or spray-drying.

The drying process affects the flavour, texture and appearance of yoghurt. The poor rheology of the re-hydrated, dried yoghurt is due to the destruction of the gel structure. Consequently, additives such as sucrose, dextrose, stabilisers, sequestering agents, calcium coprecipitate, organic acids and acidogen are used to improve the texture of freeze-dried yoghurt upon reconstitution with water (Tamine and Robinson, 1999). The microstructure of freeze-dried yoghurt is shown in Fig 2.2.
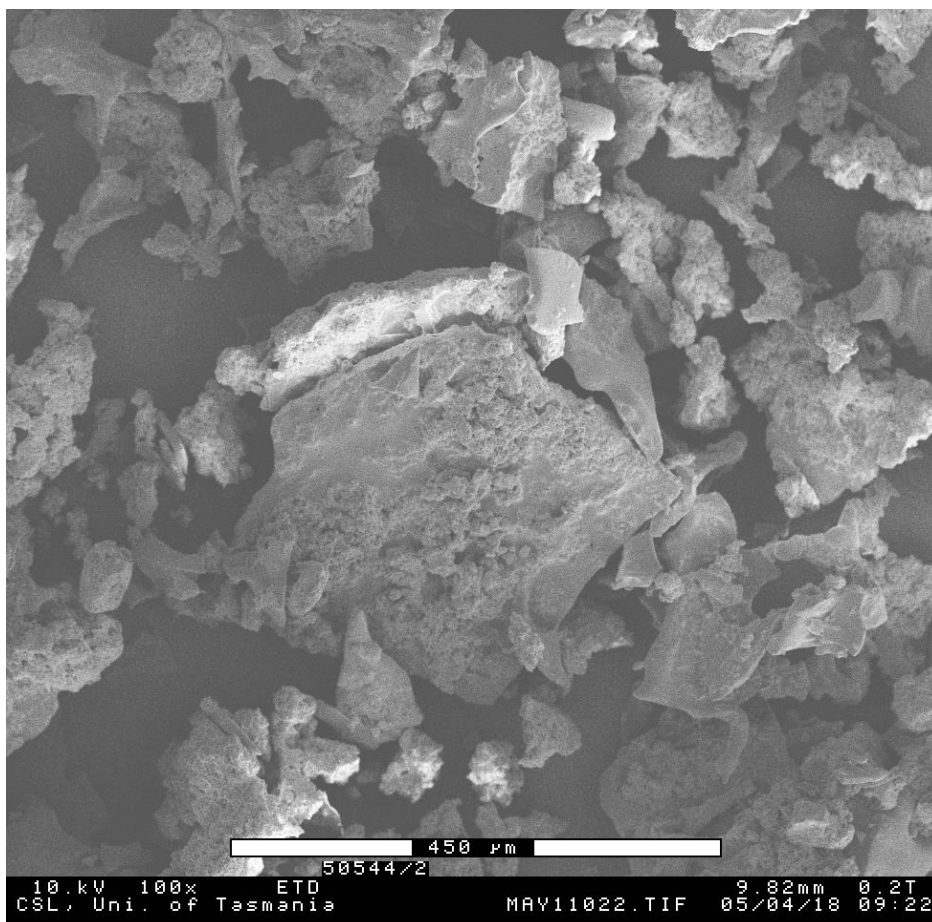
Fig. 2.2 Microstructure of freeze-dried yoghurt (**Electroscan ESEM 2020, Wilmington, Massachusetts, USA**)

2.1.8 Freeze-drying

Freeze-drying is a method of preserving food. In freeze-dried yoghurt the viability of microorganisms is preserved during processing and storage. The process involves freezing the yoghurt containing microbial cultures, followed by the removal of water at freezing temperatures and reduced pressure. During freeze-drying, water is removed from the biological material by sublimation. Damage to composition of the cell membrane in prokaryotic cells is minimized as water is sublimated from a solid state directly to a gaseous state.
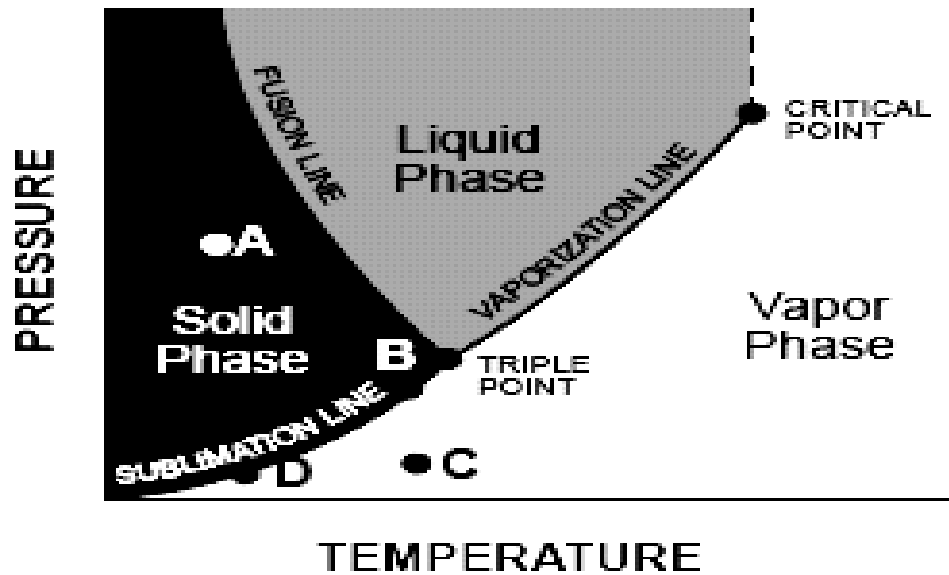
Fig. 2.3aPhase diagram for water
(Source: www.labconco.com)

The first stage of freeze-drying process is the pre-freezing. The product is frozen at a temperature much lower than 0ºC to ensure that it is completely solid. The product must be frozen to below the eutectic point (the temperature at which all water contained in the product is solid) as shown in Fig 2.3a and 2.3b (point A). Rapid cooling is desirable when freeze-drying microorganisms as the size of ice crystals is reduced.

The second stage of freeze-drying is primary drying. This involves the removal of free ice by sublimation. The sublimation process leaves what appears as a dry product. During this stage the temperature of the product must be balanced between the temperature that keeps the product frozen, and the temperature maintaining the vapour pressure of the product. The temperature is then increased to just below the critical temperature (point B) and pressure is reduced. However, the product may still have a significant amount of water that is bound. The final stage is known as secondary drying which is necessary to remove the bound water by desorption (Baker, 1997).

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During freeze-drying, water is removed from a freeze-dryer using a vacuum pump. The vacuum also reduces the pressure of the area surrounding the product to the region shown as 'point C', Fig 2.3b. The vapour pressure shown at 'point D' must be lower than the product to ensure that molecules move the moisture collector.
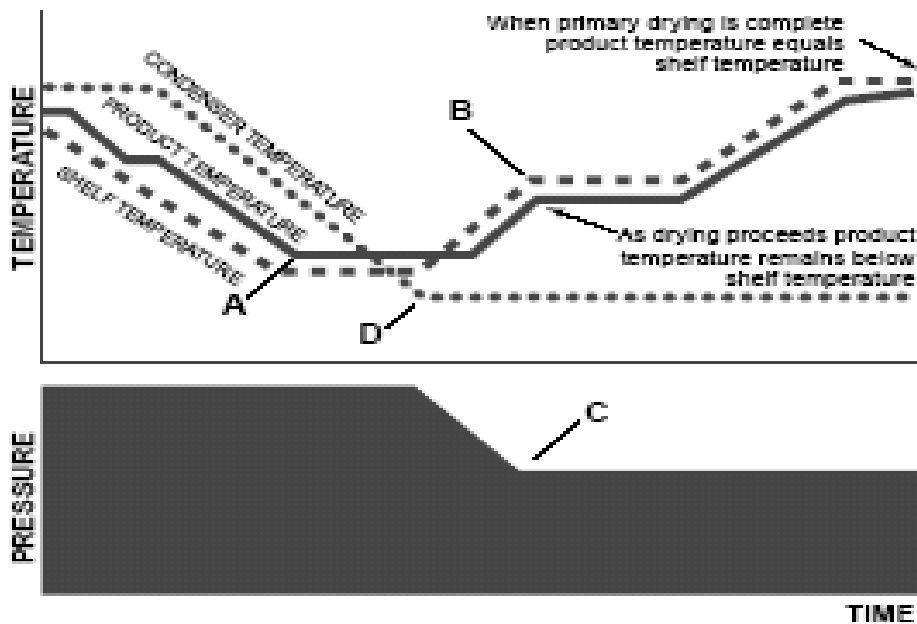
Fig. 2.3b Typical sublimation cycle used by freeze-dryers with shelves
 (Source: www.labconco.com)

2.1.9 The effects of freeze-drying on the microstructure of yoghurt

Set yoghurt has a texture which is similar to jelly due to the coagulation of milk during fermentation. Freeze-drying removes the water in this gel resulting in the formation of a powder. Techniques must be developed to overcome the problems caused to the microstructure of yoghurt during the freeze-drying process. Kailasapathy and Rybka (1997) found that the texture of reconstituted freeze-dried yoghurt was poorer than that of the fresh yoghurt. Attempts were made by these researchers to improve the texture by re-incubating freeze-dried yoghurt. However these efforts were unsuccessful.

Bhullar et al. (2002) investigated the effects of ingredient supplementation on textural characteristics and microstructure of yoghurt. Bhullar et al. found that the addition of 2% whey protein concentrate allowed for the formation of firmer yoghurt than the control (plain yoghurt without supplementation). The microstructure of yoghurt supplemented with whey protein concentrate had a more regular and dense protein network when compared to yoghurt containing whey powder and skim milk powder.

2.2 Probiotic organisms

2.2.1 Health benefits associated with the consumption of probiotic organisms

'Probiotic' is a Greek word, which can be translated as 'for life'. In 1965, Lilly and Stillwell defined 'probiotics' as 'microorganisms that have a growth promoting effect against other microorganisms'. While this definition was originally used to describe a supplement added to an animal feed, at present the word 'probiotic' has relevance to humans. Probiotics are defined as 'mono or mixed-cultures of live microorganisms which transit the gastrointestinal tract and upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition' (Fuller, 1993; Schaafsma, 1996; Tannock et al., 2000).

Human gut microflora is influenced by factors such as poor hygiene, consumption of processed foods (most of which are heat sterilised by cooking), exposure to stress, and antibiotic therapy (Sullivan, 2001). There has been extensive research focusing on the health benefits of ingesting viable probiotic organisms (Table 2.2). Ingestion of viable probiotic organisms may reduce or eliminate ailments such as colon irritation, constipation and travellers' diarrhoea (Van Niel et al., 2002).

Table 2.4 Alleged health benefits associated with the consumption of viable probiotic population

Intestinal effects	Immune system effects	Other effects
-Relieve effects, promote recovery from diarrhoea (rotavirus, travellers' and antibiotic-induced)	-Enhance specific and non-specific immune response	-Optimise effects of vaccines (e.g. rotavirus vaccine, typhoid fever vaccine)
Siitonen et al. (1990), Oksanen et al. (1990), Isolauri et al. (1991)	-Reduce chance of infection from common pathogens (Salmonella, Shigella)	-Synthesize nutrients (folic acid, niacin, riboflavin, Vitamins B6 & B12
-Produce lactase, alleviate symptoms of lactose intolerance and malabsorption	Shahani et al. (1979)	Alm (1982)
Gade et al. (1989)	-Stimulate gastrointestinal immunity	-Reduce risk of certain cancers (colon, bladder)
-Relieve constipation	-Inhibit growth of pathogen and translocation	-Lower serum cholesterol concentrations
Seki et al. (1978)	Friend et al. (1984)	-Reduce blood pressure in hypertensive individuals
-Treat colitis	Majamaa et al. (1997)	-Treat food allergies
Nanji et al. (1994), Kruis et al. (1997)		-Detoxify carcinogens
		-Suppress tumours
		-Increase nutrient bioavailability
		-Improve urogenital health
		Aso et al. (1992), Sanders (1999)

2.2.2 Genus Lactobacillus

Lactobacilli are native to habitats such as the mucosal membrane of humans and animals, mainly oral cavity, intestine or vagina (Duffy et al., 2005). At present, 56 species of the genus *Lactobacillus* have been identified (Table 2.3). These organisms have complex nutritional requirements and are strictly fermentative, aero-tolerant or anaerobic, aciduric or acidophilic.

2.2.2.1 Lactobacillus acidophilus

L. acidophilus is non-spore forming rods that occur singly, in pairs and in short chains. Liong and Shah (2005a) found *L. acidophilus* 33200 to have superior tolerance to bile salts and acid than several other probiotic organisms. In vitro studies found *L. acidophilus* 32000 to effectively remove cholesterol from fermentation broths (Liong and Shah, 2005b). *L. acidophilus* DDS-1 (LA1) has been found to suppress cancerous

Table 2.5 Species of genus *Lactobacillus*

*Lactobacillus acidophilus**	*L. bifermentans*	*L. hilgardii*
*L. amylovourus**	*L. casei**	*L. kefir*
*L. crispatus**	*L. coryneformis*	*L. malofermentans*
*L. delbrueckii**	*L. curvatus*	*L. oris*
L. gallinarum	*L. graminis*	*L. parabuchnery*
*L. gasseri**	*L. homohiochii*	*L. ruteri**
*L. helveticus**	*L. intestinalis*	*L. pontis*
L. gensenii	*L. murinus*	*L. vaginalis*
*L. johnsonii**	*L. paracasei**	*L. suebicus*
L. kefiranofaciens	*L. rhamnosus**	*L. vaccinostercus*
L. aviarius	*L. sake**	*L. sanfracisco*
L. farciminis	*L. agilis*	*L. confuses*
*L. salivarius**	*L. pentosus*	*L. fructosus*
L. mali	*L. plantarum**	*L. halotolerans*
L. ruminis	*L. brevis**	*L. viridescens*
L. sharpeae	*L. buchneri*	*L. kandlery*
L. acetotolerans	*L. collinoides*	*L. minor*
L. hamsteri	*L. fermentum**	*L. caucasicus**
L. alimentarius	*L. fructivorans*	*L. leichmannii**

(Source: Wood and Holzapfel, 1995)

*Considered to be human probiotics

tumours by stimulating the immune system, increasing levels of interleukin-1 α (IL-1 α) and tumour necrosis factor-alpha (TNF-alpha) (Rangavajhyala et al., 1997). Strains of *L. acidophilus* including MUH-41, O-61, L-1, 43121 and La-5 have been added to food products such as yoghurt and buttermilk (Nighswonger et al., 1996). Commonly used *L. acidophilus* strains added to commercial food products such as yoghurt are *L. acidophilus* are La-5 (Christian Hansen) and *L. acidophilus* LAFTI L10 (DSM Food Specialties) (Talwalkar and Kailasapathy, 2004).

2.2.2.2 Lactobacillus casei

L. casei is a Gram-positive organism found in the reproductive and intestinal tracts of humans and animals as well as in fermented plant products (Kandler and Weiss, 1986). *L. casei* is non-motile and rod-shaped which is 0.7-1.1 x 2.0-4.0µm. Lactic acid is the major metabolic end product produced by these facultative anaerobes as a result of their strictly fermentative metabolism (Axelsson, 1998; Kandler and Weiss, 1986). During fermentation, *L. casei* also produces lactic acid from pentoses via the 6-phosphogluconate/phosphoketolase pathway and hexose sugars via the Embden-Meyerhof pathway (Axelsson, 1998). *L. casei* strain Shirota was found to modulate the composition and metabolic activity of the intestinal flora (Spanhaak et al., 1998). *L. casei* ASCC 292 was found to remove cholesterol via various mechanisms in the presence of the prebiotics fructooligosaccharides (FOS) and maltodextrin (Loing and Shah, 2005b-).

2.2.2.3 Lactobacillus rhamnosus

L. rhamnosus is a rod-shaped, Gram positive probiotic organism. *L. rhamnosus* is recognised for its ability to reduce the severity and duration of acute diarrhoea caused by rotavirus infections in infants and young children (Majamaa et al., 1995). Grant and Salminen (1998) found *L. rhamnosus* GG to alleviate intestinal inflammation and normalise increased intestinal permeability. *L. rhamnosus* GG has been found to reduce the side effects associated with diarrhoea such as abdominal distress, stomach cramps and flatulence. Other therapeutic properties that this organism provides its human host include benefits against allergic conditions, eczema, bacterial and fungal infections (Kalliomaki, et al., 2001; Alvarez-Olmos et al., 2001),

2.2.3 Bifidobacterium spp.

Bifidobacterium spp. are found in humans, animals, wastewater and fermented milk (Gomes and Malcata, 1999). The name 'Bifidobacteria' originated from the observation of microorganisms in Y-shaped or bifid form. Bifidobacteria are non-spore forming, non-motile, Gram-positive and catalase-negative anaerobes. They are saccharolytic organisms that produce acetic and lactic acids without generation of CO_2 except during degradation of gluconate. When Bifidobacteria ferment carbohydrates, acetic and lactic acids are produced in the molar ratio of approximately 2:3. Although they are strictly anaerobic organisms some species have a level of tolerance to oxygen in certain media (Ballongue, 1998).

Bifidobacterium spp. in the adult colon is among the most abundant probiotic organisms that populate the colon (Finegold et al., 1983) Bifidobacteria are found in the mouth and the intestinal tract of humans and animals. Infants contain large populations of *Bifidobacterium* spp., especially those that are breast-fed. These organisms are usually among the first to colonise the large intestine due to the 'bifidus factor' which is present in the breast milk which stimulates the selective growth of *Bifidobacterium* spp. (Mackie et al., 1999). Bifidobacteria added to yoghurt include *B. adolescentis, B. animalis, B. bifidum, B. infantis, B. longum* and *B. thermophilum.* Species of the genus *Bifidobacteria* are shown in Table 2.4. Strains of bifidobacteria used in food applications include *B. lactis* Bb-12 (Christian Hansen), *B. lactis* LAFTI™ B94 (DSM Food Specialties) and *B. longum* Bb536 (Morinaga Milk Industry Co.) (Talwalkar and Kailasapathy, 2004).

Table 2.6 Species of genus *Bifidobacterium*

B. bifidum*	*B. pseudolongum*	*B. coryneformes*
B. longum*	*B. cuniculi*	*B. asteroides*
B. infantis*	*B. choerinum*	*B. indicum*
B. breve*	*B. animalis**	*B. gallicum*
B. adolescentis*	*B. thermophilum*	*B. ruminatium*
B. catenulatum	*B. boum*	*B. mercicum*
B. pseudocatenlatum	*B. magnum*	*B. saeculare*
B. denticum	*B. minimum*	*B. angulatum*
B. globosum	*B. subtile*	*B. suis*
B. gallinarum	*B. pullorum*	*B. licheniformis**

(Source: Sgorbati et al., 1995)
*Considered to be human probiotics

2.2.3.1 Bifidobacterium longum

B. longum has been extensively studied for its potential to protect humans from carcinogens such as methyl quinolines (Reddy and Rivenson, 1993), heterocyclic amines (Sreekumar and Hosono, 1998), nitrosamines (Grill et al., 1995) and azomethane (Singh et al., 1997). *B. longum* 536 added to yoghurt was found to improve intestinal health by reducing the population of *Clostridium* spp. and reducing the level of putrefactive substances (Ogata et al., 1999).

2.2.4 Viability of probiotic organisms in freeze-dried yoghurt

A minimum of one million probiotic organisms per gram of a product should be present (Kurmann and Rasic, 1991). Previous work done by researchers involving enumeration of probiotic organisms and yoghurt bacteria found that freeze-drying had an adverse affect on viability. In studies undertaken by Rybka and Kailasapathy (1995), one batch of yoghurt fermented with *Lactobacillus acidophilus*, *Bifidobacterium* spp. and *Streptococcus thermophilus* while a second batch of yoghurt was fermented with *L. acidophilus, Bifidobacterium* spp., *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus.* After 96 h of freeze-drying at -50°C, bacterial populations in freeze dried samples were 0.25 to 2 log lower than those taken from yoghurt that was not freeze dried. Rybka and Kailasapathy (1995) also found that *L. delbrueckii* ssp. *bulgaricus* was the most sensitive bacteria to freeze-drying. Viable counts of *Lactobacillus acidophilus* and *Bifidobacterium* spp. in the yoghurt powders were present in less than the recommended minimum of one million colony forming units per gram (cfu/g) after 27 days of storage. Even after re-incubation of the yoghurt powder, there was no increase in viable population of yoghurt

bacteria.

The viability of probiotic organisms is reduced during the processing and storage of freeze-dried yoghurt due to oxygen content, high temperature, low pH, low water activity and elevated solute concentration (Shah, 2000; Carvalho et al., 2004). Probiotic strains vary in their susceptibility to freeze-drying. It was found that population of lactic acid bacteria in commercial ABT yoghurt did not decrease significantly during freeze-drying (48 h at -40°C). After 21 days of storage only *L. acidophilus* was present at viable quantities. After 31 days of storage no lactic acid bacteria were detected.

2.3 Prebiotics

2.3.1 Substrate for probiotic organisms

A prebiotic is defined as 'a non digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that can improve host health (Gibson and Roberfroid, 1995). Prebiotics have been added to probiotic yoghurt as a food source for improving the ability of beneficial bacteria colonising the gut.

2.3.2 Oligosaccharides

There are several types of oligosaccharides that have been shown to be prebiotics using in *vitro* models, animal models and human trials. Oligosaccharides that have been studied include lactulose, fructooligosaccharides, galacto-oligosaccharides, soybean oligosaccharides and lactosucrose, isomalto-oligosaccharides, gluco-oligosaccharides, and xylo-oligosaccharides (Rastall and Gibson, 2002).

2.3.3 Hi-maize

Hi-maize, derived from high amylose maize (corn) is also a prebiotic that improves the survival of some probiotic organisms through the digestive process. As a prebiotic, Hi-maize is also used as a fuel for native bacteria in the colon. Hi-maize in the presence of the probiotic organism *Clostridium butyricum*, was found to significantly decrease the number of crypt foci, an indicator for colon cancer, better than *C. butyricum* in the absence of the prebiotic (Nakanishi, et al., 2003).

2.3.4 Inulin

Inulin is a $\beta2 \rightarrow 1$-linked fructan that is most commonly obtained from roots of the chicory plant. During the extraction of inulin, liming and carbonation at alkaline pH remove proteins, peptide, anion, colloids and phosphates. Anion and cation ion exchange chromatography is then used to demineralise inulin. Finally, inulin is sterilised, concentrated and spray-dried to obtain a food grade product (Gibson et al., 2000). Inulin can be further hydrolysed (Fig. 2.4) by the enzyme inulinase to produce short-chain fructooligosaccarides (FOS) which is represented as Glu α1-2 [β Fru 1-2]$_n$ where n = 2-9 (Crittenden, 1999).

2.3.5 Fructooligosaccharides (FOS)

In addition to providing substrate for probiotic organisms in the colon, prebiotics have their own specific health benefits. The enzymic synthesis of FOS is shown in Fig. 2.4. Inulin and FOS were found to selectively stimulate the growth of *Bifidobacterium* in mixed batch and chemostat study (Wang and Gibson, 1993). A similar result was

obtained by Kleeson et al. (1997) who found a significant increase in the counts of *Bifidobacterium* in 10 senile adults that were fed 20-40g of inulin for 20 d. In another study, FOS was found to increase intestinal Riga of mice, thus improving the body's defence against invaders (Nakamura, et al., 2004).

Fruβ2↔1αGlc   *Sucrose*

Sucrose

Glucose

Fruβ2→1Fruβ2↔1αGlc   *1-Kestose*

Sucrose

Glucose

1Fruβ2→1Fruβ2→1Fruβ2↔1αGlc   *Nystose*

Sucrose

Glucose

Fruβ2→1Fruβ2→1Fruβ2→1Fruβ2↔1αGlc   *Fructosylnystose*
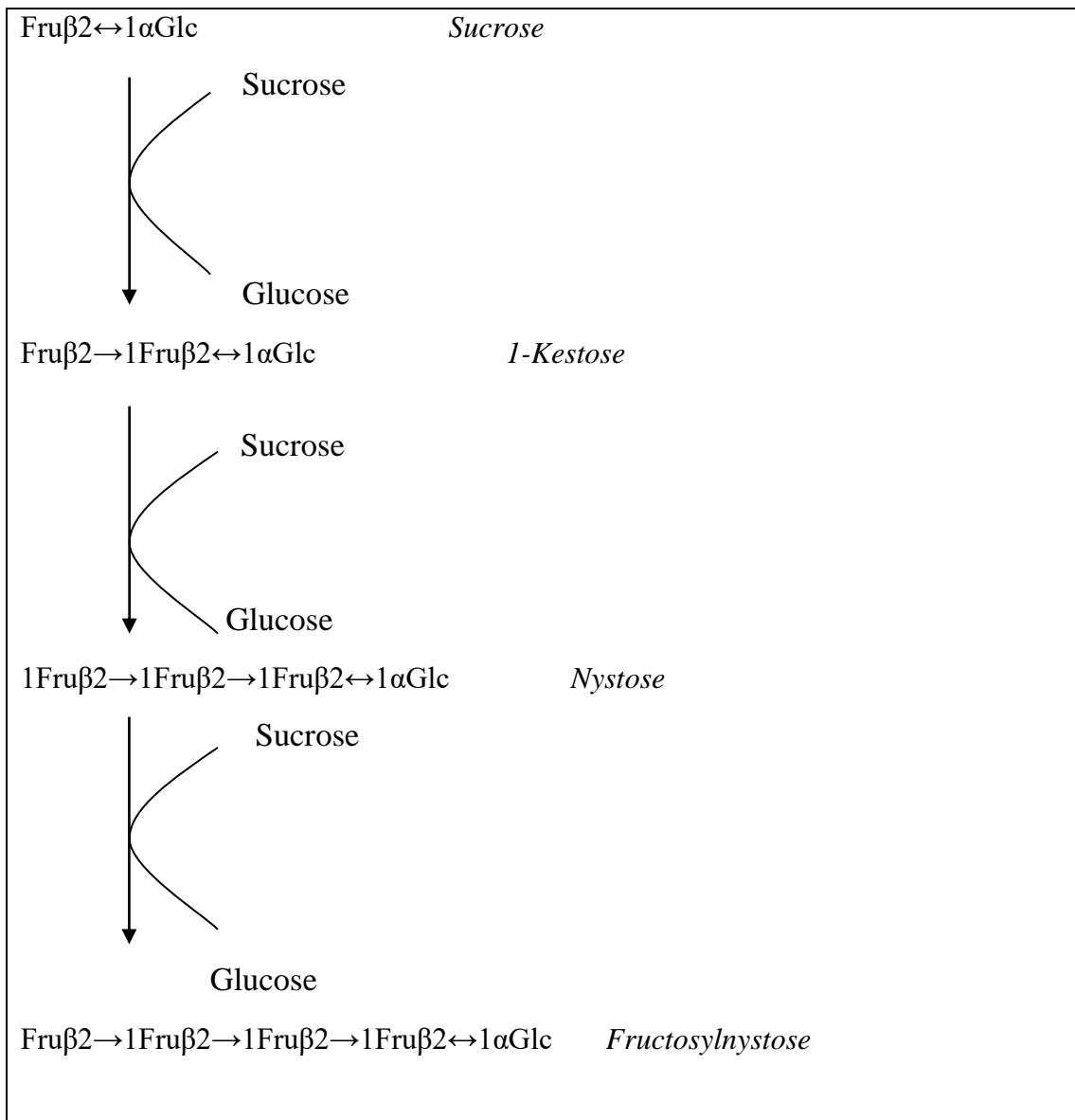
Fig. 2.4 Enzymatic synthesis of fructooligosaccharides (FOS)

(Source: Gibson et al., 2000)

2.4 Cryoprotective agents

2.4.1 Types of cryoprotectants

Cryoprotectants such as mannitol, sorbitol, sodium tripolyphosphate, xylitol, glycerol, raffinose, maltodextrin, erythritol, threitol, trehalose, glucose and fructose are reported to improve the cold tolerance of microorganisms (Thunnell, et al., 1984). Probiotic organisms are sensitive to freeze-drying due to deterioration of physiological state of the cells. A cryoprotectant is a substance that accumulates within the cells to reduce osmotic difference with the external environment (Kets, et al., 1996) or a substance that surrounds cells to improve cold tolerance. In freeze-dried yoghurt, compatible cryoprotectants are added to the yoghurt mix prior to fermentation to assist in the adaptation of probiotics to the environment. The formation of extracellular amorphous glass is believed to improve the survival of probiotic organisms in freeze-dried yoghurt containing cryoprotectants. The extent to which cryoprotection is provided may vary between cultures (Das, et al., 1976).

2.4.2 Cryoprotectants to improve survival of probiotic organisms

Ravula and Shah (2000) found that cryoprotectant 'Unipectine™ RS 150' improved the viable counts of *L. acidophilus* and *Bifidobacterium* by 1 log during storage in fermented dairy desserts. Das et al. (1976) investigated the effect of pectin as a cryoprotectant, and found an improvement in the survival of *S. thermophilus*. Morichi (1972, 1974) used cryoprotective agents to reduce bacterial cell damage in a cell suspension (Table 2.7).

Table 2.7 Effect of cryoprotectants on the survival of microorganisms[*]

Cryoprotectant	S. thermophilus	L. acidophilus	L. delbrueckii ssp. bulgaricus
L-Glutamic acid	35-40	42-63	16-21
L-Arginine	21-40	39-57	20-35
L-Lysine	6-7	4-38	1-10
DL-Threonine	7-11	6-21	6-10
Acetyl glycine	29-44	3-35	7-33
DL-Malic acid	52-59	28-66	6-15
DL-Pyrrolidine-carboxylic acid	24-48	24-56	9-11

[*]Figures are expressed as the percentage survival of the original population; (Source: Adapted from Morichi, 1972)

2.5 Microencapsulation

2.5.1 Microencapsulation of probiotic organisms to improve survival

There must be one million probiotic organisms per gram of product for a sufficient number of probiotic organism delivered to the colon for health benefits to be obtained by humans. The survival of probiotic organisms such as *L. acidophilus* and *Bifidobacterium* spp. has been found to be low in the presence of acid and bile salts (Lankaputhra and Shah, 1995). It is essential that probiotic organisms survive the transit in the intestine where they are exposed to acid and bile salts to multiply in the colon. In dried yoghurt, concentrations of solutes are increased due to the removal of water causing stress to probiotic organisms. Bacterial cells are protected from such stresses using microencapsulation.

Microencapsulation is the process of encasing an active component in a shell. For encapsulation of probiotics for use in food, the organisms are coated in a non-toxic coating material such as sodium alginate. Microencapsulation protects probiotic organisms during freezing and freeze-drying (Kearney et al., 1990; Sheu and Marshall, 1993; Kim and Yoon, 1995; Shah and Ravula, 2000). Initially, probiotic organisms that are incorporated into yoghurt must be able to survive the manufacturing process. During processing, factors such as high temperature, excessive exposure to light, low pH may all affect the viability of probiotic organism. During storage, probiotic organisms are vulnerable to oxygen and high temperature. During the production of freeze-dried yoghurt, Lactobacilli are frozen at -20°C. Sodium alginate gels improve the viability of probiotic organisms by permitting the diffusion of nutrients while shielding the microorganisms from molecules that may cause stress (Talwalkar and Kailasapathy, 2000).

Microencapsulation has been found to reduce probiotic cell death caused by oxygen toxicity (Talwalkar and Kailasapathy, 2003). Probiotic organisms have also been found to agglomerate in the centre of beads creating anaerobic regions (Beunik et al., 1989). Ravula and Shah (2001), found that the survival of encapsulated *Lactobacillus acidophilus* and *Bifidobacterium* improved by two to three log cycles per batch when compared to free probiotics. There was about 40% of *Lactobacillus* that had survived in frozen ice milk when they were entrapped in calcium alginate. These studies also showed that encapsulated probiotic bacteria were able to survive acidic conditions at pH 2.5. These results indicate that probiotics that are encapsulated should survive the highly acidic conditions encountered in the stomach. Sultana et al. (2000) had also investigated

metabolic/acidifying activity of encapsulated bacteria, and evaluated their survival under simulated gastrointestinal conditions (1.0 and 2.0% bile salts and pH 2.0, 3.0 and 4.0 at 37°C for 3 h) and in yoghurt (at 4°C for 8 wk). Encapsulated bacteria experienced a lower rate of acid production compared to free cultures as evidenced by their acidification kinetics. However, encapsulated bacteria did not show enhanced survival under acid and bile conditions.

A non-toxic coating material, such as sodium alginate, must be used for food applications. Other agents that can be used to immobilize lactic acid bacteria include kappa-carrageenan-locust bean gum (Arnaud, et al., 1991) and gellan-xanthan (Sun and Griffiths, 2000). When encapsulation agents are combined with calcium chloride, a calcium alginate gel matrix is formed, creating a suitable coat for sensitive probiotic organisms (Shah and Ravula, 2000). The emulsion and extrusion techniques are two common procedures for microencapsulating probiotic organisms. However, both techniques have their advantages and limitations (Krasaekoopt et al., 2004) as shown in Table 2.8.

2.5.2 Emulsion technique

The emulsion technique involves combining a mixture of probiotic organisms and sodium alginate with vegetable oil (Krasaekoopt et al., 2004). A water-in-oil emulsion is formed by agitation, usually with a magnetic stirrer. Calcium chloride solution is used to set the alginate beads, and is slowly added to the emulsion while stirring Shah and Ravula (2000) found that calcium alginate beads, formed using the emulsion technique improved the viability of probiotic organisms during processing and storage in frozen yoghurt.

2.5.3 Extrusion technique

The extrusion technique using hydrocolloids such as carrageenan, alginate and guar gum is the oldest and most commonly used agent for microencapsulating hydrocolloids (King, 1995). To produce calcium alginate beads using the extrusion technique, sterile sodium alginate solutions is mixed thoroughly with probiotic organisms. A syringe is then used to add the suspension into a calcium chloride solution. Upon contact with the calcium chloride solution, spherical beads are formed which are allowed to harden for several minutes.

Table 2.8 Extrusion and emulsion techniques- a comparison

	Extrusion	Emulsion
Technological feasibility	Difficult to scale up	Easy to scale up
Cost	Low	High
Simplicity	High	Low
Survival of microorganisms	80-95%	80-95%
Bead size	2-5mm	25 µm- 2mm

Source: Krasaekoopt et al. (2003)

2.5.4 Coating of encapsulated probiotic organisms

Coating of encapsulated probiotic organisms involves the application of an additional layer, providing probiotic organisms with an additional barrier (Krasaekoopt et al., 2004). The extra coat is effective in protecting sensitive organisms from oxygen and other molecules (Talwalkar and Kailasapathy, 2003). Polymer coatings such as chitosan or poly-L-lysine (PLL) can be applied to calcium alginate beads. Krasaekoopt et al. (2004) found an improvement in the survival of *L. acidophilus* and *L. casei* using a chitosan coated alginate bead in acidic conditions.

2.5.5 Effect of microencapsulation technique on bead size

Although, coating encapsulated probiotics improves survival, the additional layer may affect the overall size of the bead. Large beads in fermented foods such as yoghurt are undesirable, as they may influence the texture and mouth-feel. Beads of below 100 µm are desirable, while those with diameters greater than 0.1 mm are detectable in the mouth (Lacroix and Picot, 2003). Lee et al. (2003) reduced bead size using spray guns. Air atomizers with a variety of nozzle sizes have also been used to reduce bead size (Kwok et al., 1991).

CHAPTER 3

EFFECT OF FREEZE-DRYING AND INOCULUM SIZE ON SURVIVAL OF
LACTOBACILLUS ACIDOPHILUS, *LACTOBACILLUS CASEI*, *LACTOBACILLUS RHAMNOSUS* **AND** *BIFIDOBACTERIUM* **SPP. IN YOGHURT AND FREEZE-DRIED YOGHURT**[1]

3.1 Introduction

The large intestine contains over 400 different microbial species. The native microorganisms, which are the dominant microflora in the colon, limit the ability of pathogenic genera including *Escherichia*, *Clostridium, Salmonella* and *Campylobacter* to attach to the intestine (Ziemer and Gibson, 1998). Once the microbial balance is disturbed, intestinal bloating and diarrhoea may occur (Isolauri et al., 2002). Of the native strains of colonic microflora, the probiotic genera of *Bifidobacterium* and *Lactobacillius* have been studied extensively and it is well established that they are valuable native inhabitants of the colon (Nielsen et al., 2003)

Probiotics are defined as 'live microbial feed supplements which beneficially affect the host by improving the intestinal microbial balance' (Fuller, 1989). Ingestion of viable probiotic organisms reduces or eliminates ailments such as colon irritation, constipation and travellers' diarrhoea. Other health benefits have been reviewed (Roberfriod, 2000; Ziemer and Gibson, 1998) and include inhibition of pathogenic bacteria, synthesis of B vitamins, lowering of blood ammonia levels, cholesterol

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[1]Results described in this chapter have been published in Food Research International, volume 39, Issue 2, Pages 203-211. The paper is titled 'Effect of cryoprotectants, prebiotics and microencapsulation on survival of probiotic organisms in yoghurt and freeze-dried yoghurt'.

absorption, and inhibition of tumour formation. However, in order to provide health benefits, it is essential to have a minimum of one million viable probiotic organisms per gram of a product (Shah, 2002).

It is essential that viable probiotic organisms be added to food products for the consumer to gain the health benefit. Probiotic organisms are harvested at the end of the exponential phase of growth to ensure that they remain viable during storage in a product such as freeze-dried yoghurt. Growth curves can be constructed to identify the time at which probiotic organisms display the most rapid rate of growth and the highest number of cells.

Probiotics have a limited shelf life in conventional yoghurt (Shah et al., 1995). Freeze-drying is a process that preserves yoghurt and helps retain the viability of probiotics. During freeze-drying, the frozen water is removed by sublimation, thus reducing damage to biological structures. Previous research has found that certain strains of probiotics are better able to survive the freeze-drying process (Rybka and Kailasapathy, 1995), possibly due to differences in the surface areas of the microorganisms, and variations in cell wall and membrane composition. During the processing and storage of freeze-dried yoghurt, oxygen content, high temperature, low pH, water activity and elevated solute concentration may all deleteriously affect the viability of probiotic organisms (Shah, 2000; Carvalho et al., 2004).

The aims of this investigation were to i) select probiotic organisms based on their ability to withstand the stresses associated with freeze-drying in order to incorporate them in yoghurt and freeze-dried yoghurt, ii) construct growth curves to identify growth patterns of the selected probiotic organisms, and starter cultures *Lactobacillus delbrueckii*

spp. *bulgaricus* and *Streptococcus thermophilus,* iii) the study the effect of inoculum size on the viability of probiotic organisms during the manufacture of freeze-dried yoghurt was studied.

3.2 Materials and method

3.2.1 Construction of growth curves

3.2.1.1 Growth of S. thermophilus, L. delbrueckii ssp. bulgaricus, L. rhamnosus GG, L. acidophilus 33200, L. casei 279 and B. longum 536 in M-17 and deMann, Rogosa, Sharpe (MRS) broth

Growth curves were constructed to identify the optimal time for harvesting selected probiotic organisms. *L. delbrueckii* ssp*. bulgaricus, L. rhamnosus* GG*, L. acidophilus* 33200*, L. casei* 279 and *B. longum* 536 were activated by growing in MRS broth (Oxoid Ltd., Hampshire, United Kingdom) three times successively at 37°C for 24 h. MRS broth containing *B. longum* 536 was supplemented with filter-sterilized 0.05% w/v L-cysteine·hydrochloride. One percent of each culture at 8.5-9.0 $\log_{10}$ cfu/g was added to 200 mL of sterile MRS broth. *S. thermophilus* at 8.9 $\log_{10}$ cfu/g was added at 1% to 200 mL of sterile M-17 broth. The organisms were incubated at 37°C for 24 h and enumerated at 3 h intervals. *Lactobacillus* spp. were enumerated on MRS-agar plates that were incubated anaerobically for 48-72 h at 37°C in anaerobic jars. *S. thermophilus* was enumerated on M-17 agar incubated aerobically for 48-72 h at 37°C.

3.2.1.2 Growth of L. delbrueckii ssp. bulgaricus, L. rhamnosus GG, L. acidophilus 33200, L. casei 279 and B. longum 536 in 14% reconstituted skim milk

A 1% inoculation of *L. delbrueckii* ssp. *bulgaricus, L. rhamnosus* GG*, L. acidophilus* 33200*, L. casei* 279 and *B. longum* 536 was added to 200 mL of reconstituted skim milk (RSM; 14% w/v) that was pre-warmed to 43°C. The organisms were incubated at 43°C for 5 h in order to replicate the condition used during the manufacture of yoghurt. Each organism was enumerated at hourly intervals for 5 h. The organisms were enumerated on MRS-agar plates after anaerobic incubation for 48-72 h at 37°C.

3.2.2 Freeze-drying of probiotic organisms

3.2.2.1 Preparation of probiotic organisms

Probiotic organisms including 2 strains of *Lactobacillus acidophilus*, 4 strains of *Lactobacillus casei*, 1 strain of *Lactobacillus rhamnosus* and 6 strains of *Bifidobacterium* spp. were obtained from a number of suppliers (Table 3.1). Ten millilitre aliquots of sterile MRS broth were individually inoculated with each of the probiotic organisms. MRS broth containing *Bifidobacterium* spp. was supplemented with filter sterilized 0.05% w/v L-cysteine·hydrochloride (Sigma Chemical Co., Castle Hill, Sydney, Australia) to create an anaerobic environment. The cultures were activated by growing three times successively at 37°C for 15-18 h. For freeze-drying experiments, organisms were grown in 500 mL of MRS broth and cells were harvested by centrifugation (Sorvall RT7 refrigerated centrifuge) at $1510 \times g$ at 25°C for 25 min (Bhandari et al., 2004). Ten grams (wet weight) of freshly harvested cells were then washed and suspended in Schott bottles containing 90 mL of sterile (14% w/v) RSM.

3.2.2.2 Freeze-drying and enumeration of Lactobacillus acidophilus, Lactobacillus casei,

Lactobacillus rhamnosus and Bifidobacterium spp.

Ten grams (wet weight) of washed and harvested cells were suspended in Schott bottles containing 90 mL of sterile (14% w/v) RSM. The cell suspension was mixed thoroughly and a weighed quantity (30-50 g) was spread onto 90 mm petri dishes and freeze-dried. Probiotic organisms were freeze-dried by freezing at –80°C for 2 h using a freeze-dryer (model FD-300; Airvac Engineering Pty. Ltd., Dandenong, Australia) followed by 40 h of primary drying at –30°C and 8 h of secondary drying at -10°C. Once the freeze-drying cycle was completed, the freeze-dried yoghurt and probiotic organisms were reconstituted with the same mass of water that was removed during freeze-drying. The freeze-dried cells were allowed to stand for 30-60 min prior to enumeration

The number of the probiotic organisms was determined before and after freeze-drying by pour plating. The serial dilution was carried out in sterile peptone and water solution (0.15% w/v) and MRS agar was used for plating. MRS agar for enumeration of *Bifidobacterium* spp. was supplemented with 0.05% w/v L-cysteine·hydrochloride. The plates were then incubated anaerobically for 48-72 h at 37°C in anaerobic jars with a gas generating kit (BRD 38). Probiotic organisms were enumerated on plates containing 25-250 colony forming units per gram.

3.2.3 Effect of inoculum size on viability of probiotic organisms during the manufacture of freeze-dried yoghurt

L. acidophilus 33200, *L. casei* 279, *B. longum* 536 and *L. rhamnosus* GG were incorporated into yoghurt mixes at 2 or 4 percent. Yoghurts were made as per Fig. 3.3.3.1 and the number of organisms was enumerated before fermentation, after fermentation (prior to freeze-drying) and after freeze-drying as described in 3.2.2.2 using MRS-vancomycin agar (Tharmaraj and Shah, 2003).

3.3 Results and Discussion

3.3.1 Growth of microorganisms

3.3.1.1 Growth of microorganisms in MRS and M-17 broths

The growth of *S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. rhamnosus* GG, *L. acidophilus* 33200, *L. casei* 279 and *B. longum* 536 in MRS and M-17 broths is shown in Fig. 3.3.1.1. The population of *S. thermophilus* in M-17 broth was increased by 1.7 $\log_{10}$ cfu/g during the first 6 h of incubation with no apparent lag phase visible on the growth curve. This was possibly due to the long interval between the initial population measurement and that recorded after 3 h of incubation. After 6 hours of logarithmic growth, *S. thermophilus* had entered the stationary phase of growth. This result showed that *S. thermophilus* is most suitable for use in the manufacture of yoghurt between 6-9 h of growth. *L. rhamnosus* GG, *L. acidophilus* 33200, *L. casei* 279 and *B. longum* 536 displayed similar growth curve patterns and remained in the exponential phase for the first 9 h of incubation during which time their populations had increased by 1.8 and 2.1 $\log_{10}$ cfu/g. A clear stationary phase was visible between 12 and 18 h of incubation. This shows that these probiotic organisms were most abundant and active for use in experiments after 9-12 h of growth. *L. delbrueckii* ssp. *bulgaricus* displayed an obvious

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lag phase as the population had only increased by 0.01 $\log_{10}$ cfu/g after 3 h of incubation. However, the population of *L. delbrueckii* spp. *bulgaricus* steadily increased between 3-18 h of incubation to 2.4 $\log_{10}$ cfu/g. Due to slow growth of *Lactobacillus delbrueckii* ssp. *bulgaricus* in MRS broth, sterile RSM may be a more suitable medium for growing this organism.

3.3.1.2 Growth of microorganisms in reconstituted skim milk

The growth *L. delbrueckii* ssp. *bulgaricus, L. rhamnosus* GG*, L. acidophilus* 33200, *L. casei* 279 and *B. longum* 536 in RSM during 5 h of incubation at 43°C is shown in Fig. 3.3.1.2. *L. delbrueckii* ssp. *bulgaricus* displayed the highest increase in growth after 5 h as the population rose by 320%. This was expected as *L. delbrueckii* ssp. *bulgaricus* is used as a starter organism in fermented dairy products. *B. longum* 536 exhibited the least growth after 5 h as the population only increased by 160%, while the population of *L. rhamnosus* GG*, L. acidophilus* 33200, *L. casei* 279 rose by 180-200%. Despite the comparatively lower growth of the probiotic organisms in RSM, the growth curves showed that probiotic organisms added to a yoghurt mix continue to grow during fermentation. Adding probiotic organisms together with yoghurt starter culture may assist in improving viability during storage, as the probiotic organisms may slowly adapt to the acidic environment during fermentation.

3.3.2 Effect of freeze-drying on the viability of probiotic organisms

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Effect of freeze-drying on the viability of probiotic organisms is shown in Table 3.1. Although not statistically significant, *Bifidobacterium* spp. generally showed 7% higher survival than *Lactobacillus* spp. with the exception of *B. longum* 1941. The higher survival of *Bifidobacterium* may be attributed to differences in cell wall and membrane composition (Carvalho et al., 2004). Of the thirteen probiotic organisms, *B. infantis* 17930 and *L. rhamnosus GG* showed the greatest survival of 85.73% and 84.99%, respectively. The organisms selected for further experiments included *L. acidophilus* 33200, *L. casei* 279, *B. longum* 536 and *L. rhamnosus GG.*

3.3.3 Effect of inoculum size on the viability of probiotic organisms during the manufacture of freeze-dried yoghurt

Fig. 3.3.3.2 shows the effect of inoculum size on viability of the probiotic organisms before fermentation, after fermentation and after freeze-drying. A probiotic inoculum size of 4% ensured viability at a satisfactory level during processing. During the fermentation of milk by yoghurt starter cultures, such as *L bulgaricus* ssp. *delbrueckii* and *S. thermophilus,* the viability of the probiotic organisms also increased at 2% inoculation size. When probiotic organisms were added to the mix at the same time as yoghurt starter, the organisms were better able to adapt to the changing environment as organic acids were produced and the pH was reduced to 4.5. The stress caused by a reduced pH may have enhanced survivability during freeze-drying as a result of adaptation (Jan et al., 2001). There was less decline in the viability of probiotic organisms in freeze-dried yoghurt that had been inoculated with 4% (0.36 log) than 2% (0.59 log).

3.4 Conclusion

There was a noticeable improvement in the survival of *Bifidobacterium* spp. after freeze-drying which was most likely due to the physiological characteristic of the organisms. The optimal time to harvest *S. thermophilus* grown in M-17 broth was between 6-9 h of incubation and that for *L. rhamnosus* GG*, L. acidophilus* 33200, *L. casei* 279 and *B. longum* 536 was between 9-12 h of growth in MRS broth. *L. delbrueckii* ssp. *bulgaricus* grew better in RSM than in MRS broth. An inoculum size of between 2 and 4% ensured viability of probiotic organisms during processing and storage.
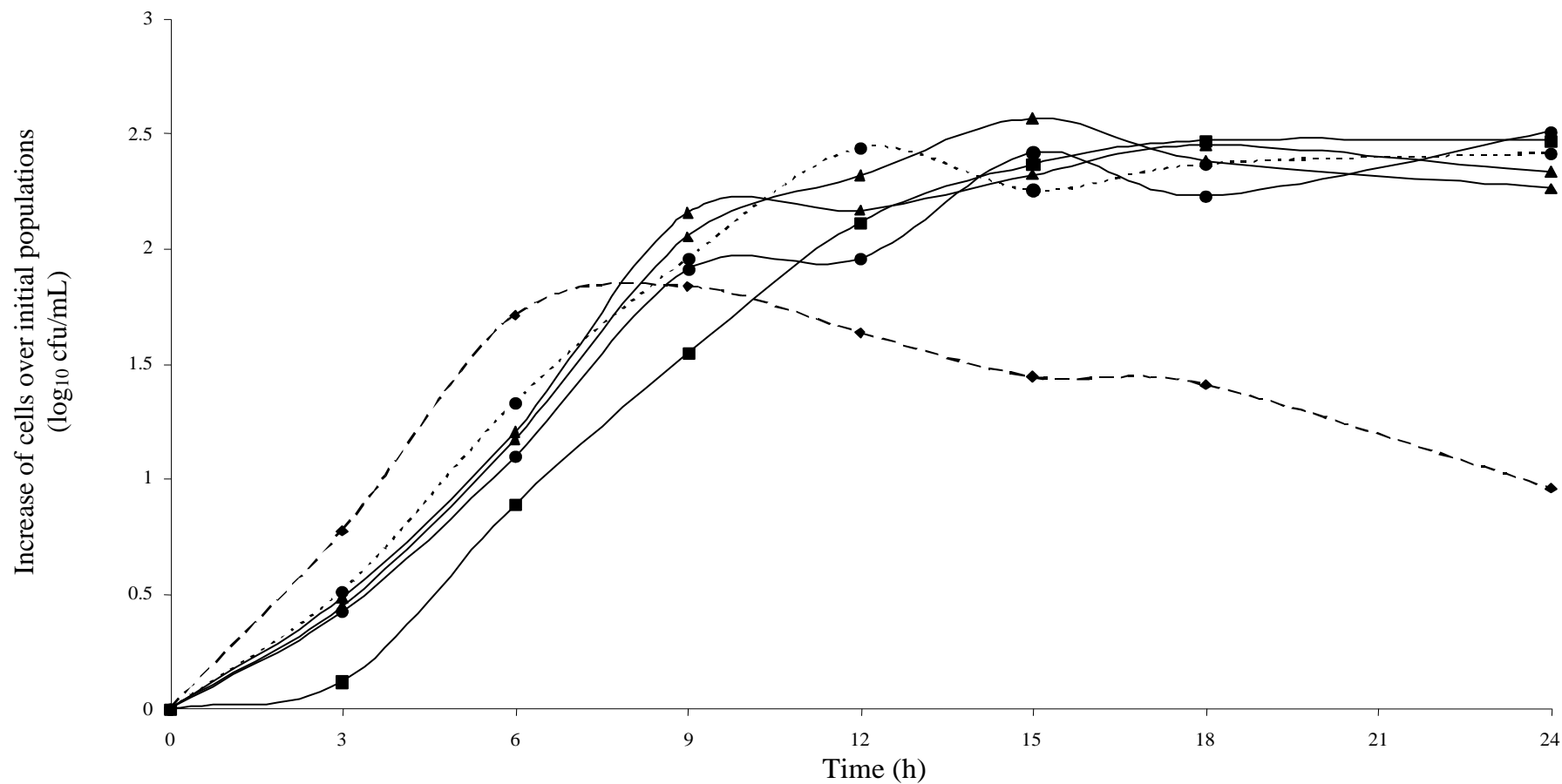
Fig. 3.3.1.1 Growth of—■— *L. delbruecki*i spp. *bulgaricus* —▲— *L. rhamnosus* GG, ----■---- *B. longum* 536, ----▲---- *L. acidophilus* 33200 and — ● — *L. casei* 279 in MRS broth and GG and ——◆—— *S. thermophilus* in M-17 broth at 37ºC
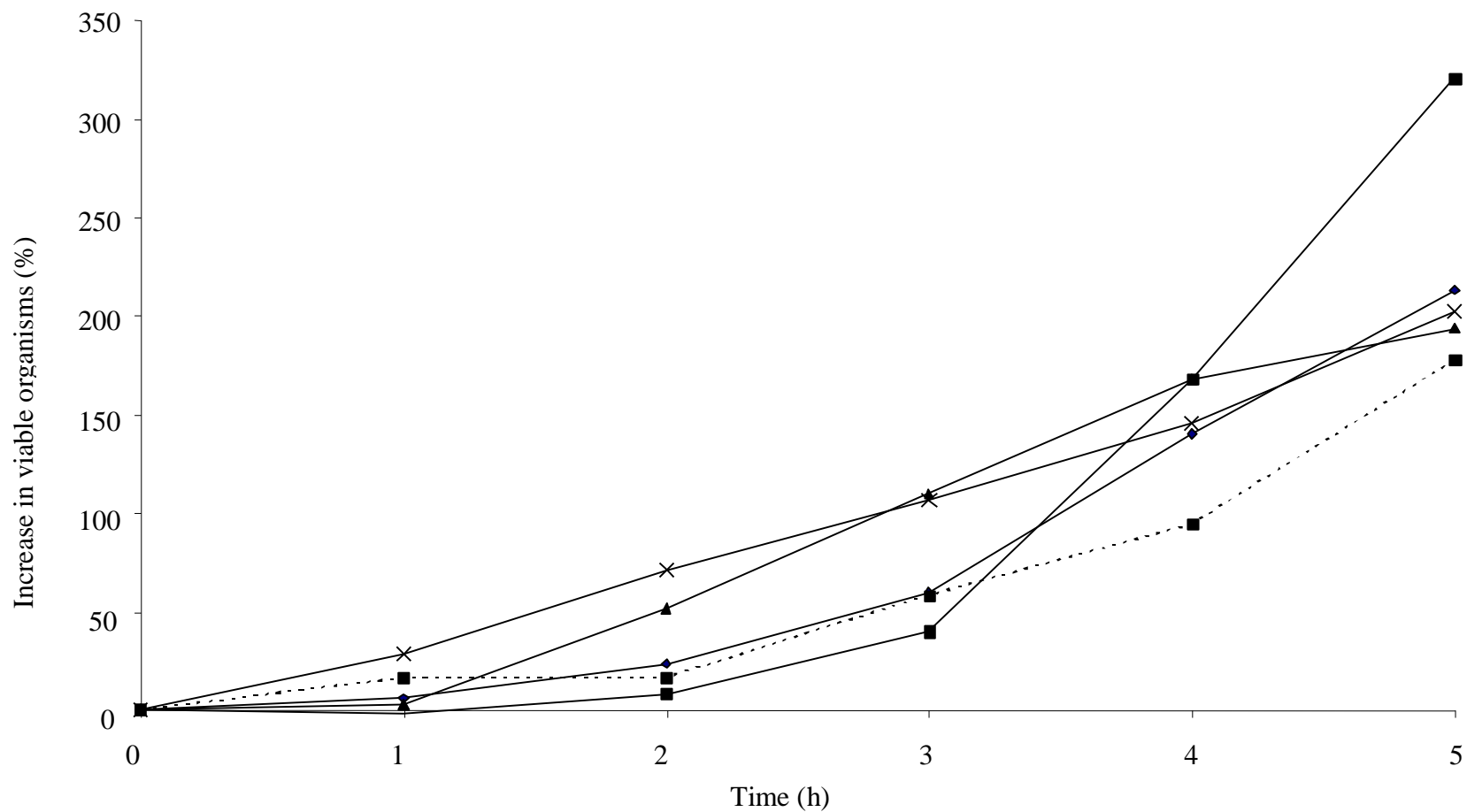
Fig. 3.3.1.2 Growth of microorganisms in RSM (14% w/v) at 42°C. The organisms are — ♦ — *L. acidophilus* 33200, — ■ — *L. delbrueckii* spp. *bulgaricus*, — ▲ — *L. casei* 279, — × — *L. rhamnosus* GG and ----■---- *B. longum* 536

Table 3.1

Effect of freeze-drying on the viability of *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus rhamnosus* and *Bifidobacterium* spp.

Micro-organism	Source	Viable counts ($\log_{10}$) cfu/g of probiotic cultures[a]		Survival (%)
		Before freeze-drying	After freeze-drying	
Lactobacillus acidophilus 4356	American Type Culture Collection	9.36 ± 0.11	9.22 ± 0.11	72.85 [b]
Lactobacillus acidophilus 33200	American Type Culture Collection	10.19 ± 0.09	10.06 ± 0.09	73.88 [b]
Lactobacillus casei 279	Australian Starter Culture Collection	9.69 ± 0.06	9.57 ± 0.05	75.34 [b]
Lactobacillus casei 292	Australian Starter Culture Collection	9.56 ± 0.03	9.42 ± 0.03	71.97 [b]
Lactobacillus casei 1520	Australian Starter Culture Collection	9.89 ± 0.16	9.50 ± 0.12	40.93 [c]
Lactobacillus casei 2607	CSIRO Starter Culture Collection	10.03 ± 0.16	9.91 ± 0.17	75.31 [b]
Lactobacillus rhamnosus GG	Isolated from a commercial product	9.76 ± 0.06	9.69 ± 0.06	85.73 [b]
Bifidobacterium bifidus 12	Victoria University Culture Collection	9.38 ± 0.02	9.30 ± 0.02	83.39 [b]
Bifidobacterium longum 536	Victoria University Culture Collection[d]	9.79 ± 0.18	9.72 ± 0.17	84.12 [b]
Bifidobacterium infantis 1912	Victoria University Culture Collection[e]	9.94 ± 0.14	9.85 ± 0.13	81.47 [b]
Bifidobacterium longum 1941	Victoria University Culture Collection[e]	10.15 ± 0.08	9.87 ± 0.13	52.21 [c]
Bifidobacterium breve 15698	American Type Culture Collection	9.57 ± 0.13	9.48 ± 0.12	80.51 [b]
Bifidobacterium infantis 17930	American Type Culture Collection	10.07 ± 0.04	10.00 ± 0.04	84.99 [b]

[a] Measurements are means ± standard error of 3 replicates for each individual strain

[b,c] Values followed by the same letter are not significantly different ($p > 0.05$)

[d] Originally obtained from Morinaga Milk Industry Co. Ltd. (Tokyo, Japan)

[e] Originally obtained from the Commonwealth Scientific and Research Organization (CSIRO), Highett, Victoria, Australia

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┌─────────────────────────────────────────────────────┐
│    Reconstitution of skim milk of 14% total solids   │
└─────────────────────────────────────────────────────┘
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┌─────────────────────────────────────────────────────┐
│              Heat treatment (85°C/30 min)            │
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                          ▼
┌─────────────────────────────────────────────────────┐
│        Cooling to incubation temperature (43°C)      │
└─────────────────────────────────────────────────────┘
                          │
                          ▼
┌─────────────────────────────────────────────────────┐
│         Addition of 4% probiotics including *L.*     │
│   *acidophilus* 33200, *L. casei* 279, *B. longum* 536│
│      and *L. rhamnosus* GG (1% of each organism)     │
└─────────────────────────────────────────────────────┘
                          │
                          ▼
┌─────────────────────────────────────────────────────┐
│   Addition of the 2% yoghurt starter organisms, *S.* │
│  *thermophilus* and *L. delbrueckii* ssp. *bulgaricus*│
└─────────────────────────────────────────────────────┘
                          │
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│                 Filling into containers              │
└─────────────────────────────────────────────────────┘
                          │
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┌─────────────────────────────────────────────────────┐
│         Incubation at 43°C till pH reaches 4.5       │
└─────────────────────────────────────────────────────┘
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                          ▼
┌─────────────────────────────────────────────────────┐
│              Refrigeration for 12 h at 4°C           │
└─────────────────────────────────────────────────────┘
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Fig. 3.3.3.1 Yoghurt manufacturing process.
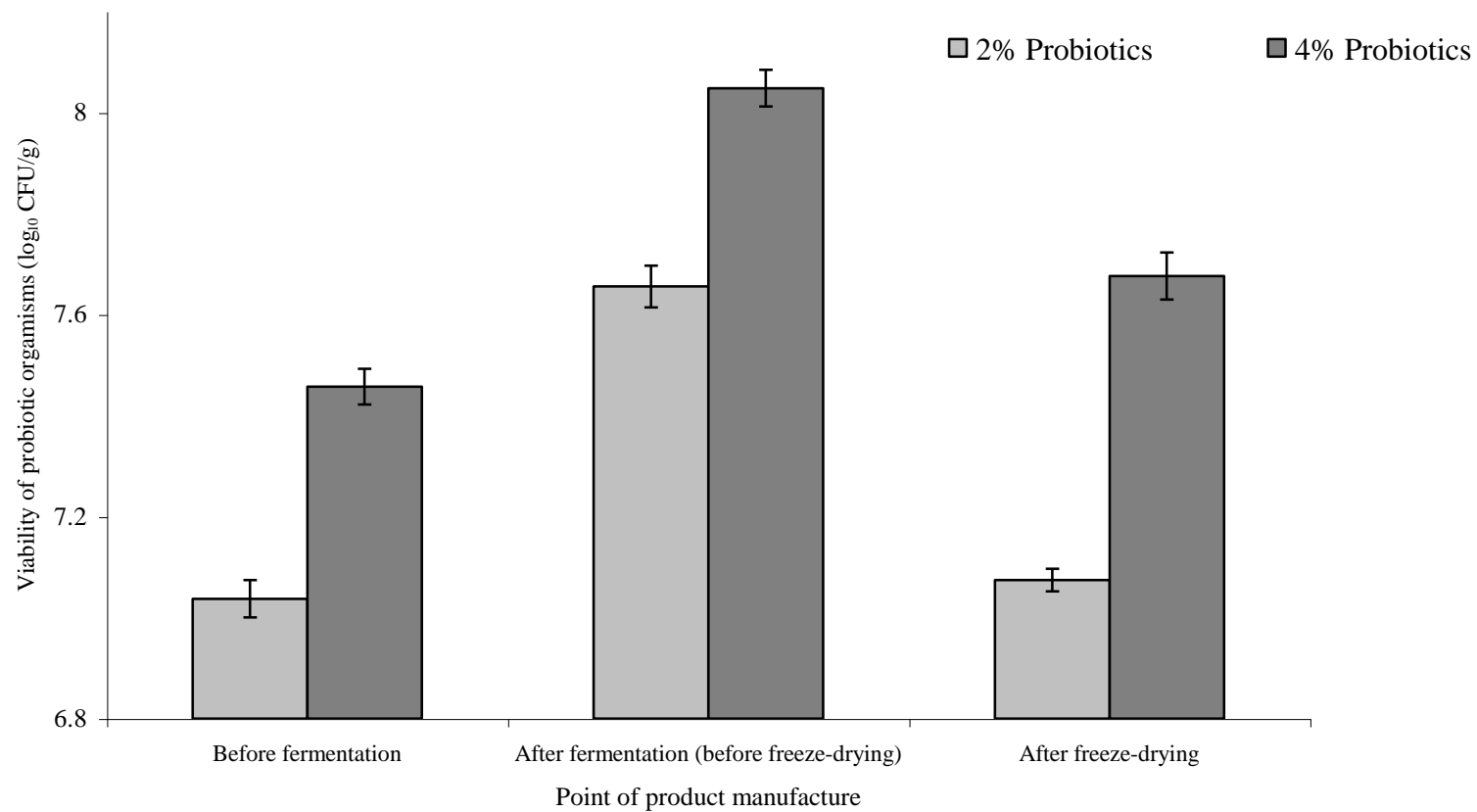
Fig. 3.3.3.2 Effect of inoculum size on viability of the combined probiotics *L. rhamnosus* GG, *L. acidophilus* 33200, *L. casei* 279 and *B. longum* 536 during the manufacture of freeze-dried yoghurt.

CHAPTER 4

THE EFFECT OF PREBIOTICS AND CRYOPROTECTANTS ON THE VIABILITY AND SURVIVAL OF PROBIOTICS IN YOGHURT AND FREEZE-DRIED YOGHURT[2]

4.1 Introduction

The human colon contains 12 $\log_{10}$ bacterial cells for every gram of gut contents (Rastall, 2004). The large bacterial population is due to favourable pH, slow transit time and the abundance of nutrients (Gibson et al., 2000). Some of these organisms including *Escherichia*, *Clostridium, Salmonella* and Campylobacter, may cause intestinal bloating and diarrhoea (Rolfe, 2000). Probiotic organisms such as *Lactobacillus* spp. and *Bifidobacterium* spp. improve intestinal health and benefit the host (Rastall, 2004). The colonic ecosystem can be controlled using prebiotics such as inulin, and fructooligosaccharides (FOS) to provide a substrate for desirable microorganisms such as probiotics (Roberfroid, 2001).

Prebiotics are defined as 'non-digestible food ingredients that may beneficially affect the host by selectively stimulating the growth and/or the activity of a limited number of bacteria in the colon' (Roberfroid, 1998). Prebiotics are classified as functional food ingredients under the categories of inulin-type fructans and synthetic fructooligosaccharides (Roberfroid, 2000). Inulin and their synthetic derivatives, the

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[2] Results described in this chapter have been published in Food Research International, volume 39, Issue 2, Pages 203-211. The paper is titled 'Effect of cryoprotectants, prebiotics and microencapsulation on survival of probiotic organisms in yoghurt and freeze-dried yoghurt'.

fructooligosaccharides (FOS), are predominantly obtained from the chicory plant (*Cichorium intybus*). Humans obtain prebiotics from dietary sources such as artichokes, onions, chicory, garlic, leeks and cereals. However, cereals do not adequately supply sufficient amounts of prebiotics for the probiotic organisms in the colon. Consequently, prebiotics can be incorporated in products such as yoghurts to supply a substrate for probiotic organisms in the colon.

Prebiotics are also used to improve the viability of probiotic organisms in yoghurt during storage. It is essential that one million probiotic organisms per gram be present in a product (Shah, 2002) in order to confer the health benefits (Le-Tien, et al., 2001) of probiotic organisms. Shin et al. (2000) found that the viability of commercial *Bifidobacterium* spp. in skim milk improved by 55.7% after 4 weeks of refrigerated storage when FOS was added.

Prebiotics are also used to protect probiotic organisms during freeze-drying as they provide extra solids. As oligosaccharides are hydrocolloids, they play a protective role towards probiotic microorganisms (Desai et al., 2004).

Some strains of probiotic organisms are sensitive to freeze-drying due to deterioration of physiological state of the cells. Cryoprotectants such as mannitol, sorbitol, sodium tripolyphosphate, xylitol, glycerol, raffinose, maltodextrin, erythritol, threitol, trehalose glucose and fructose play a role in improving the cold tolerance of microorganisms (Thunnell et al., 1984). Cold tolerance is improved as cryoprotectants accumulate within the cells and the osmotic difference with the external environment is reduced (Kets et al., 1996). Cryoprotectants added to a cell suspension also reduce injury and death. Cryoprotectants added to reduce the mortality of probiotic organisms in

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freeze-dried yoghurt effectively function by promoting the formation of extracellular amorphous ice glass instead of crystalline ice (Ravula, 2001). Amorphous ice glass is not as destructive upon the cell membrane of some probiotic organisms. The extent to which cryoprotection is provided may vary between cultures (Das et al., 1976).

The aim of this study was to investigate the effect of prebiotics 'raftilose' (FOS), 'Hi-maize' and 'raftiline' (inulin) and the effectiveness of cryoprotectants Unipectine^{TM} RS 150 and Satialgine^{TM} GCF 639 on the survival and viability of *Lactobacillus acidophilus* 33200, *Lactobacillus casei* 279, *Bifidobacterium longum* 536 and *Lactobacillus rhamnosus* GG in yoghurt and freeze-dried yoghurt.

4.2   Materials and method

4.2.1   *Preparation of probiotic organisms*

L. acidophilus 33200, *L. casei* 279, *B. longum* 536 and *L. rhamnosus* were grown in sterile deMann, Rogosa, Sharp (MRS) broth (Oxoid Ltd., Hampshire, UK) using 1% inoculum. To create an anaerobic environment, for *B. longum* 536, MRS was supplemented with filter sterilized 0.05% (w/v) L-cysteine·hydrochloride (Sigma Chemical Co., Castle Hill, Sydney, Australia). Each culture was grown and propagated three times successively for activation at 37°C for 12-15 h. Probiotic organisms were harvested by centrifuging (Sorvall RT7 Newtown, CT, USA) at $1510 \times g$ at 4°C for 15 min and the cell pellet was suspended in 14% (w/v) reconstituted skim milk (RSM).

L. rhamnosus GG was chosen for experiments involving the addition of cryoprotectants as it had the best survival on freeze-drying without a cryoprotectant (Chapter 3). *L. casei* 1520 was chosen as it had the lowest survival rate in earlier

experiments. Briefly, Schott bottles containing 10 mL of MRS broth were individually inoculated with each of the probiotic organisms. The cultures were activated by growing three times successively at 37°C for 15-18 h. Cells were harvested by centrifugation at $1510 \times g$ at 25°C for 25 min. Ten grams (wet weight) of freshly harvested cells were then washed and suspended in Schott bottles containing 90 mL of sterile (14% w/v) reconstituted skim milk (RSM).

4.2.2 Manufacture of yoghurt containing prebiotics

Yoghurt was prepared from reconstituted skim milk at 14% total solids. For yoghurt containing the prebiotics, (2 % w/v) Hi-maize (National Starch, Melbourne, Victoria, Australia), FOS (Raftilose®P95; Mandurah, NSW, Australia) or inulin (Raftiline®ST; Mandurah, NSW, Australia) were added into Schott bottles each containing RSM. The yoghurt mix was heat-treated at 85°C for 30 min and then cooled to the incubation temperature of 43°C. The heat-treated mix was inoculated with *L. acidophilus* 33200 (0.5%), *L. casei* 279 (0.5%), *B. longum* 536 (0.5%) and *L. rhamnosus GG* (0.5%). *Streptococcus thermophilus* (1%) and *Lactobacillus delbrueckii* ssp. *bulgaricus* (1%) were added to the mix which was incubated at 43°C for 3-4 h until the pH was reduced to 4.5. Fermentation was terminated by transferring the yoghurt into a refrigerator set at 4ºC. The treated yoghurt was subsequently stored at 4ºC for 4 weeks.

4.2.3 Manufacture of yoghurt containing cryoprotective agents

L. rhamnosus GG and *L. casei* 1520 (4%) together with yoghurt starter cultures were added to heat-treated and cooled (85°C for 30 min) 14% w/v RSM. Unipectine^{TM} RS 150 and Satialgine^{TM} GCF 639 (Degussa Texturant Systems, Newbury Berkshire, United Kingdom) were added at 0.0, 0.5, 1.0, 1.5, 2.0 and 2.5% to the RSM followed by incubation at 42°C for 3-4 h. Fermentation was terminated when the pH was reduced to 4.5 and the yoghurt was refrigerated for 16 h at 4°C. This procedure was repeated to investigate the effect of RS 150 on the viability of *L. acidophilus* 33200, *L. casei* 279 and *B. longum* 536 and *L. rhamnosus* GG (selected earlier, see Chapter 3).

4.2.4 Freeze-drying of yoghurt

A weighed quantity (30-50 g) of yoghurt was spread onto 90 mm petri dishes and freeze-dried (Model FD-300; Airvac Engineering Pty. Ltd., Dandenong, Australia) for 48 h at -100 kPa (40 h of primary drying at -30ºC and 8 h of secondary drying at -10ºC).

4.2.5 Enumeration of probiotic organism.

Probiotic organisms in fresh yoghurt with added prebiotics were enumerated weekly using MRS-vancomycin agar, during 4 weeks of storage using the method of Tharmaraj and Shah (2003). The probiotic organisms in freeze-dried yoghurt containing prebiotics were enumerated using MRS-vancomycin agar after 0, 1, 2, and 3 months of storage. Samples containing prebiotics were enumerated using MRS agar before and after freeze-drying. The serial dilution was carried out in sterile peptone and water solution (0.15% w/v) and MRS agar was used for plating. The plates were then incubated

anaerobically for 48-72 h at 37°C in anaerobic gas jars with a gas generating kit (BRD 38). Probiotic organisms were enumerated on plates containing 25-250 colony forming units per gram (cfu/g).

4.3 Results and discussion

4.3.1 Effect of raftilose (FOS), Hi-maize and raftiline (inulin) on the viability of L. acidophilus 33200, L. casei 279, B. longum and L. rhamnosus GG in yoghurt

The effect of prebiotics raftilose (FOS), Hi-maize and raftiline (inulin) on the viability of probiotic organisms is shown in Fig. 4.3.1. Of the three prebiotics investigated, raftilose was found to best retain the viability of selected probiotic organisms (8.70 log) in fresh yoghurt after four weeks of storage at 4ºC. The presence of these oligosaccharides reduced the cell death of the saccharolytic bacteria during storage, as prebiotics were available for their utilization. Bruno and Shah (2002) also found a marginal improvement in the viability of probiotic organisms in yoghurt containing FOS during refrigerated storage of fresh yoghurt over 4 weeks.

4.3.2 Effect of raftilose (FOS), Hi-maize and raftiline (inulin) on the viability of L. acidophilus 33200, L. casei 279, B. longum 536 and L. rhamnosus GG in freeze-dried yoghurt

The addition of prebiotics had an effect on the viability of selected probiotic organisms in freeze-dried yoghurt during 3 months, Fig. 4.3.2. The viability of the probiotic organisms decreased over 3 months storage when prebiotics were added. This finding was unexpected. One explanation may be that the prebiotics present outside of the

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cells were concentrated during freezing. Consequently, as osmotic pressure increased, probiotic cells were injured and killed (Mazur, 1984). According to Baati et al. (2000), lactic acid bacteria also suffer from stresses caused by changing environment. The oligosaccharides may not have been able to protect cells from injury, which may have contributed to the reduction in probiotic viability. Hi-maize, FOS and inulin were only helpful in improving viability of probiotic organisms in fresh yoghurt during storage (Fig. 4.3.1), and had a negative effect on their viability in freeze-dried yoghurt (Fig. 4.3.2). The improvement observed in fresh yoghurt and not freeze-dried yoghurt is possibly due to prebiotics providing extra solids, which tend to protect cells from injury. Oligosaccharides used in this study are hydrocolloids, which are reported to play a protective role towards probiotic microorganisms (Desai et al., 2004).

4.3.3 Effect of cryoprotectants Unipectine^{TM} RS 150 and Satialgine^{TM} GCF 639 on the viability of probiotics L. rhamnosus GG and L. casei 1520

The effect of cryoprotectant on the viability of *L. rhamnosus* and *L. casei* 1520 is shown in Fig.4.3.3. Unipectine™ RS 150 was more effective as a cryoprotectant than Satialgine™ GCF 639. As the concentration of the cryoprotective agents was increased (0.0 – 2.5%), there was an increase in the survival of probiotic organisms. The improvement in viability may have occurred as the cryoprotective agents can inhibit intracellular or extracellular ice formation by binding to the water. Some cryoprotectants function by replicating similar hydrogen bonding properties to water (Sano et al., 1999). Ravula and Shah (2000) also found Unipectine™ RS 150 to be superior to other types of cryoprotecants, as viable counts of *L. acidophilus* and *Bifidobacterium* were improved by

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1 log during storage in fermented dairy desserts. Das et al. (1976) also reported that pectin improved the survival of *S. thermophilus*. Unipectine™ RS 150 was selected for use in further experiments to investigate the effect of survival on *L. acidophilus, L. casei, L. rhamnosus* and *B. longum.*

4.3.4 Effect of RS 150 on the survival of L. acidophilus 33200, L. casei 279, B. longum 536 and L. rhamnosus GG

The effect of Unipectine™ RS 150 on viability of selected probiotic organisms is shown in Fig. 4.3.4. Of the selected probiotic organisms, there was an 80% improvement in the survival of *B. longum* 536 when Unipectine™ RS 150 was added, while there was a more modest improvement (30%) observed in the survival of *Lactobacillus* spp. Virkajärvi et al. (2003) also found that *L. rhamnosus* GG and *L. rhamnosus* E800 survived well during freeze-drying, whereas the survival of *B. animalis* was highly dependent on the addition of a cryoprotecant. This suggests that the degree of protection provided by Unipectine™ RS 150 varies between probiotic organisms, possibly due to differences in cell membrane composition of *Bifidobacterium* spp. and *Lactobacillus* spp.

4.4   Conclusions

The viability of probiotic organisms improved when Hi-Maize, FOS and inulin were added to fresh yoghurt. FOS was found to be the most effective of all prebiotics, in retaining the viability of probiotic organisms in fresh yoghurt. When the yoghurt was freeze-dried, the addition of FOS marginally reduced the viability of probiotic organisms. RS 150 was found to be the most effective cryoprotectant in retaining the viability of probiotic organisms. RS 150 had a strain-specific effect on survival of probiotic organisms in freeze-dried yoghurt.
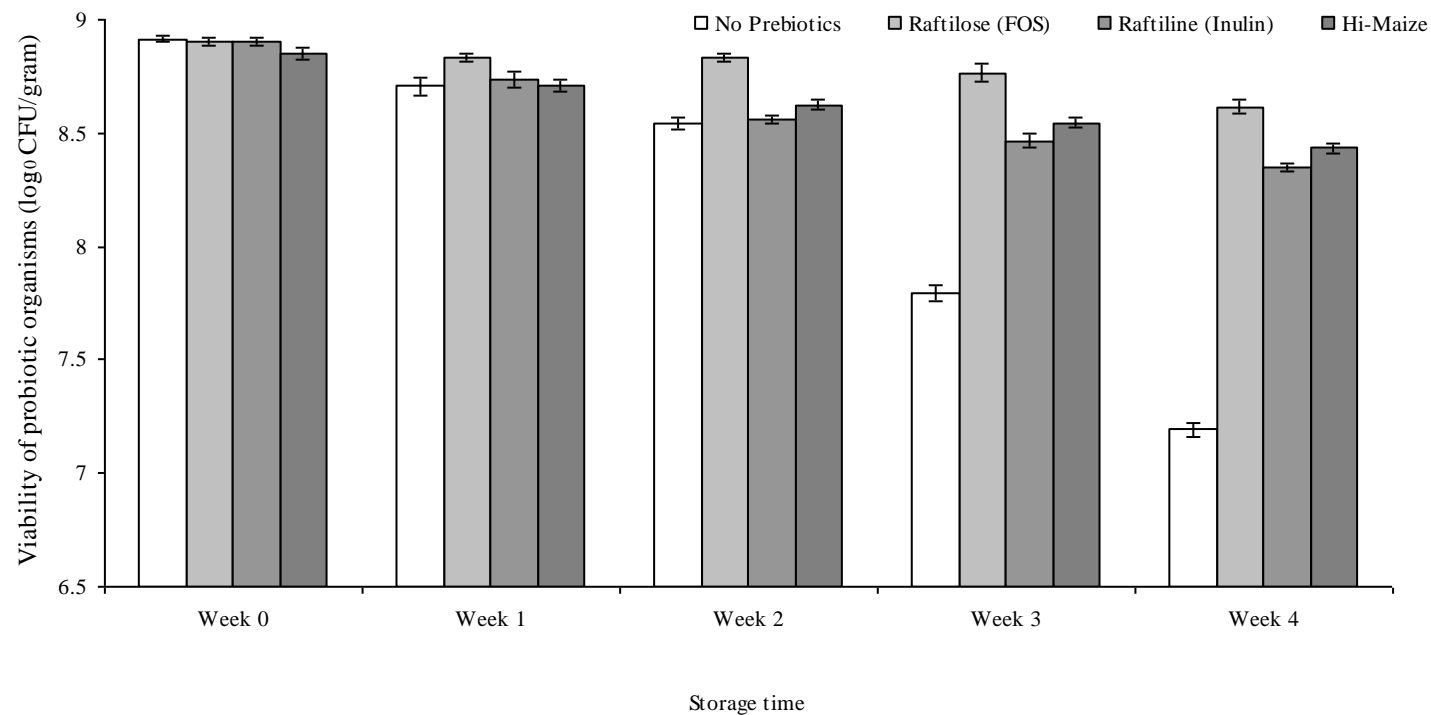
Fig. 4.3.1 Effect of prebiotics on viability of combined probiotic organisms in fresh yoghurt during four weeks of storage at 4ºC. The combined probiotics were *L. acidophilus* 33200 (LA), *L. casei* 279 (LC), *B. longum* 536 (BB) and *L. rhamnosus* (GG), and the prebiotics were raftilose (FOS), Hi-maize and raftiline (inulin)
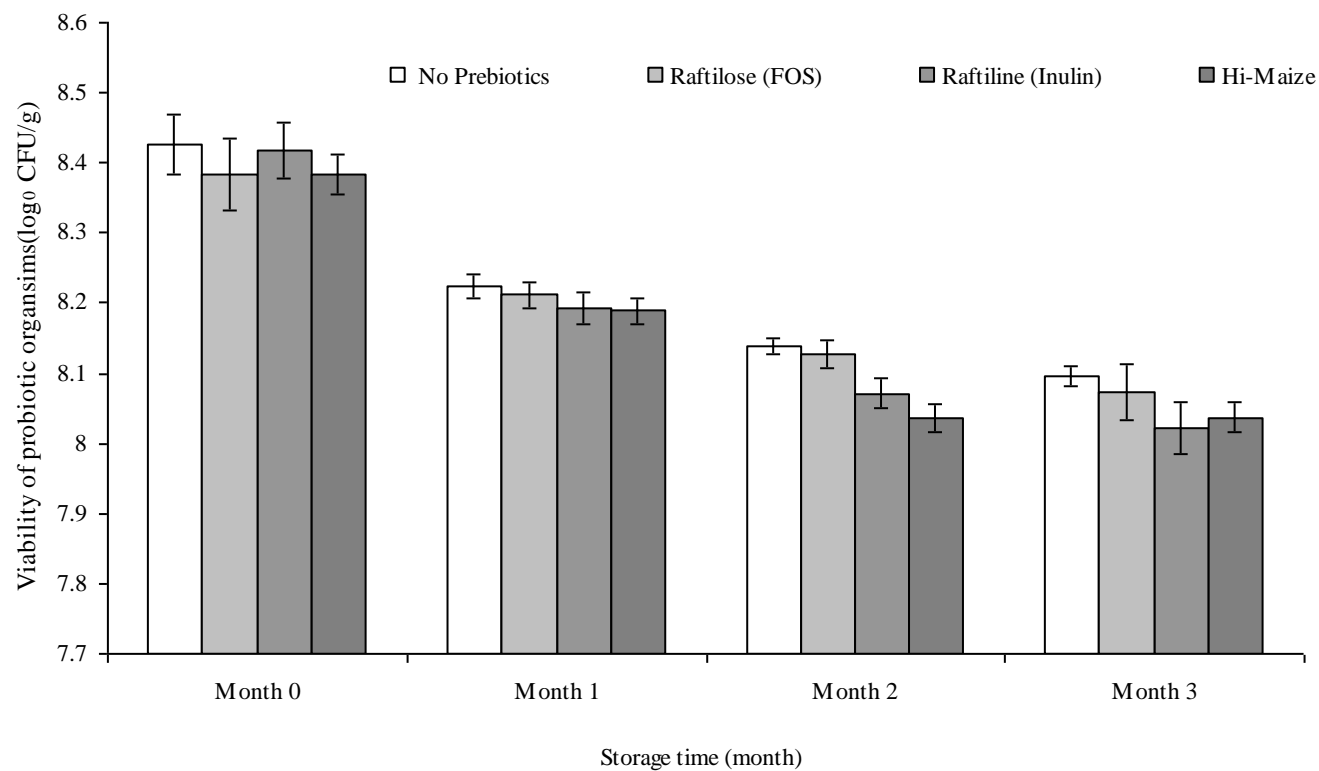
Fig. 4.3.2 Effect of prebiotics on viability of combined probiotic organisms in freeze-dried yoghurt during three months storage at 4ºC. The combined probiotics were *L. acidophilus* 33200 (LA), *L. casei* 279 (LC) *B. longum* 536 (BB) and *L. rhamnosus* (GG), and the prebiotics were raftilose (FOS), Hi-maize and raftiline (inulin).
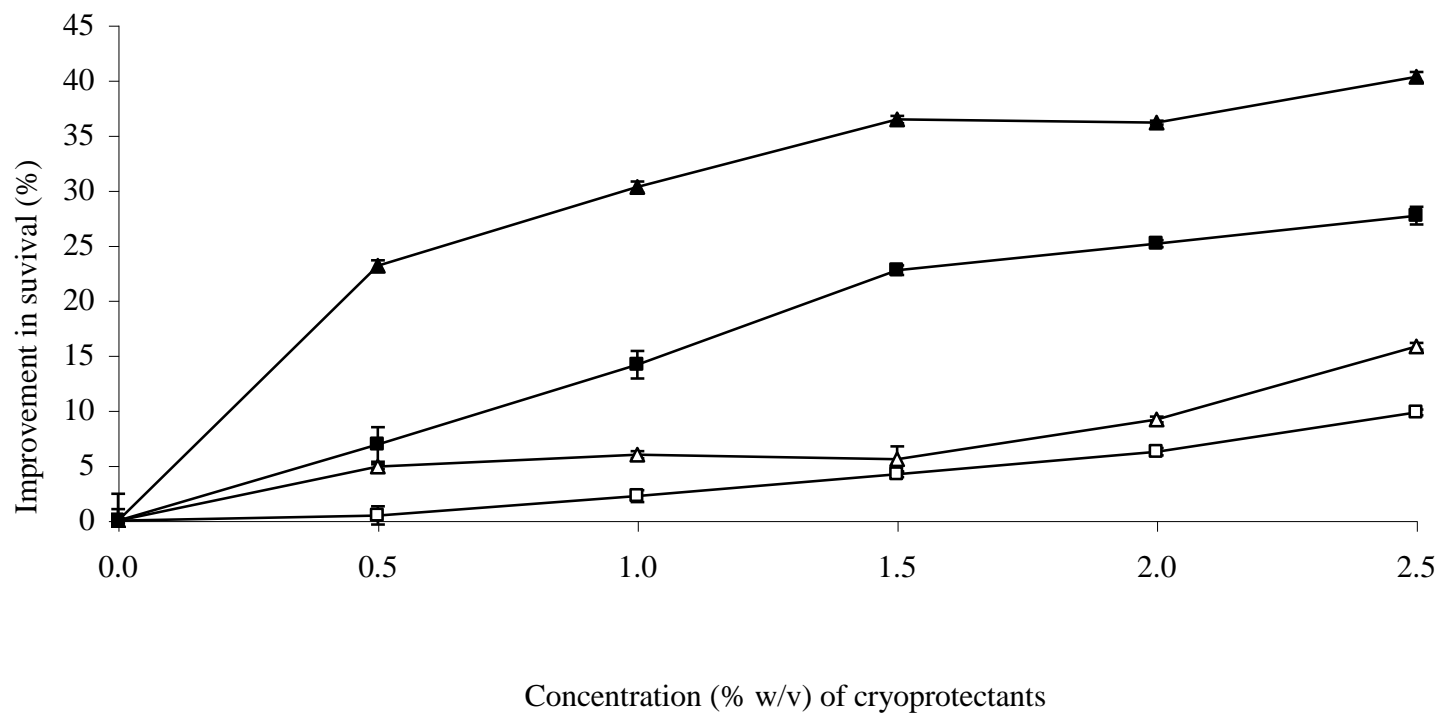
Fig. 4.3.3 Effect of cryoprotectants Unipectine^{TM} RS 150 and Satialgine^{TM} GCF 639 on the viability of probiotics *L. rhamnosus* (GG) and *L. casei* 1520 (LC). (□ LC + GCF 639, Δ GG+GCF 639, ▲ LC + RS 150, ■ GG + RS 150
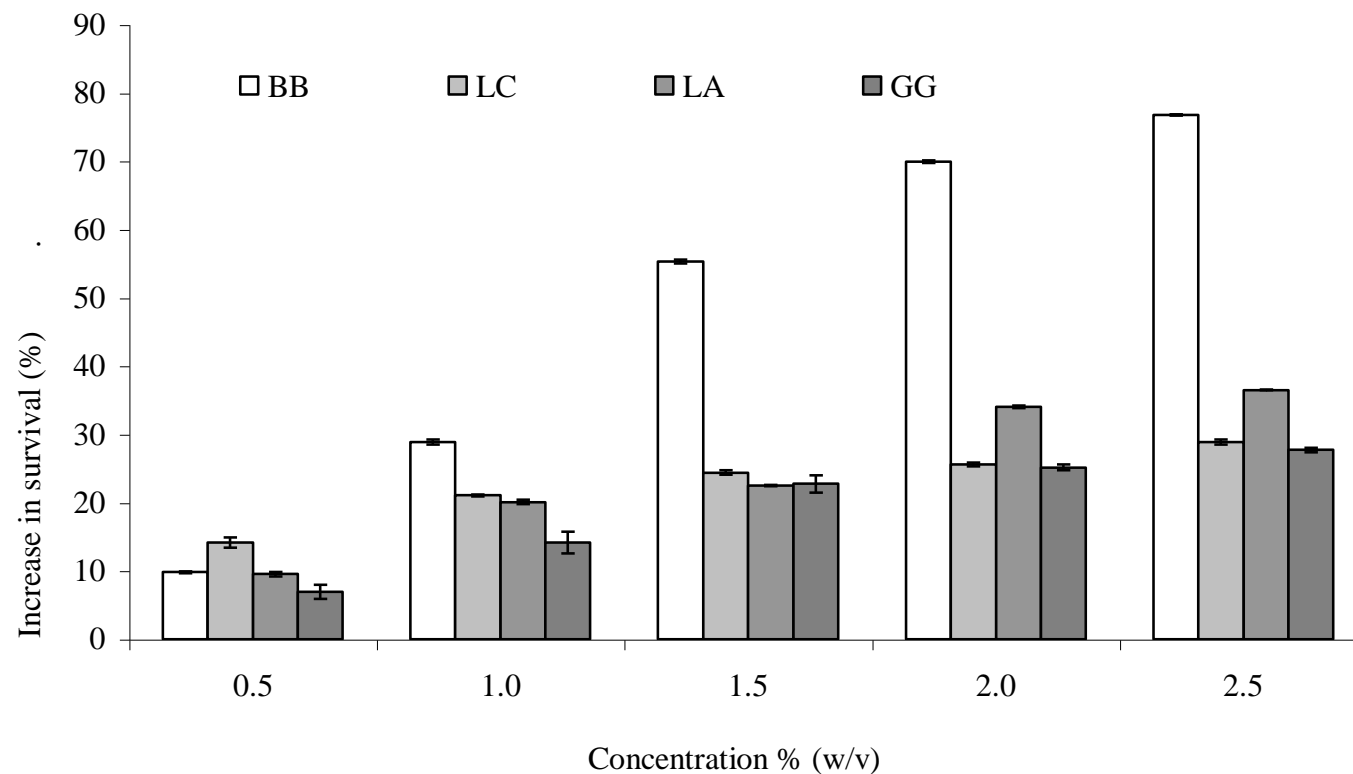
Fig. 4.3.4 Effect of cryoprotectants on the survival of *L. acidophilus* 33200 (LA), *L. casei* 279 (LC) *B. longum* 536 (BB) and *L. rhamnosus* (GG) with added cryoprotectant Unipectine RS 150 (improvement in survival is relative to the control which did not contain RS 150)

CHAPTER 5

EFFECT OF FREEZE DRYING ON THE VIABILITY OF MICROENCAPSULATED *LACTOBACILLUS ACIDOPHILUS* 33200, *LACTOBACILLUS CASEI* 279, *LACTOBACILLUS RHAMNOSUS* GG AND *BIFIDOBACTERIUM LONGUM* 536 IN YOGHURT AND FREEZE-DRIED YOGHURT[3]

5.1 Introduction

Several researchers have studied techniques to improve the viability of probiotic organisms in yoghurt during processing and storage (Dave and Shah, 1997; Akalin et al., 2002). It is essential that one million probiotic organisms per gram be present in a product (Shah, 2002) in order to confer the associated health benefits (Le-Tien et al., 2004; Marteau et al., 2001). The pH, temperature and oxygen content during the manufacture and storage of yoghurt can affect the viability of probiotic organisms Talwalkar and Kailasapathy, 2004). These factors limit the shelf-life of yoghurt to approximately six weeks of refrigerated storage. Freeze-drying is a process that can prolong the shelf-life of yoghurt and help maintain an optimum level of viable probiotic organisms.

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[3] Results described in this chapter have been published in Food Research International, volume 39, Issue 2, Pages 203-211. The paper is titled 'Effect of cryoprotectants, prebiotics and microencapsulation on survival of probiotic organisms in yoghurt and freeze-dried yoghurt'.

Freeze-drying involves the removal of frozen water by sublimation. During the manufacture of freeze-dried yoghurt, temperature and pressure are carefully controlled to allow for the formation of a glass state that minimizes damage to probiotic organisms. However, freeze-drying elevates the concentration of solutes due to the removal of water. As elevated solute concentration may stress probiotic organisms, it is necessary to protect them from such stresses in freeze-dried yoghurt. Microencapsulation is a technique used to surround probiotic organisms in a protective coating in order to improve viability.

Sodium alginate is a food grade encapsulating agent which has been used to protect probiotic organisms during processing and storage. Sodium alginate, when combined with calcium chloride, forms beads consisting of calcium alginate gel matrix. There are several methods that can be used to produce beads including the emulsion and extrusion techniques (Krasaekoopt et al., 2003). Probiotic organisms can be released from the beads by sequestering calcium ions using sodium citrate or phosphate buffer.

Encapsulation with calcium alginate has been found to improve the viability of probiotic organisms in frozen yoghurt during processing and storage (Ravula and Shah, 2000). Viability may be further improved by adjusting the concentration of sodium alginate up to 4% (Lee and Heo, 2000). The improvement in viability of probiotic organisms is attributed to the structure of the sodium alginate gel, which permits the diffusion of nutrients while shielding the microorganisms from molecules that may cause stress (Shah, 2000). Microencapsulation has been found to reduce probiotic cell death caused by oxygen toxicity (Talwalkar and Kailasapathy, 2003). Probiotic organisms have also been found to agglomerate in the centre of beads creating anaerobic regions (Beunik

et al., 1989). An additional layer can be applied to provide probiotic organisms with a barrier from molecules that may cause stress and oxygen.

An additional layer to calcium alginate beads can be achieved by applying a polymer coating such as chitosan or poly-L-lysine (PLL) to the beads. However, the coating may affect the overall size of the bead. Large beads are undesirable in yoghurt and many other products as they may detract from the mouthfeel of the product. Beads with a diameter exceeding approximately 0.1 mm (100 µm) are detectable in the mouth. Therefore, beads ranging between 15 µm and 100 µm that do not compromise the viability of probiotic organisms during processing and storage are desirable. Various techniques have been used to reduce the size of beads including the use of spray guns (Lee et al., 2003) and air atomizers with a variety of nozzle sizes (Kwok et al., 1991).

Very little work has been undertaken to select an appropriate technique to microencapsulate probiotic organisms for use in freeze-dried yoghurt. Similarly, little work has been carried out on improving the viability of probiotic organisms in freeze-dried yoghurt by applying an additional coat to calcium alginate beads.

The objectives of the present research were i) to identify a suitable concentration of sodium alginate for microencapsulating probiotic organisms, ii) to assess the effectiveness of sodium citrate and phosphate buffer in releasing freeze-dried immobilized cells, iii) to observe the effect of microencapsulating probiotic organisms using the emulsion and extrusion techniques on bead size and on the viability of probiotic organisms, iv) to investigate the effect of microencapsulation on the viability of probiotic organisms in yoghurt containing cryoprotectants and prebiotics during storage and v) to

investigate the effect of applying a coat to calcium alginate beads on the viability of probiotic organisms in yoghurt and freeze-dried yoghurt during storage.

5.2 Materials and Method

5.2.1 Preparation of probiotic bacteria

L. acidophilus 33200 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA), *L. casei* 279 was obtained from the Australian Starter Culture Collection (ASCC) (Werribee, Victoria, Australia), *B. longum* 536 was obtained from the Victoria University Culture Collection (Werribee, Victoria, Australia) but was originally obtained from Morinaga Milk Industry Co. Ltd. (Tokyo, Japan), and *L. rhamnosus* GG was isolated from a commercial product. Each of the organisms was grown in sterile deMann, Rogosa, Sharp (MRS) broth (Oxoid Ltd., Hampshire, UK) using a 1% inoculum. To create an anaerobic environment, *B. longum* 536 was supplemented with filter sterilized 0.05% (w/v) L-cysteine·hydrochloride (Sigma Chemical Co., Castle Hill, Sydney, Australia). Each culture was grown and propagated three times successively for activation at 37°C for 12-15 h. Probiotic organisms were harvested by centrifuging (Sorvall RT7 Newtown, CT, USA) at $1510 \times g$ at 4°C for 15 min and the cell pellet was suspended in 14% (w/v) in RSM.

*5.2.2 Microencapsulation of combined L. acidophilus 33200, L. casei 279, B. longum 536
and L. rhamnosus GG*

5.2.2.1 Microencapsulaton using the extrusion technique

The extrusion method of Krasaekoopt, et al. (2003) was used to encapsulate
probiotics. Briefly 100 mL of sterile sodium alginate (2, 3 and 4% w/v) was mixed with
25 mL of combined *L. acidophilus 33200, L. casei 279*, *B. longum* 536 and *L. rhamnosus*
GG (9.0-10.0 $\log_{10}$ cfu/g) and shaken thoroughly. Each suspension was added by syringe
(23 gauge) into 500 mL of sterile 0.1 M calcium chloride solutions. Spherical beads were
formed upon contact with the calcium chloride solution. The beads were kept in the
calcium chloride solution for 15 min until set.

5.2.2.2 Microencapsulation of probiotic organism using emulsion technique

The emulsion method of Ravula and Shah (2000) was used to prepare calcium
alginate beads. Briefly, 100 mL calcium alginate beads by the emulsion technique, 100
mL of sterile 3% (v/w) sodium alginate were mixed with 25 mL of suspension containing
9.0-10.0 $\log_{10}$ cfu/g of combined *L. acidophilus* 33200, *L. casei* 279 *B. longum* 536 and *L.
rhamnosus* GG. The sodium alginate and probiotic organisms were, dispensed using a
pipette, into a beaker containing 600 mL of autoclaved vegetable oil (Eta Blended
Vegetable Oil, Goodman Fielder Pty. Ltd., Melbourne, Australia) and stirred at 200 rpm
using a magnetic stirrer (IEC Industrial Equipment & Control Pty. Ltd., Melbourne,
Australia). Calcium chloride (0.1 M) was gently added down the side of the beaker until
the emulsion was broken. After 15 min, the calcium alginate beads were removed from
the aqueous phase and centrifuged at $300 \times g$ for 5 min.

5.2.2.3 Coating of calcium alginate beads with poly-L-lysine and chitosan

The emulsion technique (sec 5.2.2.2) was used to form calcium alginate beads containing the combined probiotic organisms (9.0-10.0) $\log_{10}$ cfu/g). The calcium alginate beads (15 g) were co-encapsulated by submerging in chitosan (Sigma Chemical Co., Castle Hill, Sydney, Australia) or poly-L-lysine (PLL) (Sigma Chemical Co., Castle Hill, Sydney, Australia) solutions using the modified procedure of Krasaekoopt et al. (2004). Briefly, probiotic beads combined with chitosan or PLL solutions (100 mL) were gently shaken at 100 rpm (Innova 4320 Refrigerated Incubator Shaker, Edison, NJ, USA) for 60 min to coat the calcium alginate beads. Chitosan and PLL form strong bonds with calcium alginate beads in the presence of calcium chelators (Gombotz and Wee, 1998).

5.2.2.4 Evaluation of bead size

Beads produced using the extrusion and emulsion techniques were stained with safranine and gently spread over a glass slide. The appearances of the beads were observed using a stage light microscope. The diameters of 15 beads in a randomly selected field were measured using an ocular micrometer.

5.2.3 Yoghurt preparation

Yoghurt was prepared from reconstituted skim milk at 14% total solids. Cryoprotectant 'Unipectine RS 150' (Degussa Texturant Systems, Newbury Berkshire, United Kingdom) and prebiotic 'Raftilose®P95 (Mandurah, NSW, Australia) were added at 1.5% (w/v) each. The yoghurt mix was heat-treated at 85°C for 30 min and then cooled

to the incubation temperature of 43°C. The heat-treated mix was inoculated with the combined microencapsulated probiotic organisms at 2%. *Streptococcus thermophilus* (1%) and *Lactobacillus. delbrueckii* ssp. *bulgaricus* (1%) were added to the mix which was incubated for 3-4 h until the pH was reduced to 4.5. Fermentation was terminated by transferring the yoghurt to a refrigerator.

5.2.4 Procedure used for freeze-drying

Ten grams of microencapsulated probiotic organism (9.0 $\log_{10}$ cfu/g) were suspended in 14% (w/v) RSM and frozen at -80°C for 2 h. The frozen microorganisms were then transferred to a freeze-dryer (Model FD-300; Airvac Engineering Pty. Ltd., Dandenong, Australia) for 48 h at -100 kPa (40 h of primary drying at -30°C and 8 h of secondary drying at -10°C). Probiotic organisms were enumerated before and after freeze-drying to investigate the effect of freeze-drying on viability.

5.2.5 Enumeration of probiotic organisms

The probiotic organisms were enumerated using the method prescribed by Tharmaraj and Shah (2003). *L. acidophilus* was selectively enumerated on MRS-sorbitol agar using anaerobic incubation at 37°C for 72 h. *L. casei* was enumerated on LC agar using anaerobic incubation at 37°C for 72 h. *B. longum* was selectively enumerated on MRS-NNLP (nalidixic acid, neomycin sulphate, lithium chloride and paromomycin sulphate) agar incubated at 37°C for 72 h and *L. rhamnosus GG* was enumerated on MRS-vancomycin agar using anaerobic incubation at 43°C for 72 h.

Encapsulated organisms were released from calcium alginate beads using sodium citrate and phosphate buffer prior to enumeration. The combined viability of *L. acidophilus 33200, L. casei 279, B. longum 536* and *L. rhamnosus* GG in freeze-dried yoghurt was determined using MRS-vancomycin agar (Tharmaraj and Shah, 2003) and anaerobic incubation at 37°C for 48 h.

5.2.6 Experimental outline

5.2.6.1 Effect of release agent on probiotic enumeration

The probiotic organisms were liberated from the beads by suspending in sodium citrate solution for 15 min as per Shah and Ravula (2000). Calcium alginate beads (1.5 g) containing probiotic organisms were added to 13.5 mL of sodium citrate (pH 6.9) and phosphate buffer (pH 6.9) then mixed for 10 sec using a vortex mixer (MT19 Auto Vortex mixer, Chiltern, London). One millilitre aliquot was removed at 2 min intervals for 12 min to enumerate the counts of *L. acidophilus 33200* using MRS agar.

5.2.6.2 Effect of alginate concentration on the viability of probiotic organisms after freeze-drying

To investigate the effect of sodium alginate concentration on viability of probiotic organisms after freeze-drying, sterile sodium alginate was prepared at 0, 2, 3 and 4% (w/v). Microencapsulation was carried out using the extrusion technique as described in 5.2.2.1 The microencapsulated probiotic organisms were added to RSM at 2% and freeze-dried as described in 5.2.4. The probiotic organisms were enumerated before and after

freeze-drying using MRS-vancomycin (Tharmaraj and Shah, 2003) agar and anaerobic incubation at 37°C for 48 h.

5.2.6.3 Effect of microencapsulation technique on bead size and the viability of probiotic after freeze-drying

To investigate the effect of microencapsulation technique on bead size and viability of *L. acidophilus* 33200, *L. casei* 279, *B. longum* 536 and *L. rhamnosus* GG were microencapsulated using the extrusion (5.2.2.1) and emulsion (5.2.2.2) techniques. Beads were added at 2% to 14% (w/v) RSM. The beads were freeze-dried using the procedure that was described (5.2.4). After freeze drying, the probiotic organisms were released from the beads using phosphate buffer and enumerated using MRS agar as described below.

5.2.6.4 Effect of microencapsulation, cryoprotectants and prebiotics on the viability of probiotic organisms in freeze-dried yoghurt during storage for 6 months at 4°C, 21°C and 37°C

Probiotic organisms were microencapsulated using the emulsion technique as described previously. Yoghurt containing *L. acidophilus* 33200, *L. casei* 279, *B. longum* 536 and *L. rhamnosus* GG was manufactured as shown in Fig. 5.1. The probiotic organisms were enumerated at monthly intervals for 6 months using MRS-vancomycin agar as described by Tharmaraj and Shah (2003).

5.2.6.5 Effect of coating agent and storage time on the viability of probiotic organisms in yoghurt

Probiotic organisms were coated with chitosan or PLL as described in 5.2.3. Ten grams of co-encapsulated beads were incorporated into a yoghurt mix at 2% (manufactured as described in 5.3). Yoghurt was stored in a refrigerator at 4ºC. The viability of probiotic organisms in the yoghurt was determined at weekly intervals during 4 weeks of storage using MRS-vancomycin agar.

5.2.6.6 Effect of coating agent and storage time on the viability of probiotic organisms in freeze-dried yoghurt

Ten grams of microencapsulated probiotic organism (9.0 $\log_{10}$ cfu/g) were suspended in 14% (w/v) RSM and frozen at -80ºC for 2 h. Calcium alginate beads coated with PLL or chitosan were added to yoghurt at 2%. The yoghurt was freeze-dried and stored at 4ºC, 21ºC and 37ºC. The viability of probiotic organisms in freeze-dried yoghurt was determined at monthly intervals during 4 months of storage using MRS-vancomycin agar.

5.2.7 Statistical analysis

All data are presented as mean ± standard error of 2 replicates (n =2) with 3 measurements taken each time. Data analysis was carried out using SPSS Inc. software (10.0: SPSS Inc., Chicago, IL). The significant difference between means (level of significance α = 0.05) was analysed using one-way ANOVA. Multiple comparisons between means were carried out using Tukey's test.

5.3 Results and Discussion

5.3.1 Release of L. acidophilus 33200 encapsulated within calcium alginate beads using sodium citrate, phosphate buffer or sterile water

L. acidophilus 33200 was released from calcium alginate complex by sequestering calcium ions using sodium citrate or phosphate buffer. Fig. 5.2 illustrates the time taken to release the entrapped probiotic organisms from calcium alginate beads using sodium citrate, phosphate buffer and sterile water. After 2 min, the number of liberated probiotic organisms in sodium citrate or phosphate buffer increased from 5.8 $\log_{10}$ to 8.2 $\log_{10}$ and 8.1 $\log_{10}$ cfu/g, respectively. Most of the probiotic organisms were released within 4 min using sodium citrate or phosphate buffer. However, 4 to 6 min were necessary to completely release microencapsulated probiotic organisms using sodium citrate or phosphate buffer with both agents displaying similar capabilities in releasing the probiotic organisms. As expected, sterile water was not effective in degrading calcium alginate matrix (5.8 $\log_{10}$). There results showed that either sodium citrate or phosphate buffer could be used in future experiments for releasing probiotic organisms from calcium alginate beads.

5.3.2 Effect of sodium alginate concentration on the viability of combined L. acidophilus 33200, L. casei 279, B. longum 536 and L. rhamnosus GG during freeze-drying

Effect of sodium alginate concentration on the viability of encapsulated probiotic organisms in freeze-dried yoghurt is shown in Fig. 5.3. There was 0.2-0.3 log improvement in the viability of microencapsulated probiotic organisms compared to the control (0% sodium alginate). However, there was no improvement in the survival of the

organisms beyond the sodium alginate concentration of 2%. Other researchers have found a positive effect of a higher concentration of alginate on survival of entrapped *Bifidobacterium* spp. (Sheu et al., 1993; Lee and Heo, 2000). Overall, our results show that microencapsulation provided limited protection to probiotic organisms during freeze-drying.

Calcium alginate beads produced using sodium alginate 2 and 3% (w/v) were most suitable for further experiments involving microencapsulation. The 4% (w/v) sodium alginate solution was found to be too viscous making it difficult to dispense using a syringe, which ultimately resulted in the formation of large beads.

5.3.3 Effect of the emulsion and extrusion techniques on the size of probiotic calcium alginate beads

Particles of 0.1 mm or greater are easily detected by the consumer and may have a negative affect on the mouthfeel of yoghurt. The mean diameters of calcium alginate beads manufactured using the emulsion and extrusion techniques are shown in Table 5.1. The beads manufactured using the extrusion method were spherical and had mean diameters ranging from 1.76 to 2.06 mm. These beads were larger than those manufactured using the emulsion technique, sizes ranged between 430 and 560 µm. Other researchers have reported bead size less than 50 µm using the emulsion technique (Sheu et al., 1993). Both of the encapsulation treatments used in the present study resulted in beads that may not be considered as acceptable in a commercial product.

5.3.4 Effect of the emulsion and extrusion techniques on viability of microencapsulated L. acidophilus 33200, L. casei 279, B. longum 536 and L. rhamnosus GG during freeze-drying

The effect of microencapsulation using the emulsion and extrusion techniques on viability of probiotic organisms is shown in Fig. 5.4-5.7. The emulsified beads contained the highest level of viable probiotic organisms. The viability of organisms present in these beads was between 1.0 and 1.5 $\log_{10}$ CFU/g higher than the organisms microencapsulated using the extrusion and extrusion-homogenisation techniques. Residual oil present on beads created using the emulsion technique possibly protected the probiotic organisms during freeze-drying. Shah and Ravula (2000) found that microencapsulating *L. acidophilus* and *Bifidobacterium* spp. using the emulsion technique improved viability compared to unprotected cells in frozen fermented deserts during 12 weeks of storage. Talwalkar and Kailasapathy (2003) found that large beads (2.38 mm) produced using the extrusion technique resulted in poor cell distribution which may have resulted in exposing probiotic organisms to oxygen toxicity.

5.3.5 Effect of microencapsulation, cryoprotectants and prebiotics on the viability of probiotic organisms in freeze-dried yoghurt during storage for 6 months at 4°C, 21°C and 37°C

Table 5.2 shows the viability of probiotic organisms in freeze-dried yoghurt during storage (4°C, 21°C and 37°C) for 6 months. As shown in the table, cell death was lowest in the batch containing the microencapsulated probiotics at all of three incubation temperatures during storage. After one month of storage at 37ºC, only the probiotics that

were microencapsulated remained viable to 6.84 $\log_{10}$ cfu/g. By the second month the number of viable probiotics in all four batches fell below the minimum requirement of 1 million organisms per gram. The addition of RS 150 and FOS did not improve the viability of the probiotic organisms. Cell loss was slightly greater (0.05 $\log_{10}$ cfu/g) than that of the control at 4ºC and 37ºC in the batch containing free probiotic organisms and FOS. These results were consistent with those shown in Fig 4.3.2 which showed the effect of prebiotics on viability of combined probiotic organisms in freeze-dried yoghurt during three months storage at 4ºC.

Microencapsulation was found to play a significant role in maintaining viability of probiotic organisms during 6 months of storage at 21ºC in the presence of FOS. Cells were no longer viable in batches containing FOS at the same storage temperature, which did not contain a cryoprotectant or microencapsulated probiotics. After 6 months of storage at 4ºC the probiotics in all of the batches remained viable (greater than 6.0 $\log_{10}$ cfu/g). Bruno and Shah (2003) reported a similar trend in probiotic survival during storage of freeze-dried cells at 4ºC.

5.3.6 Effect of microencapsulation and coating using PLL or chitosan on the viability of combined probiotic organisms in fresh yoghurt during storage

The viability of encapsulated and coated *L. acidophilus* 33200*, L. casei* 279*, B. longum* 536 and *L. rhamnosus* GG in yoghurt during four weeks of storage at 4ºC is shown in Fig. 5.8. Calcium alginate beads coated with chitosan were the most effective in preserving the viability of probiotic organisms with cell losses of less than 0.05 $\log_{10}$ cfu/g during storage. The viability of the combined probiotic organisms was improved

using coating with PLL or chitosan by 0.03 and 0.07 $\log_{10}$ cfu/g, respectively. This improvement is possibly due to chitosan and PLL providing the anaerobic organisms with additional protection from oxygen (Talwalkar and Kailasapathy, 2004) and organic acids. Coating with chitosan best protected probiotic organisms (8.69 $\log_{10}$ cfu/g) as viability was highest in these batches. Krasaekoopt et al. (2004) found that coating calcium alginate beads with chitosan improved viability of *L. acidophilus* and *L. casei* in simulated bile salts and gastric acid.

5.3.7 Viability of combined microencapsulated probiotic organisms in freeze-dried yoghurt during storage

The viability of microencapsulated *L. acidophilus 33200, L. casei 279, B. longum 536* and *L. rhamnosus* in freeze-dried yoghurt during four months of storage at 4ºC, 21ºC and 37ºC is shown in Figs. 5.9-5.11. The viability of combined probiotic organisms was highest (8.47 $\log_{10}$ cfu/g) for beads coated with chitosan after 4 months of storage at 4ºC (Fig. 5.8). Hsiao, Lian, & Chou (2004) also found that a storage temperature of 4ºC was effective in maintaining the viability of microencapsulated *B. longum* during storage in skim milk. After 12 weeks of storage at 21ºC, the viability of probiotic organisms coated with chitosan or PLL was higher than non-coated beads by 0.4 and 0.2 $\log_{10}$/g (Fig. 9). After 12 weeks of storage at 37ºC the probiotic organisms were no longer viable (< 6.0 $\log_{10}$ CFU/g) in each of the microencapsulation treatments (Fig. 5.10). However, the probiotic organisms did remain viable for 2-4 weeks longer when coated with PLL and chitosan. Incubation temperature affected viability of probiotic organisms possibly due to

increased metabolic activity at higher temperature during storage (Bruno and Shah, 2002).

5.4 Conclusion

Concentrations greater than 2% of sodium alginate used to microencapsulate probiotic organisms did not improve the viability of probiotic organisms during freeze-drying. Either sodium citrate or phosphate buffer solution can be used to successfully sequester calcium ions from calcium alginate beads to release probiotic organisms. The survival of *L. acidophilus* 33200, *L. casei* 279, *B. longum* 536 and *L. rhamnosus* GG in yoghurt during freeze-drying was improved using microencapsulation. Microencapsulation was found to improve the viability of probiotic organisms in freeze-dried yoghurt after 6 months of storage and 4ºC and 21ºC. The emulsion technique was the most effective method for protecting probiotic organisms during freeze-drying while coating calcium alginate beads can further improve the viability of probiotic organisms during freeze-drying. However, further work investigating the encapsulation technique is needed to reduce the size of calcium alginate beads to below 1 mm to avoid consumer detection in a product such as yoghurt.

Table 5.1 Effect of microencapsulating *L. casei* 279, *L. acidophilus* 33200, *L rhamnosus* GG and *B. longum* 536 using the emulsion and extrusion techniques on the diameter of calcium alginate beads

Probiotic organism	Diameter of probiotic beads (mm) manufactured using the emulsion, extrusion and homogenised extrusion	
	Emulsion	Extrusion
L. casei 279	$0.56^a \pm 0.21$	$1.76^b \pm 0.17$
L. acidophilus 33200	$0.52^a \pm 0.14$	$1.82^b \pm 0.32$
L. rhamnosus GG	$0.46^a \pm 0.23$	$1.79^b \pm 0.18$
B. longum 536	$0.43^a \pm 0.19$	$2.06^b \pm 0.25$

The mean diameter of 15 beads recorded over three successive days (mean $\pm$ SE, *n* =3)

[ab]The mean diameters are significantly different from other observation in that same row

Table 5.2

Viability of combined probiotics *L. acidophilus* 33200, *L. casei* 279, *B. longum* 536 and *L. rhamnosus* GG (log$_{10}$ colony forming units [CFU]/g) in freeze-dried yoghurt during storage for 6 months at 4°C, 21°C and 37°C

Storage time (Months)	Log$_{10}$ colony forming units [CFU]/g		
	4°C	21°C	37°C
Batch A (Containing unprotected probiotic organisms without FOS or RS 150)			
0	8.90 ± 0.03 [a]	8.90 ± 0.03 [a]	8.90 ± 0.03 [a]
1	8.76 ± 0.03 [a]	8.65 ± 0.05 [a]	5.30 ± 0.03[b]
2	8.65 ± 0.03 [a]	7.49 ± 0.03 [a]	0 [d]
3	8.53 ± 0.01 [a]	7.27 ± 0.03 [a]	0 [d]
4	8.58 ± 0.04 [a]	7.05 ± 0.02 [a]	0 [d]
5	8.57 ± 0.03 [a]	6.78 ± 0.05 [a]	0 [d]
6	8.40 ± 0.03 [a]	6.45 ± 0.02 [a]	0 [d]
Batch B (Containing unprotected probiotic organisms with FOS)			
0	8.93 ± 0.02 [a]	8.93 ± 0.02 [a]	8.93 ± 0.02 [a]
1	8.71 ± 0.01 [a]	7.64 ± 0.02 [b]	5.25 ± 0.03 [b]
2	8.66 ± 0.01 [a]	7.41 ± 0.02 [b]	0 [d]
3	8.66 ± 0.02 [a]	6.91 ± 0.02 [b]	0 [d]
4	8.44 ± 0.02 [a]	6.45 ± 0.02 [b]	0 [d]
5	8.34 ± 0.04 [a]	6.08 ± 0.03 [b]	0 [d]
6	8.19 ± 0.02 [a]	5.63 ± 0.03 [b]	0 [d]
Batch C (Containing unprotected probiotic organisms with FOS and RS 150)			
0	8.96 ± 0.01 [a]	8.96 ± 0.01 [a]	8.96 ± 0.01 [a]
1	8.85 ± 0.02 [a]	7.60 ± 0.01 [b]	5.47 ± 0.02 [b]
2	8.79 ± 0.02 [a]	7.48 ± 0.01 [b]	0 [d]
3	8.65 ± 0.02 [a]	6.99 ± 0.02 [b]	0 [d]
4	8.63 ± 0.03 [a]	6.65 ± 0.02 [b]	0 [d]
5	8.61 ± 0.02 [a]	6.42 ± 0.01 [b]	0 [d]
6	8.40 ± 0.03 [a]	6.19 ± 0.04 [b]	0 [d]
Batch D (Containing microencapsulated probiotic organisms with FOS and RS 150)			
0	8.93 ± 0.01 [a]	8.93 ± 0.01 [a]	8.93 ± 0.1 [a]
1	8.94 ± 0.02 [a]	8.83 ± 0.03 [a]	6.84 ± 0.4 [b]
2	8.91 ± 0.02 [a]	8.10 ± 0.02 [b]	5.46 ± 0.2 [b]
3	8.86 ± 0.03 [a]	7.85 ± 0.04 [b]	2.78 ± 1.3 [c]
4	8.85 ± 0.02 [a]	7.45 ± 0.01 [b]	0 [d]
5	8.78 ± 0.03 [a]	7.07 ± 0.02 [b]	0 [d]
6	8.67 ± 0.03 [a]	6.79 ± 0.04 [b]	0 [d]

Measurements are means ± standard error of 3 replicates for each individual strain
[a b c d e]Means that are significantly different from others ($p > 0.05$)

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┌─────────────────────────────────────────────┐
│  Reconstitution of skim milk powder to prepare │
│  reconstituted milk containing 14% total solids │
└─────────────────────────────────────────────┘
                        │
                        ▼
┌─────────────────────────────────────────────┐
│         Heat treatment (85°C/30 min)          │
└─────────────────────────────────────────────┘
                        │
                        ▼
┌─────────────────────────────────────────────┐
│   Addition of 1.5% RS 150[a] and 1.5% FOS [b]  │
└─────────────────────────────────────────────┘
                        │
                        ▼
┌─────────────────────────────────────────────┐
│    Cooling to incubation temperature (43°C)    │
└─────────────────────────────────────────────┘
                        │
                        ▼
┌─────────────────────────────────────────────┐
│  Addition of 4% probiotics including *L. acidophilus* │
│  33200, *L. casei* 279, *B. longum* 536 and *L.* │
│                *rhamnosus* GG                  │
└─────────────────────────────────────────────┘
                        │
                        ▼
┌─────────────────────────────────────────────┐
│  Addition of the 2% yoghurt starter organisms, *S.* │
│  *thermophilus* and *L. delbrueckii* ssp. *bulgaricus* │
└─────────────────────────────────────────────┘
                        │
                        ▼
┌─────────────────────────────────────────────┐
│             Filling into containers            │
└─────────────────────────────────────────────┘
                        │
                        ▼
┌─────────────────────────────────────────────┐
│      Incubation at 43°C until pH reaches 4.5   │
└─────────────────────────────────────────────┘
                        │
                        ▼
┌─────────────────────────────────────────────┐
│         Refrigeration for 12 h at 4°C          │
└─────────────────────────────────────────────┘
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Fig. 5.1 Yoghurt manufacture
[a]RS 150 was only added in batches C and D
[b]FOS was only added in batches B, C and D
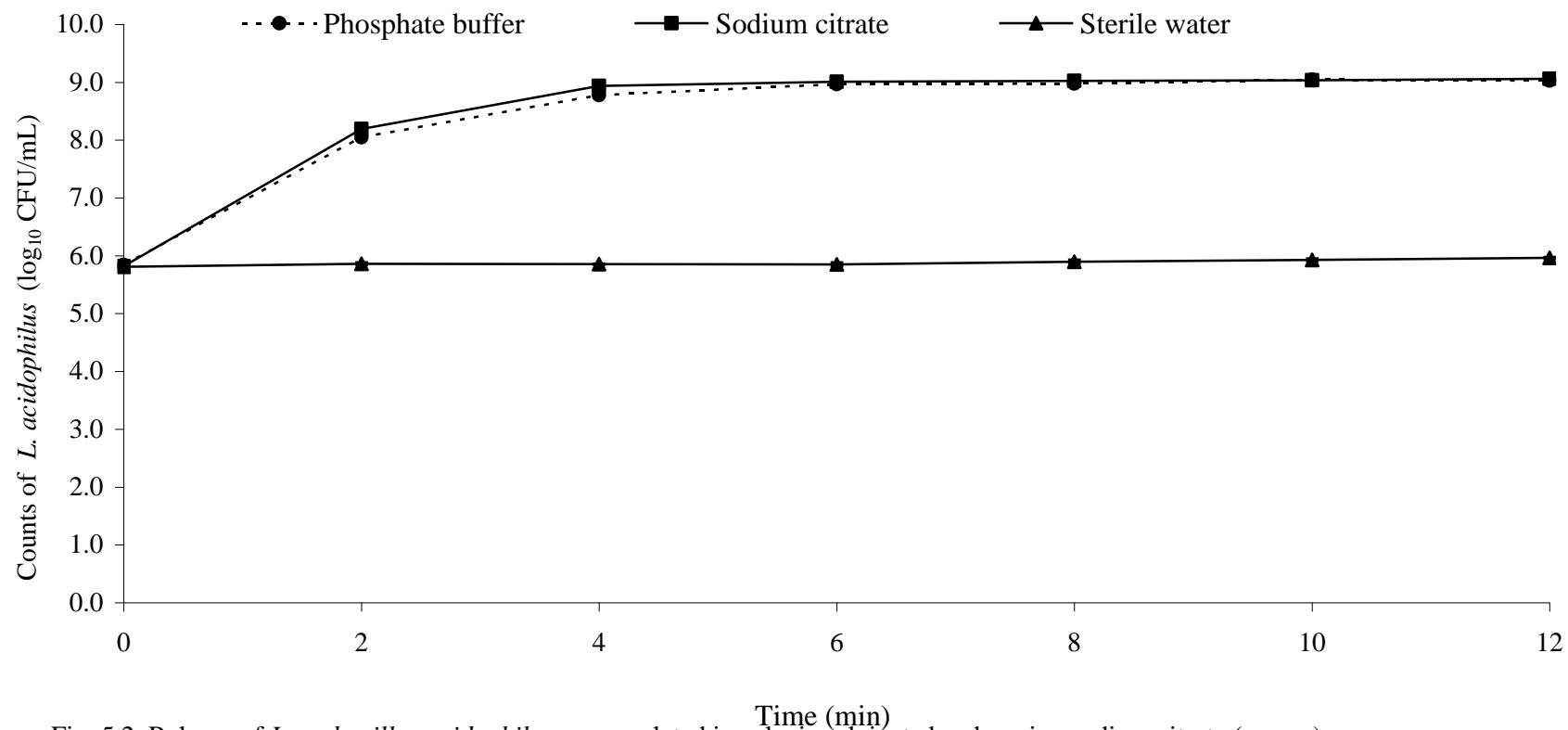
Fig. 5.2 Release of *Lactobacillus acidophilus* encapsulated in calcuim-alginate beads, using sodium citrate (—■—) phosphate buffer (---●---) and sterile water (—▲—). Results are expressed as mean ± SE, n= 3
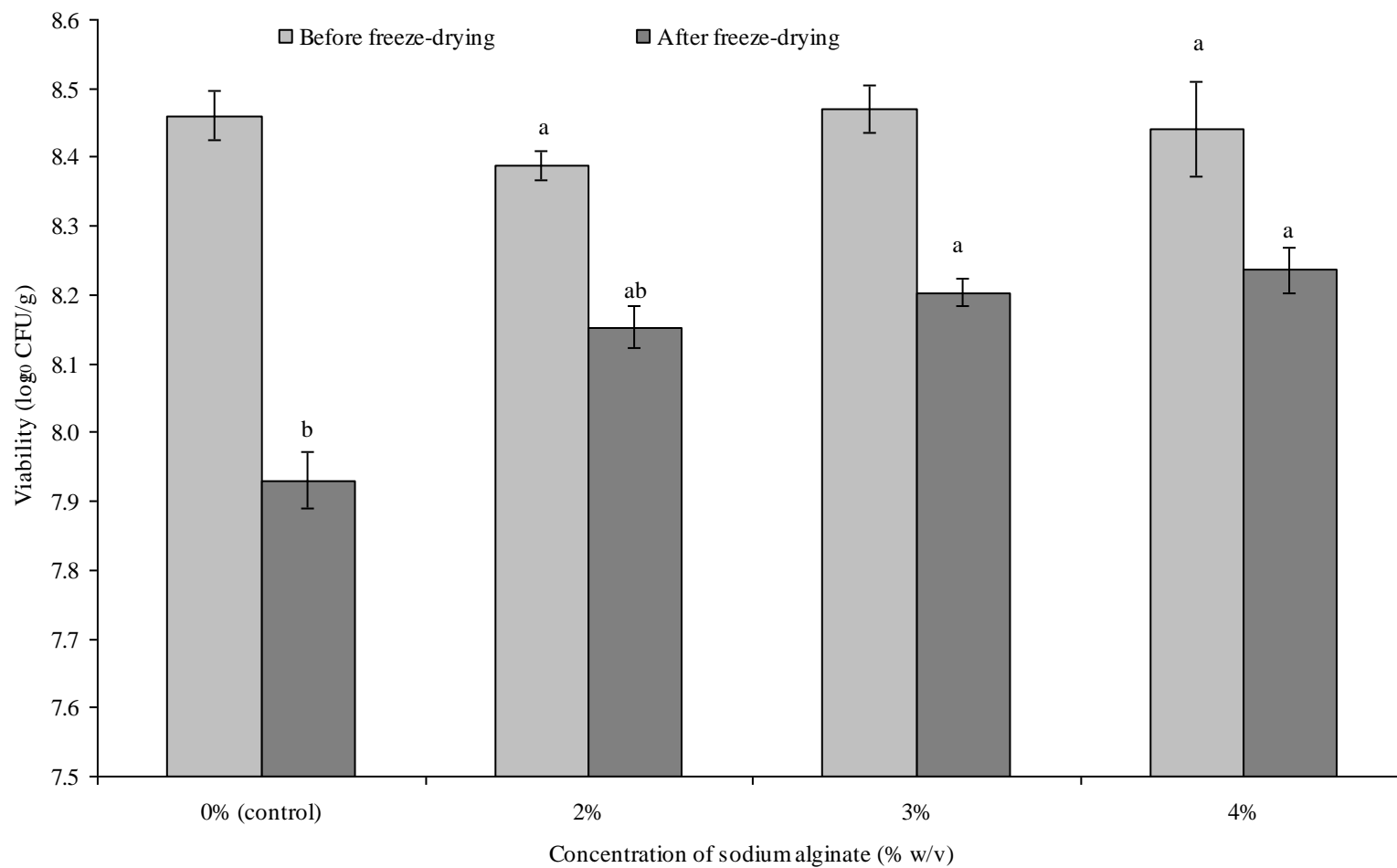
Fig. 5.3 Effect of sodium alginate concentration on the viability of combined *L. acidophilus 33200, L. casei 279, B. longum 536* and *L. rhamnosus* GG within calcium alginate beads during freeze-drying (mean ± SE, n = 3).
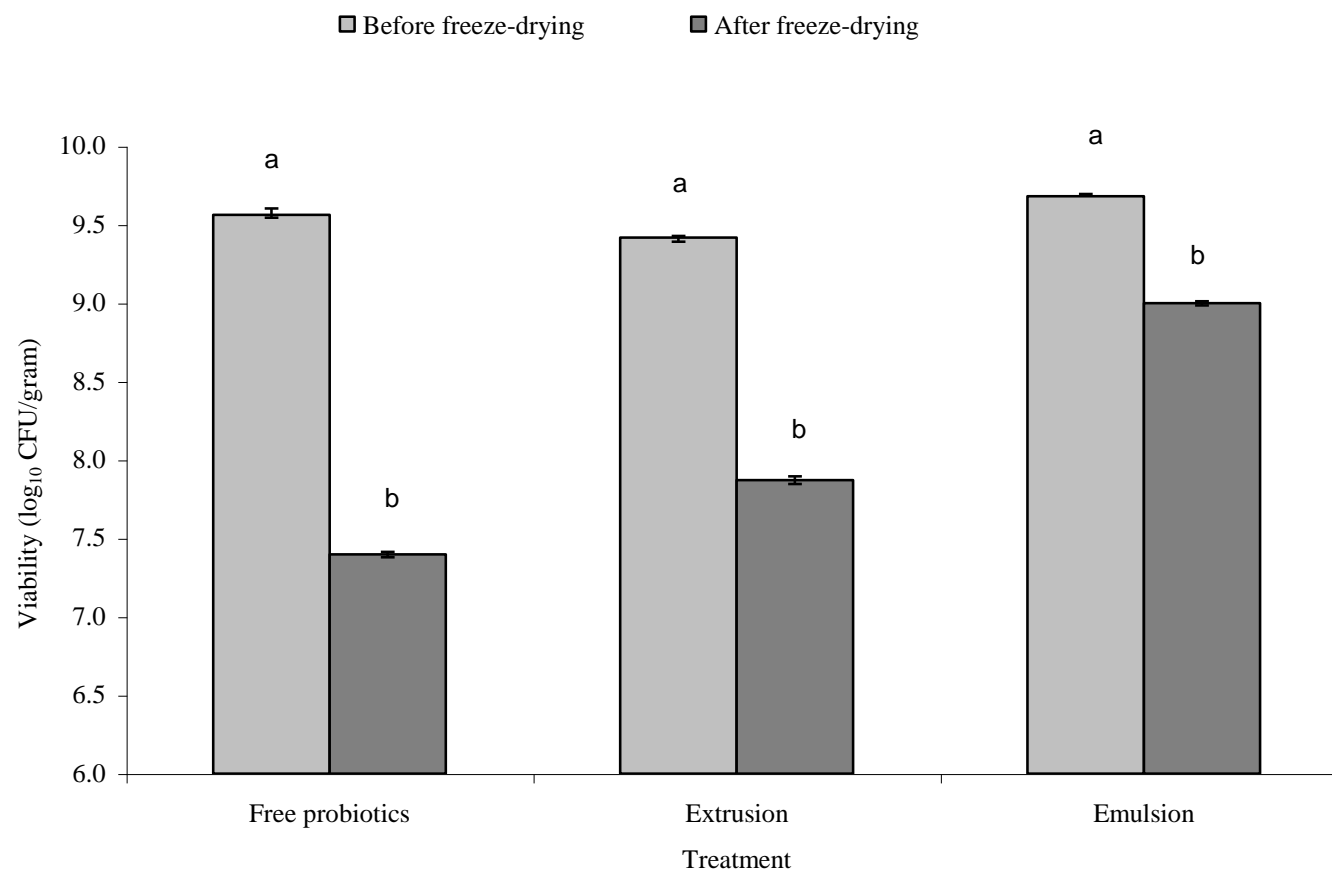
Fig.5.4 Effect of microencapsulation using the emulsion, extrusion and homogenised extrusion technique on the viability of *L. acidophilus* 33200 during freeze-drying (mean ± SE, n = 3).
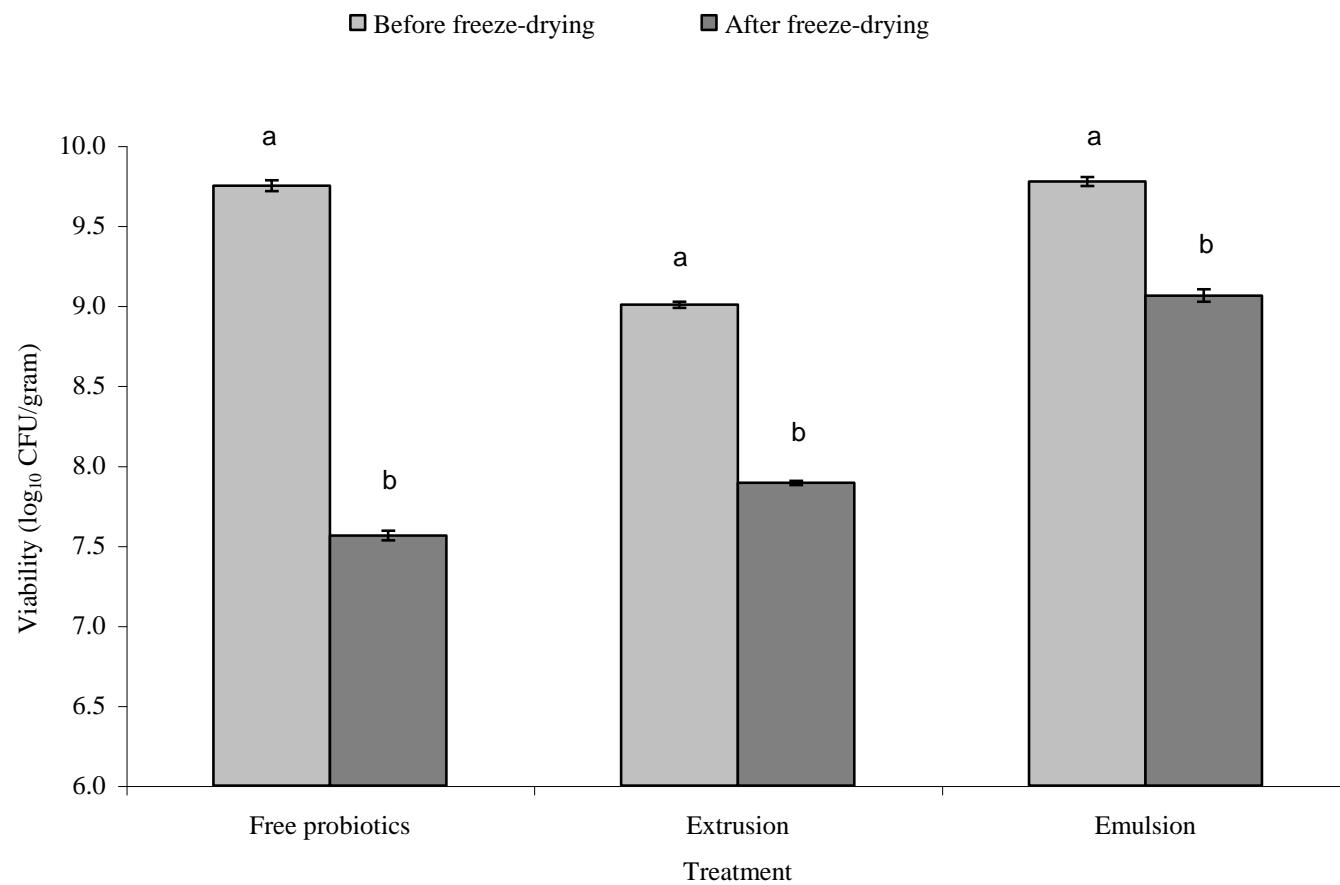
Fig. 5.5 Effect of microencapsulation using the emulsion and extrusion technique on the viability of *L. casei* 279 during freeze-drying (mean ± SE, n = 3).
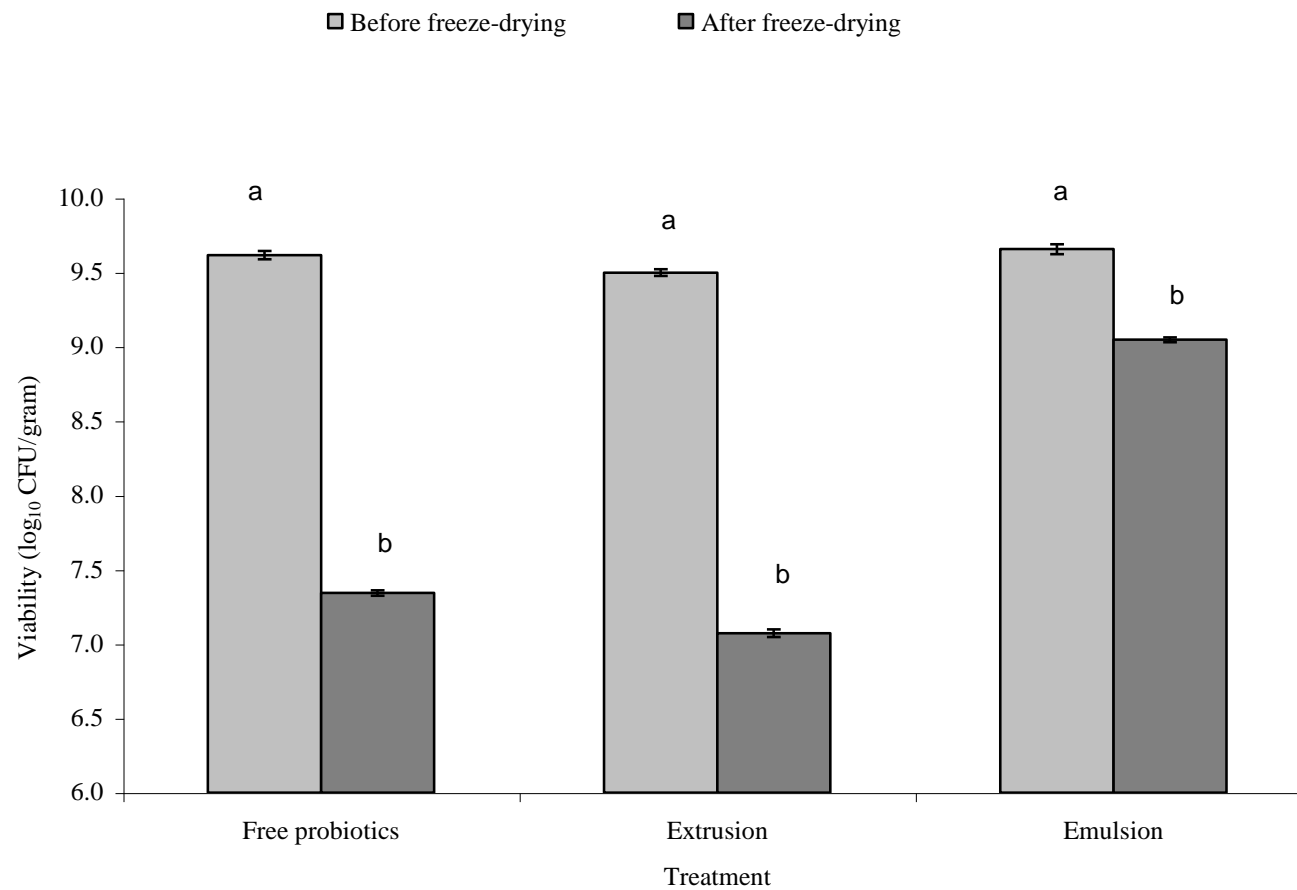
Fig. 5.6 Effect of microencapsulation using the emulsion and extrusion technique on the viability of *B. longum* 536 during freeze-drying (mean ± SE, n = 3).
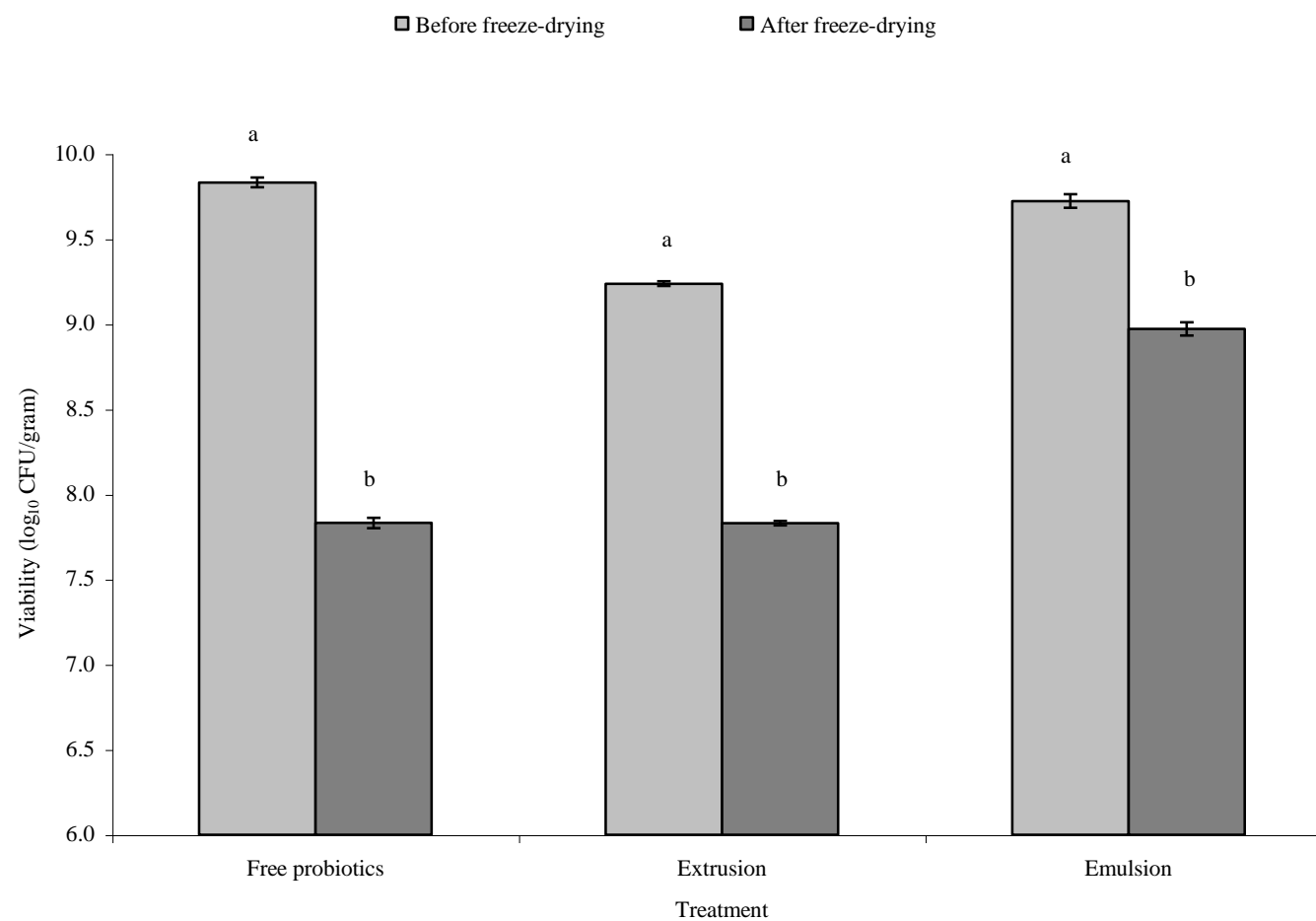
Fig. 5.7 Effect of microencapsulation using the emulsion and extrusion technique on the viability of *L. rhamnosus* GG during freeze-drying (mean ± SE, n = 3).
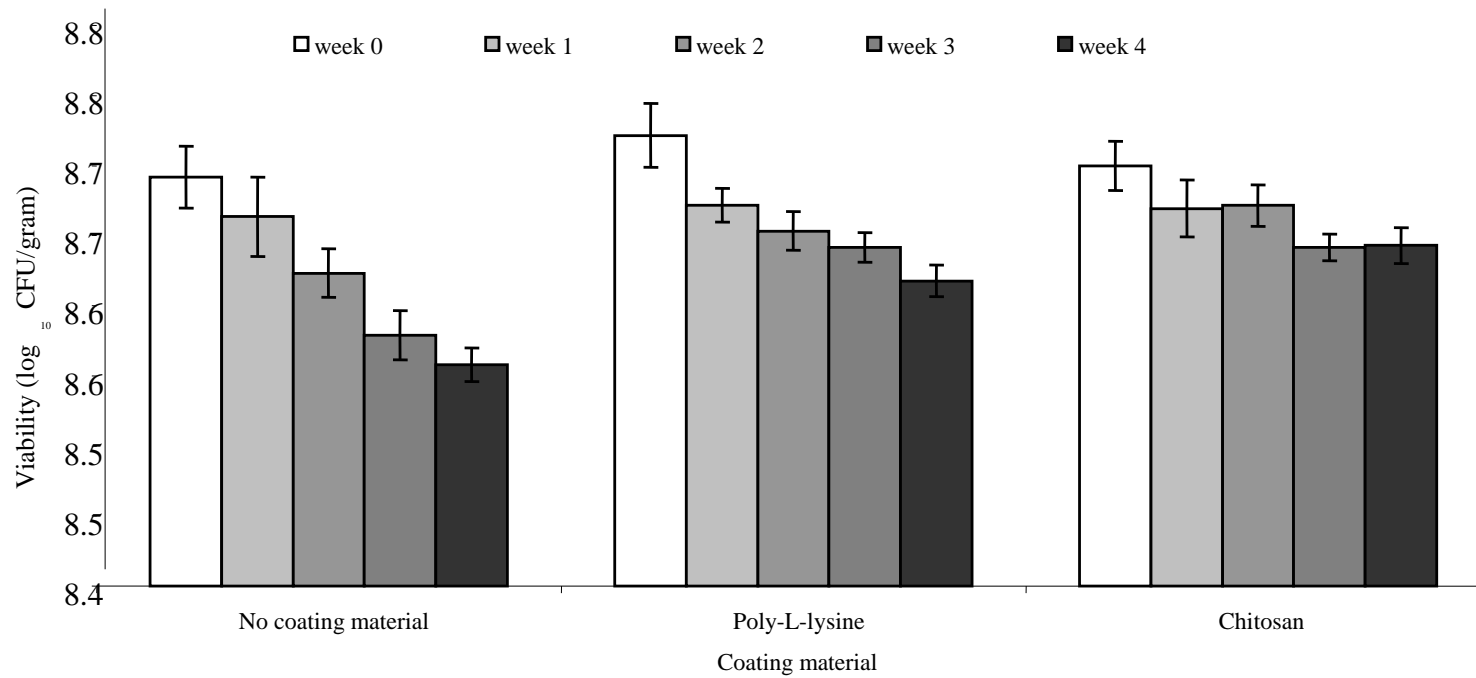
Fig. 5.8 Viability of combined *L. acidophilus* 33200, *L. casei,* 279, *L. rhamnosus* GG and *B. longum* 536 coated with poly-L-lysine and chitosan in yoghurt stored at 4°C during four weeks of storage (mean ± SE n = 3)
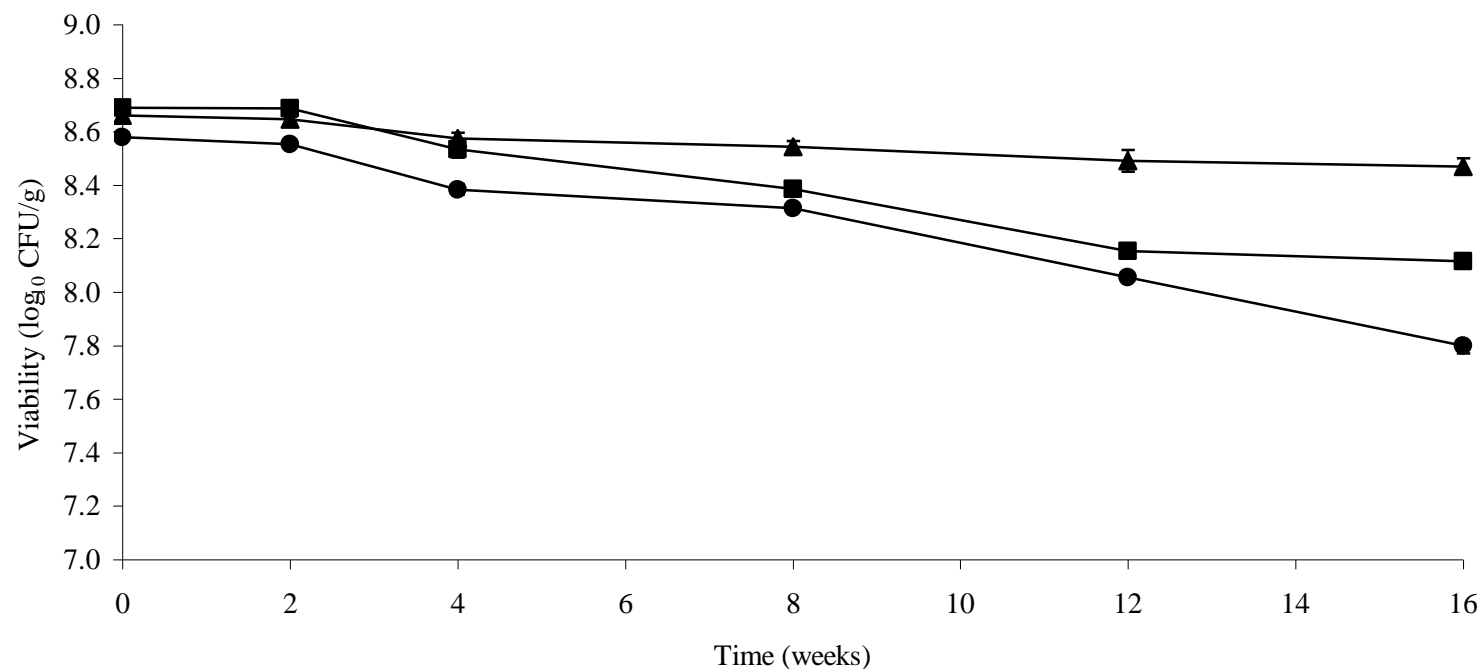
Fig. 5.9. Viability of microencapsulated probiotic organisms in freeze-dried yoghurt during storage at 4ºC. ▲ Calcium-alginate (CA) beads co-ecapsulated with chitosan, ■ CA beads co-encapsulated with poly-L-lysine, ● uncoated beads (mean ± SE, n = 3).
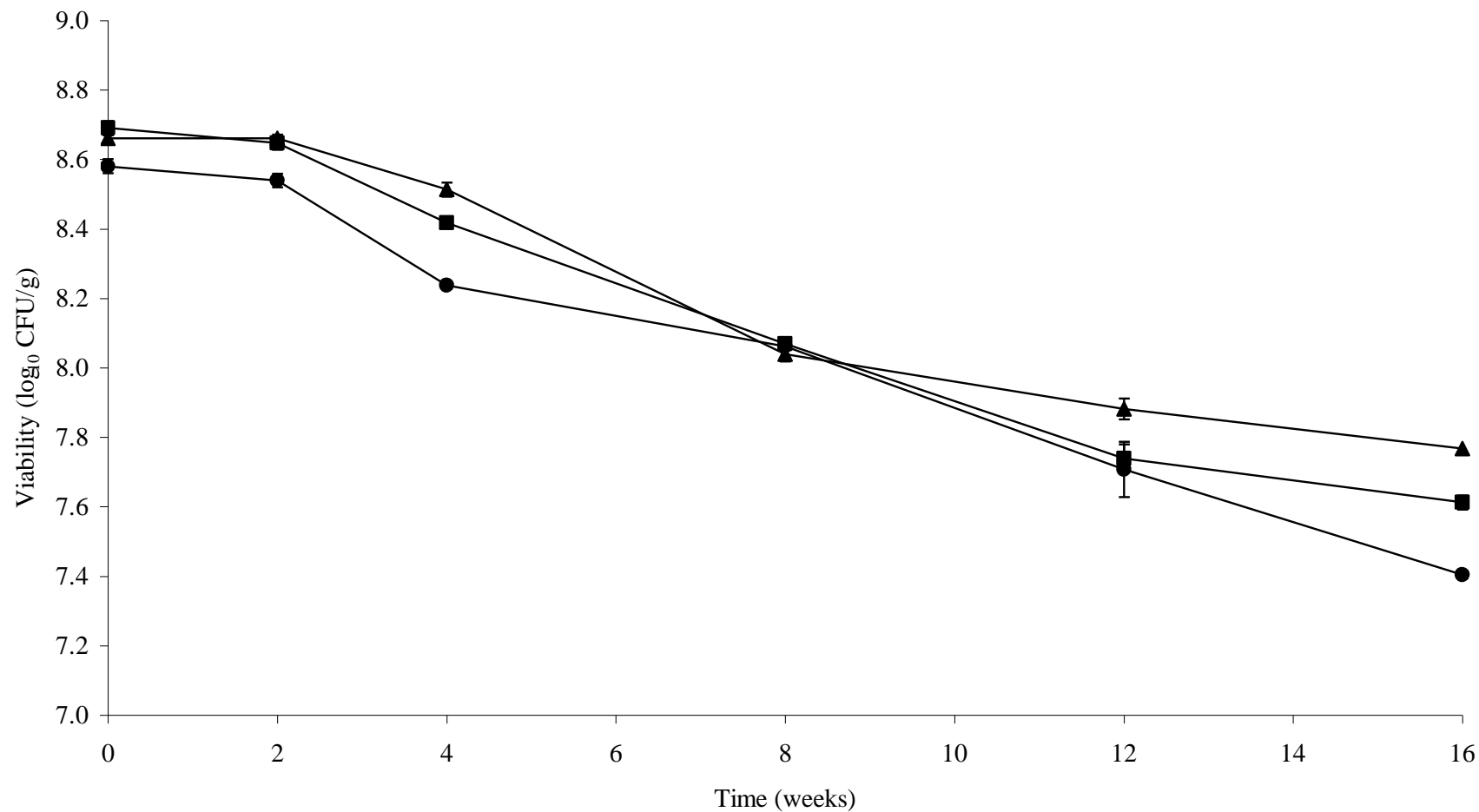
Fig. 5.10. Viability of microencapsulated probiotic organisms in freeze-dried yoghurt during storage at 21ºC. ▲ Calcium-alginate (CA) beads co-ecapsulated with chitosan, ■ CA beads co-encapsulated with poly-L-lysine, ● Uncoated beads (mean ± SE, n = 3).
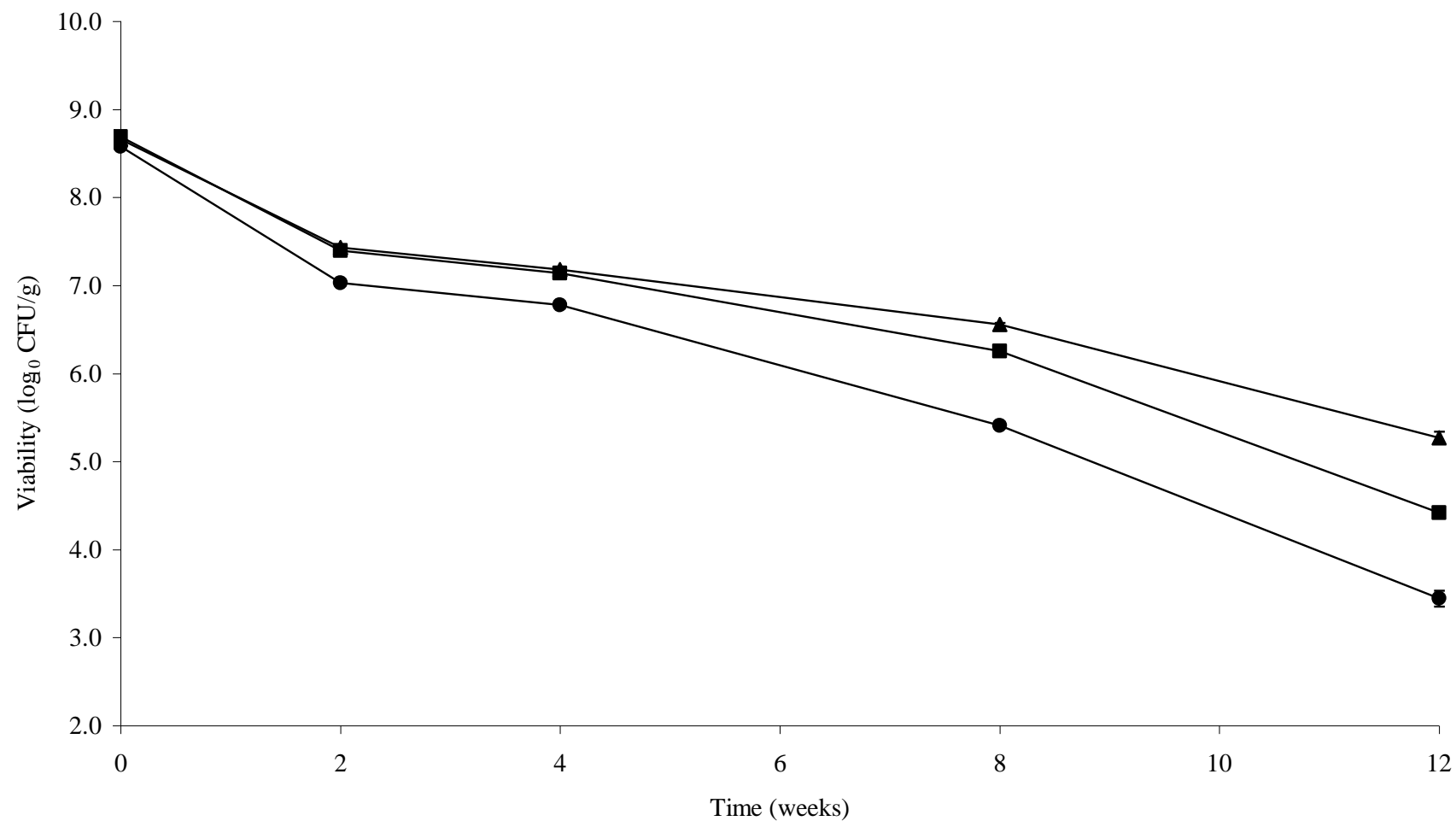
Fig. 5.11. Viability of microencapsulated probiotic organisms in freeze-dried yoghurt during storage at 37ºC. ▲ Calcium-alginate (CA) beads co-ecapsulated with chitosan, ■ CA beads co-encapsulated with poly-L-lysine, ● Uncoated beads (mean ± SE, n = 3).

CHAPTER 6

EFFECT OF HOMOGENISATION ON BEAD SIZE AND VIABILITY OF ENCAPSULATED *L. ACIDOPHILUS* 33200, *L. CASEI* 279, *B. LONGUM* 536 AND *L. RHAMNOSUS* GG

6. 1 Introduction

Microencapsulation of probiotic organisms using a non-toxic encapsulating agent such as sodium alginate is effective for protecting probiotic organisms from low pH and high oxygen environments (Talwalkar and Kailasapathy, 2003) such as those in yoghurt and frozen fermented desserts (Shah and Ravula, 2000). Microencapsulation is also effective for protecting probiotic organisms from acid and bile salts encountered during metabolisation (Chandramoui et al., 2004). However, the population of probiotic organisms may be affected during the microencapsulation process itself. Studies (Audet et al., 1998; Arnaud et al., 1992) have also found that encapsulation techniques such as the emulsion technique are conducive to forming large beads that can influence the texture and mouthfeel of products such as yoghurt.

The optimal diameter of calcium alginate beads range between 15 and 100 µm. Calcium alginate beads greater than 100 µm are detectable in the mouth, while beads less than 15 µm may not provide sufficient protection from external stress. Techniques employing spray guns (Lee et al., 2003) and air atomizers (Kwok et al., 1991) with varying nozzle sizes have been used to reduce bead size, as the emulsion technique has been found to produce exceedingly large spherical, beads with diameters ranging from 200 - 1000 µm (Poncelet et al., 1992).

The emulsion technique involved combining a mixture of probiotic organisms and sodium alginate in vegetable oil. A water-in-oil emulsion is formed by agitation, usually with a magnetic stirrer. Work has been carried out to produce uniform beads with reduced size by altering the speed, adjusting the concentration of sodium alginate and adding an emulsifier such as tween 80 (Shah and Ravula, 2000). Bead size may be further reduced by using a homogeniser to form a finer water-in-oil emulsion.

High speed mixers are most commonly used for homogenising oil and aqueous phases in the food industry. The oil and water are directly agitated by a mixing head which rotates at approximately 3600 rpm. During homogenisation, the interface between the oil and water is disrupted causing the liquids to blend together. Various mixing heads can be attached to high speed mixers to reduce droplet size by generating intense disruptive forces. High-pressure valve homogenisers create small droplets by forcing liquid through a narrow valve under high pressure. A mixture can be passed through a high-pressure valve homogeniser repeatedly to achieve the desired droplet size (McClements, 1999).

Variations in the size of calcium alginate are caused by polydisperse emulsions. Microscopic polydisperse emulsions may not be visible the naked eye. A Mastersizer™ 2000 can be used to measure particles ranging between 0.02 µm and 2000 µm by passing the particles through a laser beam (www.malvern.co.uk). A particle size distribution diagram is generated which illustrates the range of calcium alginate beads. However, due to the sensitivity of a Mastersizer™, probiotic organisms may be recorded as particles which may influence the particle size distribution.

It is essential to ensure that the selected microencapsulation technique is gentle to sensitive probiotic organisms and is effective in delivering viable organisms to the colon. The effect of homogenising sodium alginate (containing probiotic organisms) oil using high speed mixers and high pressure valve homogenisers in order to reduce the size of calcium alginate beads, has not been thoroughly investigated. The aim of this study was to determine the effect of homogenisation on the uniformity and diameter of calcium alginate beads and to investigate its effects on the survival of encapsulated *L. acidophilus* 33200, *L. casei* 279, *B. longum* 536 and *L. rhamnosus* GG.

6.2 Materials and Method

6.2.1 Preparation of probiotic bacteria

B. longum 536 was obtained from the Victoria University Culture Collection (Werribee, Victoria, Australia). The organism was originally obtained from Morinaga Milk Industry Co. Ltd. (Tokyo, Japan), *L. casei* 279 was obtained from the Australian Starter Culture Collection (Werribee, Victoria, Australia). *L. acidophilus* 33200 was obtained from the American Type Culture Collection (Manassas, VA, USA), and *L. rhamnosus* GG was isolated from a commercial product. Each of the organisms was grown in sterile MRS (deMann, Rogosa, Sharp) broth (Oxoid Ltd., Hampshire, UK) using a 1% inoculum. *B. longum* 536 was supplemented with filter sterilized 0.05% (w/v) L·cysteine hydrochloride (Sigma Chemical Co., Castle Hill, Sydney, Australia) to create an anaerobic environment. Each culture was grown and propagated three times successively for activation at 37°C for 12-15 h. Probiotic organisms were harvested by

centrifuging (Sorvall RT7 Newtown, CT, USA) at $1510 \times g$ at 4°C for 15 min and the cell pellet was suspended in saline solution followed by microencapsulation.

6.2.1.2 Microencapsulation of probiotic organism using the emulsion technique

The emulsion method of microencapsulation was used to encapsulate probiotics. Briefly, 120 mL of sterile 3% (v/w) sodium alginate was mixed with 30 mL of suspension containing 9.0-10.0 $\log_{10}$ cfu/g of combined *L. acidophilus* 33200, *L. casei* 279 *B. longum* 536 and *L. rhamnosus* GG. Fifty millilitres of the suspension of sodium alginate and probiotic organisms was gently dispensed using a pipette into a beaker containing 200 mL of vegetable oil (Eta Blended Vegetable Oil, Goodman Fielder Pty. Ltd., Melbourne, Australia) and stirred at 200 rpm using a magnetic stirrer (IEC Industrial Equipment & Control Pty. Ltd., Melbourne, Australia). Calcium chloride (0.1 M) was gently added to the side of the beaker until the emulsion was broken (Shah and Ravula, 2000)

6.2.1.3 Microencapsulation of L. acidophilus 33200, L. casei 279, B. longum 536 and L. rhamnosus GG using homogenisation (Ultra-Turrax benchtop, Avestin Inc. Piston or Silverson mixer)

The sodium alginate solution and probiotic organisms were combined with vegetable oil as described in 6.2.1.2. An emulsion was formed by homogenisation using the Ultra-Turrax benchtop homogeniser (Ika laboratory and Analytical Equipment, Staufen, Germany), Avestin Inc. piston homogeniser (Avestin, Ottawa, Canada) or Silverson mixer (Silverson Machine Ltd., Waterside, Chesham Bucks, England) by

homogenising the mixture at various speed, pressures, passes and times as described below.

To prepare alginate beads using the Ultra-Turrax benchtop homogeniser, an emulsion was formed by homogenising 200 mL mixtures (as described in 6.2.1.2) at 8,000 rpm and 13,500 rpm for 2 and 4 min. To prepare alginate beads using the Avestin Inc. piston homogeniser an emulsion was formed by passing the mixture (as described in 6.2.1.2) through the homogeniser 2 and 3 times at 50 and 100 bar of pressure. Calcium alginate beads using the Silverson mixer were formed by homogenising 200 mL of the mixtures (described in 6.2.1.2) for 2 and 4 min.

After each of the homogenisation treatments, each emulsion was transferred into a beaker. Calcium chloride (0.1M) was gently added to the side of the beaker to the emulsions while stirring using a magnetic stirrer at 200 rpm for 10 min until the emulsion was broken. Small calcium alginate beads were formed and subsequently measured using a Mastersizer™ (Hydro-2000G Malvern Instruments Limited, Worchester, UK).

6.2.1.4 Measurement of particle size of calcium alginate beads

Calcium chloride solution (0.1M) containing 1-5 g of calcium alginate beads was passed through a Mastersizer™ Hydro-2000G to measure the size of the beads. Samples were added to circulating filtered water until laser obscuration exceeded 10%. The mean particle size was expressed as d_{32} which represents the area-volume mean diameter.

6.2.2 Enumeration of L. acidophilus 33200, L. casei 279, B. longum 536 and L. rhamnosus GG within calcium alginate beads

The entrapped probiotic organisms were released from the calcium alginate beads by sequestering calcium ions with phosphate buffer at neutral pH. Once released, probiotic organisms were selectively enumerated using the techniques of Tharmaraj and Shah (2003). *L. acidophilus* was selectively enumerated on MRS-sorbitol agar using anaerobic incubation at 37°C for 72 h. *L. casei* was enumerated on LC agar using anaerobic incubation at 37°C for 72 h. *B. longum* was selectively enumerated on MRS-NNLP (nalidixic acid, neomycin sulphate, lithium chloride and paromomycin sulphate) agar incubated at 37°C for 72 h and *L. rhamnosus GG* was enumerated on MRS-vancomycin agar using anaerobic incubation at 43°C for 72 h.

6.3 Results and Discussion

6.3.1 Effect of homogenisation on bead size

The effect of homogenisation on the size of calcium alginate beads is shown in Table 6.1. The smallest beads were formed using the Ultra-Turrax benchtop homogeniser at 13,500 rpm for 4 min with modal diameters of 56.5 µm and area-volume mean diameters (d_{32}) of 39.2 µm. A homogenisation speed at 13,500 rpm was better than that at 8,000 rpm in reducing the size of calcium alginate beads. Increasing the homogenisation time from 2 to 4 min was also effective in further reducing the size of calcium alginate beads.

The largest beads were formed using an emulsion technique with modal diameters of 844 µm and d_{32} of 426 µm (Table 6.1). This was possibly due to the coarse emulsion

created using agitation with the magnetic stirrer. The particles formed using the emulsion technique are large enough to cause a gritty texture in products such as yoghurt, which may affect overall acceptability.

The calcium alginate particle size distributions are illustrated in Fig. 6.1-6.6. Beads which were formed using the traditional emulsion technique (Fig. 6.1) displayed the larges span of particle size as represented by the wide peaks. The wide span of the peaks represents the lack of uniformity in diameters of the calcium alginate beads. The narrowest spans were recorded for beads produced using the Ultra-Turrax and Silverson mixer (Fig 6.2-6.4). Variations in the diameters of these beads may have resulted from the polydisperse emulsion that was formed during homogenisation (McClements, 1999). Small particle size was also obtained using the Avestin Inc. Piston homogeniser (Fig. 6.5-6.6). This was possibly due to the effect of high pressure on the size of particles formed during the emulsifying stage. However, high pressure may have a deleterious effect on the counts of each of the probiotics organisms as described below in 6.3.2 (Ulmer et al., 2000).

6.3.2 Effect of homogenisation technique on the survival of encapsulated probiotic organisms.

The effect of homogenisation using Ultra-Turrax benchtop, Avestin Inc. Piston or Silverson mixer on survival of *L. acidophilus 33200, L. casei 279, B. longum 536* and *L. rhamnosus* GG is shown in Fig. 6.7-6.10. The counts of *L. acidophilus* 33200 were 0.40 log $\log_{10}$ cfu/g greater than the control using the Ultra-Turrax benchtop homogeniser at 8000 rpm for 2 min (Fig. 5.7), while the counts of *L. casei* 279 were 0.35 $\log_{10}$ cfu/g

greater than the control following the same treatment (Fig. 6.10). The counts for *L. rhamnosus* GG (9.12 $\log_{10}$ cfu/g) were slightly lower then the control (9.29 $\log_{10}$ cfu/g) using the Ultra-Turrax benchtop homogeniser at 8000 rpm for 2 min (Fig. 6.8). However, the initial counts for *L. rhamnosus* GG were higher than other organisms which may have influenced this result. The Ultra-Turrax and the Avestin Inc. piston homogeniser had the least impact upon the counts of probiotic organisms.

The population of *B. longum* 536 was 1.6 $\log_{10}$ cfu/g less than the control using the Silverson mixer. The Silverson mixer appeared to be the most severe treatment as it had the worst impact upon the counts of each of the probiotic organisms. The reduction in viability using Silverson mixer was possibly due to heat generated during homogenisation. Mechanical energy created during homogenisation is converted to heat energy due to the viscosity of the sodium alginate solution (McClements, 1999). Homogenisation using the Silverson mixer may have also incorporated air into the mixture, thus affecting oxygen sensitive probiotic organisms such as *B. longum* 536 (Talwalkar and Kailasapathy, 2003). Fig. 6.11 shows and image of a calcium alginate bead created using the emulsion technique.

6.4 Conclusion

It was possible to reduce the bead size below 100 µM using the Ultra-Turrax homogeniser. The Ultra-Turrax homogeniser was the most suitable for preparing calcium alginate beads with a narrow range of diameter. The population of *L. acidophilus 33200, L. casei 279, B. longum 536* and *L. rhamnosus* GG was not adversely affected during homogenisation using Ultra-Turrax benchtop and Avestin Inc homogenisers. However,

homogenisation using a Silverson mixer had a negative impact upon counts and was not suitable when preparing calcium alginate beads. However, microencapsulating probiotic organisms using these techniques was laborious and time consuming. Therefore, further research is required to develop an automated technique for mass production of calcium alginate beads in a continuous large scale operation.

Table 6.1. Effect of homogenisation with Ultra-Turrax T 25, Avestin Inc. Piston and Silverson mixer on the size (µm) of calcium alginate beads

Treatment	d (0.1) µm [a]	d (0.5) µm [a]	d (0.9) µm [a]	Span[a]	Mode µm[a]	d_{32} µm [ab]
Control	245.3 ± 6.9	733.1 ± 50.1	1375.5 ± 51.65	1.54 ± 0.03	843.8 ± 69.2	426.0 ± 7.0
Ultra-Turrax benchtop homogeniser 8000 rpm for 2 min	27.9 ± 11.0	83.0 ± 42.6	248.0 ± 154.25	2.40 ± 0.49	82.3 ± 40.8	56.9 ± 24.6
Ultra-Turrax benchtop homogeniser 8000 rpm for 4 min	36.8 ± 7.4	137.7 ± 24.6	506.6 ± 173.5	3.30 ± 0.61	127.5 ± 10.4	82.4 ± 15.6
Ultra-Turrax benchtop homogeniser 13500 rpm for 2 min	25.2 ± 2.7	83.2 ± 9.8	360.7 ± 80.88	3.98 ± 0.47	65.3 ± 2.4	52.6 ± 7.7
Ultra-Turrax benchtop homogeniser 13500 rpm for 4 min	18.6 ± 2.5	57.5 ± 12.2	209.1 ± 4.9	3.50 ± 0.87	56.5 ± 22.5	39.2 ± 4.6
Silverson Mixer for 2 min	17.8 ± 0.6	81.9 ± 17.8	224.2 ± 50.29	2.51 ± 0.06	109.3 ± 30.7	41.6 ± 4.2
Silverson Mixer for 4 min	17.6 ± 2.7	102.6 ± 2.0	290.9 ± 0.63	2.66 ± 0.07	164.8 ± 3.8	42.9 ± 4.9
Avestin Inc. Piston Homogeniser 50 bar ✕ 2 passes	47.4 ± 2.8	146.8 ± 12.5	330.6 ± 45.0	1.92 ± 0.12	166.1 ± 10.3	93.1 ± 4.9
Avestin Inc. Piston Homogeniser 50 bar × 3 passes	115.3 ± 32.6	278.2 ± 77.6	519.8 ± 132.3	1.47 ± 0.05	307.9 ± 86.7	191.6 ± 51.9
Avestin Inc. Piston Homogeniser 100 bar × 2 passes	92.4 ± 16.7	198.0 ± 26.7	444.4 ± 82.82	1.77 ± 0.10	195.0 ± 19.7	151.6 ± 25.6
Avestin Inc. Piston Homogeniser 100 bar ✕ 3 passes	60.4 ± 10.5	146.7 ± 13.0	296.9 ± 13.39	1.62 ± 0.12	158.7 ± 12.7	102.4 ± 11.2

[a]The mean of 2 determinations recorded over successive treatments ± SE

[b]Mean particle size was expressed as d_{32} which represents the area-volume mean diameter
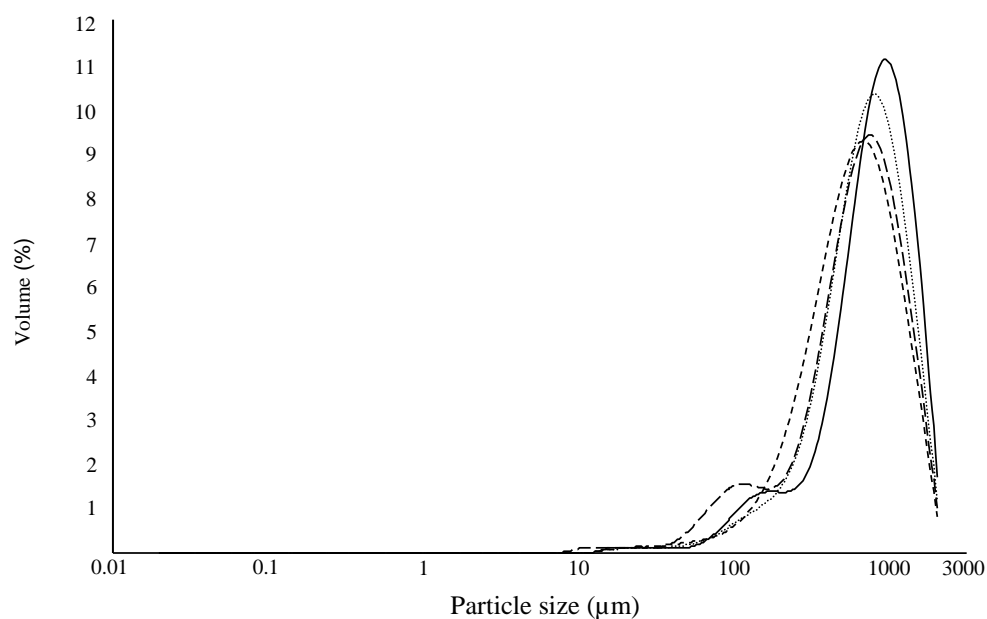
Fig. 6.1 Particle size distribution of calcium alginate beads prepared using the traditional emulsion microencapsulating technique ─ Control 'a', -----Control 'b', ── Control 'c', ···· Control 'd'
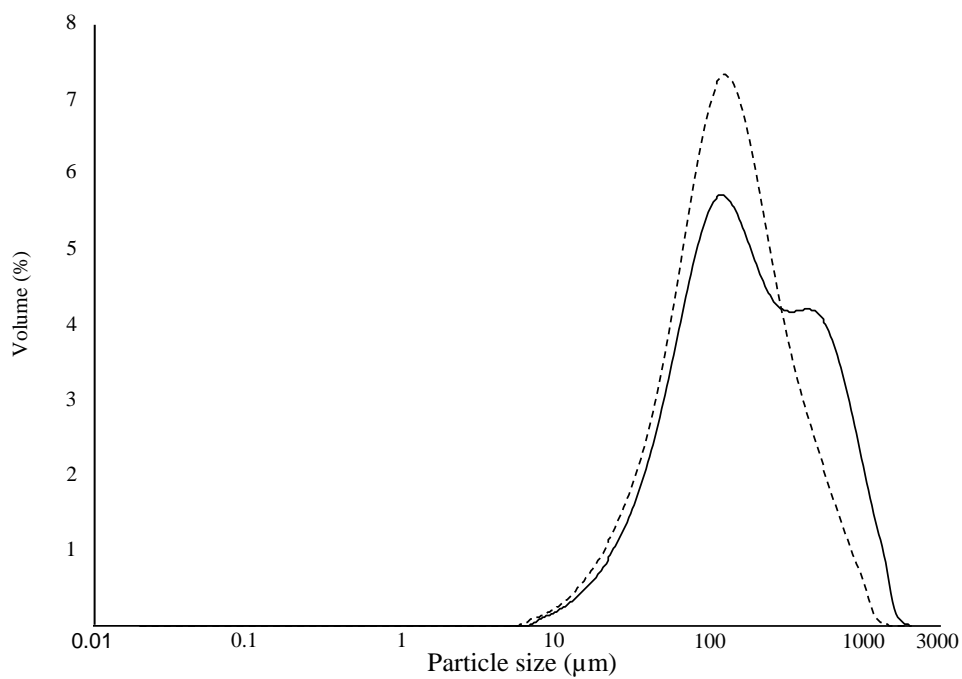
Fig. 6.2 Particle size of calcium alginate beads prepared using the Ultra-Turrax T 25 benchtop homogeniser at 8,000 rpm for 2 and 4 min, ─ 8,000 rpm for 2 min, --- 13,500 rpm for 4 min
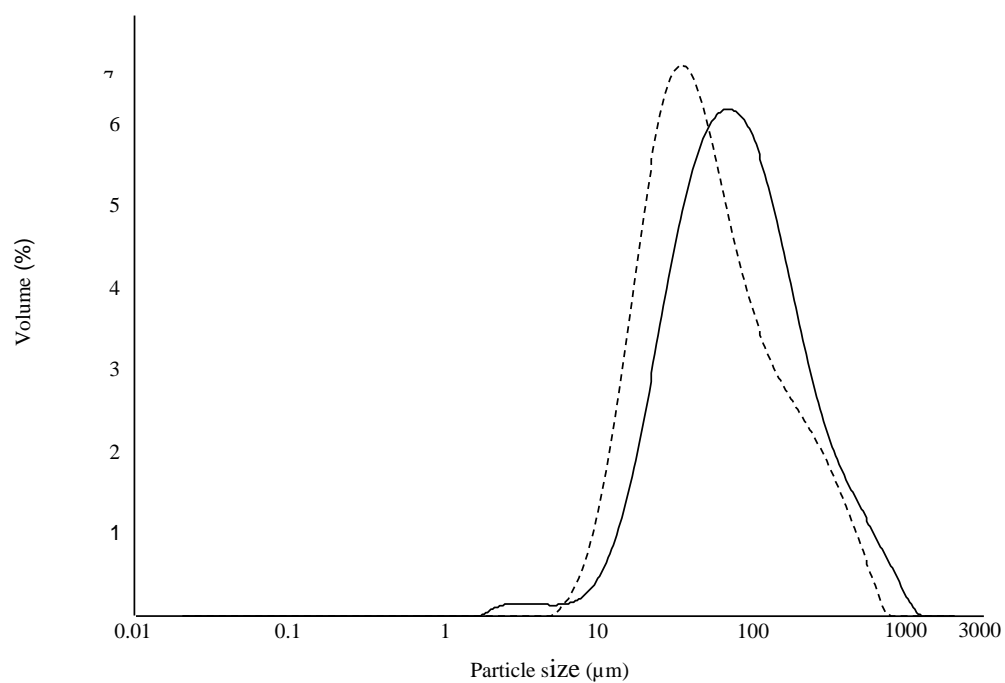
Fig. 6.3 Particle size of calcium alginate beads prepared using the Ultra-Turrax T 25 benchtop homogeniser at 13,500 rpm for 2 and 4 min, ─ 13,500 rpm for 2 min, ---13,500 rpm for 4 min.
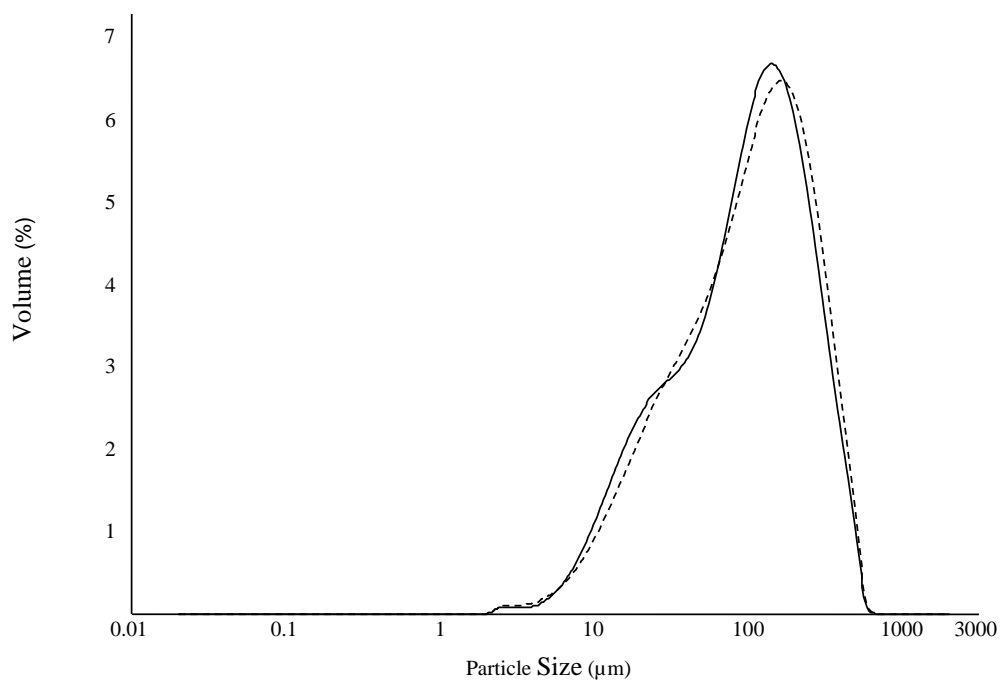
Fig. 6.4 Particle size of calcium alginate beads prepared using a Silverson Mixer for 2 and 4 min. ─ 2 min, ----4 min.
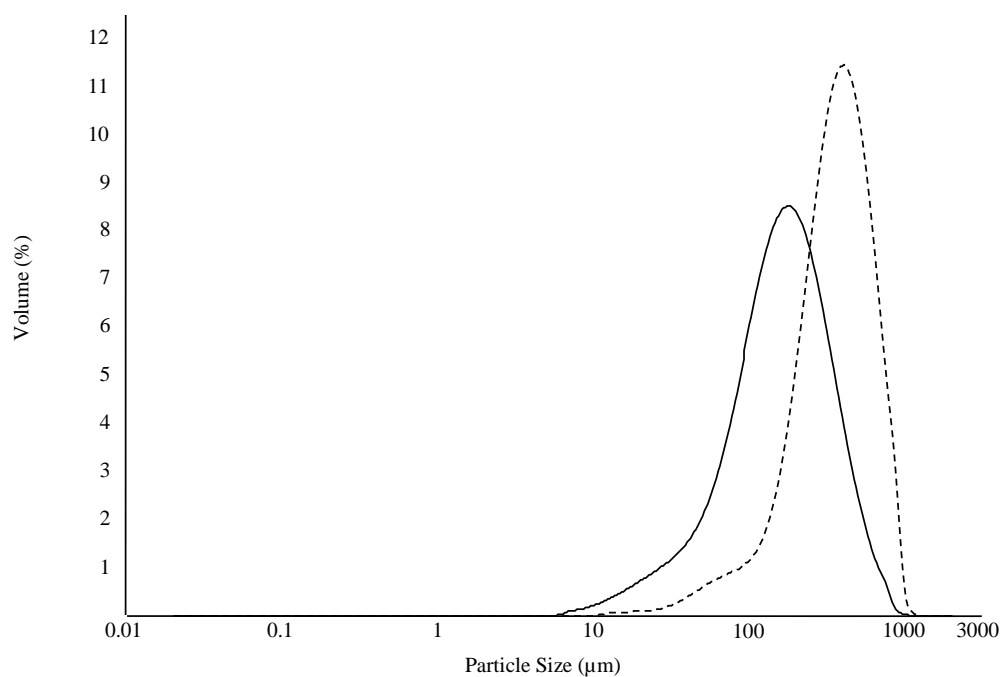
Fig. 6.5 Particle size of calcium alginate beads prepared using an Avestin Inc. Piston homogeniser at 50 bar after 2 and 3 passes, ─ 2 passes, ---- 3 passes
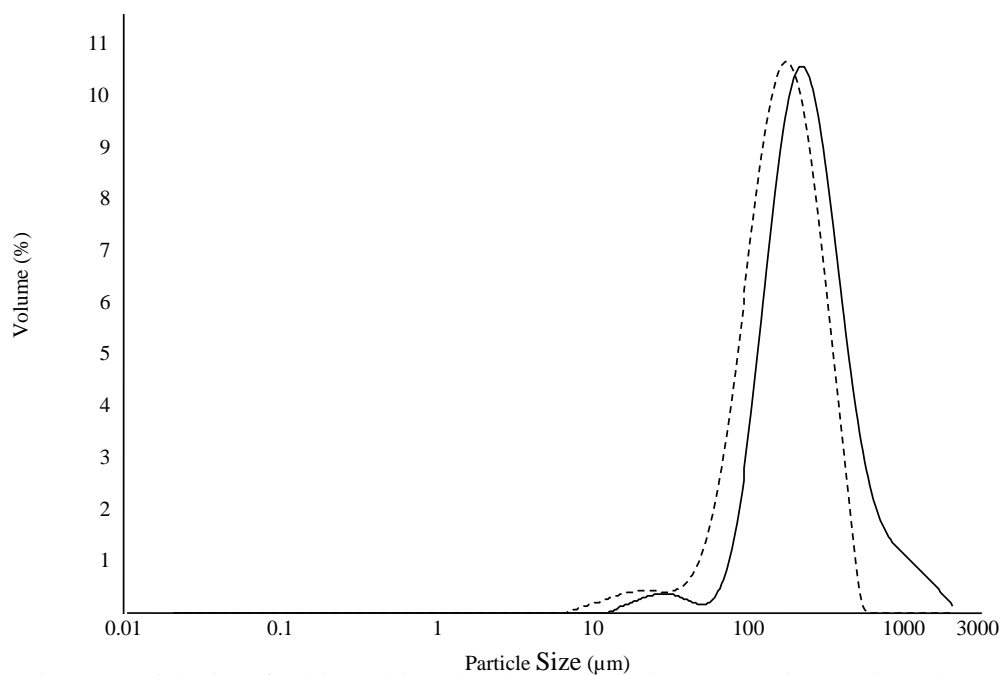
Fig. 6.6 Particle size of calcium alginate beads prepared using an Avestin Inc. Piston homogeniser at 100 bar after 2 and 3 passes, ─ 2 passes, ----3 passes
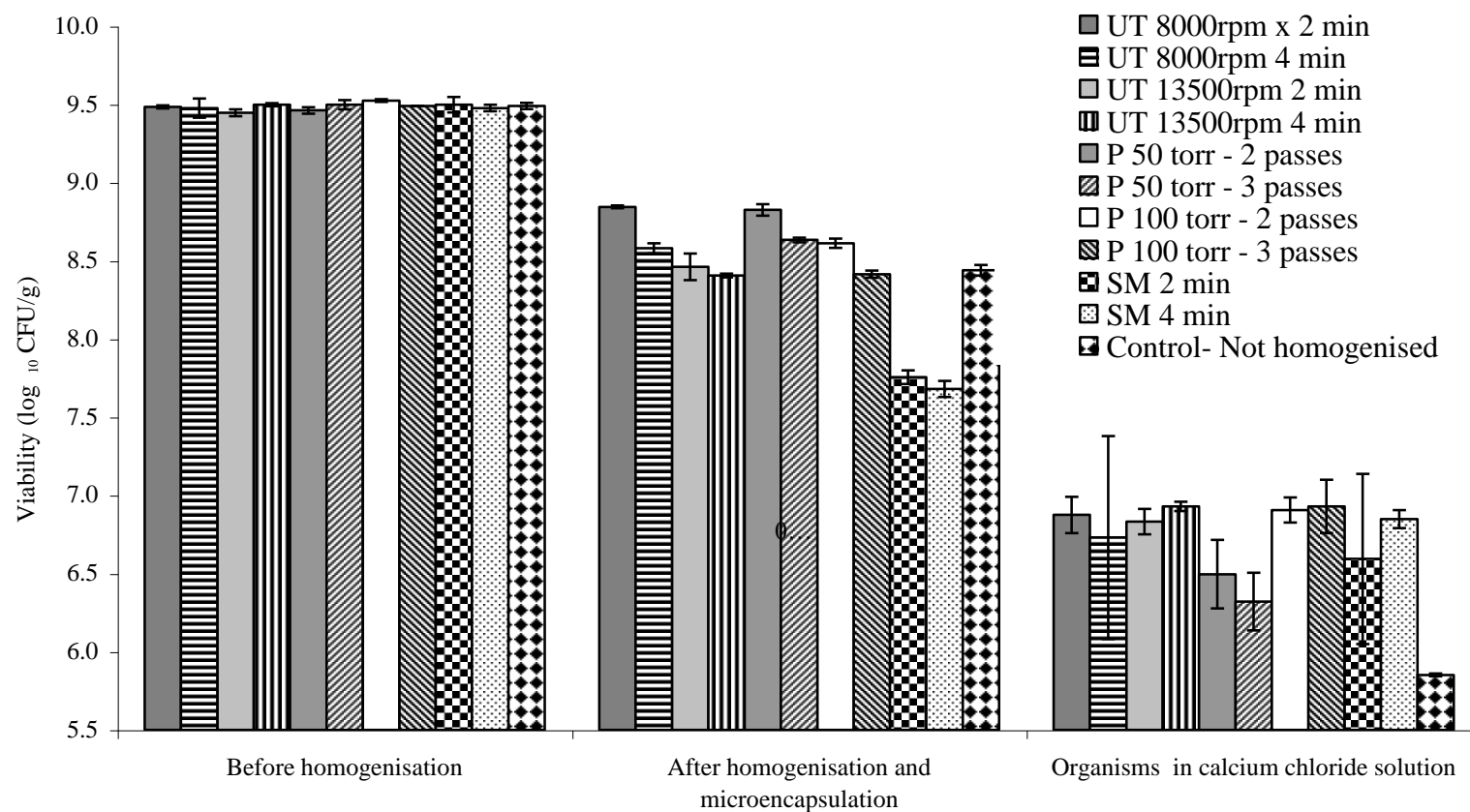
Fig. 6.7 Effect of homogenisation with Ultra-turrax T 25, Avestin Inc. Piston and Silverson mixer on the counts of *L. acidophilus* within a mixture of sodium alginate and vegetable oil during microencapsulation
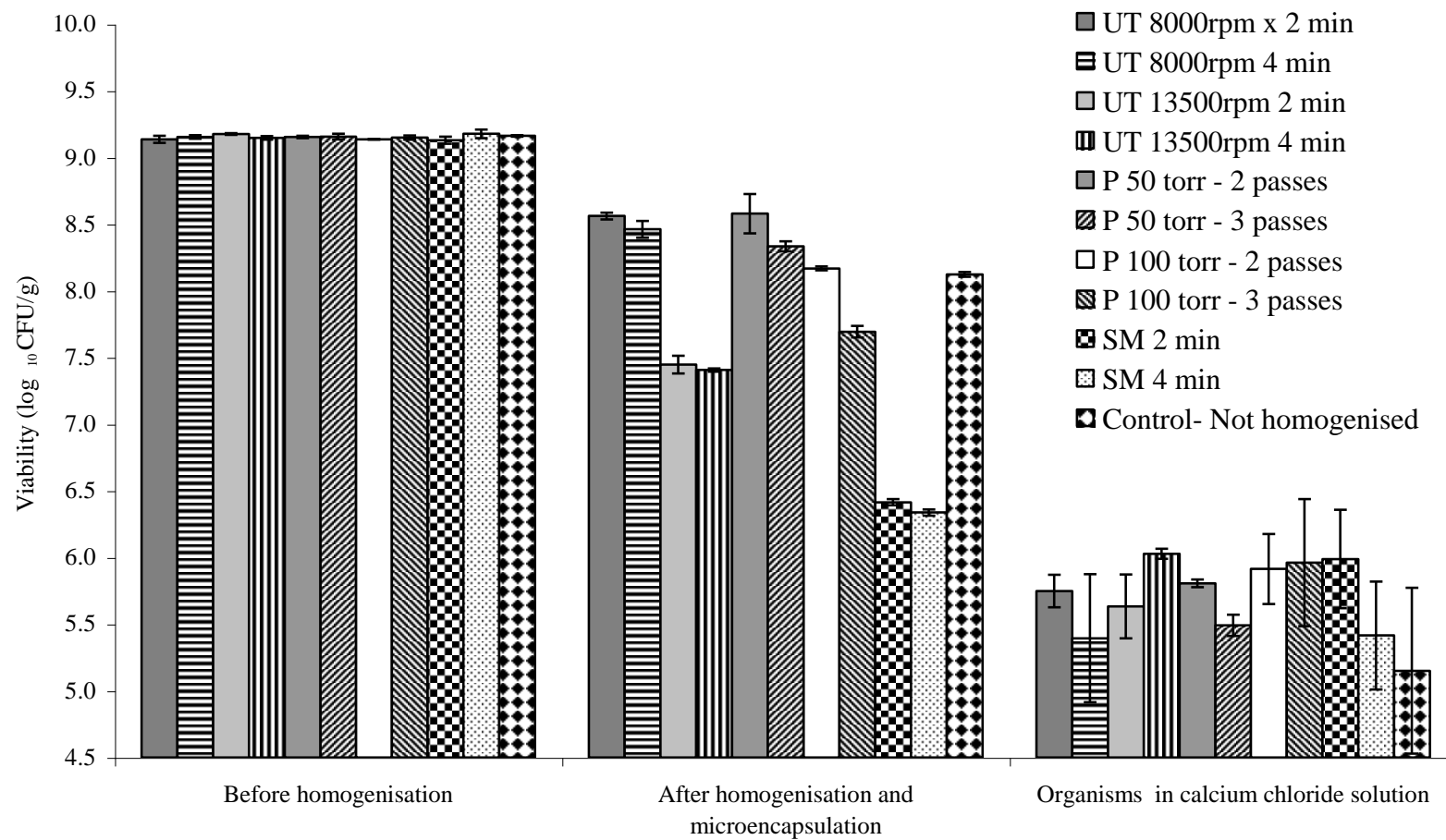
Fig. 6.8 Effect of homogenisation with Ultra-turrax T 25, Avestin inc. Piston and Silverson mixer on the counts of *B. longum* within a mixture of sodium alginate and vegetable oil during microencapsulation
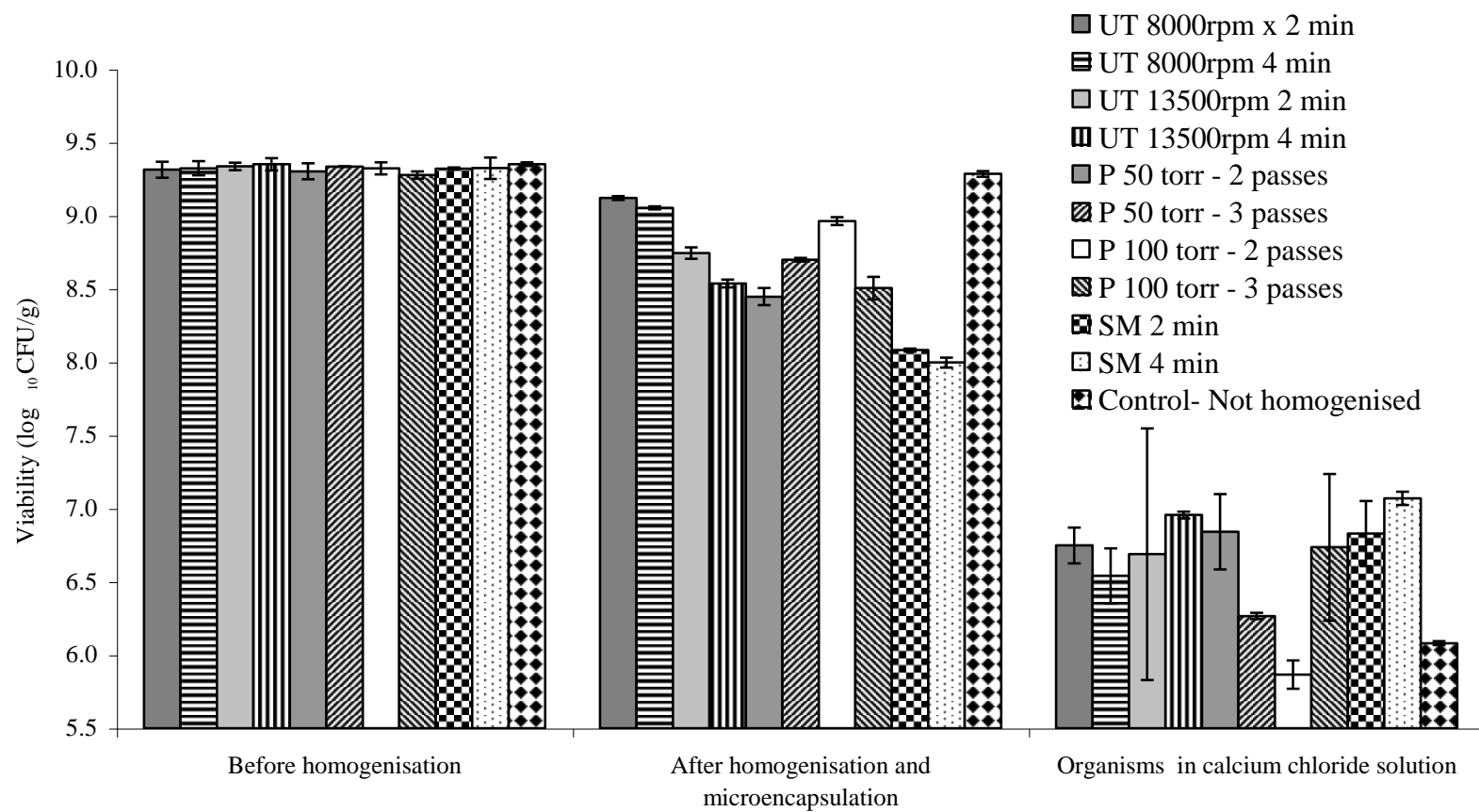
Fig. 6.9 Effect of homogenisation with Ultra-turrax T 25, Avestin inc. Piston and Silverson mixer on the counts of *Lactobacillus GG* within a mixture of sodium alginate and vegetable oil during microencapsulation
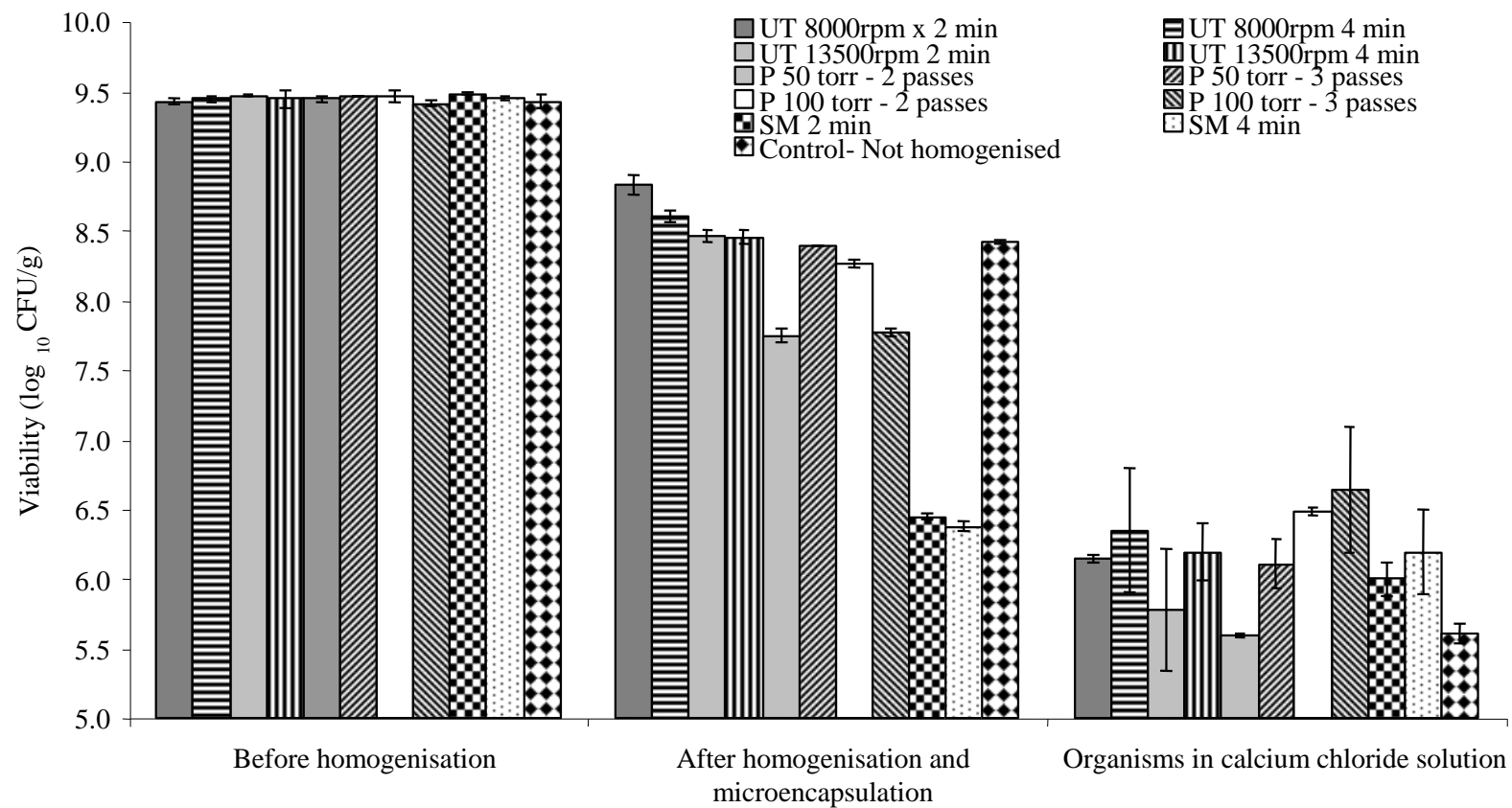
Fig. 6.10 Effect of homogenisation with Ultra-turrax T 25, Avestin inc. Piston and Silverson mixer on the counts of *L. casei* within a mixture of sodium alginate and vegetable oil during microencapsulation
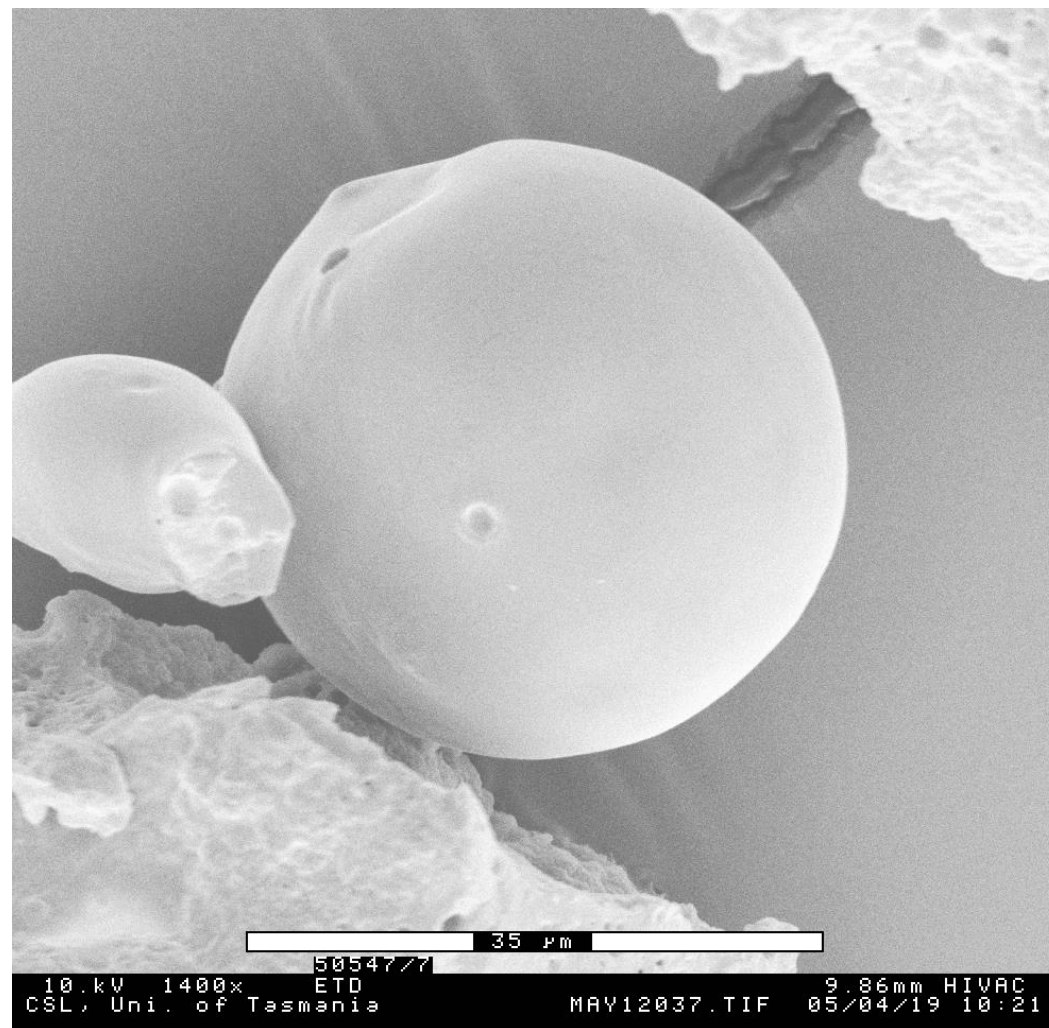
Fig. 6.11 Calcium alginate bead formed using the emulsion technique (Electroscan ESEM 2020, Wilmington, Massachusetts, USA)

CHAPTER 7

OVERALL CONCLUSIONS

It was possible to improve the viability of *Bifidobacterium* spp. *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus rhamnosus* in yoghurt and freeze-dried yoghurt using cryoprotectants, prebiotics and microencapsulation. In particular, *Bifidobacterium* spp. were found to be less susceptible to freeze-drying. The improvement in the survival of these organisms could be due to the physiological characteristic of the organisms. Probiotic organisms that were selected based on their ability to survive the freeze-drying process, were used in further experiments involving, prebiotics cryoprotectants and microencapsulation.

The optimal time for harvesting *L. rhamnosus* GG*, L. acidophilus* 33200, *L. casei* 279 and *B. longum* 536 was found to be 9-12 h. At this time, each organism was at the exponential growth phase. It was identified that an inoculum size of 2 and 4% with a cell load of approximately 10^{10} cfu/g ensured viability of probiotic organisms during processing and storage.

The addition of the prebiotics Hi-Maize, FOS and inulin to fresh yoghurt improved the viability of probiotic organisms. The most effective prebiotic for retaining the viability of probiotic organisms was FOS. However, the addition FOS marginally reduced the viability of probiotic organisms in freeze-dried yoghurt.

The viability of probiotic organisms in freeze-dried yoghurt improved when cryoprotectants GCF 639 and RS 150 were added. The most suitable cryoprotectant for

the selected probiotic organisms was RS 150. The improved survival was possibly due to its strain-specific effect on survival of probiotic organisms.

Microencapsulation proved to be useful for providing probiotic organisms with additional protection during freeze-dying. During storage, encapsulated probiotic organisms remained viable at 21°C for 6 months. However, probiotic organisms were no longer viable in freeze-dried yoghurt stored at 37°C for the same length of time.

The emulsion and extrusion techniques were assessed for their effectiveness in improving the survival of probiotic organisms. The viability of the probiotic organism microencapsulated using the emulsion technique was 1.0 CFU/g higher than organisms microencapsulated using the extrusion technique after freeze-drying. An additional coating applied to the calcium alginate beads did not significantly improve the survival of the probiotic organisms

The mean size of calcium alginate beads was lowered when homogenisation was used to form an emulsion during encapsulation. Beads less than 100 µM in diameter were created using the Ultra-Turrax homogeniser. The population of probiotic organisms was not adversely affected during homogenisation using Ultra-Turrax benchtop. However, homogenisation using a Silverson mixer exhibited a negative impact upon survival of probiotic organisms.

CHAPTER 8

FUTURE RESEARCH DIRECTIONS

Certain probiotic organisms were less susceptible to freeze-drying than others. However, due to the limited number of organisms screened in this study, future research should explore a broader range of organisms that may have an innate resistance to freeze-drying.

Cryoprotectants were useful for improving the survival of prokaryotic cells during freeze-drying. Further research should focus on the interactions between cells and compatible cryoprotective agents. A greater understanding of these protective mechanisms would also assist in the development of cryoprotective agents for specific microorganisms. The same issue of specificity is applicable to prebiotics. Currently, some food and drug manufacturers add prebiotics to products containing probiotic organisms that do not specifically target the growth of these organisms. Further research should match prebiotics with individual organisms to encourage more targeted growth of probiotics in the colon. Human trials that take into account all variables that affect the growth of probiotic organisms in the colon should be carried out.

In the future, microencapsulation may hold the key to ensuring survival of probiotic organisms during prolonged storage. Other encapsulating materials should be assessed for their ability to both protect probiotic organisms and form beads that are acceptable to consumers. In this research, the size of calcium alginate beads was reduced by using homogenisers in the process to form emulsions. However, this technique was time consuming and laborious. Future research focusing on the development of

automated techniques for mass production of calcium alginate beads in a continuous large

scale operation is recommended.

CHAPTER 9

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APPENDIX

Effect of cryoprotectants, prebiotics and microencapsulation on survival of probiotic organisms in yoghurt and freeze-dried yoghurt

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Abstract

The survival of probiotic microorganisms including *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus rhamnosus* and *Bifidobacterium* spp. was evaluated in yoghurt and freeze-dried yoghurt after processing and storage. The effectiveness of microencapsulating probiotic organisms as well as adding cryoprotectants and prebiotics in improving their viability was also investigated. The viability of *Bifidobacterium infantis* 17930 and *L. rhamnosus* GG was reduced by 0.07 log, while that of *L. casei* 1520 and *Bifidobacterium longum* 1941 was reduced by 0.28 and 0.39 log, respectively. There was a 7% improvement in the viability of *L. casei* 1520 when cryoprotectant 'Unipectine™ RS 150' was added at 2.5%(w/v). The prebiotic 'Raftilose®P95' when added at 1.5%w/v to yoghurt improved the viability of the combined selected probiotic organisms by 1.42 log during four weeks of storage at 4 °C. Microencapsulation with alginate improved viability of combined selected probiotic organisms by 0.31 log in freeze-dried yoghurt stored at 21 °C.
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Keywords: Probiotics; Prebiotics; Cryoprotectants; Freeze-drying; Yoghurt; Microencapsulation; Viability

1. Introduction

The large intestine contains over 400 different microbial species. The native microorganisms, which are the dominant microflora in the colon, limit the ability of pathogenic genera including *Escherichia*, *Clostridium*, *Salmonella* and *Campylobacter* to attach to the lumen (Ziemer & Gibson, 1998). Once the microbial balance is disturbed, intestinal bloating and diarrhea may occur. Of the native strains of colonic microflora, the probiotic genera of bifidobacteria and lactobacilli have been extensively studied and established as valuable native inhabitants of the colon.

Probiotics are defined as 'live microbial feed supplements which beneficially affect the host by improving

the intestinal microbial balance' (Fuller, 1989). Ingestion of viable probiotic organisms reduces or eliminates ailments such as colon irritation, constipation and travellers' diarrhoea. Other health benefits include inhibition of pathogenic bacteria, synthesis of B vitamins, lowering of blood ammonia levels, cholesterol absorption, and inhibition of tumour formation (Roberfroid, 2000; Ziemer & Gibson, 1998). However, in order to provide health benefits, it is essential that there is a minimum of one million viable probiotic organisms per gram of a product (Shah, 2002).

Probiotics have a limited shelf life in conventional yoghurt. Freeze-drying is a process that not only preserves yoghurt but also helps maintain a sufficient quantity of viable probiotics. During freeze-drying, the frozen water is removed by sublimation, thus reducing damage to biological structures. Previous research has found that certain strains of probiotics are better able to survive the freeze-drying process (Rybka & Kailasapathy,

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1995). Reasons for this include differences in the surface areas of the microorganisms, and variations in cell wall and membrane composition. During the processing and storage of freeze-dried yoghurt, oxygen content, high temperature, low pH, water activity and elevated solute concentration may all affect the viability of probiotic organisms (Carvalho et al., 2004). Probiotic strains vary in their susceptibility to freeze-drying.

Cryoprotectants can be added to maintain the viability of probiotic organisms during freeze-drying. Compatible cryoprotectants may be added to media or into the yoghurt mix prior to fermentation to assist in the adaptation of probiotics to the environment. As compatible cryoprotectants accumulate within the cells, the osmotic difference with their external environment is reduced (Kets, Teunissen, & de Bont, 1996). The extent to which cryoprotection is provided by any given cryoprotectant will vary between cultures (Das, Kilara, & Shahani, 1976).

Microencapsulation of probiotics can be carried out with natural polymers to reduce cell losses during processing and storage. The protective encapsulating coatings protect probiotic organisms from the digestive extremes of gastric acid and bile salts. Microencapsulation can also be used to regulate fermentation by lactic acid producing starter culture (Ravula & Shah, 2001).

Prebiotics may also aid survival of probiotic organisms during processing and storage. Shin, Lee, Pestka, and Ustunol (2000) found that the viability of commercial *Bifidobacterium* spp. in skim milk improved by 55.7% after 4 weeks of refrigerated storage when fructooligosaccharides (FOS) were added. Prebiotics are defined as 'non-digestible food ingredients that may beneficially affect the host by selectively stimulating

the growth and/or the activity of a limited number of bacteria in the colon' (Roberfroid, 1998).

It was previously reported that probiotic organisms in freeze-dried yoghurt were no longer viable after 2 months of storage at ambient temperatures (Rybka & Kailasapathy, 1995). Therefore, there is a need to study the effect of cryoprotectants, prebiotics and microencapsulation for improving the viability of probiotic organisms in freeze-dried yoghurt during storage.

In this investigation probiotic organisms were selected based on their ability to withstand the stresses associated with freeze-drying in order to incorporate them in yoghurt and freeze-dried yoghurt. The effectiveness of cryoprotectants, prebiotics and microencapsulation on the viability of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus rhamnosus* and *Bifidobacterium longum* was investigated after processing and storage of yoghurt and freeze-dried yoghurt.

2. Materials and methods

2.1. Selection of probiotic strains

2.1.1. Preparation of probiotic bacteria

Probiotic organisms including two strains of *L. acidophilus*, four strains of *L. casei*, one strain of *L. rhamnosus* and six strains of *Bifidobacterium* spp. were obtained from a number of suppliers (Table 1). Ten millilitre aliquots of sterile deMann, Rogosa, Sharpe (MRS) broth (Oxoid Ltd., Hampshire, United Kingdom) were individually inoculated with each of the probiotic organisms. MRS broth containing *Bifidobacterium* spp. was supplemented with filter sterilized

Table 1
Effect of freeze-drying on the viability of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus rhamnosus* and *Bifidobacterium* spp.

Microorganism	Source	Viable counts (log$_{10}$) CFU/g of probiotic cultures[a]		Survival (%)
		Before freeze-drying	After freeze-drying	
Lactobacillus acidophilus 4356	American Type Culture Collection	9.36 ± 0.11	9.22 ± 0.11	72.85 b
Lactobacillus acidophilus 33200	American Type Culture Collection	10.19 ± 0.09	10.06 ± 0.09	73.88 b
Lactobacillus casei 279	Australian Starter Culture Collection	9.69 ± 0.06	9.57 ± 0.05	75.34 b
Lactobacillus casei 292	Australian Starter Culture Collection	9.56 ± 0.03	9.42 ± 0.03	71.97 b
Lactobacillus casei 1520	Australian Starter Culture Collection	9.89 ± 0.16	9.50 ± 0.12	40.93
Lactobacillus casei 2607	CSIRO Starter Culture Collection	10.03 ± 0.16	9.91 ± 0.17	75.31 b
Lactobacillus rhamnosus GG	Isolated from a Commercial Product	9.76 ± 0.06	9.69 ± 0.06	85.73 b
Bifidobacterium bifidus 12	Victoria University Culture Collection	9.38 ± 0.02	9.30 ± 0.02	83.39 b
Bifidobacterium longum 536	Victoria University Culture Collection[b]	9.79 ± 0.18	9.72 ± 0.17	84.12 b
Bifidobacterium infantis 1912	Victoria University Culture Collection[c]	9.94 ± 0.14	9.85 ± 0.13	81.47 b
Bifidobacterium longum 1941	Victoria University Culture Collection[c]	10.15 ± 0.08	9.87 ± 0.13	52.21
Bifidobacterium breve 15698	American Type Culture Collection	9.57 ± 0.13	9.48 ± 0.12	80.51 b
Bifidobacterium infantis 17930	American Type Culture Collection	10.07 ± 0.04	10.00 ± 0.04	84.99 b

Values followed by the same letter are not significantly different ($p > 0.05$).
[a] Measurements are means $\pm$ standard error of three replicates for each individual strain.
[b] Originally obtained from Morinaga Milk Industry Co. Ltd. (Tokyo, Japan).
[c] Originally obtained from the Commonwealth Scientific and Research Organization (CSIRO).

0.05% w/v L-cysteine·hydrochloride (Sigma Chemical Co., Castle Hill, Sydney, Australia) to create an anaerobic environment. The cultures were activated by growing three times successively at 37 °C for 15–18 h. For freeze-drying experiments, organisms were grown in 500 mL of MRS broth and cells were harvested by centrifugation (Sorvall RT7 refrigerated centrifuge) at 1510g at 25 °C for 25 min (Bhandari, Deeth, & Krasaekoopt, 2004). Ten grams wet weight of freshly harvested cells were then washed and suspended in Schott bottles containing 90 mL of sterile (14% w/v) reconstituted skim milk (RSM).

2.1.2. Enumeration of freeze-dried L. acidophilus, L. casei, L. rhamnosus and Bifidobacterium spp.

Ten grams wet weight of washed and harvested cells were suspended in Schott bottles containing 90 mL of sterile (14% w/v) reconstituted skim milk (RSM). The cell suspension was mixed thoroughly and a weighed quantity (30–50 g) was spread onto 90 mm petri dishes. The viability of the probiotic organisms was determined before freeze-drying by pour plating. Probiotic organisms were freeze-dried by freezing at −80 °C for 2 h using a freeze-dryer (model FD-300, Airvac Engineering Pty. Ltd., Dandenong, Australia) followed by 40 h of primary drying at −30 °C and 8 h of secondary drying at −10 °C. Once the freeze-drying cycle had completed, the freeze-dried yoghurt (Fig. 1) and probiotic organisms were reconstituted with the same mass of water removed during freeze-drying. Enumeration of hydrated samples was carried out using the pour plate technique. Serial dilution was carried out in sterile peptone and water solution (0.15% w/v) and MRS agar was used for plating. Petri dishes containing Bifidobacterium spp. were supplemented with 0.05% w/v L-cysteine·hydrochloride. The plates were then incubated anaerobically for 48–72 h at 37 °C in anaerobic gas jars with gas generating kits (BRD 38). Probiotic organisms were enumerated on plates containing 25–250 colony forming units per gram. The following strains: L. rhamnosus GG, L. acidophilus 33200, L. casei 279 and B. longum 536 showed the best survival from each of individual strains investigated in this project. These probiotic organisms were used for further experiments involving cryoprotectants, prebiotics and microencapsulation.

2.2. Effect of inoculum size on viability of probiotic organisms during the manufacture of freeze-dried yoghurt

L. acidophilus 33200, L. casei 279, B. longum 536 and L. rhamnosus GG were incorporated into yoghurt mixes at 2% or 4%. Yoghurts were made as per Fig. 1 and cells were enumerated before fermentation, after fermentation (prior to freeze-drying) and after freeze-drying.
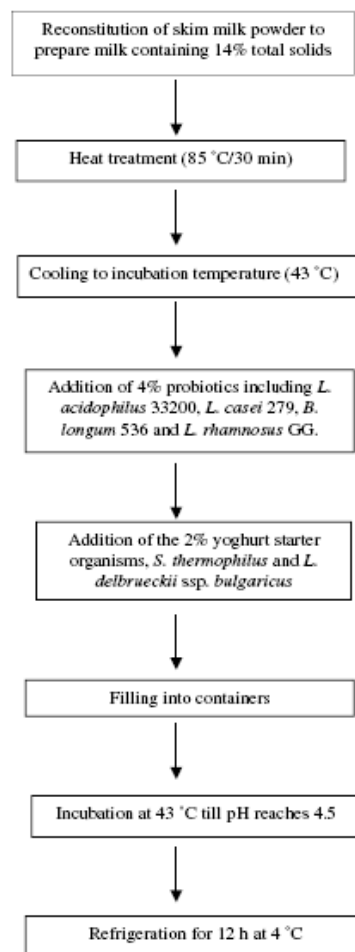
Fig. 1. Stages of yoghurt manufacture.

2.3. Effects of cryoprotectants, Unipectine™ RS 150 and Satialgine™ GCF 639 on survival of probiotic organism in freeze-dried yoghurt

L. rhamnosus GG was chosen for this experiment as it had the best survival when freeze-dried without a cryoprotectants. L. casei 1520 was chosen as a contrast to the robust GG, as it had the lowest survival rate in earlier experiments. Unipectine™ RS 150 and Satialgine™ GCF 639 (Degussa Texturant Systems, Newbury Berkshire, United Kingdom) were added to heat treated (85 °C for 30 min) 14% w/v RSM at 0.0, 0.5, 1.0, 1.5, 2.0 and 2.5%. Yoghurt starter cultures (2%) together with L. rhamnosus GG and L. casei 1520 (4%) were added to the heat treated and cooled (85 °C for 30 min) 14% w/v RSM followed by incubation at 42 °C for 3–4 h. Fermentation was terminated when

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the pH was reduced to 4.5 and the yoghurt was refrigerated for 16 h at 4 °C. Viability of probiotic organisms before and after freeze-drying was determined by enumeration. The procedure was repeated using Unipectine™ RS 150 which was found to be the superior cryoprotectant using the selected probiotic strains, *L. acidophilus* 33200, *L. casei* 279 and *B. longum* 536.

2.4. Effects of Hi-maize, FOS (Raftilose®P95) and inulin (Raftiline*ST) GCF 639 on improving the survival of probiotics organisms in yoghurt and freeze-dried yoghurt

The prebiotics (2% w/v) Hi-maize (National Starch, Melbourne, Victoria, Australia), FOS (Raftilose®P95; Mandurah, NSW, Australia) and inulin (Raftiline®ST; Mandurah, NSW, Australia) were individually incorporated into batches of fresh and freeze-dried yoghurt manufactured using *L. acidophilus* 33200 (0.5%), *L. casei* 279 (0.5%), *B. longum* 536 (0.5%), *L. rhamnosus* GG (0.5%) and yoghurt starter culture (2%). Cells were enumerated after 0, 1, 2, 3 and 4 weeks of storage at 4 °C in fresh yoghurt and after 0, 1, 2 and 3 months of storage in freeze-dried yoghurt.

2.5. Effect of microencapsulation on survival of probiotic organisms

2.5.1. Microencapsulation of probiotics

Probiotic organisms were microencapsulated using a modified method of Ravula and Shah (2000). Briefly, 100 mL of sterile 3% v/w sodium alginate (D 3247 AJAX Chemicals Ltd., Melbourne, Australia) was mixed with 25 mL of concentrated cell culture. The mixture was slowly dispensed using a pipette into a beaker containing 600 mL of vegetable oil and 1 g of Tween 80 while stirring using at 200 rpm. Calcium chloride (0.1 M) was gently added at the side of the beaker until the emulsion was broken. After 15 min, the calcium alginate beads were removed from the aqueous phase and centrifuged at 300*g* for 5 min. The calcium alginate beads were incorporated into yoghurt that was subsequently freeze-dried and vacuum-sealed. The freeze-dried yoghurt was stored at 4, 21 and 37 °C to study the effects of storage temperature on viability of probiotic organisms.

2.5.2. Enumeration of microencapsulated probiotic organisms

The microencapsulated probiotic organisms that were present in yoghurt and freeze-dried yoghurt were released from the calcium alginate beads by sequestering calcium ions with phosphate buffer at neutral pH. Once liberated, the probiotic organisms were enumerated on MRS-vancomycin agar using the method of (Tharmaraj & Shah, 2003).

3. Results and discussion

3.1. Effect of freeze-drying on the viability of unprotected probiotic organisms

Effect of freeze-drying on the viability of selected probiotic organisms is shown in Table 1. Freeze drying had a deleterious effect on the viability of all probiotic organisms. In general, *Bifidobacterium* spp. had 7% higher survival than *Lactobacillus* spp. with the exception of *B. longum* 1941. The higher survival rate of *Bifidobacterium* may be attributed to differences in cell wall and membrane composition (Carvalho et al., 2004). *Bifidobacterium infantis* 17930 and *L. rhamnosus* GG had the greatest survival of 85.73% and 84.99%, respectively, of the 13 probiotic strains after being freeze-dried. The strains selected for further experiments were *L. acidophilus* 33200, *L. casei* 279, *B. longum* 536 and *L. rhamnosus* GG.

3.2. Effect of probiotic inoculum size on viability during the processing of freeze-dried yoghurt

Fig. 2 shows the effect of inoculum size on viability of the probiotic organisms before fermentation, after fermentation and after freeze-drying. A probiotic inoculum size of 4% prior to fermentation ensured viability at a satisfactory level during processing. During the fermentation of milk by yoghurt starter, the viability of the probiotic organisms had also increased when a 2% inoculation was added to milk. When probiotic organisms were added to the mix at the same time as yoghurt starter, the organisms were better able to adapt to the changing environment as organic acids were produced and the pH was reduced to 4.5 (Fig. 2). The stress caused by a reduced pH may have enhanced survivability during freeze-drying as a result of adaptation. Consequently, there was less decline in the viability of probiotic organisms in freeze-dried yoghurt that had been inoculated with 4% (0.36 log) than 2% (0.59 log).

3.3. Selection of a suitable cryoprotectant

Fig. 3 shows the effect of cryoprotectant on the viability of *L. rhamnosus* and *L. casei* 1520. Unipectine™ RS 150 was more effective as a cryoprotectant than Satialgine™ GCF 639. The improvements in viability may have occurred as the cryoprotective agents can inhibit intracellular or extracellular ice formation by binding to the water. Ravula and Shah (2000) had also found Unipectine™ RS 150 to be a superior cryoprotectant as viable counts of *L. acidophilus* and bifidobacteria were improved by 1 log during storage in fermented dairy desserts. As the concentration of the cryoprotective agents in the milk solution was increased, there was an increase in the survival of probiotic organisms.

Fig. 2. Effect of inoculum size on viability of the combined probiotics *L. rhamnosus* GG, *L. acidophilus* 33200, *L. casei* 279 and *B. longum* 536 during the manufacture of freeze-dried yoghurt. The combined probiotics are *L. acidophilus* 33200 (LA), *L. casei* 279 (LC) *B. longum* 536 (BB) and *L. rhamnosus* (GG).
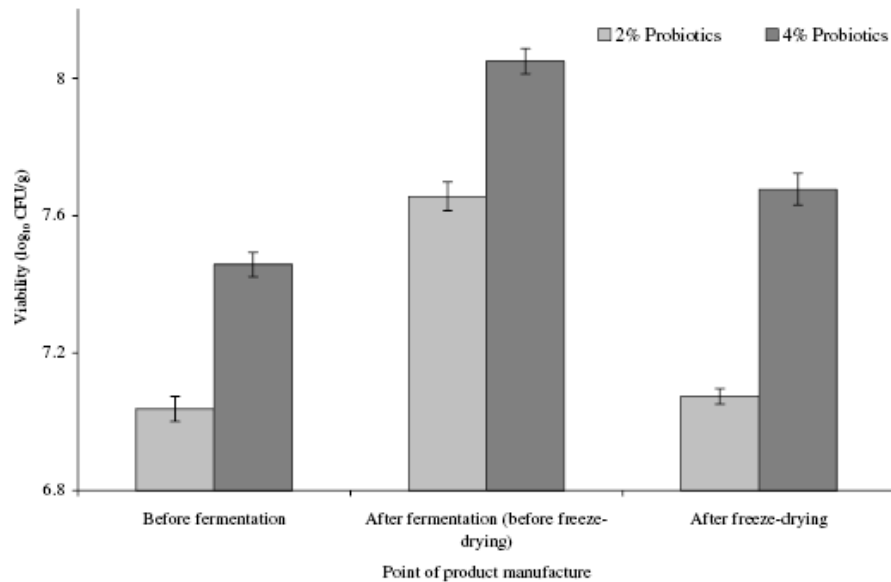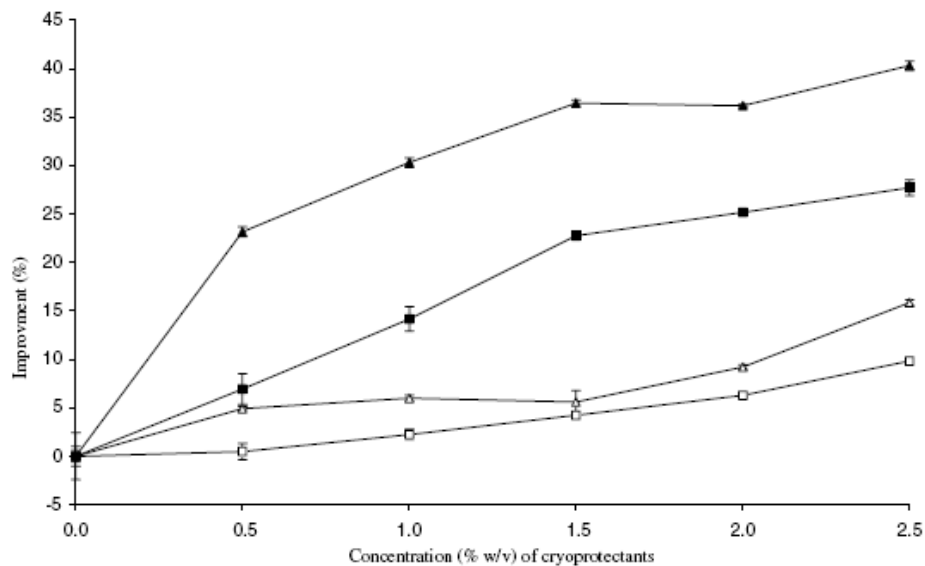

Fig. 3. Effect of cryoprotectants Unipectine™ RS 150 and Satialgine™ GCF 639 on the viability of probiotics. *L. rhamnosus* (GG) and *L. casei* 1520 (LC) (–□– LC + GCF 639, –△– GG + GCF 639, –▲– LC + RS 150, –■– GG + RS 150).

Fig. 4 shows the effect of Unipectine™ RS 150 on viability of selected probiotic organisms. Of the selected probiotic organisms, there was an 80% improvement in the survival of *B. longum* 536 when Unipectine™ RS 150 was added, while there was only 30% improvement in the survival of *Lactobacillus* spp. This suggests that the degree of protection provided by Unipectine™ RS 150 varies between probiotic organisms, possibly due to differences in cell membrane composition of *Bifidobacterium* spp. and *Lactobacillus* spp.
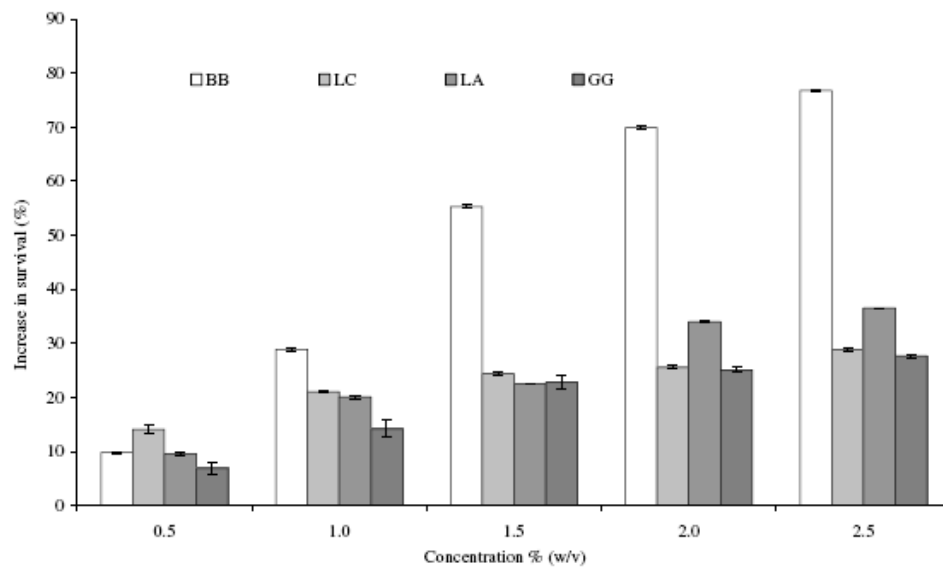
Fig. 4. Effect of cryoprotectants on the survival of *L. acidphilus* 33200 (LA), *L. casei* 279 (LC) *B. longum* 536 (BB) and *L. rhamnosus* (GG) with added cryoprotectant Unipectine RS 150 (improvement in survival is relative to the control which does not contain RS 150).

3.4. Selection of a suitable prebiotic

Fig. 5 shows the effect of prebiotics raftilose (FOS), Hi-maize and raftiline (inulin) on the viability of probi-

otic organisms. Of the three prebiotics investigated, raftilose was found to best retain the viability of selected probiotic organisms (8.70 log) in fresh yoghurt after four weeks of storage at 4 °C. The presence of these oli-
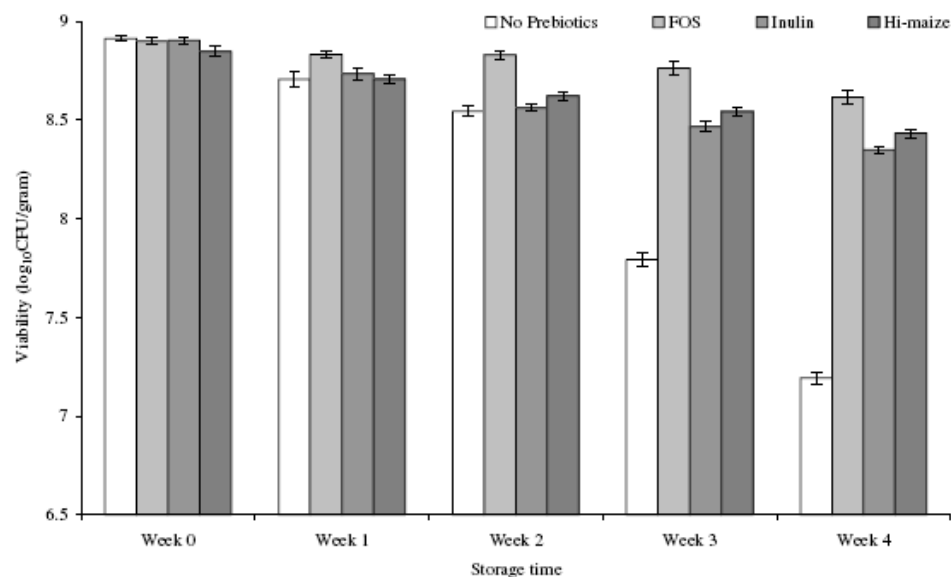

Fig. 5. Effect of prebiotics on viability of combined probiotic organisms in fresh yoghurt during four weeks of storage at 4 °C. The combined probiotics are *L. acidophilus* 33200 (LA), *L. casei* 279 (LC), *B. longum* 536 (BB) and *L. rhamnosus* (GG), the prebiotics are raftilose (FOS), Hi-maize and raftiline (inulin).

gosaccharides reduced the rate of cell death of the saccharolytic bacteria during storage, as prebiotics were available for their utilization. Bruno and Shah (2002) also found a marginal improvement in the viability of probiotic organisms in yoghurt containing FOS during refrigerated storage of fresh yoghurt over 4 weeks.

Fig. 6 shows the effect of prebiotics on the viability of selected probiotic organisms in freeze-dried yoghurt during storage for 3 months. As shown, the viability of the probiotic organisms was comparatively but not significantly lower than the control when prebiotics were added. According to Baati, Fabre-Ga, Auriol, and Blanc (2000), lactic acid bacteria suffer from stresses caused by changing environment. The oligosaccharides may not have been able to protect cells from injury, which may have contributed to the reduction in probiotic viability. The viability of probiotics in the control batch during storage remained higher (0.02–$0.07 \log$) than in each of yoghurts made with added prebiotics. Hi-maize, FOS and inulin were only helpful in improving viability of probiotic organisms in fresh yoghurt during storage (Fig. 5), and had a negative effect on their viability in freeze-dried yoghurt (Fig. 6). The improved viability in fresh yoghurt is possibly due to prebiotics providing extra solids, which tend to protect cells from injury. Oligosaccharides used in this study are hydrocolloids, which are reported to play a protective role towards probiotic microorganisms (Desai, Powell, & Shah, 2004).

3.5. Viability of free and microencapsulated probiotic organisms during storage with the addition of a prebiotic and cryoprotectant

Table 2 shows the viability of probiotic organisms in freeze-dried yoghurt during storage for 6 months at 4, 21 and 37 °C. As shown, cell death was lowest in the batch containing the microencapsulated probiotics at all of three incubation temperatures during storage. After one month of storage at 37 °C, only the probiotics that were microencapsulated had remained viable ($6.84 \log_{10} CFU/g$). By the second month the number of viable probiotics in all four batches had fallen below the minimum requirement of 1 million organisms per gram. The addition of a cryoprotectant and a prebiotic did not improve viability of the probiotic organisms. Cell loss was $0.05 \log$ greater than that of the control at 4 and 37 °C in the batch containing free probiotic organisms and FOS. These results were consistent with those shown in Fig 6. Microencapsulation, was found to play a significant role in maintaining probiotic viability during 6 months of storage at 21 °C in the presence of FOS. Cells were no longer viable in Batch B containing FOS at the same storage temperature, which did not contain a cryoprotectant or microencapsulated probiotics. After 6 months of storage at 4 °C the probiotics in all of the batches had remained viable. Bruno and
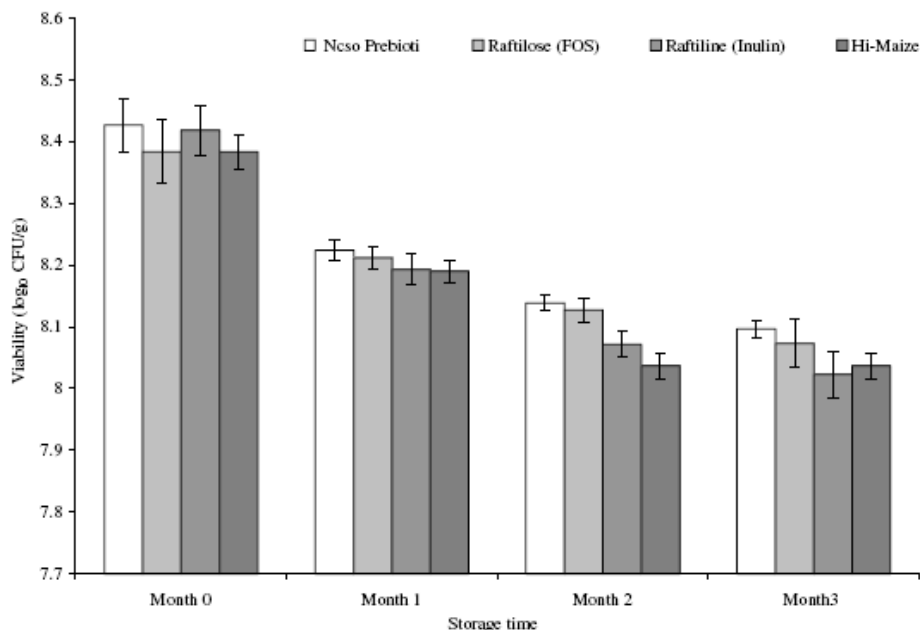

Fig. 6. Effect of prebiotics on viability of combined probiotic organisms in freeze-dried yoghurt during three months storage at 4 °C. The combined probiotics are *L. acidphilus* 33200 (LA), *L. casei* 279 (LC), *B. longum* 536 (BB) and *L. rhamnosus* (GG), the prebiotics are raftilose (FOS), Hi-maize and raftiline (inulin).

Table 2
Viability of combined probiotics L. acidophilus 33 200, L. casei 279, B. longum 536 and L. rhamnosus GG (log$_{10}$ colony forming units [CFU]/g) in freeze-dried yoghurt during storage for 6 months at 4, 21 and 37 °C

Storage time (months)	Log$_{10}$ colony forming units [CFU]/g[a]		
	4 °C	21 °C	37 °C
Batch A (containing unprotected probiotic organisms without FOS or RS 150)			
0	8.90 ± 0.03	8.90 ± 0.03	8.90 ± 0.03
1	8.76 ± 0.03	8.65 ± 0.05	5.30 ± 0.03[b]
2	8.65 ± 0.03	7.49 ± 0.03	0
3	8.53 ± 0.01	7.27 ± 0.03	0
4	8.58 ± 0.04	7.05 ± 0.02	0
5	8.57 ± 0.03	6.78 ± 0.05	0
6	8.40 ± 0.03	6.45 ± 0.02	0
Batch B (containing unprotected probiotic organisms with FOS)			
0	8.93 ± 0.02	8.93 ± 0.02	8.93 ± 0.02[b]
1	8.71 ± 0.01	7.64 ± 0.02[b]	5.25 ± 0.03[c]
2	8.66 ± 0.01	7.41 ± 0.02[b]	0
3	8.66 ± 0.02	6.91 ± 0.02[b]	0
4	8.44 ± 0.02	6.45 ± 0.02[b]	0
5	8.34 ± 0.04	6.08 ± 0.03[b]	0
6	8.19 ± 0.02	5.63 ± 0.03[b]	0
Batch C (containing unprotected probiotic organisms with FOS and RS 150)			
0	8.96 ± 0.01	8.96 ± 0.01	8.96 ± 0.01[b]
1	8.85 ± 0.02	7.60 ± 0.01[b]	5.47 ± 0.02[c]
2	8.79 ± 0.02	7.48 ± 0.01[b]	0
3	8.65 ± 0.02	6.99 ± 0.02[b]	0
4	8.63 ± 0.03	6.65 ± 0.02[b]	0
5	8.61 ± 0.02	6.42 ± 0.01[b]	0
6	8.40 ± 0.03	6.19 ± 0.04[b]	0
Batch D (containing microencapsulated probiotic organisms with FOS and RS 150)			
0	8.93 ± 0.01	8.93 ± 0.01	8.93 ± 0.1[b]
1	8.94 ± 0.02	8.83 ± 0.03	6.84 ± 0.4[c]
2	8.91 ± 0.02	8.10 ± 0.02[b]	5.46 ± 0.2[d]
3	8.86 ± 0.03	7.85 ± 0.04[b]	2.78 ± 1.3[e]
4	8.85 ± 0.02	7.45 ± 0.01[b]	0
5	8.78 ± 0.03	7.07 ± 0.02[b]	0
6	8.67 ± 0.03	6.79 ± 0.04[b]	0

[a] Measurements are means ± standard error of three replicates for each individual strain.
[b-e] Means that are significantly different from others ($p > 0.05$).

Shah (2003) reported a similar trend in probiotic survival during freeze-dried storage at 4 °C.

4. Conclusion

An inoculum size of between 2% and 4% ensured viability of probiotic organisms during processing and storage. The viability of probiotic organisms improved when Hi maize, FOS and inulin were added to yoghurt. FOS was found to be the most effective of the prebiotic in retaining the viability of probiotic organisms in yoghurt, however, when the yoghurt was freeze-dried, the addition of FOS had marginally reduced the viability of probiotics. RS 150 was found to be the most suitable cryoprotectant in retaining the viability of probiotic organisms. Microencapsulation was found to improve the viability of probiotic organisms in freeze-dried yoghurt after 6 months of storage and 4 and 21 °C.

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