STUDIES ON ENHANCING THE VIABILITY AND
SURVIVAL OF PROBIOTIC BACTERIA IN DAIRY
FOODS THROUGH STRAIN SELECTION AND
MICROENCAPSULATION

Presented as a thesis for the fulfilment of Master of
Science (Honours) of the University of Western Sydney

By

GEORGIA NGA-MUN YAM GODWARD

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Centre for Advanced Food Research, School of Science, Food and
Horticulture, University of Western Sydney, Hawkesbury, Australia
PLEASE NOTE

The greatest amount of care has been taken while scanning this thesis,

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The candidate, Georgia Nga-Mun Yam Godward, hereby declares that this submission is her own work and that, to the best of her knowledge and belief, it contains no material previously published or written by another person, nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment is made in the text.

December 2000

Signed:
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ABSTRACT

Probiotic bacteria are those bacteria from the intestine of humans that confer health benefits. Many commercial dairy products now contain adjunct probiotic bacteria; however few live up to the standard of the International Dairy Federation which suggests that a minimum of $10^7$ probiotic bacterial cells be alive at the time of consumption per gram of product.

In this study, strains probiotic bacteria from CSIRO have been selected, by exposure, for tolerance to pH as low as 2.0, bile up to 2% concentration, sucrose up to 25% concentration, oxygen in media and low storage temperatures of 5 and -20°C. *Lactobacillus acidophilus* 2401 and *Bifidobacterium infantis* 1912 were selected as strains able to survive in these conditions and were the easiest to grow on a larger scale. These two strains were then offered further protection from the adverse conditions of food processing and storage by microencapsulation in a calcium alginate and starch gel matrix. This calcium alginate and starch gel has an abundance of calcium ions in it, due to the specific type of alginate used in the encapsulation process.

Microencapsulation in food gums has been used previously to protect probiotic bacteria from cell death in frozen and refrigerated dairy products. This study showed that for *L. acidophilus* 2401 and *B. infantis* 1912 encapsulation in calcium alginate increases survival in yoghurt by 0.44 and 0.64 log respectively. Four trials were done with these strains incorporated into yoghurt, the results were inconsistent thus no conclusions can be drawn.
In Cheddar cheese the free *L. acidophilus* 2401 and *B. infantis* 1912 cells survived better than the encapsulated cells by 0.62 and 0.26 log respectively, probably due to the dense nature of the Cheddar cheese matrix combined with the encapsulation restricting the flow of the nutrients and metabolites between the outside environment and the cells. In ice cream survival of *L. acidophilus* 2401 and *B. infantis* 1912 was high whether the cells were in the free or encapsulated state, probably due to the high fat and solids nature of the ice cream combined with the low storage temperature. For ice cream, the trial results of the laboratory scale production was consistent with the survival results for yoghurt and Cheddar cheese. Survival of probiotic bacteria was also assessed in commercial ice cream and industrially produced yoghurt – these trails were performed for comparison using commercially available strains of probiotic bacteria as well as to enable sensory analysis on the products.

Incorporation of encapsulated probiotic bacteria into ice cream and Cheddar cheese was acceptable by sensory standards and largely un-noticeable in comparison with the same foods without capsules. The capsules were visible and able to be felt on the tongue when eaten in yoghurt causing the product to be disliked by the panellists. More work can be done on improving the encapsulation procedure to make the capsules smaller.
CHAPTER 1

INTRODUCTION

1.1 Background Information

Humans need fuel in the form of food for survival. Dairy foods are important for health and form a significant part of the human diet. Popular dairy foods include yoghurt, ice-cream, frozen yoghurt, cheese, cottage cheese, cream and sour cream. The health giving or therapeutic value of fermented dairy products has been utilised for many generations, dating back to Biblical days. Now known as functional foods, these are the foods that confer added health benefits or disease prevention characteristics beyond the basic nutrition of the food (Culhane, 1999; Rhodes, 1999; Roberfroid, 1999; Roberfroid, 1998a).

More recently the health giving or therapeutic value of certain dairy foods has been enhanced with the incorporation of beneficial probiotic bacteria, especially in yoghurt. This is an area of rapid growth for the Australian Dairy Industry (Australian Dairy Corporation, 2000b). The word probiotic is derived from the Greek, meaning "for life". Probiotic bacteria are the healthy bacteria naturally found in the human intestine, making up a large proportion of the gut bacteria, including the lactic acid bacteria Lactobacillus acidophilus and Bifidobacterium spp., which are integral to human health. Their primary function is in balancing the intestinal system and enhancing resistance against intestinal pathogens.
As we grow older the percentage of bifidobacteria in the intestine decreases as the presence of putrefactive and pathogenic bacteria increases leading to the production of toxic compounds in the colon (Mizota, 1996a). It naturally follows then that as the healthy bacteria decline and the pathogenic bacteria increase ill health is more likely. Thus it is important for the maintenance of good health later in life that the population of healthy bacteria are replenished as the original population declines. A popular way to do this is to consume dairy foods on a regular basis with incorporated probiotic bacteria.

Probiotic bacteria have been used to treat a wide range of human disorders including lactose intolerance, diarrhoea, food allergies, intestinal infection, constipation, gastroenteritis, hepatic encephalopathy, flatulence, colitis, gastric acidity, high blood cholesterol and cancer (Daly et al., 1998; Fuller, 1992; Kailasanath and Chin, 2000; Salminen et al., 1996). Probiotic bacteria are especially useful in the treatment of intestinal disorders due to disturbed intestinal mucosa and bacteria, intestinal inflammation and altered gut permeability. The most widely accepted therapeutic use of probiotic bacteria is for the increased tolerance of lactose in sensitive individuals. Probiotic bacteria such as Lactobacillus acidophilus and bifidobacteria produce $\beta$-galactosidase which auto-digests the lactose present in cultured dairy products. Products such as yoghurt contain less lactose than unfermented milk and are transported through the gastrointestinal tract (g.i.t.) with the living probiotic bacteria which can intra-intestinally digest the lactose thereby improving human tolerance to lactose (Daly et al., 1998).
To be effective therapeutically, dairy products containing probiotic bacteria must contain a minimum of $10^7$ cfu/g in the product (Ouweland and Salminen, 1998). Probiotic bacteria incorporated into dairy foods face difficulties of survival as conditions of production and storage are harsh and unusual from those normally experienced by them. The pH of yoghurt, for example, is often between 4.0 and 4.5 as opposed to the more alkaline conditions of the intestine. The storage temperature of refrigerated dairy products is lower than the optimal growth temperature for probiotic bacteria. The metabolites of other bacteria in the product, like the yoghurt fermenting organisms, can have an adverse effect on the survival of probiotics. The presence of oxygen may also affect the survival of bifidobacteria as they are anaerobic organisms.

It may be possible to increase the survival of probiotic bacteria in dairy foods by a number of methods including addition of growth promoters, terminating dairy product fermentation above pH 5.0, lowering fermentation temperature and increasing fermentation time (giving the population longer time to secure itself in the product) lowering storage temperature, buffering of yoghurt mix with whey protein concentrate, or applying hydrostatic pressure (Costello, 1993; Dave and Shah, 1998; Sakai et al., 1987; Shah, 2000; Supriadi et al., 1994; Tanaka and Hatanaka, 1992; Varnam and Sutherland, 1994). None of these methods has been reported to be entirely successful.
It is very important that the probiotic strains chosen for use therapeutically are able to survive the conditions of food processing and digestion. This includes low pH for the acidity of yoghurt, as well as the extremely acidic conditions of the stomach and gastro-intestinal passage. During digestion the food and associated bacteria will encounter bile, which is detrimental to the survival of bacteria. Dairy foods are stored in the refrigerator or freezer; thus survival at those temperatures is essential. Most importantly the bacteria must survive the shelf life within the conditions of the food; more commonly in frozen dairy desserts which has a high sucrose concentration and is often aerated.

It is possible to increase the survival of probiotic bacteria by the use of physical protection from adverse attributes of food and digestion. Microencapsulation is a process that forms a continuous shell of protective material around the organisms, thus aiding survival. Microencapsulation has been used to increase the survival of *B. pseudolongum* in a simulated gastric environment (Sheu and Marshall, 1993) and to increase the survival of *L. delbrueckii* ssp. *bulgaricus* incorporated into frozen desserts without affecting sensory characteristics of ice-cream (Sheu *et al.*, 1993).

It is hypothesised that selection of probiotic bacterial strains suitable for conditions of food processing and digestion improves viability and delivery of therapeutic doses and the microencapsulation of probiotic bacteria ensures viability of the organisms *in vitro* and *in vivo*. Some strain selection has been done in the past but the focus has always remained on survival in low pH conditions and survival upon exposure to bile salts, the conditions of the
intestinal tract. This research shows that survival in dairy product conditions is strain dependent and influences the ability of the product to deliver therapeutic doses of probiotic bacteria as well as the survival of those organisms in intestinal conditions of low pH and bile salts.

The conditions of dairy product processing that have not been extensively tested in relation to probiotic bacterial survival are that of low storage temperature, high sucrose concentration and oxygen incorporation. These conditions are relevant for dairy products, particularly that of low storage temperature. Most dairy products are refrigerated and some are frozen, such as ice cream and frozen yoghurt. High sucrose concentration is relevant for sweetened foods, particularly ice cream and frozen yoghurt. Likewise oxygen is most relevant for ice cream and frozen yoghurt due to the need for air incorporation into the products to achieve more than 100% over run, also in stirred yoghurt products. The presence of air in the atmosphere is unavoidable and most probiotic bacteria would be exposed to air sometime during processing of dairy products.

This study is an integrated approach to improving survival of probiotic bacteria and delivery of therapeutic doses by two methods, firstly by selecting strains able to survive conditions of low pH, high bile, high oxygen, high sucrose concentration and low storage temperature. Secondly microencapsulation was used to further protect the probiotic bacteria from those conditions and when incorporated into yoghurt, ice cream, fermented ice cream and Cheddar cheese.
1.2 Aims and Objectives

The overall aim of this research was to enhance the viability and survival of probiotic bacteria in fermented, refrigerated and frozen dairy products. The means of achieving this included the selection of robust strains able to withstand the adverse conditions of food processing and digestion as well as the microencapsulation of the selected strains of bacteria for further protection and enhancement of viability. The achievement of these goals provides the potential for significant value-adding to dairy products as well as the development of health-based dairy foods. The goal is to be able to state clearly on the packaging that the International Dairy Federation minimum of $10^7$ colony forming units of probiotic bacteria is present per gram of dairy product (Ouwehand and Salminen, 1998). In entirety this means considerable economic significance to the food industry by increasing its competitiveness in overseas markets.

The principal aims of the research were:

1. To identify strains of probiotic bacteria capable of surviving air-incorporated, acidic, bile, sucrose, refrigerated and freezing conditions.
2. To develop techniques for encapsulation of probiotic bacteria to increase their survival during manufacture and storage of selected refrigerated and frozen dairy foods.
3. To evaluate the viability of encapsulated probiotic bacteria by *in vitro* studies in acidic (to simulate gastric) and alkaline (to simulate intestinal) conditions.
4. To incorporate encapsulated probiotic bacteria into yoghurt, ice cream and Cheddar cheese and to evaluate the survival of encapsulated bacteria compared with non-encapsulated bacteria.

The individual objectives of the research were to:

1. Examine survival of probiotic bacteria after three hours in milk media adjusted to pH 2.0, 2.5, 3.0, 3.5 and 4.0.

2. Examine survival of probiotic bacteria after three hours in milk media adjusted to pH 4.5 and supplemented with bile at concentrations of 0.5, 1.0, 1.5 and 2.0% concentration.

3. Examine survival of probiotic bacteria after three hours in sucrose solution of 10, 15, 20 and 25% concentration.

4. Examine survival of probiotic bacteria after three hours storage in milk media in refrigerated and frozen conditions.

5. Examine survival of probiotic bacteria after overnight storage in broth media following deaeration or aeration of the media by vacuum pump or shaking respectively.

6. Study the effect of cysteine on the survival of probiotic bacteria in aerated or deaerated broth media.

7. Examine results of all strain selection experiments and determine which of the tested strains demonstrated high survival in all tested conditions and therefore would be most useful for incorporation into dairy products.

9. Encapsulate the selected probiotic bacterial strains in a calcium alginate shell material using the developed technique.

10. Study the effect of encapsulation on probiotic bacteria, with respect to survival in low pH and bile solutions.

11. Incorporate encapsulated and unencapsulated probiotic bacteria in dairy products including yoghurt, ice cream and Cheddar cheese.

12. Monitor the survival of encapsulated and unencapsulated probiotic bacteria in yoghurt, ice cream and Cheddar cheese over the shelf life of the dairy products, 8 weeks for yoghurt and 6 months for ice cream and Cheddar cheese.

13. Study the texture analysis profile of yoghurt with encapsulated and unencapsulated probiotic bacteria.

14. Monitor the probiotic bacterial β-galactosidase activity while encapsulated and incorporated into ice cream.

15. Submit the dairy products with encapsulated and unencapsulated probiotic bacteria to sensory studies to determine the effect of the capsules on sensory qualities of yoghurt, ice cream and Cheddar cheese.
2.1 Functional Foods

A functional food is a food or food ingredient, not necessarily a nutrient, with a demonstrated health benefit, or with the capacity to help protect against disease, beyond fundamental nutritional attributes (Culhane, 1999; Hasler, 1998; Rhodes, 1999; Roberfroid, 1998a; Roberfroid, 1998b; Roberfroid, 1999). Further than nutrition, the human diet may facilitate various specific functions of the body, both physiological and psychological (Roberfroid, 1998a; Roberfroid, 1999). There is the potential for functional foods in maintaining a state of wellbeing as well as protecting against cancer, coronary heart disease and bowel disorders (Rhodes, 1998a; Roberfroid, 1999). Functional food claims for health cannot be justified without scientific study. This requires parameters or markers, which prove a statistically and biologically significant change in the body as a result of modifying the diet to contain these functional foods. However, in most cases no parameters or markers have been identified or validated (Roberfroid, 1999).

Examples of functional foods include foods high in fibre and antioxidants. Functional foods may be fruits, vegetables, tea and red wine which all contain functional ingredients that are active in their natural environment, and may play a role in protecting the health of the human gut and heart (Rhodes, 1999; Roberfroid, 1999). Functional foods are still foods therefore they must remain
foods and not be converted to pills or capsules. The concept is that functional foods are to remain a part of the normal daily diet and contribute to optimum nutrition (Roberfroid, 1998a). The most crucial aspect of defining functional foods is that they must be demonstrated to have a beneficial effect on the consumer, beyond the expected nutritional effects, which is related to either maintenance of good health or the reduction of the risk of disease (Roberfroid, 1999).

Nutraceuticals are products isolated or purified from a food and sold as medicines with a demonstrated effect against chronic disease (Culhane, 1999). Herbal and natural products have been used for centuries by various cultures for health benefits. This industry has grown recently with many new products being sold as a "food" but making either explicit or implicit health claims. Foods used as drugs are known as "frugs". Effectively, these products, sold with the intention of use as a drug, however, do not need to conform to the rigorous regulations of labeling, manufacturing and proof of efficacy needed for drugs. Validation criteria used for promoting such products include the fact that they are natural and have been used for many centuries (Ehrnreich, 2000; Thadani, 1999).
2.2 Functional Foods and Human Health

The beneficial claims of functional foods can be defined as either "Enhanced-functional claims" or "Disease risk reduction claims". An enhanced-functional claim means that there is a positive benefit of eating specific foods or food ingredients on a particular function of the body, for example, the prevention of oxidation by the consumption of antioxidants. The disease risk reduction claim relates to the reduction in risk of contracting a disease by consuming a specific food component, for example, a claim of reduction of risk of cardio-vascular disease or of cancer. Any health claim must be based on sound scientific evidence with objectivity and consistency, and must meet scientific standards of statistical and biological significance (Roberfroid, 1999).

Functional foods could be used for improvement of a number of targets in the human body including:

1. Gastrointestinal function, including the balancing of colonic microflora and control of nutrient bioavailability, food transit time, immune activity, endocrine activity, mucosal motility and epithelial cell proliferation.

2. Antioxidant and redox systems require certain amounts of vitamins as well as non-vitamin components like polyphenols. Antioxidant and redox functions are important for all cells and tissues; however beneficial effects have not been proven except when consumed as a component of fresh fruit and vegetables.
3. Metabolism of macronutrients (carbohydrates, amino acids and fatty acids) and the related hormonal regulation e.g. insulin/glucagon balance.

4. Xenobiotic metabolic activities and their control by some food components such as glucosinolates, which are mostly non-nutritive. These functions may be important in controlling toxicity and carcinogenicity caused by chemical contaminants of the food and/or the environment.

5. Moods and behaviour as well as cognitive and physical performances may be influenced by food components, though there is a fine line between nutrition and pharmacology in this category (Roberfroid, 1998a; Roberfroid, 1999).

The population is becoming more aware of nature and the changes humans have made to our environment. Accordingly consumers are also becoming more health conscious and more attentive to the products used on and within their bodies, remembering the catch phrase: "You are what you eat". The trend now is to adopt a preventative approach to health, rather than a curative one, and to take an interest in one's "inner health" and to utilise functional foods. Eating functional foods as a part of the regular diet for health care is more accessible to the consumer (because foods are more readily available and less expensive) than prescription drugs.
As a result of people taking more responsibility for their own health, the role of probiotics, prebiotics and synbiotics are gaining increasing attention from health professionals, consumers and food producers. Several dairy foods including yoghurts, fermented milks, sour cream, buttermilk, cottage cheese, ice-cream and other frozen desserts have been or are being reformulated to contain the probiotics *L. acidophilus* and *B. bifidum* (AB culture).

The consumers’ health and well-being are not the only advantages of using probiotic organisms in food, the quality of the food itself is also improved (Hull et al., 1991). The shelf life can be extended by the same principle as inside the body - by the inhibition of pathogens or food spoilage organisms via the production of bacteriocins, hydrogen peroxide, organic acids and lowering the pH. During fermentation the presence of probiotic bacteria can also increase both the nutritional quality and the organoleptic quality of the food. The probiotics may be able to metabolise food components with adverse effects such as lactose, oligosaccharides and phytic acid. At the same time protein digestibility and vitamin levels can be increased. Meanwhile fermentation also alters both the flavour and the texture of the raw ingredients and in some cases can remove undesirable flavours (Hull et al., 1991).
2.3 The Human Intestinal System

The adult human intestine houses at least a hundred trillion viable cells, including more than 100 species. The bacteria living in the human intestinal system are referred to as intestinal bacteria or intestinal flora. The human intestinal flora consists of three broad groups. The lactic acid bacteria group includes *Bifidobacteria, Lactobacilli* and *Streptococci*. The second group is the anaerobic group, consisting of *Bacteroidaceae, Eubacteria, Peptococcaceae, Veillonella, Megasphaera, Gemmiger, Clostridia* and *Treponema*. The third group is the aerobic group, which contains the *Enterobacteriaceae, Staphylococci, Bacilli, Corynebacteria, Pseudomonas* and yeasts (Mitsuoka, 1997). A balance of microorganisms is required in the gastrointestinal tract to ensure that levels of pathogenic and abnormal microorganisms are controlled at a very low level or even excluded, that the levels of microbial enzyme activity are maintained at normal levels, that enzymic formation of toxic and carcinogenic substances is minimized and that normal enzymic production of organic acids and epithelial cell energy sources is maintained (Evans et al., 1993). The normal balance of microbes in the intestinal tract can be disrupted by diet, stress, alcohol, antibiotic and chemotherapy treatments (Evans et al., 1993).
2.3.1 Composition of the human intestinal microflora throughout life

Humans are born without intestinal flora as the gestation environment inside the mother is sterile. By the day after birth, the intestine is implanted with *Escherichia coli*, *Enterococcus*, *Lactobacillus*, *Clostridium* and *Staphylococcus*. Breast fed infants are implanted with *Bifidobacterium* at about 3 days of age and at the same time the other bacterial groups begin to decline. Within the first week of life the *Bifidobacterium* becomes predominant at $10^{10}$-$10^{11}$ cells per gram while the remaining bacterial types reduce to about 1% of the population in the gut, which stabilises at the balanced ratios contributing to good health of the child throughout life (Mitsuoka, 1997).

As the child is weaned the intestinal microflora resembles the adult flora more closely. The counts of *Bacteroides*, *Eubacterium*, anaerobic *Streptococcus* and *Clostridium* increase while the species of *Bifidobacterium* present alter from the infantile pattern consisting mostly of *B. infantis* and *B. breve* to the adult pattern including mostly *B. longum* and *B. adolescentis* (Mitsuoka, 1997).

In a healthy adult there are usually $10^7$ bacteria per gram of saliva in the oral cavity including the *Lactobacillus*, coliform-type bacteria, *Veillonelliae* and *Enterococcus*. In the stomach this number decreases to $10^2$-$10^3$ cells per gram due to the gastric acidity. This number then begins to increase again in the upper small intestine where there are $10^3$-$10^4$ cells per gram, most of which are *Lactobacillus*, *Enterococcus* and *Veillonella*. The numbers continue to increase
through the lower ileum in the small intestine where the composition of the flora begins to change and includes both types found in the upper small intestine as well as the large intestine including *Enterobacteriaceae*, *Eubacterium*, *Bacteroides* and anaerobic *Streptococcus*. Once through the ileocecal valve into the large intestine the numbers of bacteria per gram rise to at least $10^{11}$, mostly from the anaerobic group including *Bacteroides*, *Eubacterium*, anaerobic *Streptococcus*, *Clostridium* and *Bifidobacterium*. Other cells are present at a lower level of $10^5$-$10^7$ cfu/g including *Enterobacteriaceae*, *Enterococcus*, *Lactobacillus*, *Veillonella* and *Staphylococcus*. This is the composition of the fecal flora of healthy human adults (Mitsuoka, 1997).

The presence of healthy bacteria including bifidobacteria in the gut is integral to human health. As we grow older the percentage of bifidobacteria in the intestine decreases as the presence of putrefactive and pathogenic bacteria increases leading to the production of toxic compounds in the colon. These changes occur from middle to old age when the total number of bacteria in the gut tends to decrease. In some people the *Bifidobacterium* becomes undetectable while *Clostridium perfringens* may significantly increase along with *Lactobacillus*, *Enterobacteriaceae* and *Enterococcus*. This pattern may be associated with aging of physiological functions affecting the intestinal flora, which in turn may also accelerate the aging process (Mitsuoka, 1997).
2.3.2 Good, bad and neutral intestinal bacteria

The bacteria that make up the intestinal microflora fall into three categories relative to human health:

- Beneficial
- Harmful
- Neutral.

The normal microflora of the human intestinal system may include bacteria from the following genera:

- Enterobacteria
- Streptococci
- Staphylococci
- Lactobacilli
- Bacteroides
- Bifidobacteria
- Peptococci
- Clostridia
- Fusobacterium
- Eubacteria
- Vellonelliae
Table 1 lists the harmful bacteria that may be found in the human intestinal system with the toxic compounds produced by those microorganisms. Table 2 shows how those particular compounds are toxic to humans.

Although the toxic compounds listed in Tables 1 and 2 are only found in low concentrations in the colon, they may still prove dangerous due to being in contact with the intestinal mucous membrane for a lifetime (Mizota, 1996a). Due to the potentially harmful effects of the putrefactive and pathogenic bacteria in the intestine and the ability of *Bifidobacteria* to counteract these effects, it is important that a high proportion of *Bifidobacteria* is maintained in the intestine throughout life (Mizota, 1996a).
Table 1: Putrefactive and pathogenic bacteria present in the colon and the toxic compounds produced by them.

<table>
<thead>
<tr>
<th>Harmful bacteria found in the human intestinal system</th>
<th>The toxic compounds produced by harmful bacteria in the human intestinal system</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli,</em> Clostridia</td>
<td>ammonia</td>
</tr>
<tr>
<td></td>
<td>amines</td>
</tr>
<tr>
<td></td>
<td>nitrosoamines</td>
</tr>
<tr>
<td></td>
<td>phenols</td>
</tr>
<tr>
<td></td>
<td>cresols</td>
</tr>
<tr>
<td></td>
<td>indole</td>
</tr>
<tr>
<td></td>
<td>secondary bile acids</td>
</tr>
<tr>
<td></td>
<td>aglycones</td>
</tr>
<tr>
<td><em>Bacteroidaceae,</em> <em>Streptococcus faecalis</em></td>
<td>nitrosoamines</td>
</tr>
<tr>
<td></td>
<td>secondary bile acids</td>
</tr>
<tr>
<td></td>
<td>aglycones</td>
</tr>
<tr>
<td><em>Proteus</em></td>
<td>ammonia</td>
</tr>
<tr>
<td></td>
<td>amines</td>
</tr>
<tr>
<td></td>
<td>indole</td>
</tr>
</tbody>
</table>

Table 2: Effects of toxic compounds produced by putrefactive and pathogenic bacteria in the colon.

<table>
<thead>
<tr>
<th>Compounds produced by bacteria in the human intestinal system</th>
<th>Effects on the human body of the compounds produced by bacteria in the human intestinal system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia</td>
<td>liver and brain toxin</td>
</tr>
<tr>
<td>Amines</td>
<td>carcinogen</td>
</tr>
<tr>
<td>Nitrosoamines</td>
<td>carcinogen</td>
</tr>
<tr>
<td>Phenols</td>
<td>cancer promoter</td>
</tr>
<tr>
<td>Cresols</td>
<td>cancer promoter</td>
</tr>
<tr>
<td>Indole and skatole</td>
<td>carcinogens</td>
</tr>
<tr>
<td>Secondary bile acids</td>
<td>carcinogens or active colon cancer promoters</td>
</tr>
<tr>
<td>Aglycones</td>
<td>mutagenic</td>
</tr>
<tr>
<td>Estrogens</td>
<td>suspected carcinogens or breast cancer promoters</td>
</tr>
</tbody>
</table>

2.3.3 Good intestinal bacteria

Bifidobacteria are known to play an important role in general health and increasing the intestinal numbers of bifidobacteria promotes intestinal acidification and reduces ammonia concentration and urease production (Yasumita, 1996). Probiotic bacteria are helpful in maintaining a balanced microflora by producing antimicrobial substances active against pathogens, competing for nutrients and adhesion receptors and by stimulating immunity (Wang and Gibson, 1993).

A microorganism's ability to alter metabolic processes in the gut may also be considered a probiotic characteristic. Mechanisms for this may be:

- Suppressing reactions which generate toxic or carcinogenic metabolites;
- Stimulating reactions that detoxify potentially toxic substances;
- Stimulating mammalian enzymes for digestion of complex nutrients or providing a bacterial source of these enzymes; and
- Synthesising vitamins and other essential nutrients not received through diet (Rowland, 1992).

Probiotic bacteria including the Lactic Acid Bacteria (LAB) and bifidobacteria fall into the beneficial category, the presence of which is integral to maintaining human health. The beneficial effects of probiotic bacteria may result from microbial interactions in the gut or metabolic interactions in the gut. Microbial interactions include competitive colonisation and the formation of an ecosystem
within the host's gut. The intestinal ecosystem consists of three significant types of components. The biotic components include the indigenous and transient microbes as well as the gastro-intestinal epithelial cells. The abiotic components are of dietary origin and are not digested in the small intestine. The endogenous components, present in saliva and from gastric, pancreatic, hepatic and intestinal secretions, include enzymes, hormones, mucous, bile salts, urea, immunoglobulins and peptides among others. When all the components interact properly, this is compatible with the health of the host; however the ecosystem is destabilised when gastro-intestinal disorders arise (Fuller, 1992).
2.4 Probiotic Bacteria

Probiotic bacteria are the beneficial bacteria that are found in the intestines, including the lactic acid bacteria and the bifidobacteria. They are also found in cultured dairy foods, including yoghurt, buttermilk and sour cream, and are known to aid digestion and help prevent disease. Dairy products contain a number of probiotics and milk is said to be an ideal carrier and an excellent growth medium for the beneficial microbes Lactobacilli and/or bifidobacteria (Australian Dairy Corporation, 2000b).

2.4.1 History and Definition of Probiotics

Probiotic bacteria, or probiotics, are microorganisms that provide health benefits beyond inherent basic nutrition during the transit through the gastro-intestinal tract (Chin et al., 2000; Tannock et al., 2000). This definition has been evolving as more understanding is achieved (Ouwehand et al., 1999; Tannock et al., 2000) ever since the concept of probiotics was first proposed by Elie Metchnikoff in the late 1800's at the Pasteur Institute in Paris. The Nobel Prize laureate biologist postulated that one's health and longevity could benefit from the bacteria found in yoghurt, kefir and sour milk. His theory was that the microflora present in human intestines can determine the result of an invasion by enteric pathogens as well as regulating natural chronic toxaemia influencing aging and mortality. He suggested that normal "unhealthy" fermentation in the large intestine produces toxins which are absorbed and abrade the artery wall tissues causing an early death. Metchnikoff performed his own experiments and clinical
trials and decided that the use of "sour milk" stops this type of fermentation as a result of lactic acid production by the lactic bacillus (Lactobacillus spp.) in those products (Metchnikoff, 1908). Actually, fermentation has been used for thousands of years in various civilisations including the consumption of fermented milks in pre-Biblical days (Goldin, 1998; Vandenbergh, 1993).

The word probiotic literally means "for life" derived from the Greek words "pro bios" (Gismondo et al., 1999; Naidu et al., 1999). The first definition of probiotics was a substance produced by one microorganism that stimulates the growth of other microorganisms (Lilly and Stillwell, 1965). By 1974 this was revised and probiotics were defined as feed supplements that have a beneficial effect on the host via its intestinal microflora (Parker, 1974). Then in 1989 the probiotics were specified as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller, 1989). This particular definition was expanded by Havenaar and Huis in't Veld (1992) to include supplements based on food and those not in food as well as the use of mono- and mixed cultures. Vandenbergh (1993) summarised probiotics as the use of microorganisms for prevention of diseases.

The simplified definition of Salminen et al. (1998a) being "a live microbial food ingredient that is beneficial to health", still places the emphasis on the microorganisms being alive to provide the health benefits. As does the 1999 definition where a probiotic is explained as a preparation of viable micro-
organisms and/or their components or metabolic products which has a protective or beneficial effect on the health of the host either directly or indirectly through the immune system as well as improving nutritional and microbial balance in the intestinal tract (Haller et al., 1999; Hamilton-Miller et al., 1999; Naidu et al., 1999). A probiotic active substance is "a cellular complex of lactic acid bacteria (LAB) that has a capacity to interact with the host mucosa and may beneficially modulate the immune system independent of LAB's viability" (Naidu et al., 1999). The crux of the issue of probiotics now is that the benefit exerted by the microorganisms is of definite health benefit beyond nutrition, as opposed to simply protection against diseases and giving a psychological feeling of wellness (Chin et al., 2000).

2.4.2 Therapeutic potential and health benefits of probiotics

The old adage "prevention is better than cure" is still true today as prevention or reduction of disease risk is preferable to disease treatment (Salminen, 1999). Probiotics have the capacity to influence gastrointestinal physiology and function and therefore to improve the health and well being of humans (Salminen, 1999). Probiotics have been used therapeutically for many years as traditional medicine, which is now scientifically accepted (Hamilton-Miller and Gibson, 1999; Kailasapathy and Chin, 2000; Salminen, 1999). The uses of probiotics for health benefits can be divided into nutritional, physiological and antimicrobial effects (Mitsuoka, 1990; Naidu et al., 1999).
2.4.3 How probiotics work

Probiotics are believed to actively enhance health by improving the balance of intestinal microflora (Shah, 2000). There are several mechanisms by which this is achieved, including the suppression of harmful bacteria and viruses, stimulation of local and systemic immunity and modifying gut microflora metabolic activity (Dunne et al., 1999; Gismondo et al., 1999; Ouwehand et al., 1999; Tejada-Simon et al., 1999; Vandenbergh, 1993). Probiotic bacteria may show antimicrobial effects against intestinal and food-borne pathogens by preventing pathogenic adherence by competitive colonisation, establishment, replication and action. The mechanism of this anti-pathogenic effect may be through decreasing luminal pH by the production of acetic, lactic or propionic acids (short chain fatty acids, SCFA), rendering vital nutrients unavailable to pathogens, altering the redox potential of the environment, production of hydrogen peroxide or production of bacteriocins or other inhibitory substances (Fuller, 1989; Kailasapathy and Chin, 2000; Naidu et al., 1999).

Immune responses to probiotics are a focus of current research (Chin et al., 2000; Isolauri, 1999; Kailasapathy and Chin, 2000). Probiotics may cause cell-mediated immune responses including activation of reticulo-endothelial system, augmentation of cytokine pathways and stimulation of pro-inflammatory pathways such as tumour necrosis factors and interleukin regulation without being a target of the host immune system (Haller et al., 1999; Isolauri, 1999;
Mattila-Sandholm et al., 1999; Naidu et al., 1999). Probiotics may even activate macrophages directly (Tejada-Simon et al., 1999).

Probiotics are good for promoting the host's endogenous defense mechanisms as well as down-regulating hypersensitivity reactions and inflammatory responses. Many of the probiotic effects may be mediated through immune regulation, especially by controlling the balance between pro- and anti-inflammatory cytokines. The immune regulating effects of probiotics may be vital as they reduce local pro-inflammatory cytokine generation at the same time as extending the anti-inflammatory effects of probiotics beyond the gut, a useful therapy for allergies and autoimmune diseases (Isolauri, 1999).

2.4.4 Probiotic Characteristics

As opposed to antibiotics which destroy all bacteria the prefix "pro" implies a positive effect, active probiotic bacteria maintain and enhance the population of beneficial microorganisms at the expense of pathogens (Kailasapathy and Chin, 2000).

For benefits to humans, probiotics need certain properties including:

- Be of human origin,
- Remain stable and viable when exposed to acid, bile, intestinal enzymes and oxygen,
- Remain viable and active in the carrier food before consumption,
• Adhere to the host's intestinal mucosa to prolong the time the organism can influence the gastrointestinal immune function and microflora,
• Colonise the host's intestinal tract,
• Modulate immune responses,
• Be able to influence metabolic activities such as cholesterol assimilation, lactase activity and vitamin production,
• Produce antimicrobial metabolites including organic acids such as lactic acid and acetic acid, bacteriocins, hydrogen peroxide, carbon dioxide and diacetyl, and
• Show probiotic therapeutic qualities and remain safe for use in humans (non-pathogenic and non-toxic).

(Chandon, 1979; Conway, 1996; Fuller, 1989; Gibson and Roberfroid, 1995; Gilliland, 1979; Gilliland and Speck, 1977; Gismondo et al., 1999; Hove et al., 1999; Ibrahim and Bezkorovainy, 1993; Kailasapathy and Chin, 2000; Laroia and Martin, 1990; Mattila-Sandholm et al., 1999; Mishra and Lambert, 1996; Robinson and Tamime, 1990; Shahani and Dunne et al., 1999; Skaaning, 2000).

For probiotic organisms to perform their beneficial role in the intestinal tract they must be able to overcome barriers such as intestinal motility or the rate at which food material passes through the intestinal tract, gastric acidity, lysozymes, low surface tension and bile (Gilliland, 1979). All of the definitions of probiotics since 1989 (Fuller, 1989) have specified that to be useful as a probiotic the organism must be viable. Potentially useful probiotic strains must be viable and
metabolically active in the gastrointestinal tract as well as biologically active against pathogens (Kailasapathy and Chin, 2000). The probiotics should also remain viable throughout commercial production as well as in the food products for which they act as adjunct (Kailasapathy and Chin, 2000).

The organisms most commonly used as probiotics include: Lactobacillus acidophilus, L casei and the bifidobacteria including: Bifidobacterium adolescentis, B. bifidum, B. longum, B. psedulongum, B. animalis, and B. breve (Fuller, 1992; Hughes and Hoover, 1991). It is important to utilise the correct strain of probiotic bacteria for therapeutic effect since not all strains that have the same species name carry the same phenotype of probiotic qualities (Skaaning, 2000).

2.4.5 Characteristics of Lactobacillus acidophilus and Bifidobacterium

As lactic acid bacteria (LAB), L. acidophilus and the bifidobacteria are Gram-positive organisms with rod, cocci, branched or amorphic morphology. They are non spore-forming and non-motile and vary on oxygen needs from facultative anaerobes to micro-aerophiles (Finegold et al., 1983; Fuller, 1989; Gorbach, 1990; Simon and Gorbach, 1986). These bacteria are generally regarded as safe for consumption by humans (Playne and Crittenden, 1998; Salminen et al., 1998b). Table 3 lists some of the differences between Lactobacillus and Bifidobacterium spp.
Table 3: The differences between Lactobacilli and *Bifidobacterium*.

<table>
<thead>
<tr>
<th>Lactobacillus spp.</th>
<th>Bifidobacterium spp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Less anaerobic</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>Grows at $&lt;$pH 4</td>
<td>Only grows $&gt;$pH 4.5</td>
</tr>
<tr>
<td>Bacteriocin production</td>
<td>No bacteriocin production</td>
</tr>
<tr>
<td>No vitamin production</td>
<td>Produces vitamins B6 &amp; B12</td>
</tr>
<tr>
<td>No folic acid production</td>
<td>Produces folic acid</td>
</tr>
<tr>
<td>Oligosaccharides not fermented</td>
<td>Oligosaccharides fermented</td>
</tr>
</tbody>
</table>

Source: CSIRO (1993)
2.4.5.1 \textit{Lactobacillus acidophilus}

\textit{Lactobacillus} cells are usually regular rod-shapes of 0.5-1.2 X 1.0-10.0 $\mu$m in short chain formations. \textit{Lactobacillus} cells are facultative anaerobes and are sometimes micro-aerophilic. They grow poorly in air but better under reduced oxygen tension, therefore growth is generally enhanced by 5\% CO$_2$. Lactobacilli are chemoorganotrophs, requiring rich, complex media. Their metabolism is fermentative, mostly resulting in lactic acid from the fermentation of lactose. When grown in milk, the lactobacilli produce acetaldehyde, as well as diacetyl and ethanol in smaller amounts (Sneath \textit{et al}., 1986).

Despite not being considered taxonomically proteolytic, the lactobacilli grow in milk meaning that they are sufficiently proteolytic to obtain the necessary nitrogen from the milk proteins. The proteolytic ability of the \textit{Lactobacillus} may contribute to texture and flavour characteristics of cultured milk products, this may also contribute to the favourable survival conditions for other organisms in a mixed culture dairy product (Gilliland, 1989).

Many strains of \textit{Lactobacillus} species are naturally resistant to antibiotics by various mechanisms including impermeability, alteration in target molecules, enzymatic drug inactivation, enzyme coupled drug efflux and blocking. This is significant clinically since probiotic strains of \textit{Lactobacillus} are often used prophylactically or therapeutically in conjunction with antibiotics to manage intestinal diseases such as diarrhoea. The combined use of antibiotics with
probiotics for intestinal diseases is more successful than using probiotics alone; the fact that the probiotics are resistant to the antibiotics means they can be used in combination for better effect and not be killed by the antibiotic substance (Charteris et al., 1998; Salminen et al., 1998b).

_Lactobacillus acidophilus_ is not the only species currently utilised for probiotic effect. Different species may be utilised by different companies in their commercial products because strains are selected individually for probiotic effect. _L. acidophilus_ 1 was renamed as _L. johnsonii_ 1 and is the strain used by Nestlé in the LC1 products. The Nestlé strain La1 was chosen from a selection of strains including _L. acidophilus_ La3, _L. acidophilus_ La10 and _L. acidophilus_ La18 (Wahlqvist, 1997). The strains _L. acidophilus_ La-5 and _L. rhamnosus_ GG are used by Vaalia and _L. casei_ Shirota strain is used by Yakult (Schonewille, 2000).

2.4.5.2 _Bifidobacterium_ spp.

The _Bifidobacterium_ cells are rods of very varied shapes, 0.5-1.3 × 1.5-8 μm. They may be short, curved and club-shaped and are often branched into Y-shapes. The cells can be found singly or forming chains or pairs or in various arrangements like a "V" shape or in rosettes. The bifidobacteria are strictly anaerobic, some species may grow in air enriched with 10% CO₂. This genus only grows between pH 4.5 and 8.5 and they are also chemoorganotrophs. Bifidobacteria actively ferment carbohydrates producing mainly acetic and lactic acids in a molar ratio of 3:2 (vol/vol), but not CO₂, butyric acid or propionic acid.
(Dinakar and Mistry, 1994; Sneath et al., 1986). The bifidobacteria only use the 'bifidus pathway', or 'fructose-6-phosphate shunt', for metabolising hexose carbohydrates (Scardovi and Trovatelli, 1965). Compared with other sugars, the growth of *B. infantis* on glucose is lowered, the fastest growth rate is on melibiose, followed by lactose and galactose (Roy et al., 1991).

Human bifidobacteria are divided into two groups: neonate and adults. The species of bifidobacteria found in the intestines of neonates are *B. breve*, *B. infantis* and *B. bifidum*, whereas *B. adolescentis* and *B. longum* are found in adult intestines (Iwana et al., 1993). These strains found in humans are also used in probiotic products (Gainey, 1997). *B. bifidum* Bb-12 is used by Vaalia and *B. essensis* is used by Danone BIO (Schonewille, 2000).
2.5 Probiotic Bacteria in Dairy Foods

Probiotic bacteria may be incorporated into foods or available in dietary supplement form. The majority of foods containing probiotic bacteria are dairy foods due to the historical association of lactic acid bacteria with fermented milk (Sanders, 1998a). Probiotics have been incorporated into fermented milks, yoghurts, soft, semi-hard and hard cheeses, ice-cream, frozen fermented dairy desserts, quark and cottage cheese as well as salami and bread (Dinakar and Mistry, 1994; Gomes et al., 1998; Hekmat and McMahon, 1992; Hull and Studd, 1997; Laroia and Martin, 1991a; Modler et al., 1990; Stanton et al., 1998; Varnam and Sutherland, 1994).

2.5.1 Probiotic Yoghurts

Probiotic yoghurts may be produced using *L. acidophilus*, *B. bifidum, B. longum* or *L. casei* in any combination with or without the normal starter organisms for traditional yoghurt. Therapeutic yoghurt production is more difficult without the presence of the traditional starter organisms as without them acid production by therapeutic starters is slow and more care must be taken not to allow contamination. Therapeutic properties may be enhanced when both sets of starter cultures are used in combination (Varnam and Sutherland, 1994).

In Australia there is a large range of probiotic yoghurts available. Often there are not very high levels of probiotic organisms to be found in these products and their activity may be less then ideal (Dave and Shah, 1997b; Dave and Shah, 1998;
Kailasapathy and Chin, 2000; Kailasapathy and Rybka, 1997; Lankaputhra and Shah, 1997; Martin and Chou, 1992; Medina and Jordano, 1994; Rybka and Fleet, 1997; Rybka and Kailasapathy, 1995; Rybka and Kailasapathy, 1997; Samona and Robinson, 1994; Shah et al., 1995). There are international standards which require that any cultured products sold with health claims must contain a minimum of $10^7$ cfu/g of viable probiotic bacteria at the use-by date (Ouwehand and Salminen, 1998). Many cultured dairy products currently on the market fail to meet these standards because the strains of probiotic bacteria used in production cannot survive the acidity of the product (Hamilton-Miller et al., 1999; Kailasapathy & Rybka, 1997; Rybka and Kailasapathy, 1997; Shah et al., 1995).

Even if there are enough viable cells at production time, the acidity of the product can only increase when it contains lactic acid producing bacteria - eventually inhibiting their own viability (Kailasapathy and Rybka, 1997). Also resistance to acetate may be important for survival in dairy products since acetate is a major by-product of *Bifidobacterium* fermentation (Chung et al., 1999). Probiotic bacteria are unstable in yoghurt; *L. acidophilus* and *Bifidobacterium* spp. are affected by the acidity of yoghurt and a decline in the population is inevitable as growth of *L. acidophilus* ceases at pH 4.0 while that of *Bifidobacterium* spp. at pH 5.0 (Hughes and Hoover, 1995; Lankaputhra et al., 1996; Shah et al., 1995). Post-acidification of yoghurt during the storage period is a large contributor to the death of probiotic cells in the product. There are other factors contributing to
probiotic cell death including hydrogen peroxide production by the yoghurt fermenting cultures which reduces the viability of probiotic bacteria and lowers the oxygen level of the product inhibiting the survival of the anaerobic probiotic cultures (Dave and Shah, 1998; Lankaputhra et al., 1996; Shah et al., 1995).

2.5.2 Probiotic Ice Cream

Probiotic bacteria have been incorporated into ice cream and fermented ice cream which are ideal vehicles of delivery of these organisms in the human diet (Hekmat and McMahon, 1992; Laroia and Martin, 1991a; Modler et al., 1990; Ravula and Shah, 1998). The low storage temperatures of frozen dairy foods is ideal for the long term preservation of bacteria (Modler et al., 1990; Hekmat and McMahon, 1992). The frozen yoghurt type dairy desserts must adhere to the standards of yoghurt and have a pH less than or equal to 4.5, which affects the viability of *L. acidophilus* and *Bifidobacterium* spp. (Ravula and Shah, 1998). Incorporation of probiotic bacteria into ice cream, however, does not pose the same problem as the pH of 6.5-6.6 is more ideal for these organisms (Modler et al., 1990). A quality of ice cream is that of overrun, the incorporation of air, which also affects the viability of the anaerobic organisms (Modler et al., 1990; Ravula and Shah, 1998).
2.5.3 Probiotic Cheese

The incorporation of probiotic bacteria into cheese has been described by Dinakar and Mistry (1994), Hull and Studd (1997) and Stanton et al. (1998). Bifidobacteria survive well in Cheddar and Gouda cheeses, which may be the ideal delivery vehicle for probiotic bacteria in the human diet. Cheese has certain advantages over yoghurt being lower in acidity and higher in fat content offering more protection to the organisms during storage and travel through the gastrointestinal tract (Stanton et al., 1998).

Cheese starter cultures including lactic acid bacteria are required to begin the fermentation of lactose to lactic acid, which reduces the pH of the product. Later during ripening of the cheese, however, the conditions for survival of the starter cultures are not favourable. High salt in moisture, low pH, lack of fermentable carbohydrates and low storage temperature lead to a decline in numbers of the starter cultures in cheese. As the starter culture population declines, the non-starter-lactic acid bacteria (NSLAB) proliferate as the cheese ripens (Stanton et al., 1998). The adjunct bacteria incorporated for probiotic effect must then survive the relatively long ripening period of 6-24 months (Stanton et al., 1998).

Since cheese is an effective delivery vehicle for probiotic bacteria it is important for commercial application that the technology for probiotic cheese manufacture remains simple (Stanton et al., 1998). The study by Dinakar and Mistry (1994) added a commercial lyophilized culture of Bifidobacterium bifidum to Cheddar
cheese curd at the milling stage. This was done to overcome some of the obstacles of the cheesemaking process to probiotic survival such as the aerobic conditions of cheesemaking, cooking and Cheddaring, and the presence of starter culture organisms (Dinakar and Mistry, 1994). They found that the bifidobacteria did not affect the normal aerobic microflora of the Cheddar cheese and did not change the normal proteolytic activity during the ripening period (Dinakar and Mistry, 1994). However, the bifidobacteria were able to remain viable throughout the ripening period at 6 to 7°C even though the optimal temperature for growth of bifidobacteria is 37°C.
2.6 Probiotic Survival in Dairy Foods

2.6.1 Monitoring Survival Levels - Selective Enumeration Media

Several media are available for the selective enumeration of *L. acidophilus* and *Bifidobacterium* spp. For *L. acidophilus* the available media includes bile medium, Rogosa agar, cellubiose-esculin agar, agar based on X-Glu, modified Lactobacillus selective agar (mLBS), Lactobacillus selective medium (mLSM) and deMan Rogosa Sharpe (MRS) medium with salicin, maltose, raffinose or melibiose instead of glucose or dextrose (Dave and Shah, 1996; Hull and Roberts, 1984; Ingham, 1999; Shah, 2000).

For *Bifidobacterium* the available media includes modified Bifidobacterium iodoacetate medium (mBIM), MRS with cystine, bile and dicloxacillin (MRS + BCD), Reinforced Clostridial Prussian Blue agar (RCPB), Reinforced Clostridial Prussian Blue agar adjusted to pH 5 (RCPBpH5), modified Columbia agar (mCol), modified Rogosa’s agar (RMS), Arroyo, Martin and Cotton agar (AMC), lithium chloride-sodium propionate agar (LP), blood-glucose-liver agar with oxgall and gentamycin (BL-OG), modified VF-Bouillon agar plus lithium chloride, sodium lauryl sulfate, sodium propionate and neomycin sulfate, and nalidixic acid- neomycin sulfate- lithium chloride- paromomycin sulfate agar (NNLP), although some strains do not grow on this medium (Calicchia *et al.*, 1993; Dave and Shah, 1996; Ingham, 1999; Lankaputhra *et al.*, 1996; Laroia and Martin, 1991b; Onggo and Fleet, 1993; Payne *et al.*, 1999; Rybka and Kailasapathy, 1996; Samona and Robinson, 1991; Shah, 2000).
Various media are suitable for growing *L. acidophilus* and *Bifidobacterium* spp.; however, they may not be suitable for selective enumeration of one species in the presence of the other or in the presence of starter culture organisms. Another concern is that different strains within the same species display different sugar fermentation characteristics as well as tolerance to selective ingredients such as bile or antibiotics making a true determination of the viable count in dairy products more difficult (Lankaputhra *et al.*, 1996; Shah, 2000).

MRS-Maltose has been used for the selective enumeration of *L. acidophilus* from yoghurt products without *Bifidobacterium* spp. incorporated (Lankaputhra *et al.*, 1996; Shah, 2000). Different media may be more suitable for dairy products with both *L. acidophilus* and *Bifidobacterium* spp. incorporated depending on the sugar utilisation of the specific strain used in the product (Lankaputhra and Shah, 1996; Shah, 2000). A study by Lankaputhra and Shah (1996) compared sugar utilisation patterns by *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus* and different *Bifidobacterium* species. The sugars used were salicin, cellobiose, fructose, mannitol, sorbitol and glucose. Each strain of bacteria was able to use glucose and fructose; however, *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* were not able to use salicin, cellobiose, mannitol or sorbitol. *L. acidophilus* strains were all able to use each of these sugars; however, very few species of *Bifidobacterium* were able to utilise cellobiose, mannitol or sorbitol, and none could use salicin. Thus nutrient agar with salicin as the carbon source at 0.5% concentration was found suitable for selective enumeration of *L.*
acidophilus from samples containing yoghurt-fermenting cultures and Bifidobacterium spp. (Dave and Shah, 1996; Lankaputhra and Shah, 1996).

Shah (2000) describes a situation when dairy products contain both L. acidophilus and L. casei as probiotic adjuncts and explains how MRS-salicylic or MRS-sorbitol agar may be used for enumerating both organisms together. At the same time LC agar is used to determine the viability of L. casei alone and the subtraction method may then be used to determine the count of L. acidophilus alone. Likewise a subtraction method has been used for the determination of Bifidobacterium viability in a mixed culture (Shah, 2000).

2.6.2 Why Probiotic Viability is Important

All of the definitions of probiotics emphasise the importance of viability of the bacteria to have a probiotic effect (Fuller, 1989; Haller et al., 1999; Hamilton-Miller et al., 1999; Kailasapathy and Chin, 2000; Naidu et al., 1999; Salminen et al., 1998a). The probiotic bacteria must not only survive in the food during the shelf life of the product but must then survive the transit through the gastrointestinal tract and the acidity of the stomach as well as enzymes and bile salts in the intestine before reaching the site of action (Kailasapathy and Chin, 2000). Therefore there is a standard set that any foods sold with health claims due to the inclusion of probiotic bacteria must contain at least $10^7$ cfu/g viable probiotic bacteria at the use-by date (Ouwehand and Salminen, 1998; Stanton et al., 1998). If viability is important then the high numbers of viable cells is also
important since it is likely that the numbers of viable cells will decline in the food as well as in transit through the gastrointestinal tract (Martin, 1996).

There is some evidence that non-viable probiotics and bacterial components also have some health benefits; however, no comparisons have been made between the efficacy of viable cells against non-viable cells (Ouwehand et al., 1999; Ouwehand and Salminen, 1998). The best documented health effects are available for viable cells although some benefits of using non-viable cells include fewer safety concerns, no risk of infection, excellent shelf life and no risk of transfer of antibiotic resistance (Ouwehand and Salminen, 1998).

2.6.3 Reasons For Low Viability

There are several factors which have been reported to affect the viability of probiotic cultures in fermented milk products including titratable acidity, pH, hydrogen peroxide, dissolved oxygen content, storage temperature, species and strains of associative fermented dairy product organisms, concentration of lactic and acetic acids and buffers such as whey protein concentrates which have been identified to have an effect during manufacture and storage of dairy products (Clark et al., 1993; Dave and Shah, 1997c; Kailasapathy et al., 1996; Kailasapathy and Supriadi, 1996; Lankaputhra et al., 1996; Lankaputhra and Shah, 1995; Ravula and Shah, 1998; Rybka and Kailasapathy, 1995; Samona and Robinson, 1994; Shah, 2000; Shimamura et al., 1990; Shimamura et al., 1992).
The organisms' survival in dairy products is not the only issue at stake. In fact, food provides a buffer to protect the microorganism in the gut (Supriadi et al., 1994). The bacterial strains selected as probiotics must also be able to survive the gastric acidity (pH 1 - 4), the bile salts, the enzymes such as lysozymes present in the intestines, toxic metabolites including phenols produced during digestion, bacteriophage, antibiotics and anaerobic conditions. Furthermore, the human g.i.t. and stomach have the highest acidity, the probiotic bacteria must not only survive these conditions but are required to colonise the gut. Therefore probiotic strain selection is very important as many strains of L. acidophilus and Bifidobacterium spp. cannot survive these conditions (Goldin, 1998; Gopal et al., 1996; Kailasapathy and Chin, 2000; Lankaputhra and Shah, 1995; Tejada-Simon et al., 1999; Wang et al., 1999).

2.6.4 Selecting Strains Able to Survive

Within probiotic species the strain variation is large, therefore the qualities of probiotics can vary by species as well as by strain (Chung et al., 1999). Probiotic strains of human origin should be chosen as their health effects may be host specific (Vaughan and Mollet, 1999; Yaeshima, 1996). The pH of the stomach may fall as low as pH 1.5 and the bile concentrations range from 0.5 to 2.0% in the first hour of digestion although this may decrease in the second hour (Kailasapathy and Chin, 2000). Selection experiments may thus focus on the organism's resistance to conditions of low pH and bile, due to the need for the bacteria to survive the gastrointestinal tract of humans as well as the acidity of
fermented dairy products such as yoghurt (Chou and Weimer, 1999; Chung et al., 1999; Clark et al., 1993; Dunne et al., 1999; Gopal et al., 1996; Kailasapathy and Chin, 2000; Lankaputhra and Shah, 1995; Martin, 1996; Mattila-Sandholm et al., 1999; Naidu et al., 1999; Ouwehand et al., 1999; Shah, 2000; Vaughan and Mollet, 1999; Zavaglia et al., 1998).

Other factors that probiotic bacteria are selected against include oxygen due to the organisms being mostly anaerobic but being incorporated into food in an aerobic system. Also water activity for freeze dried cultures stored at room temperatures for long periods of time and freezing for incorporation into frozen dairy desserts (Chou and Weimer, 1999; Condon, 1987; Kailasapathy and Chin, 2000; Klaver et al., 1993; Shimamura et al., 1992; Zavaglia et al., 1998).

Selection may also be for certain qualities considered important for probiotics used in humans such as adherence to particular cell lines, antimicrobial activities, immune modulation, anti-allergenic effects, anti-mutagenic and anti-tumorigenic activity and nutritional advantages (Dunne et al., 1999; Ouwehand et al., 1999; Stanton et al., 1998; Vaughan and Mollet, 1999).
2.6.5 Methods of Increasing Probiotic Survival

Substances such as simple sugars, minerals and vitamins have been shown to stimulate the growth of *L. acidophilus* and bifidobacteria (Kailasapathy and Rybka, 1997). The growth of *L. acidophilus* may be stimulated by the addition of simple sugars such as glucose and fructose and minerals such as magnesium and manganese, which could be in the form of tomato juice or papaya pulp and result in higher viable counts, shorter generation times and improved sugar utilisation. A similar result can be seen with the addition of acetate or by supplementing milk with casitone, casein hydrolysate and fructose (Kailasapathy and Chin, 2000).

The growth of bifidobacteria is stimulated by vitamins, dextrin and maltose. Bifidobacteria grow poorly in milk but the survival of *B. longum* is improved with the inclusion of 0.01% bakers yeast. The use of oligosaccharides as prebiotics allows favoured growth of probiotic bacteria in the colon. Raffinose, stachyose, fructo-, isomalto- and galacto-oligosaccharides are good substrates for bifidobacteria to metabolise to acetic and lactic acids (Kailasapathy and Chin, 2000; Kailasapathy and Rybka, 1997; Mitsuoka, 1992).

In dairy products it would also be possible to increase the survival of probiotic bacteria by manipulation of production and storage conditions. This could be achieved by completing the fermentation process at a higher pH than normal (>5) allowing better survival of bifidobacteria (Varnam and Sutherland, 1994). Storing
the product at a lower temperature (below 3 - 4°C) increases the survival of AB culture (Sakai et al., 1987). Addition of whey protein concentrate and acid casein hydrolysate to yoghurt mix increases its buffering capacity, thus the probiotic cultures survive better (Dave and Shah, 1998; Shah, 2000; Supriadi et al., 1994). Application of hydrostatic pressure (200-300 MPa for 10 minutes at room temperature) to the product to prevent after-acidification would thereby maintain the number of viable LAB (Tanaka and Hatanaka, 1992). Heat treatment (5 minutes at 58°C) to prevent excess acid production and to maintain a constant acidity during storage would mean that the pH cannot reduce further and cause a decline in probiotic viability (Marshall, 1992). Lower incubation temperatures (37°C rather than 40 - 42°C) would increase incubation time and growth of bifidobacteria (Costello, 1993). Use of yoghurt starter cultures without *L. delbrueckii* subsp. *bulgaricus* can also reduce post-acidification of the product and overcome severe losses in viability of probiotic bacteria (Shah, 2000).

Storage of dairy products in glass containers rather than plastic containers would decrease the dissolved oxygen content and allow more anaerobic organisms to survive (Shah, 2000). Two-step fermentation of yoghurt where the probiotic cultures are added first and allowed to establish their populations in the first stage of fermentation, followed by a second stage of fermentation with the yoghurt starter cultures resulted in higher viability of probiotic organisms (Lankaputhra and Shah, 1997; Shah, 2000). Probiotic bacterial viability may also be achieved by stress adaptation of the culture to the harsh conditions of the
fermented dairy products, particularly the low pH of yoghurt (Shah, 2000). Fortification of products with ascorbic acid (vitamin C) or L-cysteine hydrochloride to act as an oxygen scavenger and to lower the redox potential increases the viability of L. *acidophilus* (Dave and Shah, 1997a; Dave and Shah, 1997d; Shah, 2000). Rupturing of yoghurt starter cultures to release the intracellular β-galactosidase and to reduce their viable count increases the viability of the probiotic cultures *L. acidophilus* and *Bifidobacterium* spp. (Shah, 2000; Shah and Lankaputhra, 1997). Microencapsulation is a technique, which has been used to increase survival of probiotic bacteria in human gastric and intestinal juices (Kailasapathy and Chin, 2000; Kailasapathy and Rybka, 1997; Rao et al., 1989; Shah, 2000; Sultana et al., 2000; Zavaglia, 1998).

2.6.5.1 Increasing Probiotic Survival by Adjusting Production Method

Three different methods were used by Lankaputhra and Shah (1997) to ferment yoghurt: single-step fermentation, two-step fermentation and fermentation beginning with a neutralised yoghurt mix. Two-step fermentation means introducing the probiotic bacteria into the product mix 2 hours earlier than the yoghurt culture in order for the probiotic culture to stabilise itself. The neutralised mix procedure involved adjusting the pH of the product mix from 6.6 to 6.9 before fermentation such that it should take longer to achieve pH 4.5, thereby giving more time for the probiotic cultures to grow. The results from Lankaputhra and Shah (1997) showed that the viable counts of *L. acidophilus* 2409 and *B. longum* 1941 were higher initially and were maintained higher with less cell density lost
when the yoghurt was produced by two-stage fermentation as well as in yoghurt prepared with neutralised mix. Fermentation time for the products to reach pH 4.5 was the longest for the two-step fermentation and shortest for the commercially used single-step fermentation. The neutralised mix took longer to reach pH 4.5 by 20 minutes for each 0.1 pH unit adjustment (Lankanpathra and Shah, 1997).

2.6.5.2 Increasing Probiotic Survival by Genetic Modification

2.6.5.2.1 Lactic Acid Bacteria and Genetic Modification

Since there are a number of characteristics that are ideal for a probiotic culture and it is unlikely that any particular strain would have all of those traits it would be possible to derive a new strain by genetic manipulation. The ideal probiotic culture would be a strain that could survive in the human gastro-intestinal tract as well as colonise the gut. The perfect strain should inhibit pathogens in the gut as well as contributing to the health of the host by synthesising essential nutrients not found in the diet or by auto-digesting dietary substances that the host cannot process themselves. The ideal probiotic would be suitable for cultivation on a large scale in industrial conditions as well as being suitable for preservation for storage before retail sale or incorporation into food. Crucially, the ideal probiotic strain should also be avirulent and not have any metabolic traits that could compromise the host's health. Finally, all of the mentioned ideal probiotic characteristics need to be stable properties of the cell, which if it occurs naturally would be too costly to screen for. It would be possible to derive the perfect strain
by gene technology. The initial microbe would be chosen for the target of the probiotic. A human originating strain would be used since the human gastro-intestinal tract is unique in terms of the large proportion of it being free from permanent colonisation as well as having large populations in the terminal ileum and large bowel. Colonisation of probiotics in the g.i.t. is ideal; however, the perfect strain would need to be able to overcome gastric acidity, mucus secreting mucosa lining the entire g.i.t. as well as rapid peristaltic propulsion of digesta through the small bowel. The nature of the probiotic would also be determined by whether the strain is to provide long-term colonisation in the gut to be able to continually exert its influences on the bowel or if frequent delivery is desired to specific locations within the g.i.t. in prescribed doses, which would give more choice in the original microbe. The most important consideration with genetic manipulation of gastro-intestinal microflora would be the stability of the recombinant DNA, ensuring that the plasmids cannot be transferred to other organisms in the gut (Tannock, 1992).

Some possible probiotic developments by gene technology would be strains that can immunise, particularly from mother to infant while feeding, delivery strains of other characteristics such as specific molecules to particular regions of the intestinal tract. Another development could be of probiotics for nutrition, such that the host can utilise more energy from the diet. The three crucial factors to assess genetically modified gut organisms for would be the access, expression and damage. Access means the probability of the organism escaping and
entering the human body, surviving and entering susceptible tissues. Expression relates to how efficiently the new gene will be expressed and damage is an estimation of the probability that the product of the foreign gene will cause physiological damage to the host (Tannock, 1992). Regulation and consumer acceptance again become the prime issues thus more inventive ways of increasing survival of probiotics must be investigated.

2.6.5.2.2 Regulation of Genetically Modified Foods

Regulation of genetically modified foods is not uniform around the world. Australia is in the stage of developing standards, which may require industry to determine whether foreign DNA is present in highly refined ingredients, processing aids, food additives and flavourings. The proposed regulation requires labelling of food and food ingredients where novel DNA and/or protein is present in the final food or where the food has altered characteristics. Some exemptions would be highly refined food where the effect of the refining process is to remove novel DNA and/or protein; food additives unless the foreign DNA and/or protein is still present in the final food; flavours present in a concentration less than or equal to 0.1% in the final food; and food prepared at the point of sale e.g. restaurants. Due diligence and verifiable documentation are required for compliance, especially for highly refined food. The crux of the regulation system is food safety, which is addressed at "world-best-practice level" of the Australia New Zealand Food Authority (ANZFA) (McMahon and McNiece, 2000).
2.7 Microencapsulation of Probiotic Bacteria to Improve Survival in Dairy Foods

2.7.1 Encapsulation Definition

Encapsulation is defined as "the process of forming a continuous, thin coating around encapsulants (solid particles, droplets of liquids or gas cells) which are wholly contained within the capsule wall as a core of encapsulated material" (King, 1995). The distinction is made from entrapment, which refers to "the trapping of encapsulants within or throughout a matrix (e.g. a gel or crystal etc)", different from encapsulation as some of the entrapped ingredients may be exposed at the surface of the particle (King, 1995). The material to be coated is called the active, internal phase, fill or core material while the coating material is referred to as the shell, coating, wall material, carrier or encapsulant and may vary in thickness and number of layers (Bhandari and D'Arcy, 1996; Dziezak, 1988).

Microencapsulation as a strict term would apply to particles of size 0.2-5000 µm while those larger than 5000 µm are classified as macro- and those smaller than 0.2 µm are classified as nano-microcapsules. If the core material is very large then the process is referred to as "coating" (Bhandari and D'Arcy, 1996). Ideally the encapsulated particle is spherical; however, this is influenced by the structure of the core material (Dziezak, 1988).
Encapsulated food ingredients include enzymes, flavours, flavour enhancers, sweeteners, antioxidants, food preservatives, acidulants, amino acids, colourants, edible oils, fats, leavening agents, vitamins, minerals, salts and microorganisms including lactic acid bacteria. Microencapsulation is not only used in the food industry, the process also has applications in the biotechnology, pharmaceutical and chemical industries (Bhandari and D'Arcy, 1996; Kim and Baianu, 1991).

2.7.2 Applications of Encapsulation

The original use of microencapsulation, in 1954, was for carbonless copy paper where a colourless dye was chemically microencapsulated, by coacervation, in particles less than 20 μm. The encapsulated dye was spread in a very thin layer on the back of a paper coversheet and the page underneath was similarly coated with a colourless reagent. When a person wrote on the coversheet the pressure of the pen or pencil nib ruptured the capsules releasing the dye which in turn reacted with the reagent on the page underneath to produce a coloured image (Dziezak, 1988; Jackson and Lee, 1991).

Encapsulation can be used for many applications in the food industry including stabilising the core material, controlling the start of a reaction, providing sustained or controlled release (both time of release and rate of release), to mask flavours, colours or odours, to indicate pressure, to extend shelf life or protect components against nutritional loss, to convert a liquid into solid, to
reduce volatility or to separate reactive materials (Dziezak, 1988; Johnson et al., 1996; Kim and Baianu, 1991).

Lactic acid bacteria, including *Lactococcus lactis* and *Lactobacillus helveticus*, have been immobilised in calcium alginate beads to extend the shelf life of raw milk and products made from raw milk by inhibiting the psychrotrophic bacteria normally found in raw milk by about 50% (Champagne, 1990). Also immobilised cells used in fermented dairy products can be inoculated at high rates to possibly reduce the fermentation time and then be recovered and re-used (Larisch et al., 1994).

Culture bacteria must be alive to provide benefits to their hosts. Therefore, they must not only survive transit through the gastro-intestinal tract but also in the storage food. Encapsulation has been used for food ingredients that require protection from certain factors such as processing temperature, moisture or other components of the food system (Pothakamury and Barbosa-Canovas, 1995). Likewise probiotic bacteria requiring viability during passage through the gastrointestinal tract have been encapsulated to ensure this (Gobbetti et al., 1998; Rao et al., 1989). Gobbetti et al. (1998) produced calcium alginate encapsulated bifidobacteria to be incorporated into Crescenza cheese.
An alternative reason for encapsulation of food ingredients is for delayed release such as for artificial sweeteners protected from high temperatures to prevent degradation but released such that the sweetness is detected at the end of baking (Dziezak, 1988). It is important that at certain stimuli the encapsulated material can be released at a controlled rate (Pothakamury and Barbosa-Canovas, 1995). In the case of probiotic bacteria and foods, controlled release means that the bacteria will not be lost due to exposure to the product and that once inside the host the bacteria could theoretically be released from encapsulation over a prolonged period of time allowing a continuous renewal of the population in the gut.

Controlled release of encapsulated ingredients in foods may be via a number of mechanisms:

- Pressure-activated release: the active ingredient is released when pressure is applied to the wall.
- Diffusion-controlled release: the active ingredient is allowed to diffuse through the polymer or through pores within the polymer.
- Osmotically-controlled release: when a large osmotic pressure is created inside the capsule the active ingredient is released.
- Barrier-controlled release: the active ingredient will escape once the critical part of the barrier or encapsulant is removed e.g. wall permeability, wall thickness or concentration.
- Solvent-activated release: the active ingredient is released when a solvent contacts the food and the microcapsule swells.

- pH-controlled release: the active ingredient is released at a particular pH.

- Temperature-sensitive release: the active ingredient is released due to a temperature change.

- Melting-activated release: when the food is heated and the wall material melts the active ingredient is released.

- Combined systems: the active ingredient is released due to a combination of mechanisms.

The actual mechanism of release may be one of the described methods or a combination, which will utilise certain kinetics. Ideally the kinetics of the system may be either zero-, half- or first-order. The constant release rate of zero-order is used when the core material is a pure substance and must be released as such. Generally, half-order release is used for matrix particles and first-order release is used when the encapsulated material is a liquid. In reality kinetics is not so simple because the release rate may not be zero-, half- or first-order. However there are release rate equations available to assist in the design of controlled release systems (Pothakamury and Barbosa-Canovas, 1995).
The release mechanism will be related to the wall material and the application as well as the core material. The wall material should be capable of being hardened rapidly into a thin film around the product. The coating material should be non-reactive with the encapsulated product, nor should the two be able to be mixed together. The wall material should also withstand storage, handling and the environment it is to be used in (Johnson et al., 1996). Depending on the application, the cells may not need to be released. In such a case, alginate beads containing lactic cultures have been coated with poly-L-lysine membrane to minimize cell release (Hyndman et al., 1993).

2.7.3 Encapsulating Materials

The encapsulating material chosen for a particular application would depend on the physical and chemical properties of the core material as well as the method used for capsule formation. It should be non-reactive with the core material and insoluble (Jackson and Lee, 1991). Shell materials used in the food industry fall into the general classifications of fats and/or emulsifiers, low molecular weight sugars and polymers, which may then be categorised into synthetic or natural polymers (King, 1995).
The coating material may be gums such as gum arabic, gelatine, gellan gel, sucrose, maltodextrin, β-cyclodextrin, modified starches, starches from various sources, corn syrup solids, common salt, vegetable and milk proteins, waxes or fats, carbohydrates, gums, locust bean gum, agarose, alginate, polyethyleneimine (PEI)-modified alginate, carageenan, chitosan, phosphoguargum, cellulose acetate phthalate (CAP), proteins or lipids (Brodelius and Nilsson, 1983; Büyükgüngör, 1992; Camelin et al., 1993; Champagne, 1990; Champagne et al., 1993; Dainty et al., 1986; Gobbetti et al., 1998; Groboillot et al., 1993; Hari et al., 1996; Hyndman et al., 1993; Jankowski et al., 1997; Joung et al., 1987; Kebary et al., 1998; Kenyon, 1995; Kim et al., 1996; Maharaj et al., 1984; Monshipouri and Price, 1995; Nastruzzi et al., 1994; Pothakamury and Barbosa-Canovas, 1995; Rao et al., 1989; Wang and Hettwer, 1982).

Ionotropic gels refer to those that depend on the ionic composition of the surrounding material for the stability of the carrier network, including alginate, carrageenan, chitosan, gellan gel and phosphoguargum, which are reversible and are the most popularly used for the immobilisation of whole microbial cells (Büyükgüngör, 1992). There is an advantage in using natural polymers for encapsulation of microorganisms, which is that the reagents are non-toxic and the procedures are gentle to the organisms, (Chitosan has an inhibitory action on lactococci) (Hyndman et al., 1993; Jankowski et al., 1997). Bifidobacteria have been shown to survive better in capsules of alginate rather than that of κ-carrageenan (Kebary et al., 1998).
Each carrier material has various encapsulation efficiencies and release properties as well as chemical influences on the core material depending on its own qualities, such as the oxidation of flavour compounds. A carrier material would be chosen for a particular application depending on a variety of factors, including molecular weight, solubility, film formation, diffusibility, crystallinity and glass transition. An important consideration for encapsulation in the food industry is that of flavour and texture contributions of the carrier material to the final product, as well as cost of the procedure (Bhandari and D'Arcy, 1996).

2.7.3.1 Calcium Alginate

Alginate has been used for the immobilisation of bacterial, algal, fungal, plant and mammalian cells (Smidsrød and Skjåk-Bræk, 1990). While many encapsulating materials are available, calcium alginate is commonly used for encapsulation of microbial cells, lactic acid bacteria in particular (Dainty et al., 1986; Yoo et al., 1996). Alginate is non toxic and used elsewhere in the food industry as a food additive as well as being a primary ingredient for anti-peptic drugs and as a drug carrier (Hari et al., 1996; Prevost and Divies, 1992; Smidsrød and Skjåk-Bræk, 1990). The advantages of using alginate over other encapsulating materials is that alginate is inexpensive and easy to use, it forms gels rapidly with divalent metal ions such as Ca++, it is thermally stable, reversible and has a high retention of cell viability (Dainty et al., 1986; King, 1995; Larisch et al., 1994; Lee and Heo, 2000; Levy and Edwards-Levy, 1996; Monshipouri and Price, 1995; Prevost and Divies, 1992; Sheu and Marshall, 1993).
Alginate encapsulation is easily reversible because the calcium alginate gel structure is dependent on ionic interactions. Therefore, it may be easily disrupted by cation chelating agents such as phosphate, involving a complex interaction of cation type, concentration and pH of the solution (Champagne et al., 1993; Dainty et al., 1986; Hari et al., 1996).

Alginate is a naturally occurring polymer produced mainly from *Laminaria hyperborea*, *Macrocystis pyrifera* and *Ascophyllum nodosum* as well as from *Laminaria digitata*, *Laminaria japonica*, *Eclonia maxima*, *Lesonia negrescens* and *Sargassum* sp. (Smidsrød and Skjåk-Bræk, 1990). Alginate consists of β-D-mannuronic acid (M) and α-L-glucuronic acid (G) units linked by 1,4-glycosidic bonds. The polymers form an irregular pattern of blocks along the chain with regions of M or G units, homopolymeric regions, interspersed with MG blocks, regions of alternating structure (Hannoun and Stephanopoulos, 1986; Smidsrød and Skjåk-Bræk, 1990). The monovalent alginate is water soluble; however, upon exposure to polyvalent cations, such as calcium ions, cross linking is achieved and a polymer is formed (Hannoun and Stephanopoulos, 1986). The majority of cells encapsulated with calcium alginate are able to remain viable after freeze drying and their activity persists after a long storage (Hannoun and Stephanopoulos, 1986; Morin et al., 1992).
The diffusion coefficient of both ethanol and glucose in calcium alginate is reported to be almost the same as that in water, only 9% slower. However the diffusion coefficient of oxygen in barium alginate was found to be only 25% of that in water. Diffusion of some nutrients allows the entrapped cells to continue growing while control of diffusion coefficients of other factors allows greater control over the density of cell growth inside the capsule. While growth may be desirable to prevent enzyme deactivation, excess growth may cause cell leakage, support breakup, reduced product yields as well as an accumulation of toxic by-products of the cell inside the capsule (Hannoun and Stephanopoulos, 1986).

2.7.4 Encapsulation Methods

A number of encapsulation methods are available and are used variously depending on the sample to be encapsulated as well as the encapsulating material and the end use of the encapsulated product (Bhandari and D'Arcy, 1996). Different encapsulating methods will also be able to produce different sized capsules. Some common methods of encapsulation include stationary extrusion, submerged nozzle, centrifugal extrusion, centrifugal suspension-separation, vibrating nozzle, rotating disk, spray drying, spray coating using a fluidised bed, air suspension, interfacial polymerisation, phase separation-coacervation, interfacial polymerization, electrostatic deposition, pan coating, solvent evaporation, membrane emulsification, molecular inclusion via β-cyclodextrin, cocrystallisation and in situ polymerisation (Bhandari and D'Arcy,
1996; Green et al., 1996; Groboillot et al., 1993; Hyndman et al., 1993; Johnson, 1996; Muramatsu and Kondo, 1995; Rao et al., 1989; Schlameus, 1995; Sparks et al., 1995).

In the food industry a variety of encapsulation methods have been employed previously. The use of any particular method is dependent upon economics, sensitivity of the core material, desired size of the capsules, physical/chemical properties of both the core and shell materials, application of the final food ingredient as well as release mechanism (Jackson and Lee, 1991).

2.7.4.1 Spray Drying
Spray drying is the most common method of encapsulation in the food industry since it is highly economical. Other advantages include the high stability of most food-grade coating materials, rapid solubilisation of the capsules due to their small size and the equipment is readily available since the method is so common. Some disadvantages of spray drying are that the coating material must have high viscosity at high concentrations thus the choice of materials is limited, the heat drying method may cause degradation and/or oxidation of some active materials, the active material may be able to form azeotropes with the solvent and this method cannot be used for intermediate moisture foods. This method has been used to encapsulate sugars, polysaccharides, starches, proteins, vitamins, pigments, fats, oils, flavour compounds and leavening agents. Coating materials used in spray drying include carbohydrates such as dextrins, sugars,
starches, modified starches and gums such as gum arabic or gum acacia, or proteins such as gelatin and soy protein, and cellulose esters and ethers (Dziezak, 1988; Jackson and Lee, 1991; Kim and Baianu, 1991; King, 1995).

The spray drying process involves forming an emulsion or suspension of the core and shell materials mixing of the emulsion with circulating hot dry air in a drying chamber, which then dries the liquid droplets as the moisture evaporates on contact with the hot air. The remaining solids from the shell material entrap the core material into capsules. Since this method is very rapid, it is useful for heat sensitive food ingredients (Dziezak, 1988; Jackson and Lee, 1991; Kim and Baianu, 1991).

2.7.4.2 Coacervation

Also called phase separation, this is the technique used for the very first encapsulation for the manufacture of carbonless copy paper. The process is not widely used in the food industry since it is complex and costly and very few food grade polymers are available for shell materials. The advantage is that coacervation is very efficient and results in microcapsules of a larger variety of sizes than any other method (Bhandari and D'Arcy, 1996; Dziezak, 1988; Jackson and Lee, 1991). Aqueous phase separation has been used for the microencapsulation of citrus essential oils, vegetable oils, vitamin A and flavours. All core materials of aqueous phase separation are hydrophobic and therefore a water soluble wall material such as gelatin, gum acacia or gum arabic is used.
Non-aqueous phase separation allows the encapsulation of water-soluble core materials using a hydrophobic wall material such as ethylcellulose or styrene-maleic acid copolymers (Bhandari and D'Arcy, 1996; Dziezak, 1988; Jackson and Lee, 1991).

The procedure for encapsulation by coacervation involves firstly mixing three immiscible phases: the continuous phase (water), the core material and the shell material. By changing pH, temperature or ionic strength phase separation (coacervation) of the coating material is achieved, which entraps the core material. The coating is then solidified by thermal, cross-linking or desolvation techniques (Dziezak, 1988; Jackson and Lee, 1991; King, 1995; Bhandari and D'Arcy, 1996).

2.7.4.3 Centrifugal Extrusion Encapsulation

Centrifugal extrusion encapsulation is used for specialty applications requiring protection of food ingredients. The core materials must be a liquid at room temperature with viscosity low enough to form droplets when the extruded stream from the nozzle breaks up. Some core materials may be vegetable oils, hard fats, flavour oils, vitamins, micronutrients, acids, dyes, seasonings, aqueous systems and air. Suitable shell materials may be gelatin, sodium alginate, carrageenan, starches, cellulose derivatives, gum arabic, fats/fatty acids, waxes, polyethylene glycol or a mixture depending on the requirements of the product (Dziezak, 1988; Schlameus, 1995).
The process itself requires a centrifugal extrusion device, which has nozzles as concentric orifices out of a rotating cylinder head. The liquid core material is pumped through the inner tube while the liquid shell material is fed through the outer vessel of the same tube forming an inner rod of the core material surrounded by the shell material. The two materials are co-extruded and as the device rotates the extruded rods break into droplets, which form capsules (Dziezak, 1988; Schlameus, 1995).

2.7.4.4 Rotational Suspension Separation

The rotating disk method of encapsulation is favoured for food and flavour applications as it is extremely rapid, requiring only seconds. It is a continuous, high production rate process suitable for applications ranging in size from 30 μm to several millimetres. The products are small enough to avoid adding grittiness to food products and have been used for preservation of flavours and aromas in frozen foods, released during cooking. This method is also used for encapsulating enzymes and reducing oxygenation (Dziezak, 1988; Sparks et al., 1995).

The process involves forming a suspension of the core material in the liquid coating material and passing this suspension over a rotating disk under conditions giving a film of the coating liquid at the edge of the disk which is much thinner than the diameter of the core particles. In practice, two different types of particles are formed at the edge of the rotating disk: those that are core particles
surrounding by shell material, and smaller particles that are just droplets of shell material. All the particles formed are hardened but a sieve separates the particles that are only shell material, which are then recycled (Dziezak, 1988; Sparks et al., 1995).

2.7.4.5 Emulsion Encapsulation/Entrapment

The emulsion encapsulation/entrapment method had been used extensively for the formation of microcapsules for pharmaceutical use before being used in the food industry. By this process an active material is added to a hydrocolloidal solution as the aqueous phase. The aqueous phase is added to a large volume of oil and the mixture is homogenised to form an emulsion. The water soluble polymer must then be insolublised, or cross-linked, forming tiny gels within the oil phase (King, 1995).

The traditional method of preparing calcium alginate capsules was by adding calcium chloride dropwise to a sodium alginate solution; however other methods have been used for specific application. Muramatsu and Kondo (1995) had the problem of not being able to use a water-in-water type dispersion thus Ca-EDTA was used in place of calcium chloride to provide the calcium ions for gelation (Muramatsu and Kondo, 1995). The method developed by Sheu and Marshall (1993) used a water/oil emulsion in which the alginate (3%) and culture mixture was added dropwise to vegetable oil containing 0.2% Tween 80. The capsules were then hardened with calcium in the form of calcium chloride. The beads
could be collected by centrifugation and washed with sterile water. This method has been used for the preservation of probiotic bacteria in dairy products (Sheu et al., 1993; Sheu and Marshall, 1993).

Yoo et al. (1996) performed the encapsulation procedure in the reverse order, first mixing the cells with sterile calcium chloride solution and dropping this cell suspension through a blunt ended needle into 0.6% sodium alginate solution emulsified with 0.1% Tween 20. The excess sodium alginate was removed by washing with sterile saline and the capsules formed were left to harden in a 1% calcium chloride solution (Yoo et al., 1996).
2.8 Summary of Literature Review

Some research has been done showing the therapeutic effectiveness of live probiotic bacterial cells as opposed to the usage of non-viable cells or cellular components for probiotic effect. Although the work on non-viable cells and cellular components is not complete, many results suggest that for a therapeutic probiotic effect the cells must be alive and active (Ouwehand et al., 1999; Ouwehand and Salminen, 1998). As a result, many researchers focus on maintaining viability of probiotic bacterial cells.

The probiotic bacterial cells must reach the site of action alive and active in order to exert a therapeutic effect on the host. Research to date has focused on maintaining viability of the probiotic bacterial cells during transit through the gastrointestinal tract to the site of action. Much work has been done on the resistance and tolerance of probiotic bacteria to low pH and high bile concentration conditions, similar to those of the gastrointestinal tract.

More work needs to be done regarding the survival of probiotic bacteria in foods, during processing as well as during the shelf life of the product. Many different conditions may be encountered by the probiotic bacteria depending in the food it is incorporated into, such as air, very low storage temperatures and sucrose for dairy dessert type products. It is possible to select strains suitable for these conditions as well as those of the gastrointestinal tract because there is a need for maintaining viability in the foodstuffs that probiotic bacteria are incorporated
into. The International Dairy Federation now says that to be therapeutically effective a product claiming to contain probiotic bacterial cells must do so at a rate of $10^7$ cfu/g (Ouwehand and Salminen, 1998).

Some work has also been done studying the causes of low probiotic bacterial survival in dairy products, particularly yoghurt. Little work has been done on the survival of probiotic bacteria in other dairy products. The fact that a standard for the minimum survival rate of $10^7$ cfu/g exists acknowledges that it is possible to lose probiotic bacterial cells in the food product over the shelf life duration (Ouwehand and Salminen, 1998). Many techniques have been attempted in the quest for improving survival of probiotic bacterial cells in dairy products, particularly yoghurt. Methods for improving survival that have been researched are mostly dependent on the food product itself, altering processing or storage conditions.

A method for direct protection of the probiotic organisms has been proposed. Microencapsulation has been used by many researchers for varied purposes. Probiotic bacteria have been microencapsulated before and this has been shown to increase survival in adverse conditions of low pH and low temperatures (Sheu et al., 1993; Sheu and Marshall, 1993). An integrated study is needed to research the various strain selection criteria and the utilisation of microencapsulation in improving the survival of probiotic bacteria in dairy.
products and the sensory effects of incorporating encapsulated probiotic bacteria into dairy food.
CHAPTER 3

MATERIALS AND METHODS

3.1 Media Preparation.

RSM (Reconstituted Skim Milk): This includes 9.5% skim milk powder, 0.5% yeast extract and 2% glucose. For 100mL: 9.5g skim milk powder, 0.5g yeast extract was dissolved in 92mL distilled water and autoclaved at 121°C for 15 min. A separate solution of 25% glucose (25g glucose in 100mL distilled water) was mixed and autoclaved at 121°C for 15 min. After cooling, 8mL of the 25% glucose solution was aseptically added to the milk and yeast extract such that the final concentration of glucose was 2%.

MRS (deMan-Rogosa-Sharpe) Broth: Consists of: 20g/L glucose, 10g/L peptone, 10g/L lemco powder, 5g/L yeast extract, 1mL/L Tween 80, 2g/L dipotassium hydrogen phosphate, 5g/L sodium acetate, 2g/L triammonium citrate, 0.2g/L magnesium sulphate (.7 H₂O), 0.05g/L manganese sulphate (.4 H₂O). The media is available in powdered form from Oxoid and was used in this fashion according to the manufacturers instructions which requires 52g powdered MRS media per litre of distilled water. Once the media was dissolved in the water, it was sterilised by autoclaving for 15 min at 121°C.

MRS agar: MRS broth and 15g/L agar were warmed in a microwave oven on medium power to melt the agar. The media was then sterilised in an autoclave, and poured into petri plates after the media was cooled to approximately 50°C.
MRS-C (MRS-Cysteine): MRS broth prepared as above, with 0.05% L-cysteine hydrochloride added as dry weight with powdered MRS broth.

NGYC (Non-fat milk, Glucose, Yeast extract, Cysteine): A solution of 12% non-fat dry milk, 2% glucose and 1% yeast extract were steam sterilised in an autoclave. A solution of 10% L-cysteine-hydrochloride was also sterilised for 15 min at 121°C. After cooling the L-cysteine hydrochloride was added to a final concentration of 0.05% in the media.

Phosphate Buffered Saline (PBS): 9g/L NaCl, 0.2g/L KH₂PO₄ (anhydrous), 2.9g/L Na₂HPO₄ (anhydrous), 2g/L KCl, were dissolved in distilled water, pH was 7.2. The solution was autoclaved at 121°C for 15 min.

MRS-Salvin: Used as media for the selection of L. acidophilus from a mixed culture, the MRS media was made up with its individual components with salicin instead of glucose: 10g/L salicin, 10g/L peptone, 10g/L lemco powder, 5g/L yeast extract, 1mL/L Tween 80, 2g/L dipotassium hydrogen phosphate, 5g/L sodium acetate, 2g/L triammonium citrate, 0.2g/L magnesium sulphate (.7 H₂O), 0.05g/L manganese sulphate (.4 H₂O), 15g/L agar. This was warmed in a microwave to melt the agar and autoclaved at 121°C for 15 min. After sterilisation the media was cooled to approximately 50°C and poured into petri dishes and left to solidify before use.
Peptone water: Consists of 10g/L peptone, 5g/L NaCl. It comes in the form of a pre-mixed powder from Oxoid. Made up to the manufacturer's instructions it requires 15g/L peptone water powder in distilled water. After the solution was dissolved it was autoclaved at 121°C for 15 min.

Phosphate buffer, (pH 7.0): 1M disodium phosphate was added to the 1M monosodium phosphate until pH 7.0 was reached; this required much more disodium phosphate solution than monosodium phosphate solution. Phosphate buffer was commonly used in concentrations of 0.1M and 0.2M. 1M buffer was diluted to these concentrations with distilled water before sterilisation at 121°C for 15 min.
3.2 Starter Cultures.

The probiotic cultures were obtained from the CSIRO (Commonwealth Scientific and Industrial Research Organisation (Australia)) Starter Culture Collection, the names and numbers of the strains are given in Table 4.

3.3 Starter Culture Activation

The probiotic starter cultures were obtained from CSIRO as freeze dried samples in small glass ampules. The ampules were carefully broken and opened aseptically. RSM was used to mix the culture by pasteur pipette aspirations until no lumps were visible. The culture was then added to 10mL RSM and incubated at 37°C until coagulated (18 - 72 h). This culture was diluted 1/10 with fresh RSM and stored in a freezer as the stock.

For experimental work a loopful of stock was streaked onto MRS agar, MRS-C agar for *Bifidobacterium* to lower the redox potential (Dave and Shah, 1997a), and incubated anaerobically at 37°C for 48 - 72 h. A single colony was used to inoculate MRS broth, MRS-C broth for *Bifidobacterium*, or NGYC as appropriate for the test.
Table 4: Organism name and number of probiotic bacteria (CSIRO Starter Culture Collection).

<table>
<thead>
<tr>
<th>Organism Name</th>
<th>Organism Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bifidobacterium breve</td>
<td>1900</td>
</tr>
<tr>
<td>Bifidobacterium bifidum</td>
<td>1909</td>
</tr>
<tr>
<td>Bifidobacterium infantis</td>
<td>1912</td>
</tr>
<tr>
<td>Bifidobacterium animalis</td>
<td>0941</td>
</tr>
<tr>
<td>Bifidobacterium pseudolongum</td>
<td>1944</td>
</tr>
<tr>
<td>Bifidobacterium longum</td>
<td>1971</td>
</tr>
<tr>
<td>Bifidobacterium adolescentis</td>
<td>1978</td>
</tr>
<tr>
<td>Bifidobacterium thermophilum</td>
<td>1991</td>
</tr>
<tr>
<td>Lactobacillus acidophilus</td>
<td>2400</td>
</tr>
<tr>
<td>Lactobacillus acidophilus</td>
<td>2401</td>
</tr>
<tr>
<td>Lactobacillus acidophilus</td>
<td>2404</td>
</tr>
<tr>
<td>Lactobacillus acidophilus</td>
<td>2409</td>
</tr>
<tr>
<td>Lactobacillus acidophilus</td>
<td>2415</td>
</tr>
<tr>
<td>Lactobacillus casei</td>
<td>2603</td>
</tr>
</tbody>
</table>
3.4 Viability Tests

Each culture strain was grown in MRS media as well as in NGYC media for exposure to conditions of low pH, high bile concentration, high sucrose concentration, high oxygen content and low storage temperature. For the low pH, high bile and low temperature tests the cells were grown in NGYC, for the high oxygen content and high sucrose concentration the cells were grown in MRS broth. Wherever possible the survival tests were carried out in milk media for its buffering action as the aim of these tests was to determine which strains would be most appropriate for use as adjuncts in dairy foods.

3.4.1 Preliminary Tests

3.4.1.1 Comparing Survival of Low pH Conditions in Milk Versus Broth Media.

An initial test for survival in low pH was performed on L. casei 2603 comparing survival in low pH broth media with survival in low pH milk media. The cells were grown to late log phase in both MRS broth and NGYC at 37°C over 48 hours. The coagulation in the milk media was disturbed by vortexing and then each culture was divided into fifteen 10mL samples. The media in each sample container was adjusted to pH 2.0, 2.5, 3.0, 3.5 and 4.0 with 5M HCl or 1M NaOH. The tests were performed in triplicate. pH adjustment was achieved without contamination from a pH meter probe, the sixteenth jar of each culture sample contained the pH meter probe and was used to determine the number of drops of acid or alkali needed in the container to obtain the required pH. The drops of acid were counted until the container with the pH meter probe inside reached pH 4.0, then the same number of
drops of acid were added to each of the fifteen sample containers and mixed. Three samples were put aside and labelled pH 4.0 while the drops were counted again for the remaining twelve samples to be re-adjusted to pH 3.5. This was repeated until there were three samples of each pH 4.0, 3.5, 3.0, 2.5 and 2.0. The samples were then incubated at 37°C for three hours (Lankaputhra and Shah, 1995). After each hour of incubation the containers were shaken and a sample was taken for enumeration allowing comparison of the viable count with counts from before the test began. The containers were vortexed and a sample of 100µL was taken and mixed with 900µL peptone water for a 1/10 dilution. The dilution series was continued decimally until a dilution of 10⁻⁶ was obtained. 100µL of each of the 10⁻⁴, 10⁻⁵ and 10⁻⁶ dilutions were then spread plated on MRS agar, MRS-C for Bifidobacterium. After two days of anaerobic incubation at 37°C the colonies for each hour of the viability test were counted and averaged for comparison with the count recorded at the start of the test. Following the results of this test where in general the cell viability decreased the most in the MRS broth media, it was decided to perform the actual low pH test in milk media.

3.4.1.2 Comparing Survival of L. acidophilus 2400 in Bile Extract Versus Bile Salts

Chou and Weimer (1999) discussed selection of probiotic strains able to survive in bile salts and retain their ability to grow. On the other hand, Lankaputhra and Shah (1995) tested for survival after exposure to bile extract. An initial test was done to compare survival of L. acidophilus 2400 after exposure to bile salts (Bile Salts No.3, Oxoid) and bile extract (Porcine Bile
Extract, Sigma). MRS broth was supplemented with filter sterilised bile salts and bile extract to 0.5% and 1.0% final concentration. A cell count was taken before exposure as well as after 3 hours by spread plating serial dilutions. Following this test where no survival was observed in bile salts, the remaining bile selection tests were carried out in filter sterilised bile extract.

3.4.2 Strain Selection Tests in Low pH

The cells were grown to late log phase in NGYC at 37°C over 48 hours. The coagulated media showed that the cells had grown. The coagulation was disturbed by vortexing and then each culture was divided into fifteen 10mL samples. The media in each sample container was adjusted to pH 2.0, 2.5, 3.0, 3.5 and 4.0 with 5M HCl or 1M NaOH. The tests were performed in triplicate so there were three containers of each pH value. pH adjustment was achieved without contamination from a pH meter probe, the sixteenth jar of coagulated culture sample contained the pH meter probe and was used to determine the number of drops of acid or alkali needed in the container to obtain the required pH. The drops of acid were counted until the container with the pH meter probe inside reached pH 4.0, then the same number of drops of acid were added to each of the fifteen sample containers and mixed. Three samples were put aside and labelled pH 4.0 while the drops were counted again for the remaining twelve samples to be re-adjusted to pH 3.5. This was repeated until there were three samples of each pH 4.0, 3.5, 3.0, 2.5 and 2.0. The samples were then incubated at 37°C for three hours (Lankaputhra and Shah, 1995). After each hour of incubation the containers were shaken and a sample was taken for enumeration allowing comparison of
the viable count with counts from before the test began. The containers were vortexed and a sample of 100µL was taken and mixed with 900µL peptone water for a 1/10 dilution. The dilution series was continued decimally until a dilution of 10⁻⁶ was obtained. 100µL of each of the 10⁻⁴, 10⁻⁵ and 10⁻⁶ dilutions were then spread plated on MRS agar, MRS-C for *Bifidobacterium*. After two days of anaerobic incubation at 37°C the colonies for each hour of the viability test were counted and averaged for comparison with the count taken at the start of the test.

### 3.4.3 Strain Selection Tests in High Bile Concentration

The cells were grown to late log phase in NGYC at 37°C for 24-48 hours; the growth of the cells was indicated by the coagulation in the media. The cultures were vortexed to disperse the coagulated lumps and then made into twelve 10mL aliquots. The media in each sample container was adjusted to pH 4.5 with 5M HCl or 1M NaOH by counting the drops of acid or alkali required in the thirteenth sample jar, containing a pH meter probe, to be adjusted to pH 4.5. Filter sterilised bile extract (Sigma) at 15% concentration was added to a final concentration of 0.5%, 1.0%, 1.5% and 2.0% by calculating the volume of 15% stock bile extract required in the 10mL aliquot to make the final concentrations of 0.5, 1.0, 1.5 and 2.0%. The tests were performed in triplicate so there were three jars for each bile concentration. The samples were incubated at 37°C for three hours (Lankaputhra and Shah, 1995). After each hour of incubation the containers were mixed and a sample was taken for enumeration allowing comparisons of viability to be made between times of incubation and viability before the test. The containers were
vortexed and a sample of 100μL was taken and mixed with 900μL peptone water for a 1/10 dilution. The dilution series was continued decimally until a dilution of 10⁻⁶ was obtained. 100μL of each of the 10⁻⁴, 10⁻⁵ and 10⁻⁶ dilutions were then spread plated on MRS agar, MRS-C for *Bifidobacterium*. After two days of anaerobic incubation at 37°C the colonies for each hour of the viability test were counted and averaged for comparison with the count taken at the start of the test.

3.4.4 Strain Selection Tests in Low Storage Temperature

The cells were grown to late log phase in NGYC after which the coagulation of the cultures was disturbed by vortexing. The samples were divided into 6 containers, which were incubated in triplicate at 5°C and -20°C for three hours. After three hours incubation, the frozen samples were defrosted with cold running tap water so that a sample could be taken for enumeration. Viability was compared with before exposure to the cold temperatures. The containers were vortexed and a sample of 100μL was taken and mixed with 900μL peptone water for a 1/10 dilution. The dilution series was continued decimally until a dilution of 10⁻⁶ was obtained. 100μL of each of the 10⁻⁴, 10⁻⁵ and 10⁻⁶ dilutions were then spread plated on MRS agar, MRS-C for *Bifidobacterium*. After two days of anaerobic incubation at 37°C the colonies for each hour of the viability test were counted and averaged for comparison with the count taken at the start of the test.
3.4.5 Strain Selection Tests in High Sucrose Concentration

The cultures were grown to late log phase in MRS broth, supplemented with 0.05% L-cysteine hydrochloride for *Bifidobacterium* spp. The cultures were divided into twelve 1mL aliquots. The cells were collected by micro-centrifugation at 4000 rpm for 3 min and then washed once by resuspension in an equal volume of sterile PBS (Prasad et al., 1998). The samples were re-centrifuged, then resuspended in triplicate in sucrose solution at concentrations of 10%, 15%, 20% and 25%. The samples were incubated at 5°C for three hours. After each hour of incubation the cells were remixed and a sample was taken for enumeration allowing comparison of viability throughout the time of exposure to high sucrose concentrations. The containers were vortexed and a sample of 100μL was taken and mixed with 900μL peptone water for a 1/10 dilution. The dilution series was continued decimally until a dilution of $10^{-6}$ was obtained. 100μL of each of the $10^{-4}$, $10^{-5}$ and $10^{-6}$ dilutions were then spread plated on MRS agar, MRS-C for *Bifidobacterium*. After two days of anaerobic incubation at 37°C the colonies for each hour of the viability test were counted and averaged for comparison with the count taken at the start of the test.

3.4.6 Strain Selection Tests in High Oxygen Content

The cells of each strain were grown to late log phase in MRS broth and then diluted 1/10 with fresh media. To six of the twelve samples 0.05% L-cysteine hydrochloride was added to lower the redox potential and to act as an oxygen scavenger (Dave and Shah, 1997). Three samples with cysteine and three samples without cysteine were deaerated by a vacuum pump for one hour.
while the remainder were left standing. The samples (one each of: aerated with cysteine; aerated without cysteine; deaerated with cysteine and deaerated without cysteine) were then incubated overnight at 5°C, 25°C and 45°C. After overnight incubation each sample was enumerated and the oxygen content was measured using an oxygen probe. Comparisons between viability and oxygen content were made before and after the overnight incubations. The containers were vortexed and a sample of 100μL was taken and mixed with 900μL peptone water for a 1/10 dilution. The dilution series was continued decimally until a dilution of $10^{-6}$ was obtained. 100μL of each of the $10^{-4}$, $10^{-5}$ and $10^{-6}$ dilutions were then spread plated on MRS agar, MRS-C for *Bifidobacterium*. After two days of anaerobic incubation at 37°C the colonies for each hour of the viability test were counted and averaged for comparison with the count taken at the start of the test.

### 3.5 Viable Counts

Serial decimal dilutions were made in sterile peptone water. *Lactobacillus* enumeration was performed on MRS agar. The media was supplemented with 0.05% L-cysteine hydrochloride (Sigma) (MRS-C) for *Bifidobacterium* spp. Incubation was at 37°C for 48 - 72 h with Anaerogen (Oxoid) and the use of an anaerobic indicator (Oxoid). For every sample three separate dilutions were enumerated and averaged for the viable cell count.
3.6 Encapsulation

Encapsulation of probiotic bacteria was performed by the method as described by Sultana et al. (2000). A mixture containing 2% alginate (Germantown), 2% Hi-maize™ resistant starch (Starch Australasia Ltd.) and 0.1% culture was made in one litre sterile milli Q water. The alginate (Manucol DM) was poured gently and steadily into 800mL sterile milli Q water with a strong vortex on a magnetic stirrer being careful not to leave any lumps. The alginate was left overnight in a refrigerator for the solution to settle. The next morning the alginate solution was removed from cold storage and allowed to return to room temperature at which time 20g Hi-maize™ resistant starch was mixed in using long stirring rods. At the same time the culture bacteria was suspended in sterile milli Q water. The culture suspension was added to the starch/alginate mix and made up to 1L with sterile milli Q water.

Two litres of canola oil (Crisco) was emulsified with 4mL (0.2%) Tween 80 in a very large beaker with 5L capacity. The culture/alginate/starch mixture was added to the oil and emulsified. Approximately two litres of 0.1M calcium chloride was added quickly along the side of the beaker, mixed vigorously and then the mixture was allowed to separate into oil and water phases. During the phase separation period the alginate/starch/culture, which was made hydrophobic by the oil, dropped as beads to the bottom of the calcium chloride layer. The oil layer was drained and the encapsulated culture beads were collected by low speed centrifugation (350 x g, 15 min), washed once with an equal volume of 0.9% saline, and stored at 5°C. The beads were
collected by size using 500µm and 150µm steel sieves, drained overnight inside a pan in a refrigerator to remove as much calcium chloride as possible. For freeze drying the capsules were resuspended in the smallest possible volume of 12% non fat dry milk (dissolved in distilled water and sterilised by autoclaving for 15 min at 121°C), frozen overnight and then freeze dried.

Encapsulation of both *L. acidophilus* and *Bifidobacterium* in a single capsule via the same process was referred to as co-encapsulation, the procedure for this was exactly as described above but with the two strains of bacteria mixed together before encapsulation in sodium alginate.
20g alginate + 20g Hi-maize resistant starch + 1g freeze dried culture

↓

Mix gradually into 1L milli Q water

↓

Add alginate/starch/culture mixture to 2L canola oil + 4mL Tween 80

↓

Mix well to emulsify

↓

Gently add 2L 0.1M CaCl₂ down the side of the beaker, stir quickly

↓

Allow phase separation of oil and water

↓

Alginate/starch/bacterial beads drop down into CaCl₂ layer

↓

Drain off oil

↓

Collect encapsulated bacterial beads by centrifugation: 350 x g for 15 min

↓

Collect beads by passing through steel sieves of 500μm and 150μm

↓

Store at 5°C or resuspend in 12% milk for freeze drying

Figure 1: Flow diagram for encapsulation method.
3.7 Optimisation of Encapsulation Conditions

3.7.1 Sterilisation of Alginate Solution

Three studies, Sheu and Marshall (1993), Jankowski et al. (1993) and Champagne et al. (1993), were used as the basis for setting up procedures for encapsulation using sodium alginate. The latter two studies specified the use of an autoclave for sterilising the sodium alginate solution at 121°C for 15 minutes. This step was included initially in the protocol for encapsulation. The method for encapsulation was followed with an alginate suspension that was not sterilised and a second suspension of alginate that was sterilised in an autoclave at 121°C for 15 minutes.

3.7.2 The Effect of Oil to Alginate Ratio on Capsule Yield

Sheu and Marshall (1993) recommended the use of 5 parts of oil (250mL) in a large 800mL beaker, with 0.2% Tween 80 for 1 part (50mL) of 2% alginate solution. In order to scale up the encapsulation method, an initial volume of 100mL alginate solution was used and the oil and Tween 80 were proportionally doubled, these conditions were trialled in triplicate, in order to produce capsules without the inclusion of probiotic bacteria. An experiment was then set up (in triplicate) where the encapsulation ingredients maintained the same proportions with the addition of the 2% alginate/culture mixture (L. casei). The volume of the oil was varied as follows; 50mL, 100mL, 200mL. The volume of the other materials was varied accordingly.
3.7.3 Effect of Tween 80 Concentration on Bacterial Viability

An experiment was set up, in triplicate, to vary the concentration of the detergent in the encapsulation procedure. An additional encapsulation experiment was set up to determine the effect of lecithin, as an emulsifier, on encapsulation (as a substitute emulsifier), compared with different concentrations of Tween 80. Encapsulation was carried out with variations in the detergent/emulsifier, using 50mL of 2% alginate mixture with 0, 0.1 and 0.2% Tween 80, 1% lecithin and 250mL oil.

3.7.4 Effect of Starch (Hi Maize™) on Encapsulation Yield and Bacterial Viability

The encapsulation protocol of Sheu and Marshall (1993) (based on 250mL oil and the other materials kept in proportion) was modified and used as the basis for studying the effect of starch on the entrapment of bacteria together with sodium alginate. For this experiment a 100mL solution of 2% alginate (with 0.1% *L. casei*) was used (with no added starch) as a control. Three other alginate/0.1% *L. casei* solutions were prepared by adding starch at the following concentrations: 1, 2 and 4%. 200mL oil with 0.2% Tween 80 was used for dropping the alginate/starch/culture mixture into the oil. After dropping, it was given a vigorous mixing at the highest speed to emulsify the alginate-oil mixture to a creamy consistency. Next 0.1M calcium chloride (150mL) was added quickly to the mix while it was stirring slowly and then allowed to stand. The capsules formed were recovered as described earlier and the yield and the viability of incorporated *L. casei* cultures were determined.
3.7.5 Effect of Starch (Hi Maize™) and Glycerol on Preserving Bacterial Viability in Frozen Storage Conditions

The experiments to study the effect of glycerol and starch were carried out with cultures of *L. casei*. Encapsulation was done with 2% sodium alginate alone, with 2% each of sodium alginate and starch, and with sodium alginate and starch plus 15, 20 and 30% glycerol. The experiment was repeated twice using 50mL and 100mL of 2% alginate volumes. In the tested conditions the encapsulation procedure was carried out by dropping the alginate into 250mL oil with 0.2% Tween 80, except for encapsulation with 30% glycerol where the beads were recovered by dropping directly into 0.1M calcium chloride solution. Following encapsulation with glycerol the capsules were frozen at -20°C for four months and the viability was assessed both before and after freezing by enumeration.

3.8 Examination of Alginate Beads

Examination of the alginate beads using a light microscope and the ocular and stage micrometers was performed to determine the sizes of the capsules. The outer surface and cross sections were examined by scanning electron microscopy (SEM) by the freeze fracture/cold block technique where unfixed samples were placed on a metal block and snap frozen in liquid nitrogen. Examination was performed using a Philips Electron Microscope (model 505) at an accelerating voltage of 15.0 kV.
3.9 Releasing Bacteria From Alginate Beads

For enumeration of encapsulated bacteria the capsules were solubilised by the method of Sheu and Marshall (1993). One gram of capsules was suspended in 9mL 0.1M phosphate buffer and homogenised in a stomacher blender for 30 min. Serial decimal dilutions were made in 0.1M phosphate buffer and spread plated on MRS or MRS-C agar and incubated anaerobically at 37°C for 48 - 72 hours.

3.10 Acidification Kinetics of Encapsulated Cultures

To determine if the encapsulated cells were still metabolically active and if nutrients and metabolites could permeate the capsule wall an experiment was performed comparing the time taken for free and encapsulated cells to acidify reconstituted skim milk medium. Free cells and encapsulated cells were inoculated into the medium at 2.5%; a duplicate of the encapsulated cells was inoculated at 5%. The cells were enumerated at time 0 and the pH of the medium recorded. The pH of the medium was recorded regularly for 48 hours at which time the cells were enumerated again.

3.11 Viability Studies of Encapsulated Bacteria

The encapsulated bacteria were exposed to conditions of low pH and high bile concentration in NGYC media, similar to the strain selection experiments with the free cells. This was to determine if encapsulation was providing further protection from these conditions.
3.11.1 Survival of Encapsulated Bacteria in Low pH

In thirty-six containers, 0.1g of encapsulated bacteria was added to 1mL aliquots of NGY media (NGYC media without the cysteine) and left overnight in a refrigerator at 5°C. The next day 100mL of fresh NGYC media was adjusted to pH 4.0 dropwise with 5M HCl. 5mL of the pH 4.0 NGYC was added to twelve sample containers of encapsulated bacteria in the NGY medium, without cysteine. This process was repeated for pH 3.0 and 2.0 media for each encapsulated strain.

There were twelve samples for each pH level because there were triplicate samples for 4 sampling periods: initial then 1, 2 and 3 hours after inoculation. Immediately the 0 hour samples had 5mL 0.2M phosphate buffer added and were transferred to a plastic bag and mixed in a Stomacher bagmixer machine for 30 min to allow for the beads to dissolve and release the bacteria. Serial decimal dilutions were made in 0.1M phosphate buffer and 0.1mL of three consecutive dilutions were spread plated on MRS or MRS-C agar. This was repeated after 1, 2 and 3 hours of incubation at 37°C for comparison of the viable count with before the test began.

3.11.2 Survival of Encapsulated Bacteria in High Bile Concentration

For each encapsulated strain there were twenty-four containers, 0.5g of encapsulated bacteria was added to 1mL aliquots of NGY media (NGYC media without the cysteine) and left overnight in a refrigerator at 5°C. The next day 100mL of fresh NGY media was adjusted to pH 4.5 dropwise with 5M HCl. 5mL of the pH 4.5 NGY media was added to the sample containers
of encapsulated bacteria in the 1mL medium. The containers with *Bifidobacterium* strains had 5μL of 10% cysteine added to lower the redox potential (Dave and Shah, 1997). Filter sterilised bile extract (Sigma) was added to final concentrations of 1.0% and 2.0%.

There were twelve samples for each bile concentration because there were triplicate samples of 4 sampling periods: initial then 2, 4 and 6 hours after inoculation. Immediately the 0 hour samples had 15mL 0.2M phosphate buffer added and were transferred to a plastic bag and mixed in a Stomacher bagmixer machine for 30 min to allow for the beads to dissolve and release the bacteria. Serial decimal dilutions were made in 0.1M phosphate buffer and 0.1mL of three consecutive dilutions were spread plated on MRS or MRS-C agar. This was repeated after 2, 4 and 6 hours of incubation at 37°C for comparison of the viable count with before the test began.

### 3.12 Preparation of Cultures for Incorporation into Dairy Products

The stocks prepared from the original CSIRO cultures were used to streak MRS agar plates. After anaerobic incubation at 37°C for 2 - 3 days a single colony was used to inoculate starter broth cultures in MRS for *Lactobacillus* strains or MRS-C for *Bifidobacterium* strains incubated at 37°C for 2 - 3 days. The starter cultures were used to inoculate larger volumes of broth grown at 37°C over 48 - 72 hours. The cultures were centrifuged at 6000 x g for 10 min, washed with an equal volume of 0.9% saline, and re-centrifuged. The pellet was resuspended in the smallest possible volume of RSM and frozen at
-20°C overnight. The cultures were then freeze dried for incorporation as free cells or for encapsulation.

Probiotic dairy products were also made with commercial cultures obtained from DSM Food Specialties (Sydney) and Wisby cultures of *L. acidophilus* and *Bifidobacterium* (obtained from Dairy Farmers). The commercially available cultures arrived in either frozen or freeze dried form. The frozen cultures were freeze dried in the laboratory for incorporation into products as free cells. For incorporation into products as encapsulated cells the freeze dried cultures were encapsulated by the method described above.

Collected encapsulated cultures were also resuspended in the smallest possible volume of RSM and frozen overnight at -20°C before being freeze dried for incorporation into probiotic dairy products.

### 3.13 Incorporation of Free and Encapsulated Cultures into Dairy Products

Both encapsulated and non-encapsulated bacteria were incorporated into yoghurt, ice cream and Cheddar cheese for examination of shelf life and cell survival.
3.13.1 Yoghurt Production in the Laboratory

Due to problems with enumerating both *L. acidophilus* and *Bifidobacterium* spp. in the presence of *L. delbrueckii* ssp. *bulgaricus* a single yoghurt starter organism was used. Yoghurt was prepared using whole milk standardised for 18% solids non-fat (SNF) using skim milk powder (SMP). The calculations for required volumes of milk and SMP were performed using a Pearson Square (Tamime and Robinson, 1999) as shown in Appendix 1. Whole milk was heated to 45°C and a sample removed for blending in the SMP, which was then returned to the container. Heating was continued to 80°C and the yoghurt mix held between 80-85°C for 20 min before cooling quickly in an ice-bath to 45°C when the bacteria were added. The yoghurt starter culture used was *Streptococcus salivarius* ssp. *thermophilus*, obtained from DSM Food Specialties and used in quantities recommended by the producers. Usually 4 - 5 units (U) inoculates 1000L and the cultures arrived in 2U sachets which were weighed, and simple calculations were performed to determine the weight of starter culture required. Probiotic bacteria to be added were both commercial strains of *L. acidophilus* and *Bifidobacterium* spp. obtained from DSM Food Specialties and the selected CSIRO strains from the earlier viability studies. The probiotic bacteria were added as either free cells or encapsulated beads at the same time as the yoghurt starter culture was added. Fermentation was at 40-42°C until a pH of 4.5 was reached at which time the yoghurt was transferred to 5°C and the probiotic bacteria were enumerated the next day then weekly for 8 weeks.
Yoghurt production was replicated in the laboratory, the first trial used *L. acidophilus* 2401 and *B. infantis* 1912 under free, encapsulated and co-encapsulated states. The second trial compared survival of *L. acidophilus* 2401 and *B. infantis* 1912 under free, freshly encapsulated, encapsulated freeze dried and co-encapsulated freeze dried states.

There were two samples taken from each yoghurt type weekly for enumeration. 10g of yoghurt was weighed directly into a plastic bag and mixed with 40mL phosphate buffer for the samples containing encapsulated bacteria or peptone water for the samples containing free probiotic bacteria. The yoghurt was mixed with the diluent in a Stomacher bagmixer machine for 20 min to allow the bacteria to be released from the encapsulating material. The samples containing free probiotic bacteria were treated in the same way to mix the yoghurt with the diluent. Serial decimal dilutions were performed in the same diluent and 0.1mL of three consecutive dilutions were spread plated on MRS-C (to enumerate both *L. acidophilus* and *Bifidobacterium*) and MRS-Salacin (to enumerate *L. acidophilus*) agar for enumeration. The plates were inverted and incubated at 37°C for 48 hours before counting.
Culture → Whole milk → SMP

**Raw Materials:** Yoghurt culture obtained from DSM Foods, probiotic culture prepared in the laboratory. Whole milk and SMP from the store.

**Heating:** Milk heated to 45°C

**SNF Standardisation:** The correct amount of SMP is added to the milk as derived by Pearson Square method.

**Heating:** Heating of milk and SMP continued to 80°C and yoghurt mix held at 80-85°C fro 20 min.

**Cooling:** Quickly cool milk and SMP to 45°C in an ice-bath, followed by **Inoculation** with starter culture and probiotic culture (if used).

**Packaging:** Distribute yoghurt mixture into individual cups for incubation and setting.

**Incubation:** Incubation of yoghurt mixture at 40-42°C until pH 4.5 is reached. Monitor pH every 0.5 hour and check temperature.

**Cooling:** Once pH 4.5 is reached, transfer yoghurt cups to refrigerator and store at 5°C.

Figure 2: Flow process chart for the production of yoghurt
3.13.2 Yoghurt Production at Dairy Farmers

Commercial yoghurt was produced at the Dairy Farmers Ltd. plant in Wetherill Park, Sydney. As a part of a normal weekly production run 10L buckets of unfermented pasteurised traditional natural yoghurt mix was taken. Probiotic bacteria was then mixed into the yoghurt mix in both free and encapsulated states. The yoghurt mix was aliquoted into 200mL cups which were heat sealed in the commercially available tubs. Fermentation was carried out at 40-42°C until a pH of 4.5 was reached at which time the yoghurt was transferred to 4°C overnight. The following day samples were taken for enumeration and the probiotics continued to be enumerated weekly for 8 weeks using the same method described for the laboratory trial yoghurt.

The first trial at Dairy Farmers Ltd. used commercial strains of *L. acidophilus* and *Bifidobacterium* obtained from DSM Food Specialties and compared survival between the free and encapsulated freeze dried states. The second trial at Dairy Farmers Ltd. used *L. acidophilus* 2401 and *B. infantis* 1912 grown and prepared in the laboratory. The second industrial trial also paralleled the procedure using commercial strains of *L. acidophilus* and *Bifidobacterium* obtained from Wisby, one of the suppliers of probiotic bacteria to Dairy Farmers Ltd. (personal communication, David Wong).
3.13.3 Ice Cream Production in the UWS-H Pilot Plant

The composition of ice cream requires 45% whole milk, 22.1% cream, 7.4% SMP, 15% sugar, 0.5% stabiliser, 0.04% flavour and 10% water. To make 100kg of ice cream 45kg whole milk, 28kg cream, 7.4kg SMP, 15kg sugar, 500g stabiliser, 40g flavour and 10L distilled water was used. After all of the ingredients had been weighed out the necessary machinery, including the pasteuriser and the homogeniser, was sanitised. First the milk and cream were added to the pasteuriser and heated slowly, once the temperature reached 45°C the SMP was added with one half of the amount of sugar and the water. The remaining sugar was mixed with the stabiliser before being added to the pasteuriser when the temperature reached 60°C. Heating was continued to 80°C and held at that temperature for 5-10 sec when the machine was tripped and the flavour added. The ice cream mix was homogenised while the temperature remained above 75°C. After homogenisation the ice cream mix was cooled to 45°C for the addition of the cultures. The ice cream mixture was separated into 3 batches of 20kg each. Three types of ice cream were made: one with 20g each of free *L. acidophilus* and *Bifidobacterium* spp. (DSM Food Specialties); one with 500g each of encapsulated *L. acidophilus* and *Bifidobacterium* spp. (DSM Food Specialties); one with 0.08U (1.2g) *S. salivarius* ssp. *thermophilus* (DSM Food Specialties) as well as 500g each of encapsulated *L. acidophilus* and *Bifidobacterium* spp. (DSM Food Specialties). The ice cream with *S. salivarius* ssp. *thermophilus* was fermented at 40-42°C for six hours, until pH 4.7 and then all three types of ice cream mixes were stored at 4°C overnight.
The next day the ice cream mixes were frozen in a Tetra Pack Hoyer freezer aiming for 100% over run before the ice cream was packed and stored at -20°C.

3.13.3.1 Determination of Over Run

Before freezing it is necessary to determine the weight of the ice cream mix, as the ice cream mix is frozen more and more air is incorporated thus the frozen ice cream will be lighter than the same volume of ice cream mix. Weighing of the frozen ice cream was necessary to determine the over run. The formula for over run is as follows (Arbuckle, 1986):

\[
\text{over run} = \frac{\text{weight of ice cream mix} - \text{weight of same volume ice cream}}{\text{weight of same volume ice cream}} \times 100\%
\]

3.13.3.2 Determination of Fat in Ice Cream

The Babcock method was used for the determination of percentage fat content in the ice cream mix in duplicate. To do this 9.0±0.1g of well mixed ice cream mix was weighed into a Babcock cream flask. 15mL of Minnesota reagent was added, approximately 1mL at a time, while the flask was continually swirled keeping the contents well mixed. The flask was then placed in a larger beaker containing hot water (~80°C) for 10 min with occasional swirling to ensure complete mixing of the contents. Hot water was then added to the flask until the top of the fat layer was ~1-2cm from the top of the flask. The flasks were centrifuged for 30 sec - 1 min and the percentage fat determined using calipers on the graduated scale (Arbuckle, 1986).
Vanilla Stabiliser Sugar Water SMP Cream Whole Milk

Sanitise equipment: pasteuriser and homogeniser

Pasteurise: milk and cream to 80°C

Add ingredients: At 45°C add SMP, ½ sugar and water

Add ingredients: At 60°C add remaining sugar, mixed with stabiliser

Pasteurise: Continue heating to 80°C, hold for 5-10 sec, add flavour

Homogenise: ice-cream mix 3000psi, 500psi

Cool: to 45°C and Inoculate probiotic and starter cultures

Ferment: yoghurt culture containing ice-cream mix to pH 4.7

Store: at 4°C overnight to age ice-cream mix

Freeze: ice-cream mix in Tetra Pak Hoyer freezer at 80psi aiming for 100% over run

Package: ice-cream in containers, Harden and Store ice-cream at -20°C

Figure 3: Flow process chart for the production of ice cream
3.13.3.3 Determination of Total Solids in Ice Cream

Total solids in the ice cream was measured by measuring the weight lost due to the evaporation of water at 105°C (Arbuckle, 1986). Aluminium moisture dishes were pre-dried in an air oven at 105°C for at least one hour and stored in a desiccator to cool. Handling of the aluminium dishes was with the use of tongs to avoid transfer of moisture from hands to the dish. In duplicate, the cooled and dried dishes were placed onto an analytical balance and each weight recorded. The balance was tared and approximately 5g of ice cream mixture weighed into the dish and the exact weight recorded. The dish and samples were dried at 105°C overnight. The next day the dishes and samples were stored in a desiccator again to cool before reweighing the dish and sample and calculating percentage of total solids by the following equation:

\[
\% \text{ Total Solids} = \frac{\text{dried weight}}{\text{sample weight}} \times 100\%
\]
3.13.4 Ice Cream Production in the Laboratory

"Home made" ice cream was made in the laboratory using a benchtop domestic ice cream maker (Philips HR 2303) and the recipe for Premium Vanilla Ice Cream from Breville. 1 egg, ¾ cup white sugar, ½ cup milk and 1 teaspoon vanilla essence were mixed in a blender until the sugar was dissolved. 2 cups of cream was then added to the blender, mixed until just combined and refrigerated overnight.

Four batches of ice cream were made with the probiotic culture incorporated into the product in different states. Free, freshly encapsulated, encapsulated and freeze dried, co-encapsulated and freeze dried. The cultures were mixed in to the ice cream mix to obtain a high initial cell count and the mix was refrigerated for another hour.

The ice cream maker was turned on according to instructions and the ice cream mix poured into the mixing bowl. The ice cream mix was frozen in the ice cream maker, with mixing, for one hour. The ice cream was then dispensed into 200mL cups and stored at -20°C for six months. The samples were monitored monthly for cell viability. Over run, total solids and fat content were also determined by the methods described.
3.13.5 Cheddar Cheese Production

10L raw milk was pasteurised by heating to 72°C and holding this temperature for 15 sec. The milk was then cooled quickly to 30°C and transferred to a cheese making vat with constant stirring. The acidity of the milk was determined by titration with 0.1M NaOH of 9mL milk with 2 drops phenolphthalein indicator until a permanent faint pink colour was obtained. While stirring 2.5mL 0.1M calcium chloride solution was added to the milk in the vat and 0.3mL annato for colour. Half of the pre-activated cheese culture (DSM Food Specialties, 1 sachet equivalent for 20L) was added in UHT milk. After 10 min 2.5mL rennet was added in distilled water and spread evenly over the surface. Stirring was continued for 1 min and the stirring paddle removed. After 40 min the consistency of the curd was examined and then cut with wire knives and left standing for 10 min during which time the titratable acidity was determined. During heating of the curd and whey to 38°C over 55 min the titratable acidity was monitored each 25 min. At an acidity level of 0.16 – 0.17% the whey was drained and collected for determination of quantity of cells lost in the whey. The curd was banked up on one side of the vat to knit and turned for Cheddaring until the whey coming out of the curd reached an acidity of 0.55 – 0.65%. The curd was milled into small pieces and left sitting for 20 min. Dry salt was mixed with the milled curd to a final concentration of 2.5% of the expected yield, 25g for an expected yield of 1kg. The salted curd was hooped in cheesecloth and pressed overnight at room temperature. The next day the cheese was removed from the mould into a polyethylene bag for vacuum packaging and stored at 10 - 12°C.
Figure 4: Flow process chart for Cheddar cheese production
3.14 Analysis of Probiotic Dairy Products

3.14.1 Yoghurt

3.14.1.1 Probiotic Enumeration From Yoghurt

Each individual yoghurt tub was mixed thoroughly with a sterile spatula. 10g yoghurt was weighed directly into a stomacher bag and diluted 1:2 with 0.2M phosphate buffer, such that the encapsulated probiotics would be in a final concentration of 0.1M phosphate buffer. The samples with free probiotics were diluted 1:2 with peptone water. The diluted samples were mixed for 30min in the Stomacher Bagmixer Blender machine. Serial decimal dilutions were made in the same diluent and spread plated on MRS-C and MRS-Salicin agar and incubated anaerobically at 37°C for 48 - 72 h.

3.14.1.2 Yoghurt Texture Analysis

The texture of the yoghurt was analysed near the beginning of the trial, in the middle and at the end of the storage period using a texture analyser and the method provided by the manufacturer (Stable Micro Systems). The samples of set yoghurt were tested in the setting cup immediately after removal from cold storage.

The yoghurt was subjected to texture profile analysis using a Texture Analyser TA-XT2 (Stable Micro Systems). The Texture Profile Analysis (TPA) required two passes into the product to 15mm at a speed of 1mm/s using a force of 5.0g. There was a pause in between the two passes, each of which represents the action of the jaw when biting into food. The TPA generated a twin-peaked curve showing the required force to compress the food over time.
The curve was used to give an accurate assessment of the yoghurt texture characteristics:

- **Hardness**: The force needed to complete the first compression cycle; given as the height of the first peak of the TPA curve.

- **Fracturability**: Also known as brittleness, is defined as the force at which the product fractures and is found on the curve as the force of the significant break in the TPA curve on the first peak. A sample with a higher degree of hardness will fracture.

- **Cohesiveness**: The ratio of the positive force areas under the second peak of the TPA curve compared with the first peak of the curve. It represents the strength of the internal bonds making up the body of the sample, if the sample is more Cohesive than Adhesive then the sample sticks together as the probe is removed from the sample and none of the product will remain on the probe.

- **Adhesiveness**: The negative force area, or negative curve, after the first peak represents the work necessary to remove the probe from the sample. If the sample is more Adhesive than Cohesive then some of the sample will stick to the probe as it is removed from the sample.

- **Springiness**: Or elasticity, is the distance that the food itself returns toward its original height during the pause between the two bites.

- **Gumminess**: Is defined as the product of Hardness X Cohesiveness. This represents the extent of manipulation required to disintegrate the product into a form suitable for swallowing.

- **Chewiness**: Is defined as the product of Gumminess X Springiness (which is Hardness X Cohesiveness X Springiness). This represents the time, or
number of chews, required to disintegrate the product into a form suitable for swallowing (Boume, 1982).

3.14.1.3 Yoghurt Sensory Analysis

Sensory analysis was performed on the yoghurts made with Wisby cultures at Dairy Farmers Wetherill Park. The sensory panel was composed of 20 members, for each replicate study, of the School of Food Science and Technology's students and staff. The panel was untrained but the majority had previous experience of sensory evaluation of students' products, including dairy products. Sensory analysis was performed twice during the storage period of the yoghurt made at Dairy Farmers for the first trial. These tests were undertaken at weeks 2 and 5 of the shelf life; a third test was planned for week 8, however some samples had started to grow mould, so the test was aborted.

The participants were presented with the sample yoghurts together with a "standard" yoghurt made at Dairy Farmers Wetherill Park on the same day as the free and encapsulated yoghurts were made, and an unstructured scaling questionnaire (Appendix 3) asking about the qualities of the product. The process used a series of horizontal lines marked with degrees of intensity of characteristics of smoothness/grittiness, acidity, appearance, flavour, aftertaste and overall liking.
3.14.2 Ice Cream

3.14.2.1 Probiotic Enumeration From Ice Cream

10g ice cream was weighed directly into a stomacher bag and diluted 1:2 with 0.2M phosphate buffer such that the encapsulated probiotics would be in a final concentration of 0.1M phosphate buffer. The samples with free probiotics were diluted 1:2 with peptone water. The diluted samples were mixed for 30min in the Stomacher Bagmixer Blender machine. Serial decimal dilutions were made in the same diluent and spread plated on MRS-C and MRS-Salicin agar and incubated anaerobically at 37°C for 48 - 72 h.

3.14.2.2 Ice Cream Sensory Analysis

Sensory analysis was performed on the experimental ice cream made with commercial cultures in the UWS-H Pilot Plant. The sensory panel was composed of 20 members, of the School of Food Science and Technology's students and staff. The panel was untrained but the majority had previous experience of sensory evaluation of students' products, including dairy products.

The participants were presented with the ice cream samples made in the UWS-H pilot plant together with a Savings brand vanilla ice cream as a reference, and an unstructured scaling questionnaire (Appendix 4) asking about the qualities of the product. The process used a series of horizontal lines marked with degrees of intensity of characteristics of smoothness/grittiness, acidity, appearance, flavour, aftertaste and overall liking.
3.14.2.3 Ice Cream β-Galactosidase Assay

β-galactosidase activity was measured using a chromogenic substrate o-nitrophenyl-β-D-galactopyranoside (ONPG) (Hekmat and McMahon, 1992). Five grams of frozen ice cream was assayed for the bacterial cell count. In order to release the encapsulated bacteria, the sample was mixed with 4.5ml of 0.1M phosphate buffer. This sample was incubated for 30 min at 37°C and diluted ten fold in 0.1M phosphate buffer (pH 7.0), containing 0.001M MgSO₄ and 0.05M β-mercaptoethanol. Then 1ml of the diluted sample was withdrawn, and two drops of chloroform and one drop each of 0.1% sodium dodecyl sulfate and ONPG were added to it. The reaction assay mixture was vortexed for 10s and then incubated at 37°C for a period until the yellow colour appeared (15-20min); the reaction was stopped quickly by adjusting the solution to pH 11 by adding 0.5ml of 1M Na₂CO₃. At this pH the enzyme is inactivated. The samples were centrifuged at 16266 × g, and the optical density (420 nm) was recorded using a Beckman DU-65 spectrophotometer.

The following formula was used:

\[
β-Galactosidase\text{ (units/ml)} = 1000 \frac{A_{420}}{TV}
\]

where \( t \) = time of reaction, \( v \) = vol. of sample used

\( A \) = absorbance at 420 nm
3.14.3 Cheddar Cheese

3.14.3.1 Probiotic Enumeration From Cheddar Cheese

1g cheese was weighed and transferred to a stomacher bag and diluted 1:20 with 0.1M phosphate buffer. The samples with free probiotics were diluted 1:20 with peptone water. The diluted samples were mixed for 30min in the Stomacher Bagmixer Blender machine. Serial decimal dilutions were made in the same diluent and spread plated on MRS-C and MRS-Salicyl agar and incubated anaerobically at 37°C for 48 - 72 h.

3.14.3.2 Cheddar Cheese Sensory Analysis

Sensory analysis was performed on the Cheddar cheese made with commercial cultures. The sensory panel was composed of 20 members, of the School of Food Science and Technology's students and staff. The panel was untrained but the majority had previous experience of sensory evaluation of students' products, including dairy products.

The participants were presented with the Cheddar cheese samples made with commercial cultures together with a sample of Coon brand Cheddar cheese, and an unstructured scaling questionnaire (Appendix 5) asking about the qualities of the product. The process used a series of horizontal lines marked with degrees of intensity of characteristics of smoothness/grittiness, acidity, appearance, flavour, aftertaste and overall liking.
CHAPTER 4

RESULTS AND DISCUSSION

4.1 Strain Selection

4.1.1 Preliminary Experiments

4.1.1.1 Comparison of Survival of \textit{L. casei} 2603 in Low pH Broth Versus Milk Media

The initial test performed with \textit{L. casei} 2603 comparing survivability in low pH milk and broth media generally showed that a larger decrease in cell viability was found when the cells were incubated in broth rather than in milk media. The results are shown in Table 5 where the initial count, final count and count decrease are represented in log numbers. Performing the test in broth media caused a greater cell viability loss compared with milk, showing clearly that the remainder of the low pH survival tests should be performed in milk media.

The milk media appeared to be protective of the cells compared with the broth. The objective was to determine which cells would survive better when incorporated into dairy foods, thus it was more appropriate for the remaining tests to be completed in milk media for a more accurate view of how the tested strains would survive in dairy products.
Table 5: The effects of NGYC and MRS on the viability of *L. casei* 2603 over three hours under different pH conditions.

<table>
<thead>
<tr>
<th></th>
<th>NGYC</th>
<th>MRS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial count (log)</td>
<td>Final count (log)</td>
</tr>
<tr>
<td><strong>pH 2.0</strong></td>
<td>10.3±0.3</td>
<td>9.0±0.6</td>
</tr>
<tr>
<td><strong>pH 2.5</strong></td>
<td>10.8±0.4</td>
<td>9.9±0.3</td>
</tr>
<tr>
<td><strong>pH 3.0</strong></td>
<td>10.0±0.9</td>
<td>8.6±0.2</td>
</tr>
<tr>
<td><strong>pH 3.5</strong></td>
<td>10.0±0.7</td>
<td>8.9±0.8</td>
</tr>
<tr>
<td><strong>pH 4.0</strong></td>
<td>10.6±0.4</td>
<td>9.7±0.7</td>
</tr>
</tbody>
</table>

Average determination, n = 3

NGYC = Non-fat milk, glucose, yeast extract and cysteine media.

MRS = deMan, Rogosa, Sharpe broth.
4.1.1.2 Comparison of Survival of \textit{L. acidophilus} 2400 in Bile Salts Versus Bile Extract.

Table 6 shows the results of survival of \textit{L. acidophilus} 2400 in media supplemented with bile salts compared with bile extract. Bile salts are a much more concentrated version of bile than the bile extract, thus did not allow any survival. The Oxoid Bile Salts No.3 is extremely inhibitory to growth and is used as a selective inhibitory agent and is effective at less than one third of the bile concentration quoted in selective media preparations from the Oxoid Manual (Bridson, 1995). Bile extract (with its less inhibitory nature than bile salts) was used for the bile resistance selection tests.

Chou and Weimer (1999) studied the effect of individual bile salts on the survival of \textit{L. acidophilus} strains by supplementing MRS agar adjusted to pH 4.0, 5.0, 6.0 or 7.0 with 0.3% glycocholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid or oxgall individually. Their results showed that most strains grew between pH 5 – 7 with individual bile salts; however, at pH 4.0 all strains were inhibited by glycocholic acid and oxgall but most strains were able to grow with glycodeoxycholic acid.
Table 6: The effects of bile salts versus bile extract on the viability of *L. acidophilus* 2400 after 3 h, expressed as log numbers.

<table>
<thead>
<tr>
<th>Bile concentration</th>
<th>Bile Salts No.3 (Oxoid)</th>
<th>Bile Extract (Sigma)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial count (log)</td>
<td>Final count (log)</td>
</tr>
<tr>
<td></td>
<td>Initial count (log)</td>
<td>Final count (log)</td>
</tr>
<tr>
<td>0.5 %</td>
<td>7.0±0.3</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>1.0 %</td>
<td>9.5±0.4</td>
<td>0.0±0.0</td>
</tr>
</tbody>
</table>

Average determination, n = 3
4.1.2 Strain Selection Tests in Low pH

For probiotic organisms to perform their beneficial role in the intestinal tract they must be able to overcome high acidity. The gastrointestinal tract can vary in levels of acidity and the pH may fall as low as 1.5 (Lankaputhra and Shah, 1995). The probiotic bacterial strains obtained from CSIRO were exposed to viability tests in media adjusted to pH 2.0, 2.5, 3.0, 3.5 and 4.0. The most significant decrease in viability for all the tested probiotic bacterial strains was in pH 2 (Tables 7a & 7b). At pH 2.0 *B. pseudolongum* 1944 was robust enough to not decrease in cell count over 3 h. *L. acidophilus* 2409 showed the smallest decrease in cell count of the *Lactobacillus* strains, decreasing from 11.2 to 10.0 log cfu/ml over 3 h, a 1.2 log reduction. The poorest surviving organisms were *B. animalis* 1941 decreasing from 7.26 to 3.60 log cfu/mL, a log reduction of 3.66. *L. acidophilus* 2401 decreased from 11.4 to 9.94 log cfu/mL, a log reduction of 1.46.

The *Lactobacillus* strains tested all showed only 1 – 2 log reduction demonstrating the natural resistance against low pH conditions of the organism. On the other hand the survival of the *Bifidobacterium* strains varied widely (Berrada et al., 1991; Chung et al., 1999; Clark et al., 1993; Ibrahim and Bezkorovainy, 1993; Martin and Chou, 1992), with both high and low log reduction results - indicating the variability in survival of the strains tested and possibly the presence or absence of a more resistant cell wall material (Shah and Jelen, 1990).
Table 7a: Log reduction of probiotic strains after 3h in pH 2.0, 2.5 and 3.0 media

<table>
<thead>
<tr>
<th>ORG</th>
<th>2.0</th>
<th></th>
<th></th>
<th>2.5</th>
<th></th>
<th></th>
<th>3.0</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>F</td>
<td>D</td>
<td>I</td>
<td>F</td>
<td>D</td>
<td>I</td>
<td>F</td>
<td>D</td>
</tr>
<tr>
<td>La 2400</td>
<td>11.4</td>
<td>9.94</td>
<td>1.44</td>
<td>11.1</td>
<td>10.0</td>
<td>1.00</td>
<td>11.9</td>
<td>10.0</td>
<td>1.84</td>
</tr>
<tr>
<td>La 2401</td>
<td>11.3</td>
<td>9.69</td>
<td>1.65</td>
<td>11.4</td>
<td>9.66</td>
<td>1.77</td>
<td>10.4</td>
<td>9.05</td>
<td>1.31</td>
</tr>
<tr>
<td>La 2404</td>
<td>10.6</td>
<td>9.47</td>
<td>1.17</td>
<td>10.5</td>
<td>8.41</td>
<td>2.08</td>
<td>8.98</td>
<td>6.93</td>
<td>2.05</td>
</tr>
<tr>
<td>La 2409</td>
<td>11.2</td>
<td>10.0</td>
<td>1.17</td>
<td>9.05</td>
<td>8.32</td>
<td>0.72</td>
<td>9.53</td>
<td>8.96</td>
<td>0.57</td>
</tr>
<tr>
<td>La 2415</td>
<td>10.9</td>
<td>9.46</td>
<td>1.45</td>
<td>10.2</td>
<td>8.90</td>
<td>1.33</td>
<td>10.6</td>
<td>9.26</td>
<td>1.34</td>
</tr>
<tr>
<td>Lc 2603</td>
<td>11.4</td>
<td>10.1</td>
<td>1.35</td>
<td>10.8</td>
<td>9.96</td>
<td>0.85</td>
<td>11.0</td>
<td>9.63</td>
<td>1.32</td>
</tr>
<tr>
<td>Ban 1941</td>
<td>7.26</td>
<td>3.6</td>
<td>3.66</td>
<td>7.26</td>
<td>7.42</td>
<td>0</td>
<td>7.26</td>
<td>7.50</td>
<td>0</td>
</tr>
<tr>
<td>Bbr 1900</td>
<td>9.65</td>
<td>7.00</td>
<td>2.65</td>
<td>9.65</td>
<td>7.00</td>
<td>2.65</td>
<td>9.65</td>
<td>6.75</td>
<td>2.90</td>
</tr>
<tr>
<td>Bi 1912</td>
<td>11.1</td>
<td>10.1</td>
<td>1.03</td>
<td>8.38</td>
<td>6.52</td>
<td>1.86</td>
<td>8.38</td>
<td>7.85</td>
<td>0.53</td>
</tr>
<tr>
<td>Bp 1944</td>
<td>5.97</td>
<td>6.70</td>
<td>0</td>
<td>5.97</td>
<td>6.79</td>
<td>0</td>
<td>5.97</td>
<td>6.88</td>
<td>0</td>
</tr>
<tr>
<td>Bt 1991</td>
<td>11.1</td>
<td>6.67</td>
<td>4.39</td>
<td>8.89</td>
<td>8.19</td>
<td>0.70</td>
<td>8.89</td>
<td>8.43</td>
<td>0.46</td>
</tr>
</tbody>
</table>

Average determination, n = 3

La = Lactobacillus acidophilus
Lc = L. casei
Bbr = Bifidobacterium breve
Bi = B. infantis
Ban = B. animalis
Bp = B. pseudolongum
Bt = B. thermophilum

I = Initial cell count (log)
F = Final cell count (log)
D = Log decrease (the difference between I and F).
Table 7b: Log reduction of probiotic strains after 3h in pH 3.5 and 4.0 media

<table>
<thead>
<tr>
<th>ORG</th>
<th>3.5</th>
<th></th>
<th>4.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>F</td>
<td>D</td>
</tr>
<tr>
<td>La 2400</td>
<td>11.2</td>
<td>9.97</td>
<td>1.27</td>
</tr>
<tr>
<td>La 2401</td>
<td>10.2</td>
<td>9.11</td>
<td>1.08</td>
</tr>
<tr>
<td>La 2404</td>
<td>7.56</td>
<td>6.89</td>
<td>0.67</td>
</tr>
<tr>
<td>La 2409</td>
<td>9.22</td>
<td>9.04</td>
<td>0.18</td>
</tr>
<tr>
<td>La 2415</td>
<td>11.1</td>
<td>9.97</td>
<td>1.16</td>
</tr>
<tr>
<td>Lc 2603</td>
<td>11.0</td>
<td>9.88</td>
<td>1.11</td>
</tr>
<tr>
<td>Ban 1941</td>
<td>7.26</td>
<td>7.52</td>
<td>0</td>
</tr>
<tr>
<td>Bbr 1900</td>
<td>9.65</td>
<td>7.57</td>
<td>2.07</td>
</tr>
<tr>
<td>Bi 1912</td>
<td>8.38</td>
<td>7.67</td>
<td>0.71</td>
</tr>
<tr>
<td>Bp 1944</td>
<td>5.97</td>
<td>6.67</td>
<td>0</td>
</tr>
<tr>
<td>Bt 1991</td>
<td>8.89</td>
<td>8.41</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Average determination, n = 3

La = Lactobacillus acidophilus

Lc = L. casei

Bbr = Bifidobacterium breve

Bi = B. infantis

Ban = B. animalis

Bp = B. pseudolongum

Bt = B. thermophilum

I = Initial cell count (log)

F = Final cell count (log)

D = Log decrease (the difference between I and F).
A pH level of 3.0 is the most commonly used in *in vitro* studies of probiotic survival due to the cell viability decrease being more pronounced at pH 2 and lower (Garriga *et al.*, 1998; Gupta *et al.*, 1996; Hood and Zottola, 1988; Lankaputhra and Shah, 1995; Prasad *et al.*, 1998; Suskovic *et al.*, 1997). Most strains decreased by a similar log count in all of the tested pH levels. The trend for each individual strain was a decrease of approximately the same log value for each pH level tested (Tables 7a & 7b). When exposed to various pH levels it would be expected for a single strain to decrease a little in pH 4 and viability to reduce significantly in pH 2; however this was not observed in the present study. *L. acidophilus* 2400 decreased by log 1.44, 1.00, 1.84, 1.27 and 1.24 for pH levels 2.0, 2.5, 3.0, 3.5 and 4.0 respectively. For *L. acidophilus* 2401 the decrease ranged from log 0.87 in pH 4.0 to 1.77 in pH 2.5, while for strain 2404 the range was no decrease in pH 4.0 to a decrease of log 2.08 in pH 2.5. The *L. acidophilus* strain 2409 decreased only log 0.18 in pH 3.5 and log 1.17 in pH 2.0, whereas strain 2415 decreased log 1.09 in pH 4.0 and log 1.45 in pH 2.0. The variation for *L. casei* 2603 was from log 0.85 in pH 2.5 to log 1.35 in pH 2.0.

Of the *Bifidobacterium* strains tested, *B. animalis* 1941 showed the greatest variation in results with no decrease in viability for pH 2.5, 3.0, 3.5 and 4.0 but decreasing 3.66 log in pH 2.0 (Tables 7a & 7b). *B. thermophilum* 1991 also showed a large variation in results, decreasing only 0.42 log in pH 4.0 but losing 4.39 log in pH 2.0. *B. pseudolongum* 1944 showed the least variation with no decline in viability for any pH tested. *B. breve* 1900 showed a range of viability loss of 1.72 log for pH 4.0 to 2.90 log in pH 3.0. The results for *B.*
*infantis* 1912 ranged from a loss of 0.44 log in pH 4.0 to a decrease of 1.86 log in pH 2.5.

Some of the results of the present study contrast with those of Lankaputhra and Shah (1995) who used the same strains and tested survival in pH 1.5, 2.0, 2.5 and 3.0 up to 3 h. For the *Lactobacillus* strains 2400, 2401, 2404, 2405, 2409 and 2415 Lankaputhra and Shah (1995) found that over the three hours of testing the viability decrease in pH 2 was at least 4 log and between 0 - 2 log decrease in pH 3. For the *Bifidobacterium* strains Lankaputhra and Shah (1995) observed in most cases a decrease of at least 5 log for pH 1.5, 2.0, 2.5 and 3.0 for *B. bifidum* 1900 and 1901, *B. adolescentis* 1920, *B. breve* 1930, *B. longum* 20097 and *B. thermophilum* 20210. The exceptions were *B. infantis* 1912, *B. longum* 1941 and *B. pseudolongum* 20099 which decreased less than 2 log in pH 3.0 over three hours. Some of the CSIRO strains have been reclassified since the Lankaputhra and Shah (1995) study thus *B. bifidum* 1900 in their study is the *B. breve* 1900 in the present study and the *B. longum* 1941 in their study is the *B. animalis* 1941 of the present study. As demonstrated by the present study and others, the survival rates of Bifidobacteria in low pH conditions is strain specific (Berrada et al., 1991; Chung et al., 1999; Clark et al., 1993; Ibrahim and Bezkorovainy, 1993; Lankaputhra and Shah, 1995; Martin and Chou, 1992). The results of the present study differs from that of Gopal *et al.* (1996) due to the reclassification of *B. longum* 1941 as *B. animalis* 1941, recently by CSIRO Starter Culture Centre. This reclassification occurred after Gopal's work was published.
The Prasad et al. (1998) study used a similar method of testing for probiotic bacterial survival in low pH media but with a different media; the method of adjusting the pH of the media to different pH levels with HCl, then incubating the mixtures in 37°C and enumerating the cells at 0, 1, 2 and 3 h of incubation. In contrast to the present study, Prasad et al. (1998) performed the low pH viability study in phosphate buffered saline (PBS) probably for its buffering action as well as to simulate the gastro-intestinal juices whereas the present study used milk based media to buffer the pH shock as well as to simulate survival of probiotic bacteria in acidic dairy products. Chou and Weimer (1999) selected strains able to survive in pH 3.5 for 90 minutes due to the report of Berrada et al. (1991) reporting that the time from entrance to release from the stomach for food is 90 minutes. Thus probiotic bacteria selected for survival in acidic conditions must be able to survive for at least 90 minutes; however some digestive processes take longer.
4.1.3 Strain Selection Tests in High Bile Concentration

Probiotic bacteria in food must also be able to survive the bile of the human intestine which can vary in concentration with the type of food consumed and may range between 0.5 – 2.0% in the first hour of digestion but decrease after that (Davenport, 1977). The present study concurs with that of Lankapurthra and Shah (1995) to show that the higher the bile concentration, the greater the decrease in cell counts. The largest decrease in cell count was observed with 2% bile (Table 8). In 2% bile, \textit{B. pseudolongum} 1944 showed the best survival, with no decrease in cell count. \textit{L. acidophilus} 2401 showed the strongest survival of the \textit{Lactobacillus} strains, decreasing in cell count from log 6.72 to 6.46 cfu/mL over 3 h, a log reduction of 0.26.
Table 8: Log reduction of probiotic cultures after 3h in 0.5, 1.0, 1.5 and 2.0% bile concentration

<table>
<thead>
<tr>
<th>ORG</th>
<th>0.5%</th>
<th>1.0%</th>
<th>1.5%</th>
<th>2.0%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>F</td>
<td>D</td>
<td>I</td>
</tr>
<tr>
<td>La 2400</td>
<td>8.42</td>
<td>8.28</td>
<td>0.14</td>
<td>8.42</td>
</tr>
<tr>
<td>La 2401</td>
<td>6.72</td>
<td>6.67</td>
<td>0.05</td>
<td>6.72</td>
</tr>
<tr>
<td>La 2404</td>
<td>7.08</td>
<td>6.29</td>
<td>0.79</td>
<td>7.08</td>
</tr>
<tr>
<td>La 2409</td>
<td>6.61</td>
<td>5.93</td>
<td>0.68</td>
<td>6.61</td>
</tr>
<tr>
<td>La 2415</td>
<td>8.07</td>
<td>7.51</td>
<td>0.56</td>
<td>8.07</td>
</tr>
<tr>
<td>Lc 2603</td>
<td>8.42</td>
<td>8.36</td>
<td>0.07</td>
<td>8.42</td>
</tr>
<tr>
<td>Ban 1941</td>
<td>8.72</td>
<td>8.94</td>
<td>0</td>
<td>8.72</td>
</tr>
<tr>
<td>Bbr 1900</td>
<td>9.65</td>
<td>8.66</td>
<td>0.99</td>
<td>9.65</td>
</tr>
<tr>
<td>Bi 1912</td>
<td>7.72</td>
<td>7.84</td>
<td>0</td>
<td>7.72</td>
</tr>
<tr>
<td>Bp 1944</td>
<td>6.30</td>
<td>8.15</td>
<td>0.15</td>
<td>8.30</td>
</tr>
</tbody>
</table>

Average determination, n = 3

La = *Lactobacillus acidophilus*

Lc = *L. casei*

Bbr = *Bifidobacterium breve*

Bi = *B. infantis*

Ban = *B. animalis*

Bp = *B. pseudolongum*

Bt = *B. thermophilum*

I = Initial cell count (log)

F = Final cell count (log)

D = Log decrease (the difference between I and F).
The lowest surviving organisms were *B. breve* 1900 decreasing from log 9.65 to 6.28 cfu/mL, 3.37 log reduction, and *L. casei* 2603 decreasing from log 8.42 to 4.7 cfu/mL, 3.72 log reduction. In comparison the Lankaputhra and Shah (1995) study found that all the strains tested in 1.0 and 1.5% bile decreased between 0.5 and 6 log cycles over the three hour test period.

*L. acidophilus* 2400 decreased in the range of 0.14 to 0.85 log in 0.5 to 1.5% bile concentration, the decrease for 2.0% bile was 0.75 log. *L. acidophilus* 2401 also showed the largest viability decrease in 1.5% of 0.35 log, while the lowest was 0.05 log in 0.5% bile. *L. acidophilus* 2404 decreased 0.63 log in 1.0% up to 1.04 log in 2.0% bile. *L. acidophilus* 2409 lowered 0.44 log in 1.0% and 0.77 log in 2.0% bile. *L. acidophilus* 2415 lost 0.54 log in 1.0% bile and up to 1.96 log in 2.0% bile. The *L. casei* 2603 followed the general trend of losing more cells with higher concentration bile, decreasing by only 0.07 log in 0.5% and up to 3.73 log in 2.0% bile.

The results of the present study both concur and conflict with those of Gopal *et al.* (1996) who studied tolerance of probiotic bacterial strains by the ability to grow in the presence of 0.3% oxgall. Gopal *et al.* (1996) quantified the inhibition of the cultures represented by a coefficient of inhibition. The *L. acidophilus* strains 2400, 2401, 2404 and 2409 displayed results similar to the current study. However the *Bifidobacterium* strains had conflicting results in the two studies where Gopal *et al.* (1996) found that *B. longum* 1941 had a high coefficient of inhibition indicating lower bile tolerance whereas in the
present study the same strain (reclassified by CSIRO) *B. animalis* 1941 survived very well in bile of all tested concentrations.

*B. animalis* 1941 did not lose any cells at all in the bile viability test. The same was true for *B. infantis* 1912 in 0.5%, *B. pseudolongum* 1944 in 1.0, 1.5 and 2.0%, as well as *B. thermophilum* 1991 in 0.5, 1.0 and 1.5% bile. *B. infantis* 1912 lost only 0.16 log in 1.0% and 0.73 log in 2.0% bile. *B. pseudolongum* 1944 in 0.5% only decreased 0.15 log and *B. thermophilum* 1991 only dropped 0.26 log in 2.0% bile. *B. breve* 1900 decreased 0.99 log, 0.72, 0.97 and 3.37 log in 0.5, 1.0, 1.5 and 2.0% bile respectively.

The results of the present study reflect the findings of others showing that *Bifidobacterium* strains vary widely in their ability to survive in bile (Berrada *et al.*, 1991; Chung *et al.*, 1999; Clark *et al.*, 1993; Clark and Martin, 1994; Ibrahim and Bezkorovainy, 1993; Lankaputhra and Shah, 1995). Survival of probiotic strains in bile alone may not be significant without the factor of acidified media as was used in the present study. Chou and Weimer (1999) explained that acid and bile can have both a separate and a combined effect on the growth of bacteria.
4.1.4 Strain Selection Tests in Low Storage Temperature

Probiotic bacteria as dairy food adjuncts must be able to survive the low storage temperatures of the frozen and refrigerated dairy foods. This study found that low storage temperatures do not have such a defined role on the survival of probiotic bacteria as acid or bile. There was very little change in viability after storage in low temperatures (Table 9). During refrigerated storage, L. acidophilus strains 2400 and 2404, L. casei 2603, B. animalis 1941, B. pseudolongum 1944 and B. thermophilum 1991 showed no reduction in cell count. The remaining organisms were L. acidophilus 2401 decreasing from log 6.72 to 5.30 cfu/mL, L. acidophilus 2409 dropping from log 6.61 to 6.34 cfu/mL, L. acidophilus 2415 reducing from log 8.19 to 8.11 cfu/mL, B. breve 1900 decreasing from log 9.65 to 8.82 cfu/mL and B. infantis 1912 reducing from log 7.72 to 7.42 cfu/mL.

In frozen storage, L. acidophilus 2400, L. acidophilus 2415, L. casei 2603, B. animalis 1941, B. pseudolongum 1944 and B. thermophilum 1991 did not decrease in cell count (Table 9). The other organisms were L. acidophilus 2401 decreasing from log 6.72 to 5.95 cfu/mL, L. acidophilus 2404 dropping from log 7.08 to 6.9 cfu/mL, L. acidophilus 2409 decreasing from log 6.61 to 5.0 cfu/mL, B. breve 1900 decreasing from log 9.65 to 8.21 cfu/mL and B. infantis 1912 reducing from log 7.72 to 7.47 cfu/mL.
Table 9: Log reduction of probiotic cultures after 3h storage in refrigerated and freezing temperatures.

<table>
<thead>
<tr>
<th>ORG</th>
<th>4°C</th>
<th>-20°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>F</td>
</tr>
<tr>
<td>La 2400</td>
<td>8.42</td>
<td>8.43</td>
</tr>
<tr>
<td>La 2401</td>
<td>6.72</td>
<td>5.30</td>
</tr>
<tr>
<td>La 2404</td>
<td>7.08</td>
<td>7.27</td>
</tr>
<tr>
<td>La 2409</td>
<td>6.61</td>
<td>6.34</td>
</tr>
<tr>
<td>La 2415</td>
<td>8.19</td>
<td>8.11</td>
</tr>
<tr>
<td>Lc 2603</td>
<td>7.35</td>
<td>7.49</td>
</tr>
<tr>
<td>Ban 1941</td>
<td>7.26</td>
<td>7.58</td>
</tr>
<tr>
<td>Bbr 1900</td>
<td>9.65</td>
<td>8.82</td>
</tr>
<tr>
<td>Bi 1912</td>
<td>7.72</td>
<td>7.42</td>
</tr>
<tr>
<td>Bp 1944</td>
<td>5.97</td>
<td>7.01</td>
</tr>
</tbody>
</table>

Average determination, n = 3

La = *Lactobacillus acidophilus*

Lc = *L. casei*

Bbr = *Bifidobacterium breve*

Bi = *B. infantis*

Ban = *B. animalis*

Bp = *B. pseudolongum*

Bt = *B. thermophilum*

I = Initial cell count (log)

F = Final cell count (log)

D = Log decrease (the difference between I and F).
The strains appear to be similarly affected by refrigerated and freezing temperatures, except for two strains, which had a much larger viability loss in freezing compared with refrigerated temperatures. *L. acidophilus* 2409 decreased 1.61 log in the freezer compared with 0.27 log in the refrigerator. Likewise *B. breve* 1900 lost 1.44 log when frozen compared with 0.83 log when refrigerated. On the other hand *L. acidophilus* 2401 fell only 0.76 log when frozen but 1.41 log when refrigerated. *B. infantis* 1912 decreased 0.25 and 0.30 log in the freezer and refrigerator respectively. The only other strain to record a loss was *L. acidophilus* 2415 decreasing only 0.08 log in the refrigerator, otherwise viable counts were maintained in cold storage.

Other studies that have been performed on the survival of probiotic bacteria in low storage temperatures have been for a longer duration of weeks and months. Often these studies are in a refrigerated or frozen dairy product such that temperature is not the only factor affecting survival (Gilliland and Lara, 1988; Hekmat and McMahon, 1992; Kailasapathy and Supriadi, 1996; Lankaputhra *et al.*, 1996; Laroia and Martin, 1991a; Rybka and Kailasapathy, 1995). The studies that take weeks and months to complete would be more appropriate for screening of probiotic bacterial strains for the purposes of food adjuncts with long shelf lives. The present low temperature storage survival study however was a simple preliminary screening test as a part of the battery of survival tests for probiotic bacterial strains suitable for use in dairy foods.
4.1.5 Strain Selection Tests in High Sucrose Concentration

Dairy desserts including ice cream are often high in sucrose. The probiotic bacterial strains used in those foods can be selected for survival in high sucrose concentrations as the osmotic pressure associated with sucrose may be a cause of cell death during the food storage period. Little variability was observed between the different concentrations of sucrose tested indicating that sucrose concentration does not have a significant influence on probiotic bacterial survival.

The highest sucrose concentration studied was 25% (Table 10) where *B. pseudolongum* 1944 showed the best survival of the *Bifidobacterium*, decreasing from 7.69 to 7.48 log cfu/mL. *L. acidophilus* 2404 showed the strongest survival, decreasing from 7.32 to 7.19 log cfu/mL over 3 h in 25% sucrose. The poorest surviving organisms were *B. thermophilum* 1991 decreasing from 8.89 to 8.25 log cfu/mL and *L. acidophilus* 2400 decreasing from 11.1 to 9.78 log cfu/mL.

The only strain to not decrease in viable count was *B. infantis* 1912 in 10% sucrose; the range of cell loss was 0.40 to 0.49 log for that organism in sucrose at 15 - 25% sucrose concentration. For *B. animalis* 1941 the range was 0.15 to 0.30 log reduction, 0.11 to 0.70 log for *B. breve* 1900, 0.20 to 0.26 log for *B. pseudolongum* 1944 and 0.48 to 0.88 log for *B. thermophilum* 1991.
Table 10: Log reduction of probiotic cultures after 3 h in 10, 15, 20 and 25% sucrose concentration.

<table>
<thead>
<tr>
<th>ORG</th>
<th>10%</th>
<th>15%</th>
<th>20%</th>
<th>25%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>F</td>
<td>D</td>
<td>I</td>
</tr>
<tr>
<td>La 2400</td>
<td>11.4</td>
<td>9.82</td>
<td>1.60</td>
<td>10.6</td>
</tr>
<tr>
<td>La 2401</td>
<td>11.2</td>
<td>8.96</td>
<td>2.27</td>
<td>10.9</td>
</tr>
<tr>
<td>La 2404</td>
<td>7.32</td>
<td>7.17</td>
<td>0.14</td>
<td>7.32</td>
</tr>
<tr>
<td>La 2409</td>
<td>10.9</td>
<td>9.86</td>
<td>1.08</td>
<td>11.0</td>
</tr>
<tr>
<td>La 2415</td>
<td>11.0</td>
<td>10.1</td>
<td>0.90</td>
<td>11.1</td>
</tr>
<tr>
<td>Lc 2603</td>
<td>9.18</td>
<td>8.43</td>
<td>0.75</td>
<td>9.18</td>
</tr>
<tr>
<td>Ban 1941</td>
<td>8.43</td>
<td>8.13</td>
<td>0.30</td>
<td>8.43</td>
</tr>
<tr>
<td>Bbr 1900</td>
<td>8.04</td>
<td>7.66</td>
<td>0.37</td>
<td>8.04</td>
</tr>
<tr>
<td>Bi 1912</td>
<td>8.38</td>
<td>9.12</td>
<td>0</td>
<td>8.38</td>
</tr>
<tr>
<td>Bp 1944</td>
<td>7.69</td>
<td>7.45</td>
<td>0.26</td>
<td>7.69</td>
</tr>
<tr>
<td>Bt 1991</td>
<td>8.89</td>
<td>8.10</td>
<td>0.79</td>
<td>8.89</td>
</tr>
</tbody>
</table>

Average determination, n = 3

La = Lactobacillus acidophilus
Lc = L. casei
Bbr = Bifidobacterium breve
Bi = B. infantis
Ban = B. animalis
Bp = B. pseudolongum
Bt = B. thermophilum
I = Initial cell count (log)
F = Final cell count (log)
D = Log decrease (the difference between I and F).
In general, *L. acidophilus* did not survive as well as the *Bifidobacterium* in sucrose concurring with the results of Hekmat and McMahon (1992) who studied the survival of the two organisms in ice cream with 12.5% sugar. *L. acidophilus* 2400 range of cell loss was from 0.83 to 1.74 log, for strain 2401: from 0.86 to 2.27 log, for strain 2404: 0.13 to 0.23 log, for strain 2409: 0.92 to 1.08 log and for strain 2415: 0.90 to 1.60 log decrease. *L. casei* 2603 decreased 0.75, 0.90, 0.73 and 0.63 log in 10, 15, 20 and 25% sucrose respectively.

### 4.1.6 Strain Selection Tests in High Oxygen Content

Oxygen is incorporated into ice cream during processing as the over run is increased to 100%. As anaerobic organisms *L. acidophilus* and the bifidobacteria do not appear suited to the ice cream environment. The responses to oxygen content coupled with incorporation of cysteine as an oxygen scavenger and temperature were strain dependent (Tables 11a and 11b). No patterns can be established except strain by strain. *B. breve* 1900 survived best at 5 °C with oxygen and cysteine but much lower survival was detected at 45 °C. *B. infantis* 1912 survived best at 25 °C regardless of oxygenation or cysteine concentration. The lowest survival was at 45 °C deaerated with cysteine. *B. animalis* 1941 showed between 2 and 3 log decrease in all conditions; the highest survival was in 5 °C deaerated without cysteine and the poorest survival was in 25 °C aerated without cysteine. The results are shown in Table 12.
Table 11a: Initial and final log cell counts of probiotic bacterial strains after overnight storage at 5, 25 and 45°C with and without the addition of 0.05% L-cysteine hydrochloride and treated by vacuum pump for deaeration.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Initial count</th>
<th>Deaerated</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>cfu/mL (log)</td>
<td>5°C</td>
<td>25°C</td>
<td>45°C</td>
<td>5°C</td>
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<td>45°C</td>
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<td>-c</td>
<td>+c</td>
<td>-c</td>
<td>+c</td>
<td>-c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2400</td>
<td>7.06</td>
<td>7.11</td>
<td>7.17</td>
<td>6.97</td>
<td>6.95</td>
<td>5.60</td>
<td>4.00</td>
</tr>
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<td>8.02</td>
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<td>4.00</td>
<td>4.00</td>
<td>7.35</td>
<td>5.78</td>
</tr>
<tr>
<td>2404</td>
<td>7.98</td>
<td>7.64</td>
<td>7.79</td>
<td>7.79</td>
<td>7.95</td>
<td>4.00</td>
<td>4.00</td>
</tr>
<tr>
<td>2409</td>
<td>7.79</td>
<td>7.65</td>
<td>7.80</td>
<td>7.82</td>
<td>7.63</td>
<td>7.61</td>
<td>7.98</td>
</tr>
<tr>
<td>2415</td>
<td>7.71</td>
<td>7.53</td>
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<td>7.28</td>
<td>7.69</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>2603</td>
<td>8.37</td>
<td>8.39</td>
<td>8.00</td>
<td>9.08</td>
<td>9.05</td>
<td>8.08</td>
<td>8.51</td>
</tr>
<tr>
<td>1900</td>
<td>7.53</td>
<td>7.02</td>
<td>6.67</td>
<td>6.66</td>
<td>6.80</td>
<td>4.48</td>
<td>3.00</td>
</tr>
<tr>
<td>1941</td>
<td>9.35</td>
<td>7.31</td>
<td>7.41</td>
<td>7.24</td>
<td>7.31</td>
<td>6.47</td>
<td>7.11</td>
</tr>
<tr>
<td>1944</td>
<td>7.44</td>
<td>7.54</td>
<td>7.99</td>
<td>7.98</td>
<td>7.91</td>
<td>6.00</td>
<td>5.93</td>
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<tr>
<td>1991</td>
<td>8.88</td>
<td>7.70</td>
<td>7.60</td>
<td>8.83</td>
<td>9.02</td>
<td>9.18</td>
<td>9.09</td>
</tr>
</tbody>
</table>

2400 = *L. acidophilus* 2400  
2401 = *L. acidophilus* 2401  
2404 = *L. acidophilus* 2404  
2409 = *L. acidophilus* 2409  
2415 = *L. acidophilus* 2415  
2603 = *L. casei* 2603  
1900 = *B. breve* 1900  
1912 = *B. infantis* 1912  
1941 = *B. animalis* 1941  
1944 = *B. pseudolongum* 1944  
1991 = *B. thermophilum* 1991  
+c = with 0.05% L-cysteine hydrochloride  
-c = without 0.05% L-cysteine hydrochloride
Table 11b: Initial and final log cell counts of probiotic bacterial strains after overnight storage at 5, 25 and 45°C with and without the addition of 0.05% L-cysteine hydrochloride and left standing.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Initial count (log)</th>
<th>5°C</th>
<th>25°C</th>
<th>45°C</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>cfu/mL</td>
<td>+c</td>
<td>-c</td>
<td>+c</td>
</tr>
<tr>
<td>2400</td>
<td>7.06</td>
<td>7.12</td>
<td>6.99</td>
<td>7.00</td>
</tr>
<tr>
<td>2401</td>
<td>8.02</td>
<td>4.00</td>
<td>5.90</td>
<td>7.57</td>
</tr>
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<td>7.48</td>
<td>7.65</td>
</tr>
<tr>
<td>2603</td>
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<td>7.95</td>
<td>8.28</td>
<td>9.07</td>
</tr>
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<td>1900</td>
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<td>7.20</td>
<td>7.10</td>
<td>5.99</td>
</tr>
<tr>
<td>1912</td>
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<td>8.08</td>
<td>8.16</td>
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<td>7.17</td>
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<td>1991</td>
<td>8.88</td>
<td>5.00</td>
<td>8.35</td>
<td>9.03</td>
</tr>
</tbody>
</table>

2400 = L. acidophilus 2400
2401 = L. acidophilus 2401
2404 = L. acidophilus 2404
2409 = L. acidophilus 2409
2415 = L. acidophilus 2415
2603 = L. casei 2603
1900 = B. breve 1900
1912 = B. infantis 1912
1941 = B. animalis 1941
1944 = B. pseudolongum 1944
1991 = B. thermophilum 1991

+c = with 0.05% L-cysteine hydrochloride
-c = without 0.05% L-cysteine hydrochloride
Table 12: Log cell counts of probiotics after overnight storage in deaerated or shaking conditions with and without the addition of cysteine.

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>La 2401</th>
<th>La 2409</th>
<th>Lc 2603</th>
<th>Bi 1912</th>
<th>Bp 1944</th>
</tr>
</thead>
<tbody>
<tr>
<td>+O₂ 5°C +c</td>
<td>7.64</td>
<td>7.36</td>
<td>8.01</td>
<td>8.34</td>
<td>7.72</td>
</tr>
<tr>
<td>+O₂ 5°C -c</td>
<td>8.62</td>
<td>7.16</td>
<td>8.26</td>
<td>8.74</td>
<td>7.06</td>
</tr>
<tr>
<td>+O₂ 25°C +c</td>
<td>7.85</td>
<td>7.99</td>
<td>9.01</td>
<td>9.66</td>
<td>8.29</td>
</tr>
<tr>
<td>+O₂ 25°C -c</td>
<td>7.92</td>
<td>8.06</td>
<td>9.12</td>
<td>9.31</td>
<td>8.93</td>
</tr>
<tr>
<td>+O₂ 45°C +c</td>
<td>5.00</td>
<td>7.95</td>
<td>7.48</td>
<td>5.98</td>
<td>5.00</td>
</tr>
<tr>
<td>+O₂ 45°C -c</td>
<td>4.85</td>
<td>5.00</td>
<td>7.11</td>
<td>4.81</td>
<td>4.00</td>
</tr>
<tr>
<td>-O₂ 5°C +c</td>
<td>7.66</td>
<td>7.85</td>
<td>7.53</td>
<td>8.36</td>
<td>7.02</td>
</tr>
<tr>
<td>-O₂ 5°C -c</td>
<td>7.55</td>
<td>7.76</td>
<td>7.89</td>
<td>8.40</td>
<td>6.93</td>
</tr>
<tr>
<td>-O₂ 25°C +c</td>
<td>7.91</td>
<td>7.53</td>
<td>9.01</td>
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<td>7.81</td>
</tr>
<tr>
<td>-O₂ 25°C -c</td>
<td>8.96</td>
<td>7.38</td>
<td>9.00</td>
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<td>7.78</td>
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<tr>
<td>-O₂ 45°C +c</td>
<td>2.00</td>
<td>6.00</td>
<td>7.63</td>
<td>6.73</td>
<td>7.04</td>
</tr>
<tr>
<td>-O₂ 45°C -c</td>
<td>4.53</td>
<td>5.00</td>
<td>7.70</td>
<td>6.39</td>
<td>5.00</td>
</tr>
</tbody>
</table>

n = 3

La = *Lactobacillus acidophilus*

Lc = *L. casei*

Bi = *B. infantis*

Bp = *B. pseudolongum*

+O₂ = with shaking

-O₂ = deaerated

+c = with cysteine 0.05%

-c = without cysteine
B. *pseudolongum* 1944 survived equally well at 5 and 25 °C but viability declined at 45 °C; the highest survival was in 5 °C deaerated without cysteine and the lowest survival was in 45 °C aerated without cysteine. *B. thermophilum* 1991 survived equally well at 25 and 45 °C with a decrease in cell count at 5 °C; the highest survival was at 45 °C deaerated with cysteine and the lowest survival was in 5 °C aerated with cysteine.

*L. acidophilus* 2400 showed almost no change in cell counts in 5 and 25 °C but displayed consistent 3 log decreases at 45 °C except for the deaerated with cysteine, the highest survival was seen at 25 °C aerated without cysteine. *L. acidophilus* 2401 showed a varied response surviving well at 45 °C and in 5 °C when deaerated with cysteine or when aerated without cysteine and at 25 °C aerated with cysteine. *L. acidophilus* 2404 survived well in all conditions except for when deaerated at 45 °C with and without cysteine. *L. acidophilus* 2409 showed very little change in viability after every oxygen viability test, the highest cell count was at 45 °C deaerated without cysteine, the lowest survival was at 45 °C aerated with cysteine. *L. acidophilus* 2415 survived well in 5 and 25 °C but the cell count declined at 45 °C, the highest survival was in 25 °C deaerated without cysteine. *L. casei* 2603 was largely unaffected by the oxygen content tests and was able to grow at 25 °C in all conditions, the lowest survival was at 5 °C aerated with cysteine.

Table 12 shows the results of the oxygen study repeated with the aerated samples being shaken for proper aeration instead of simply being left standing overnight. This table also shows no real patterns for survival in oxygen and
cysteine. Oxygen content does not have such a defined role on the survival of probiotic bacterial strains. The influence of oxygen varies strain by strain as found by Condon (1987) for lactic acid bacteria and Shimamura et al. (1990) for bifidobacteria responses to oxygen. Lactic acid bacteria have a reaction to oxygen, either a good reaction or a bad reaction. The detrimental reaction of lactic acid bacteria to oxygen may be indirect, as a result of toxic levels of hydrogen peroxide accumulation, which is a product of some flavoprotein oxidases of lactic acid bacteria. Other metabolic changes may occur in lactic acid bacteria when exposed to oxygen such as sugar metabolism and an oxidation stress response to sub-lethal levels of hydrogen peroxide (Condon, 1987; Shimamura et al., 1992).

4.1.7 Selecting Strains for Further Study

The results of each of the strain selection tests were analysed, the strains that showed low log reduction in multiple tests were considered to be stronger than others that showed greater log reduction in the same tests. The strains ideal for further study were narrowed down to 6 strains initially as a result of the strain selection experiments: *L. acidophilus* 2401, 2409, *L. casei* 2603, *B. infantis* 1912, *B. pseudolongum* 1944 and *B. thermophilum* 1991. Two strains, *L. acidophilus* 2401 and *B. infantis* 1912, were chosen for further study of microencapsulation and dairy product incorporation because they were both easy to grow. Further study was also performed on the commercially available strains from Wisby and DSM Food Specialties, mainly for comparison with the selected CSIRO strains *L. acidophilus* 2401 and *B. infantis* 1912.
4.2 Microencapsulation

4.2.1 The Process of Microencapsulation

Initially the starch/alginate/culture mix was added to the oil drop by drop through a dropping funnel, which was scaled up using a peristaltic pump. However the protocol used for encapsulation required the starch/alginate/culture/oil mixture to be emulsified. Therefore the dropwise addition of one to the other was replaced with the complete addition of one to the other in one step.

4.2.2 Effect of Sterilisation on the Alginate Solution

Initially the alginate solution was autoclaved for maintaining sterility because other calcium alginate encapsulation studies had done so (Champagne et al., 1993; Jankowski et al., 1993). Several attempts at encapsulation using sterilised sodium alginate led to failure to achieve the capsule/bead formation. The detrimental effect of heat during sterilisation of the alginate solution was considered a possible reason for the failure to develop capsules. This experiment resulted in the recovery of capsules only in the alginate that was not sterilised; no capsules were formed with the sterilised alginate solution. Hence for the encapsulation procedures, all solutions and glassware used were sterilised while the alginate was weighed and handled under sanitised conditions. This meant that the sterility of the encapsulating system could not be assured; however, sterilisation of all the encapsulating equipment and reagents resulted in no contamination of the encapsulated product. Thus it was assumed that the high grade alginate that was used for encapsulating was uncontaminated.
Literature information is not clear as to why the alginate was sterilised by other researchers. Alginate polymer loses its ability to form a firm gel when heated, the gelling properties are lost which in the current study resulted in capsules failing to form properly during encapsulation.

4.2.3 Yield and Viability of Microencapsulated Bacteria

4.2.3.1 Oil to Alginate Ratio and Yield of Capsules

During scaling up of the microencapsulation procedure of Sheu and Marshall (1993) the capsule recovery decreased (measured by wet weight) when the quantity of alginate solution was increased with the use of the same volume of oil. Thus an experiment was designed using 100mL alginate solution and varying the volume of oil: 50mL, 100mL and 200mL. The recovery of the capsules increased (measured by wet weight) when the oil was doubled in volume (Table 13) with 100mL alginate solution. The results suggest that the recovery of capsules would be increased more by using a higher ratio of oil to alginate.

During encapsulation with varied oil volumes for 100mL alginate solution the viability of *L. casei* in the capsules decreased as the volume of oil increased. The viability of the cells recovered when 50mL oil was used was $4 \times 10^{11}$ cfu/g; the viability decreased for 100mL oil sample to $7 \times 10^{10}$ cfu/g and for the 200mL oil sample it was $2 \times 10^{10}$ cfu/g. The results from this experiment show that the recovery of capsules increased with the increase of the oil to alginate ratio. However, the viability of the cells was higher in the capsules recovered from the experiment where the lower oil volume (50mL) was used.
Preliminary results have shown that good capsule formation did not occur when the oil to alginate ratio was 5:1. It could depend on the type of alginate used. Further, there was a concern that the oil residue will remain in the capsules, hence increase the final fat content of the product and that is very undesirable. Higher oil residue will affect the viability of bacteria since a higher concentration of emulsifier is required. Hence, a modified ratio of oil to alginate was used in this study.
Table 13: Microencapsulation yield measured as wet weight of capsules recovered with varying volumes of oil for 100mL alginate mix

<table>
<thead>
<tr>
<th>Volume of oil used to drop alginate solution (mL)</th>
<th>50</th>
<th>100</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total wet weight of capsules recovered (g)</td>
<td>10.78 ± 3.5</td>
<td>80.5 ± 2.1</td>
<td>109.3 ± 1.4</td>
</tr>
</tbody>
</table>

Results are the average of three determinations
4.2.3.2 Effect of Tween 80 Concentration on Bacterial Viability and Yield of Capsules

Table 14 shows the results of viability studies of *L. casei* during encapsulation under the varying concentrations of Tween 80 and lecithin. The study of the effect of the concentration of Tween 80 showed that there is no substantial difference between 0.1% and 0.2% Tween 80 concentrations on the yield of capsules. The yield of capsules (*L. casei*) emulsified with lecithin was higher than that obtained with the use of Tween 80 (Table 14). However the texture of the capsules produced with lecithin was different from those produced with Tween 80.

The results of the present study concur with those of Sheu and Marshall (1993) to show that 0.2% Tween 80 was indeed the suitable percentage of emulsifier to use during encapsulation for maintaining cell viability. Although lecithin allowed a higher capsule recovery by weight, the use of this emulsifier had a detrimental effect on the texture of the capsules, the capsules formed with lecithin produced an unfavourable stringy texture and varying shapes. This would require more research if lecithin was to be further considered as an emulsifier for encapsulation.
Table 14: Effect of different concentrations of Tween 80 and Lecithin in 50mL 2% alginate on encapsulation yield and viability of *L. casei*.

<table>
<thead>
<tr>
<th>Emulsifier and Concentration</th>
<th>Yield of Capsules (g)</th>
<th>Log cfu/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% Tween 80</td>
<td>13 ± 1.2</td>
<td>11.78 ± 0.2</td>
</tr>
<tr>
<td>0.1% Tween 80</td>
<td>37 ± 2.3</td>
<td>10.53 ± 0.5</td>
</tr>
<tr>
<td>0.2% Tween 80</td>
<td>35 ± 1.6</td>
<td>10.60 ± 0.9</td>
</tr>
<tr>
<td>1% Lecithin</td>
<td>49 ± 0.2</td>
<td>10.30 ± 1.1</td>
</tr>
</tbody>
</table>

Results are the average of three determinations.
4.2.3.3 Effect of Starch (Hi Maize™) on Encapsulation Yield and Bacterial Viability

The results (Table 15) of the analysis of the effect of microencapsulation of *L. casei* with Hi-Maize™ starch (supplied by Starch Australia Ltd) indicated a positive role on survival of the bacteria. Hi Maize™ starch is also known as resistant starch and is a prebiotic used for increasing the viability of probiotic bacteria. The results (Table 15) show that adding 1 - 2% starch with the encapsulation procedure increases the viability of the probiotic bacteria. However it appears that this is the optimal concentration as the viable count in the recovered encapsulation decreased when the starch concentration was increased to 4%. The encapsulation yield was also maximised with 2% starch. Hi Maize™ starch is used as a prebiotic food supplement for probiotic bacteria. The starch also protects the bacteria by binding them in the capsule matrix. During the microencapsulation procedure the starch is also used for oil/water reversion with alginate.

Wang *et al.* (1999) utilised amyllose maize starch granules to increase the survival of *Bifidobacterium* cells during exposure to low pH and bile acids in a mouse digestive tract. This is relevant to the present study which aims to increase the survival of probiotic bacteria in dairy foods and the human digestive tract. Wang *et al.* (1999) ascribed the increased survival of *Bifidobacterium* to adherence of the cells to the starch granules. Encapsulation of probiotic bacterial cells, especially *Bifidobacterium*, with starch in the encapsulating material provides extra protection to the cells for resistance to low pH and bile acids, which was tested in further experiments.
Table 15: Effect of concentration of starch on the yield of encapsulated *L. casei*

<table>
<thead>
<tr>
<th>Starch (%)</th>
<th>Yield of Capsules (g)</th>
<th>Log cfu/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>38 ± 2.0</td>
<td>8.60 ± 0.1</td>
</tr>
<tr>
<td>1</td>
<td>54 ± 3.6</td>
<td>11.96 ± 0.2</td>
</tr>
<tr>
<td>2</td>
<td>60 ± 2.6</td>
<td>11.65 ± 0.0</td>
</tr>
<tr>
<td>4</td>
<td>52 ± 4.0</td>
<td>9.46 ± 0.3</td>
</tr>
</tbody>
</table>

Average of three determinations, mean ± standard deviation.
4.2.3.4 Preserving Bacterial Viability in Frozen Storage Conditions

The effect of different concentrations of glycerol on cell survivals in the presence and absence of starch in microencapsulation was studied. Glycerol is used routinely for cryo-preservation of bacterial cultures. The concentrations of glycerol chosen were 15% and 20% because they are used routinely to freeze cultures in growth media directly at −20°C.

Encapsulating with calcium alginate together with starch did not improve the recovery of cells after storage in frozen conditions after four months (Table 16). The addition of glycerol at 15% with calcium alginate and starch was no improvement to the recovery of cells however when the glycerol concentration was increased to 20% the cell recovery was improved. At 30% glycerol there was virtually no cell loss after storage in frozen conditions. The higher the concentration of glycerol, the more protection is given to the cells in the frozen storage conditions however the use of higher concentrations of glycerol is more difficult due to its viscosity.
Table 16: Effect of incorporating starch into calcium alginate encapsulation and the addition of glycerol at varying concentrations on preserving bacterial cell viability of *L. casei* during frozen (-20°C) storage for four months.

<table>
<thead>
<tr>
<th>Encapsulation Conditions</th>
<th>Log cfu/g</th>
<th>Log cfu/g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-Freezing</td>
<td>Post-Freezing</td>
</tr>
<tr>
<td>2% Na-Alginate</td>
<td>8.08 ± 0.02</td>
<td>6.20 ± 0.07</td>
</tr>
<tr>
<td>2% Na-Alginate + 2% starch</td>
<td>10.00 ± 0.05</td>
<td>6.60 ± 0.06</td>
</tr>
<tr>
<td>2% Na-Alginate + 2% starch + 15% Glycerol</td>
<td>12.08 ± 0.06</td>
<td>8.30 ± 0.02</td>
</tr>
<tr>
<td>2% Na-Alginate + 2% starch + 20% Glycerol</td>
<td>12.36 ± 0.10</td>
<td>10.60 ± 0.03</td>
</tr>
<tr>
<td>2% Na-Alginate + 2% starch + 30% Glycerol</td>
<td>12.40 ± 0.07</td>
<td>12.15 ± 0.01</td>
</tr>
</tbody>
</table>

Average of three determinations ± standard deviation.
4.2.4 Examination of Alginate Beads

The dimensions of the beads were determined using a light microscope and the ocular and stage micrometers. The size and shape of the beads were determined from the electron photomicrographs, as well as by using the light microscope. The encapsulation procedure used in this study resulted in bead size of 0.5mm to 1mm, and only a small proportion of the microbeads were <500 μm. Size segregation was carried out using 1mm, 500 μm and 150 μm sieves. The large size as well as the inconsistency of capsule sizes were due to the manual method of encapsulation. After this work was completed an encapsulating machine was commissioned by the SouthWest Research Institute in San Antonio, Texas. It is anticipated that by manufacturing the capsules with a machine, the capsule sizes will be smaller and more consistent in size. Although the larger beads would provide more protection to the cells in dairy products, the smaller capsules that are still big enough to provide protection for the cells would minimise coarseness of texture and sensory interference when incorporated into foods (Lee and Heo, 2000).

The shape of the beads was generally spherical; sometimes elliptical shaped capsules were observed as well (Fig 5). The beads featured in the present study had a similar shape to those shown by Dainty et al. (1986), Green et al. (1996), Hari et al. (1996), Rao et al. (1989) and Shah and Ravula (2000); however, the capsules produced by Sheu and Marshall (1993) were a pronounced tear drop shape.
The scanning electron photomicrographs show the pores in the alginate matrix forming the capsule wall. The starch molecules embedded in the cavities and the bacterial cells within the alginate are evenly distributed within the beads. The starch grains were present in the cavities (Fig 6a), while the bacteria were distributed randomly in the alginate matrix (Figs 6b & c). With an even distribution of both starch grains and bacterial cells in the encapsulating matrix a more uniform product is possible. An even distribution may also assist in the protection of the cells and, with the starch grains in such close proximity to the bifidobacteria, this may also assist in increasing survival.
Fig 5: Scanning electron photomicrograph of microcapsules
Fig 6: Section of alginate microcapsule showing: a) the starch grains in cavities, b) *L. acidophilus* and c) *B. infantis* located in the alginate matrix.
b)
4.2.5 Acidification Kinetics of Encapsulated Cultures

Acidification kinetics of encapsulated *L. casei* & *L. acidophilus* were established over a period of 48 hrs, after inoculating as free cells or encapsulated culture at 2.5 and 5.0% into reconstituted skimmed milk medium and incubated at 37°C. The viable count for both the cultures was taken at the beginning (>10⁷) and at the end (>10¹¹) of the experiment. The results (Fig. 7) show that the encapsulated bacterial cells are active and the ability to produce acid is similar to the free cell cultures, as measured by the pH changes over a period of time. However the time taken for the encapsulated cells to arrive at the same pH is longer than that taken by the free cells.

The encapsulated cultures were able to acidify a milk medium, since probiotic bacterial growth leads to acidification this was a clear indication that the bacteria remained viable and active while encapsulated. However it was noted that the encapsulated cultures took twice as long to reach the same pH as the free cultures, which reached pH 4 after 22 hours incubation. This could be due to the slow uptake of nutrients and slow release of the metabolites across the encapsulating alginate shell of the capsule. A similar pattern was also observed by Larisch *et al.* (1994). They found that the alginate/poly-L-lysine encapsulated cells took 17% longer than free lactococci to reduce the pH of milk to 5.5. It has been suggested that the size of the microbeads affect the rates of mass transfer and activity of the immobilised bacteria (Larisch *et al.*, 1994).
Figure 7: Kinetics of acidification with encapsulated and free cells of *L. casei* and *L. acidophilus* during growth in reconstituted skim milk at 37°C.

L.c = *L. casei*

L.a = *L. acidophilus*

# = encapsulated cells

* = free (unencapsulated) cells

2.5% = Inoculated with 2.5% culture

5% = Inoculated with 5% culture
4.3 Viability Studies of Encapsulated Bacteria

4.3.1 Survival of Encapsulated Bacteria in Low pH

Initially the survival experiment was carried out with freeze-dried encapsulated cultures to monitor survival under low pH conditions. No survivals were recorded by incubating encapsulated freeze dried cultures directly in pH adjusted MRS broth (pH 2, 3, 4 and 6) over a period of 0, 1, 2, 3 and 4 hours. The initial cell count was $>10^8$ cfu/g of freeze-dried cultures. The freeze-dried bacterial cells do not represent the normal metabolic status of probiotic cells, nor the condition of the cells as they are challenged in the gut by gastric acidity. Hence, the freeze-dried encapsulated cells were then re-hydrated in 9.5% reconstituted skim milk powder, after weighing out the aliquot, and leaving overnight in the refrigerator. The reason for no growth or survival of encapsulated freeze dried cultures even at pH 6.0 is because, the cultures obtained from commercial suppliers as frozen concentrate were freeze dried first, then encapsulated and freeze dried again to a powder, for easy incorporation. The effect of freeze drying the cultures twice significantly reduced the viability of the bacteria.

The acid survival experiment was repeated with a modification, where the overnight cultures were not only re-hydrated but also given a pre-incubation time. The re-hydrated cultures were pre-incubated for 2 hours at 37°C, before being resuspended in the low pH medium for testing the survivals. An analysis of cell survival at pH 2 was carried out after a time period of 1h and 2h. The result of the analysis recorded cell survivals, as indicated in Table 17.
Bacterial survival was recorded for both the DSM commercial strains - *L. casei* and *L. acidophilus*; it was observed that there was a survival of \( >10^7 \) cfu/g. There was a decrease of 3 log in viability from the original cell count (\( >10^{10} \) cfu/g) in the freeze-dried encapsulated bacteria, suggesting that encapsulation did not offer significant protection to these cells.
Table 17: Survival (log cfu/g) of encapsulated commercial (DSM Food Specialties) L. casei and L. acidophilus in pH 2 after 1 and 2 hours.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Initial count</th>
<th>1 h</th>
<th>2 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. acidophilus</td>
<td>10.00</td>
<td>7.64</td>
<td>7.59</td>
</tr>
<tr>
<td>L. casei</td>
<td>10.00</td>
<td>7.81</td>
<td>7.75</td>
</tr>
</tbody>
</table>

Average of three determinations, n = 3.
A few strains including *L. acidophilus* 2401, 2409, *L. casei* 2603 and *B. infantis* 1912 were chosen for encapsulation and exposure to low pH after the strain selection tests in low pH. Experiments using selected strains (*L. acidophilus* 2409 and *B. infantis* 1912) were carried out in order to study the effect of encapsulation on the bacteria under acidic and alkaline conditions. The survival of encapsulated *L. acidophilus* 2409 in acid conditions (Table 18) shows that there is a similar decrease of the bacterial log cfu/g in NGYC at pH 4.0 and pH 3.0. Incubation of cultures for 3h at pH 4.0 and 3.0 resulted in a decrease of about 2 log cycles, while at pH 2.0 there was a loss of 5 log cfu/g. *B. infantis* 1912 showed only a small decrease at pH 4.0 and at pH 3.0, while there was a decrease of 3 log cycles at pH 2.0 after 3 h of exposure under similar conditions.

The results suggest that encapsulated *L. acidophilus* 2409 was more sensitive to low pH conditions than encapsulated *B. infantis* 1912, which did not decrease more than 1 log in pH 3 and pH 4 conditions. The results were similar to those of the low pH survival studies carried out on the free cells of the same strains during the strain selection experiments, suggesting that encapsulation did not provide significant protection of the bacteria in very low pH conditions. This indicates that the capsule wall of alginate is highly permeable to small molecules and ions including hydrogen ions, probably due to the pores in the capsule wall. Although Figure 7 indicates a slowing down of diffusion across the encapsulating wall this is more likely to relate to larger molecules such as nutrients and cell metabolites while smaller molecules such as hydrogen ions are still readily permeable.
Table 18: Survival of encapsulated *L. acidophilus* 2409 and *B. infantis* 1912 over three h of exposure to low pH media.

<table>
<thead>
<tr>
<th>pH</th>
<th>0h</th>
<th>1h</th>
<th>2h</th>
<th>3h</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>11.78</td>
<td>11.72</td>
<td>11.58</td>
<td>6.51</td>
</tr>
<tr>
<td>3.0</td>
<td>12.70</td>
<td>12.62</td>
<td>10.18</td>
<td>10.08</td>
</tr>
<tr>
<td>4.0</td>
<td>12.72</td>
<td>12.38</td>
<td>12.00</td>
<td>10.40</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pH</th>
<th>0h</th>
<th>1h</th>
<th>2h</th>
<th>3h</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>11.56</td>
<td>11.04</td>
<td>8.85</td>
<td>8.45</td>
</tr>
<tr>
<td>3.0</td>
<td>11.74</td>
<td>11.64</td>
<td>11.36</td>
<td>11.00</td>
</tr>
<tr>
<td>4.0</td>
<td>11.69</td>
<td>11.60</td>
<td>11.60</td>
<td>11.46</td>
</tr>
</tbody>
</table>

Average of three determinations
The results of the present study concur with those of Rao et al. (1989) who found that encapsulated *B. pseudolongum* in simulated gastric juice (pH 1.33) decreased in viability. The decrease in viability was attributed to the ability of the gastric juice to permeate surface pinholes in the microsphere. Rao et al. (1989) were able to resolve this problem by coating the capsules with an outer layer of 1% beeswax with 1 – 2% stearic acid, which allowed higher survival rates of *B. pseudolongum* and made the capsules less permeable to oxygen.

### 4.3.2 Survival of Encapsulated Bacteria in High Bile Concentration

A few strains were chosen for encapsulation and exposure to bile after the strain selection tests in high bile concentration. Survival of *L. acidophilus* 2409, *B. infantis* 1912 and *L. casei* 2603 was monitored after exposure to (NGYC medium containing) 1% and 2% bile extract (Table 19). Survivals were recorded 2 hourly up to 6 h. The results indicated that cell death over time is similar for all the three strains. *L. acidophilus* 2409 and *L. casei* 2603 showed a decrease of 2 log cycles as compared to the initial cell count, while *B. infantis* 1912 showed only a slight decrease in numbers as compared to the initial cell count.

Encapsulation appears to have been beneficial to *L. casei* 2603 and *B. infantis* 1912 during exposure to high bile concentrations, compared with the results of the same strains as free cells in the same conditions. *L. acidophilus* 2409 was more sensitive than *L. casei* 2603 and *B. infantis* 1912. The alginate shell may be less permeable to bile than hydrogen ions thus encapsulation with calcium alginate and starch offers greater protection to the
probiotic bacterial cells from exposure to bile than to low pH conditions. The results concur with those of Lee and Heo (2000) who showed that *B. longum* survival decreased as the capsules were left longer in the bile solution. They also showed that the concentration of alginate in the encapsulating matrix and bead size has an effect on the survival rate, the higher the alginate concentration and bead size the slower the death rate. Thus alginate is a protective agent against bile extract for probiotic bacterial cells. A balance would need to be found for larger bead size for cell protection and apparent graininess in a food product and between increasing the concentration of alginate in the encapsulating matrix but maintaining an easy to use encapsulating system.
Table 19: Log survival of encapsulated *L. acidophilus* 2409, *B. infantis* 1912 and *L. casei* 2603 over six h of exposure to 1 and 2% bile solution

<table>
<thead>
<tr>
<th>Strain</th>
<th>Bile conc (%)</th>
<th>Time of Incubation (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. acidophilus</em> 2409</td>
<td>1</td>
<td>7.53 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7.54 ± 0.6</td>
</tr>
<tr>
<td><em>B. infantis</em> 1912</td>
<td>1</td>
<td>6.54 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6.86 ± 0.3</td>
</tr>
<tr>
<td><em>L. casei</em> 2603</td>
<td>1</td>
<td>10.26 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>10.36 ± 0.4</td>
</tr>
</tbody>
</table>

Average of three determinations ± standard deviation.
4.4 Incorporation of Probiotic Bacteria into Dairy Products

Both encapsulated and non-encapsulated bacteria were incorporated into dairy products for evaluation of the viability of the bacteria over the shelf life of the product. The products assessed were yoghurt, ice cream, and Cheddar cheese. The trials were performed on both laboratory and pilot plant scales where the effect of encapsulated bacteria and growth factors on physical, functional and sensory properties of the products were examined using a texture analyser and sensory panel.

4.4.1 Yoghurt

4.4.1.1 Probiotic Survival in Yoghurt

Overall, the results for probiotic survival in yoghurt were inconclusive. Survival of free and encapsulated probiotic bacteria in yoghurt is very much strain dependent, similar to the strain selection experiments and testing of survival of encapsulated strains under different conditions. The first trial for yoghurt was done on a lab scale (200mL cups) and compared the survival of free cells with freshly encapsulated and co-encapsulated cells in yoghurt made with *S. thermophilus* as the only starter culture (Table 20). For free *L. acidophilus* 2401 the log decrease after 8 weeks storage was 2.98, from 9.32 to 6.34, compared with the encapsulated cells which showed a 2.85 log decrease, from 7.60 to 4.75 and the co-encapsulated cells which recorded only 1.19 log decrease, from 7.45 to 6.26. For free *B. infantis* 1912 the log decrease after 8 weeks storage was 3.11, from 9.20 to 6.09, compared with the encapsulated cells which showed a 2.86 log decrease, from 7.57 to 4.71 and the co-encapsulated cells which recorded only 0.69 log decrease, from
7.90 to 7.21. Clearly the co-encapsulated cells were the best survivors in this trial. In the lab trial 1, the free counts were greater than the encapsulated ones in 0 week, which was due to the effect of freeze drying on the viability of encapsulated cultures.

The results of the first laboratory trial suggested that *L. acidophilus* 2401 and *B. infantis* 1912 were slightly protected from the yoghurt by encapsulation allowing a reduction in the cell decrease as compared with the free cells (Table 20). In the same trial the results indicated that co-encapsulation was beneficial to both strains as the cell decrease was less than half that of the encapsulated type. Co-encapsulation appears to have been beneficial to both *L. acidophilus* 2401 and *B. infantis* 1912 as survivals increased compared with both free cells and separately encapsulated cells. It has been demonstrated previously that bifidobacteria benefit from co-culturing with proteolytic species such as *L. acidophilus* as Bifidobacteria have no proteolytic activity of their own (Klaver et al., 1993).
Table 20: Log cfu/g of yoghurt (first laboratory trial) made with *L. acidophilus* 2401 and *B. infantis* 1912 under free, encapsulated and co-encapsulated states over 8 weeks storage at 5°C.

<table>
<thead>
<tr>
<th>Probiotic Cultures in Different States</th>
<th>STORAGE PERIOD (WEEKS)</th>
<th>LOG DECREASE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Free A</td>
<td>9.32</td>
<td>6.90</td>
</tr>
<tr>
<td>Free B</td>
<td>9.20</td>
<td>7.36</td>
</tr>
<tr>
<td>En A</td>
<td>7.60</td>
<td>6.85</td>
</tr>
<tr>
<td>En B</td>
<td>7.57</td>
<td>6.88</td>
</tr>
<tr>
<td>Co A</td>
<td>7.45</td>
<td>6.57</td>
</tr>
<tr>
<td>Co B</td>
<td>7.90</td>
<td>7.60</td>
</tr>
</tbody>
</table>

Average of three determinations, n = 3.

Free = Unencapsulated cultures

En = Encapsulated culture

Co = Co-encapsulated culture

*A = L. acidophilus* 2401

*B = Bifidobacterium infantis* 1912
The second trial was done on a lab scale (200mL cups) and compared free
cells with freshly encapsulated cells as well as freeze dried encapsulated and
freeze dried co-encapsulated cells (Table 21). This time the freeze dried
encapsulated cells survived the best with *L. acidophilus* 2401 decreasing only
0.57 log, from 6.08 to 5.51 and *B. infantis* 1912 decreasing 0.58 log, from 5.90
to 5.32. The free *L. acidophilus* 2401 cells decreased by 1.44, from 7.87 to
6.43, compared with the freshly encapsulated cells with a decrease of 1 log,
from 7.30 to 6.30 and the freeze dried co-encapsulated cells decreased in cell
count from 7.04 to 6.08 log, a 0.96 log decrease. The free *B. infantis* 1912
decreased 1.08 log, from 7.08 to 6.00 while the freshly encapsulated cells
decreased only 0.44, from 6.78 to 6.34 and the freeze dried co-encapsulated
cells decreased 1.33 log, from 7.15 to 5.82.

In the second laboratory trial the best survivors were the cells that were
encapsulated separately and then freeze dried before being incorporated into
the yoghurt (Table 21). Freeze drying of the bacterial capsules shrinks the
capsules by removing the water and therefore much of the bulk surrounding
the cells. Incorporation of freeze dried encapsulated cells into yoghurt
products is more likely to be commercially successful than incorporation of
freshly encapsulated cells for this reason. The smaller the capsules the less
likely they are to interfere with the sensory qualities of the product. On the
other hand freeze drying requires further processing of the capsules before
incorporation into the products, which would be less acceptable for
commercial application due to the additional costs involved with extra
processes.
Table 21: Log cfu/g of yoghurt (second laboratory trial) made with *L. acidophilus* 2401 and *B. infantis* 1912 under free, freshly encapsulated, encapsulated freeze dried and co-encapsulated freeze dried states over 8 weeks storage at 5°C.

<table>
<thead>
<tr>
<th>Probiotic Cultures in Different States</th>
<th>STORAGE PERIOD (WEEKS)</th>
<th>LOG DECREASE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Free A</td>
<td>7.87</td>
<td>7.28</td>
</tr>
<tr>
<td>Free B</td>
<td>7.08</td>
<td>6.48</td>
</tr>
<tr>
<td>En A</td>
<td>7.30</td>
<td>7.18</td>
</tr>
<tr>
<td>En B</td>
<td>6.78</td>
<td>6.75</td>
</tr>
<tr>
<td>En(FD) A</td>
<td>6.08</td>
<td>6.08</td>
</tr>
<tr>
<td>En(FD) B</td>
<td>5.90</td>
<td>5.86</td>
</tr>
<tr>
<td>Co(FD) A</td>
<td>7.04</td>
<td>6.85</td>
</tr>
<tr>
<td>Co(FD) B</td>
<td>7.15</td>
<td>6.86</td>
</tr>
</tbody>
</table>

Average of three determinations, n = 3.

Free = unencapsulated cultures

En = Freshly encapsulated culture

En(FD) = Encapsulated freeze dried culture

Co(FD) = Coencapsulated freeze dried culture

*A = L. acidophilus* 2401

*B = Bifidobacterium infantis* 1912
Freeze drying would be expected to contribute to lower survival due to the additional stress put on the cells. However the higher survival rate in the second laboratory trial was for the cells that were freeze dried before product incorporation. In this case freeze drying may have acted as a stress adaptation process such those cells able to survive the process of freeze drying were more resistant to the conditions found in yoghurt.

In the first industrial trial at Dairy Farmers using commercial *L. acidophilus* and *Bifidobacterium* from DSM Food Specialties the free cells actually survived better than the encapsulated (freeze dried) cells (Table 22). A reason for this could be that the commercial cultures are already selected for resistance against acid in yoghurt combined with the stress on the cells of the encapsulation procedure which may have been a hindrance to survival of these cultures. The free *L. acidophilus* decreased 0.44 log, from 7.72 to 7.28, compared with the encapsulated cells decreasing 0.95 log, from 7.41 to 6.46. The free *Bifidobacterium* decreased only 0.61 log, from 7.52 to 6.91, compared with the encapsulated cells which decreased 0.76 log, from 7.91 to 7.15.

The results of the first industrial trial show that these commercial strains do not require extra protection as survival of the free cells was high (Table 22). Also the encapsulated cells of the same strains decreased further than the free cells over the same time period suggesting that encapsulation was deleterious to the survival of these cells in some way, perhaps restricting flow of nutrients or metabolites.
Table 22: Log cfu/g of yoghurt made at Dairy Farmers with *L. acidophilus* and *Bifidobacterium* spp. (DSM Food Specialties) incorporated as free and encapsulated cells over eight weeks storage at 7°C.

<table>
<thead>
<tr>
<th>Probiotic Cultures in Different States</th>
<th>STORAGE PERIOD (WEEKS)</th>
<th>LOG DECREASE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Free A</td>
<td>7.72</td>
<td>7.70</td>
</tr>
<tr>
<td>Free B</td>
<td>7.52</td>
<td>7.15</td>
</tr>
<tr>
<td>En(FD) A</td>
<td>7.41</td>
<td>7.32</td>
</tr>
<tr>
<td>En(FD) B</td>
<td>7.91</td>
<td>7.73</td>
</tr>
</tbody>
</table>

Average of three determinations, n = 3.

Free = Unencapsulated culture

En = Encapsulated culture

FD = Freeze Dried culture

A = *L. acidophilus*

B = *Bifidobacterium*
Commercial strains are continually selected against adverse conditions of food manufacture and storage thus should already be able to survive in yoghurt. The selective pressure on commercial probiotic strains may be so specific to the food it is meant for use in, yoghurt for example, that the strains were not suitable for encapsulation.

The second industrial trial at Dairy Farmers used *L. acidophilus* 2401 with *B. infantis* 1912 and a parallel trial used Wisby strains of *L. acidophilus* and *Bifidobacterium* which are used by Dairy Farmers in their manufacture (Table 23). Encapsulation did not assist survival over the eight weeks shelf life for any strain. The free *L. acidophilus* 2401 decreased 0.73 log, from 8.41 to 7.68 while the encapsulated decreased 1.36 log, from 7.08 to 5.72. The free *B. infantis* 1912 decreased 0.56 log, from 7.76 to 7.20 while the encapsulated decreased 1.38 log, from 8.20 to 6.82.

The trial with the Wisby cultures was different because the *L. acidophilus* arrived in frozen form and had to be freeze dried before any manipulations began for consistency between the trials. After encapsulation it was freeze dried again putting a large stress on the bacteria and making the cell recovery very low (Table 23). After performing a cell count on the available cultures before starting the trial, both free and encapsulated, it was aimed to inoculate each batch of yoghurt with similar initial counts between A and B cultures. The amount inoculated in each case depended on both the cfu/g of freeze dried culture and the number of grams available for use. The cfu/g and the grams available were multiplied together to determine the number of cells
available for inoculation. Although the Wisby cultures were used commercially by Dairy Farmers for probiotic dairy products, these cultures do not appear to have been selected for acid resistance as the DSM Food Specialties strains had been. The results given in the tables indicates the average of three determinations and the trials were replicated. There was not much variation in the results.
Table 23: Log cfu/g of yoghurt made at Dairy Farmers with *L. acidophilus* 2401 and *B. infantis* 1912 and the parallel trial with *L. acidophilus* and *Bifidobacterium* spp. from Wisby, as free and encapsulated cultures over eight weeks storage at 7°C.

<table>
<thead>
<tr>
<th>Probiotic Cultures in Different States</th>
<th>STORAGE PERIOD (WEEKS)</th>
<th>LOG DECREASE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Free 2401</td>
<td>8.41</td>
<td>8.32</td>
</tr>
<tr>
<td>Free 1912</td>
<td>7.76</td>
<td>7.59</td>
</tr>
<tr>
<td>En(FD) 2401</td>
<td>7.08</td>
<td>7.04</td>
</tr>
<tr>
<td>En(FD) 1912</td>
<td>8.20</td>
<td>8.18</td>
</tr>
<tr>
<td>Free A</td>
<td>9.30</td>
<td>9.20</td>
</tr>
<tr>
<td>Free B</td>
<td>8.62</td>
<td>8.45</td>
</tr>
<tr>
<td>En(FD) A</td>
<td>3.70</td>
<td>3.04</td>
</tr>
<tr>
<td>En(FD) B</td>
<td>4.30</td>
<td>4.23</td>
</tr>
</tbody>
</table>

Average of three determinations, n = 3.

Free = Unencapsulated cultures

En = Encapsulated culture

FD = Freeze Dried culture

2401 = *L. acidophilus* 2401

1912 = *Bifidobacterium infantis* 1912

A = *L. acidophilus* (Wisby)

B = *Bifidobacterium* spp. (Wisby)
The free *L. acidophilus* (Wisby) dropped (Table 23) 1.38 log, from 9.30 to 7.92 while the encapsulated decreased by 1.85 log, from 3.70 to 1.85. The free *Bifidobacterium* (Wisby) decreased by 0.93 log, from 8.62 to 7.69 while the encapsulated version dropped only 0.26 log, from 4.30 to 4.04.

The second industrial trial at Dairy Farmers was also unsuccessful in demonstrating that encapsulation could assist the survival of probiotic bacterial strains in yoghurt except for the Wisby strain of *Bifidobacterium* (Table 23). The selected strains *L. acidophilus* 2401 and *B. infantis* 1912 also did not benefit from encapsulation in the Dairy Farmers yoghurt even though the opposite was true for the laboratory scale yoghurts. This may mean that the different processes and ingredients used between the laboratory and industrial trials may have an effect on the level of protection given to the cells by encapsulation.

The industrial procedure for yoghurt production involves the use of more than one starter culture, although due to commercial confidence the particular cultures used by Dairy Farmers for the production of their traditional natural yoghurt is unknown. Compared with the laboratory procedure for yoghurt production which used only *S. thermophilus* as the fermenting culture, the cultures used in the industrial process may have had a large influence on the survival of *L. acidophilus* and *Bifidobacterium* by the production of additional metabolites and possibly post-acidification. The other culture commonly used as a yoghurt starter is *L. delbrueckii* ssp. *bulgaricus* which interferes with the survival of probiotic bacteria. *S. thermophilus* is required to produce the
necessary acidity as acidophilus and bifidobacter produce much less acid on their own.

4.4.1.2 Texture Profile Analysis of Yoghurt

The texture of the yoghurt was analysed for springiness, cohesiveness, chewiness, gumminess and hardness at the start of the trial, in the middle and at the end of the storage period using a texture analyser (see appendix 2 for results). The samples of set yoghurt from the laboratory trial as well as the industrial trials were tested in the setting cup immediately after removal from cold storage.

ANOVA performed on the results for yoghurt made in the laboratory (shown in Table 24) showed no significant differences between the yoghurts made with cultures treated in different ways (free, freshly encapsulated, encapsulated freeze dried or co-encapsulated freeze dried) for springiness, cohesiveness, chewiness or gumminess. The yoghurt made with free probiotic cultures was significantly (p<0.01) harder than the yoghurts made with the encapsulated and co-encapsulated cultures.

ANOVA performed on the results for yoghurt made at Dairy Farmers (trial 1, DSM cultures) (shown in Table 25) showed no significant differences (p>0.01) between the Standard Dairy Farmers production yoghurt (Standard) and that with free (Free) and encapsulated (Encapsulated) probiotic bacteria incorporated, for springiness, cohesiveness, chewiness, gumminess or hardness.
ANOVA performed on the results for yoghurt made at Dairy Farmers (trial 2, \textit{L. acidophilus} 2401 and \textit{B. infantis} 1912 and Wisby cultures) (shown in Table 26) showed no significant differences (p>0.01) for springiness, cohesiveness, chewiness, gumminess or hardness between the yoghurts made with different cultures, free or encapsulated. In the earlier studies (laboratory trial and Dairy Farmers trial 1), it was found that there was no significant variation in the texture profile parameters up to 4 weeks of storage, hence the texture profile analysis data starts at 4 weeks of storage which is the average storage period of yoghurt.
Table 24: Texture Profile Analysis results of yoghurt made in the laboratory with *L. acidophilus* 2401 and *B. infantis* 1912.

<table>
<thead>
<tr>
<th>Week</th>
<th>Free</th>
<th>Freshly Encapsulated</th>
<th>Encapsulated (Freeze Dried)</th>
<th>Co-Encapsulated (Freeze Dried)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Springiness (g)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.973</td>
<td>0.877</td>
<td>0.965</td>
<td>0.907</td>
</tr>
<tr>
<td>5</td>
<td>1.008</td>
<td>0.699</td>
<td>0.979</td>
<td>0.885</td>
</tr>
<tr>
<td>8</td>
<td>0.984</td>
<td>0.891</td>
<td>0.984</td>
<td>0.896</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cohesiveness (g)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.337</td>
<td>0.332</td>
<td>0.313</td>
<td>0.297</td>
</tr>
<tr>
<td>5</td>
<td>0.600</td>
<td>0.302</td>
<td>0.347</td>
<td>0.325</td>
</tr>
<tr>
<td>8</td>
<td>0.331</td>
<td>0.337</td>
<td>0.281</td>
<td>0.311</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chewiness (g)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>314.376</td>
<td>236.647</td>
<td>228.689</td>
<td>229.922</td>
</tr>
<tr>
<td>5</td>
<td>280.433</td>
<td>167.929</td>
<td>278.17</td>
<td>241.977</td>
</tr>
<tr>
<td>8</td>
<td>353.625</td>
<td>204.692</td>
<td>207.113</td>
<td>228.134</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Gumminess (g)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>322.989</td>
<td>269.734</td>
<td>236.901</td>
<td>253.59</td>
</tr>
<tr>
<td>5</td>
<td>303.000</td>
<td>240.356</td>
<td>284.233</td>
<td>273.318</td>
</tr>
<tr>
<td>8</td>
<td>359.374</td>
<td>229.818</td>
<td>210.481</td>
<td>254.614</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hardness (g)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>957.3</td>
<td>812.6</td>
<td>756.2</td>
<td>853.1</td>
</tr>
<tr>
<td>5</td>
<td>1090.9</td>
<td>795.2</td>
<td>819.3</td>
<td>840.6</td>
</tr>
<tr>
<td>8</td>
<td>1086</td>
<td>681.4</td>
<td>749.9</td>
<td>818.4</td>
</tr>
</tbody>
</table>
Table 25: Texture Profile Analysis results of yoghurt made at Dairy Farmers (trial 1) with *L. acidophilus* and *Bifidobacterium* spp. (DSM Food Specialties).

<table>
<thead>
<tr>
<th>Week</th>
<th>Standard Dairy Farmers Production Yoghurt</th>
<th>Free</th>
<th>Encapsulated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Springiness (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.971</td>
<td>0.971</td>
<td>0.971</td>
</tr>
<tr>
<td>5</td>
<td>0.979</td>
<td>1.005</td>
<td>0.957</td>
</tr>
<tr>
<td>8</td>
<td>0.965</td>
<td>1.003</td>
<td>0.899</td>
</tr>
<tr>
<td></td>
<td>Cohesiveness (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.451</td>
<td>0.417</td>
<td>0.651</td>
</tr>
<tr>
<td>5</td>
<td>0.426</td>
<td>0.41</td>
<td>0.406</td>
</tr>
<tr>
<td>8</td>
<td>0.368</td>
<td>0.406</td>
<td>0.341</td>
</tr>
<tr>
<td></td>
<td>Chewiness (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>114.159</td>
<td>64.841</td>
<td>42.059</td>
</tr>
<tr>
<td>5</td>
<td>135.388</td>
<td>104.451</td>
<td>101.911</td>
</tr>
<tr>
<td>8</td>
<td>130.642</td>
<td>82.408</td>
<td>107.145</td>
</tr>
<tr>
<td></td>
<td>Gumminess (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>117.608</td>
<td>66.8</td>
<td>43.33</td>
</tr>
<tr>
<td>5</td>
<td>138.339</td>
<td>103.896</td>
<td>106.453</td>
</tr>
<tr>
<td>8</td>
<td>135.333</td>
<td>82.189</td>
<td>119.226</td>
</tr>
<tr>
<td></td>
<td>Hardness (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>283.1</td>
<td>160.1</td>
<td>66.6</td>
</tr>
<tr>
<td>5</td>
<td>324.6</td>
<td>253.7</td>
<td>262.3</td>
</tr>
<tr>
<td>8</td>
<td>367.5</td>
<td>202.5</td>
<td>349.6</td>
</tr>
</tbody>
</table>
Table 26: Duplicated Texture Profile Analysis results of yoghurt made at Dairy Farmers (trial 2) with *L. acidophilus* 2401 & *B. infantis* 1912 and *L. acidophilus* & *Bifidobacterium* spp. (Wisby).

<table>
<thead>
<tr>
<th>Week</th>
<th>Free CSIRO</th>
<th>En CSIRO</th>
<th>Free Wisby</th>
<th>En Wisby</th>
<th>Springiness (g)</th>
<th>Cohesiveness (g)</th>
<th>Chewiness (g)</th>
<th>Gumminess (g)</th>
<th>Hardness (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.968</td>
<td>0.888</td>
<td>0.92</td>
<td>0.865</td>
<td>0.965</td>
<td>0.323</td>
<td>0.283</td>
<td>111.522</td>
<td>125.358</td>
</tr>
<tr>
<td></td>
<td>0.963</td>
<td>0.843</td>
<td>0.965</td>
<td>0.965</td>
<td>0.965</td>
<td>0.283</td>
<td>0.283</td>
<td>110.726</td>
<td>125.386</td>
</tr>
<tr>
<td>5</td>
<td>0.966</td>
<td>0.906</td>
<td>0.917</td>
<td>0.917</td>
<td>0.917</td>
<td>0.328</td>
<td>0.289</td>
<td>110.371</td>
<td>126.024</td>
</tr>
<tr>
<td></td>
<td>0.963</td>
<td>0.906</td>
<td>0.917</td>
<td>0.917</td>
<td>0.917</td>
<td>0.289</td>
<td>0.289</td>
<td>110.371</td>
<td>126.024</td>
</tr>
<tr>
<td>6</td>
<td>0.976</td>
<td>0.939</td>
<td>0.928</td>
<td>0.928</td>
<td>0.928</td>
<td>0.348</td>
<td>0.289</td>
<td>117.544</td>
<td>125.529</td>
</tr>
<tr>
<td></td>
<td>0.904</td>
<td>0.939</td>
<td>0.928</td>
<td>0.928</td>
<td>0.928</td>
<td>0.289</td>
<td>0.289</td>
<td>117.544</td>
<td>125.529</td>
</tr>
<tr>
<td>7</td>
<td>0.931</td>
<td>0.883</td>
<td>0.907</td>
<td>0.897</td>
<td>0.907</td>
<td>0.345</td>
<td>0.287</td>
<td>117.83</td>
<td>125.529</td>
</tr>
<tr>
<td></td>
<td>0.931</td>
<td>0.883</td>
<td>0.907</td>
<td>0.897</td>
<td>0.907</td>
<td>0.287</td>
<td>0.287</td>
<td>117.83</td>
<td>125.529</td>
</tr>
<tr>
<td>8</td>
<td>0.927</td>
<td>0.776</td>
<td>0.92</td>
<td>0.803</td>
<td>0.92</td>
<td>0.34</td>
<td>0.282</td>
<td>107.714</td>
<td>120.433</td>
</tr>
<tr>
<td></td>
<td>0.933</td>
<td>0.864</td>
<td>0.885</td>
<td>0.888</td>
<td>0.885</td>
<td>0.282</td>
<td>0.282</td>
<td>107.714</td>
<td>120.433</td>
</tr>
<tr>
<td>4</td>
<td>0.323</td>
<td>0.283</td>
<td>0.31</td>
<td>0.307</td>
<td>0.307</td>
<td>0.328</td>
<td>0.289</td>
<td>111.522</td>
<td>126.024</td>
</tr>
<tr>
<td>5</td>
<td>0.348</td>
<td>0.289</td>
<td>0.47</td>
<td>0.297</td>
<td>0.297</td>
<td>0.345</td>
<td>0.289</td>
<td>110.371</td>
<td>125.529</td>
</tr>
<tr>
<td>6</td>
<td>0.34</td>
<td>0.287</td>
<td>0.321</td>
<td>0.281</td>
<td>0.281</td>
<td>0.341</td>
<td>0.287</td>
<td>117.83</td>
<td>125.529</td>
</tr>
<tr>
<td>7</td>
<td>0.275</td>
<td>0.23</td>
<td>0.323</td>
<td>0.279</td>
<td>0.279</td>
<td>0.275</td>
<td>0.23</td>
<td>108.9</td>
<td>125.529</td>
</tr>
<tr>
<td>8</td>
<td>0.31</td>
<td>0.283</td>
<td>0.332</td>
<td>0.309</td>
<td>0.309</td>
<td>0.355</td>
<td>0.286</td>
<td>107.714</td>
<td>120.433</td>
</tr>
<tr>
<td>4</td>
<td>125.164</td>
<td>111.522</td>
<td>110.726</td>
<td>150.677</td>
<td>150.677</td>
<td>149.941</td>
<td>110.726</td>
<td>111.522</td>
<td>126.024</td>
</tr>
<tr>
<td>5</td>
<td>147.05</td>
<td>117.544</td>
<td>76.159</td>
<td>127.553</td>
<td>127.553</td>
<td>149.02</td>
<td>117.544</td>
<td>117.544</td>
<td>126.85</td>
</tr>
<tr>
<td>6</td>
<td>148.543</td>
<td>117.83</td>
<td>118.749</td>
<td>154.544</td>
<td>154.544</td>
<td>128.502</td>
<td>117.83</td>
<td>117.83</td>
<td>126.85</td>
</tr>
<tr>
<td>7</td>
<td>113.153</td>
<td>108.9</td>
<td>110.023</td>
<td>129.103</td>
<td>129.103</td>
<td>113.153</td>
<td>108.9</td>
<td>108.9</td>
<td>110.023</td>
</tr>
<tr>
<td>4</td>
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<td>125.586</td>
<td>120.357</td>
<td>156.088</td>
<td>156.088</td>
<td>135.756</td>
<td>130.967</td>
<td>130.967</td>
<td>151.462</td>
</tr>
<tr>
<td>5</td>
<td>189.153</td>
<td>129.264</td>
<td>83.022</td>
<td>149.947</td>
<td>149.947</td>
<td>186.829</td>
<td>129.264</td>
<td>129.264</td>
<td>149.936</td>
</tr>
<tr>
<td>6</td>
<td>149.091</td>
<td>125.529</td>
<td>125.807</td>
<td>148.219</td>
<td>148.219</td>
<td>142.179</td>
<td>125.529</td>
<td>125.529</td>
<td>147.603</td>
</tr>
<tr>
<td>7</td>
<td>121.582</td>
<td>123.389</td>
<td>121.348</td>
<td>146.246</td>
<td>146.246</td>
<td>121.582</td>
<td>123.389</td>
<td>123.389</td>
<td>146.246</td>
</tr>
<tr>
<td>8</td>
<td>134.261</td>
<td>120.433</td>
<td>126.927</td>
<td>164.421</td>
<td>164.421</td>
<td>176.113</td>
<td>124.785</td>
<td>124.785</td>
<td>164.421</td>
</tr>
<tr>
<td>4</td>
<td>400.8</td>
<td>443.4</td>
<td>398.4</td>
<td>506.0</td>
<td>506.0</td>
<td>474.5</td>
<td>447.2</td>
<td>447.2</td>
<td>494.1</td>
</tr>
<tr>
<td>5</td>
<td>485</td>
<td>452</td>
<td>176.5</td>
<td>505.1</td>
<td>505.1</td>
<td>488.8</td>
<td>452</td>
<td>452</td>
<td>495.4</td>
</tr>
<tr>
<td>6</td>
<td>439.1</td>
<td>439.1</td>
<td>362.2</td>
<td>528</td>
<td>528</td>
<td>450.1</td>
<td>439.1</td>
<td>439.1</td>
<td>528</td>
</tr>
<tr>
<td>7</td>
<td>441.5</td>
<td>440.5</td>
<td>375.9</td>
<td>533.3</td>
<td>533.3</td>
<td>441.5</td>
<td>440.5</td>
<td>440.5</td>
<td>533.3</td>
</tr>
<tr>
<td>8</td>
<td>432.8</td>
<td>425.7</td>
<td>382.6</td>
<td>592.8</td>
<td>592.8</td>
<td>496</td>
<td>433.8</td>
<td>433.8</td>
<td>592.8</td>
</tr>
</tbody>
</table>
If there had been increasing values for the results of the texture profile analysis over time indicate a strengthening of the gel structure over the storage period. In yoghurt the gel strength would increase over the storage period due to casein micelle aggregation with calcium during post-acidification. As the pH drops during post-acidification calcium is released. At the same time β-casein becomes positively charged and αs1-casein remains negatively charged. As the two oppositely charged caseins attract each other a stronger network is developed (Varnam and Sutherland, 1994).

The incorporation of calcium alginate in the form of probiotic bacteria capsules into the yoghurt samples probably accelerated the strengthening of the yoghurt matrix. This would occur as the excess calcium ions of the encapsulating material that were not bound in the structure of the calcium alginate gel were released into the yoghurt. This process could be a result of either the presence of a salt able to sequester the calcium ions from the gel or the presence of negatively charged ions or carbonyl radicals attracting the calcium ions. The water binding capacity of the yoghurt coagulum would then be increased following the stabilisation of the protein network, which retards free movement of water in the product, increases the hydration level of proteins and binding of water of hydration (Varnam and Sutherland, 1994).
4.4.1.3 Sensory Analysis of Yoghurt

The average results of all the panellists for the sensory analysis of yoghurt is shown in Table 27. The results of the sensory analysis showed that with regard to mouthfeel the yoghurt with encapsulated bacteria was grittier than the standard yoghurt with no probiotic bacteria. Also the yoghurt with encapsulated probiotic bacteria was grittier than that with free probiotic bacteria incorporated. The standard yoghurt and yoghurt with free probiotic bacteria were regarded with similar smoothness to each other (Figure 8). However it was found that all three types of yoghurt were regarded as more gritty later in the shelf life due to nodule formation; all three samples increased in grittiness in approximately the same way from week 2 to week 5. The standard yoghurt on both days of testing had the same smoothness as the yoghurt with free probiotic bacteria in week 5; however the yoghurt with free probiotic bacteria from the week 2 was different in smoothness. The standard yoghurt and the yoghurt with free probiotic bacteria had the same smoothness in week 2. The results of the sensory analysis performed on yoghurts made at Dairy Farmers show that the capsules containing probiotic bacteria can be felt as grittiness in the mouth.
Table 27: Average results for the sensory analysis of yoghurt made at Dairy Farmers (trial 1) with commercial (DSM Food Specialties) probiotic bacteria incorporated as free or encapsulated cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Smoothness</th>
<th>Acidity</th>
<th>Appearance</th>
<th>Flavour</th>
<th>Aftertaste</th>
<th>Overall Liking</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>3.96</td>
<td>9.085</td>
<td>3.885</td>
<td>7.67</td>
<td>6.31</td>
<td>8.15</td>
</tr>
<tr>
<td>S2</td>
<td>5.41</td>
<td>8.625</td>
<td>4.53</td>
<td>8.295</td>
<td>7.235</td>
<td>8.105</td>
</tr>
<tr>
<td>F1</td>
<td>2.68</td>
<td>7.25</td>
<td>3.715</td>
<td>8.39</td>
<td>6.98</td>
<td>8.97</td>
</tr>
<tr>
<td>F2</td>
<td>5.77</td>
<td>9.375</td>
<td>4.83</td>
<td>8.435</td>
<td>8.075</td>
<td>7.795</td>
</tr>
<tr>
<td>E1</td>
<td>9.26</td>
<td>7.26</td>
<td>4.52</td>
<td>6.385</td>
<td>5.59</td>
<td>6.85</td>
</tr>
<tr>
<td>E2</td>
<td>12.91</td>
<td>8.955</td>
<td>6.69</td>
<td>7.245</td>
<td>7.245</td>
<td>4.26</td>
</tr>
</tbody>
</table>

S = Standard yoghurt

F = Yoghurt with free probiotic bacteria

E = Yoghurt with encapsulated probiotic bacteria

1 = First day of sensory analysis, week 2

2 = Second day of sensory analysis, week 5

Scale:

Smoothness: 0 = very smooth, 15 = very gritty

Acidity: 0 = none, 15 = very acidic

Appearance: 0 = very white, 15 = very coloured

Flavour: 0 = very off, 15 = excellent

Aftertaste: 0 = none, 15 = intense

Overall Liking: 0 = dislike, 15 = like
Figure 8: Tukey’s means comparison test results for smoothness of yoghurt.

\[ \begin{array}{cccccc}
E2 & E1 & F2 & S2 & S1 & F1 \\
\end{array} \]

\( \alpha = 0.05 \)

E = Encapsulated probiotic bacteria in yoghurt

F = Free probiotic bacteria in yoghurt

S = Standard Dairy Farmers yoghurt

1 = First day of sensory analysis, week 2

2 = Second day of sensory analysis, week 5
The acidity of the yoghurt was not affected by the addition of probiotic bacteria either in the free or encapsulated states. The acidity of all the yoghurts was similar regardless of treatment for all three types of yoghurt (Figure 9). Probiotic bacteria incorporated into yoghurt in the free or encapsulated state apparently did not affect the sensory perception of acidity of the product.

The appearance of the yoghurt with encapsulated probiotic bacteria was only different from the standard yoghurt and that with free probiotic bacteria at week 5 (Figure 10). The standard yoghurt and the yoghurt with free probiotic bacteria were regarded as similar to each other in appearance. All yoghurts were considered more discoloured at week 5 rather than week 2 by the panellists. The colouration of the yoghurt with encapsulated probiotic bacteria was a result of the addition of capsules. The alginate itself is slightly coloured but the majority of the colour came from the skim milk that the capsules were resuspended in before freeze drying. The skim milk itself was coloured due to caramelisation during sterilisation.
Figure 9: Tukey’s means comparison test results for acidity of yoghurt.

| F2 | S1 | E2 | S2 | E1 | F1 |

$\alpha = 0.05$

E = Encapsulated probiotic bacteria in yoghurt

F = Free probiotic bacteria in yoghurt

S = Standard Dairy Farmers yoghurt

1 = First day of sensory analysis, week 2

2 = Second day of sensory analysis, week 5
Figure 10: Tukey’s means comparison test results for appearance of yoghurt.

\[
\begin{array}{cccccc}
E2 & F2 & S2 & E1 & S1 & F1 \\
\end{array}
\]

\[\alpha = 0.05\]

E = Encapsulated probiotic bacteria in yoghurt

F = Free probiotic bacteria in yoghurt

S = Standard Dairy Farmers yoghurt

1 = First day of sensory analysis, week 2

2 = Second day of sensory analysis, week 5
Overall there was no difference in the flavour of the yoghurts. The flavour of the standard yoghurt was considered slightly better than the yoghurts with both free and encapsulated probiotic bacteria incorporated but the differences were not significant ($p<0.01$) enough to be displayed on the graph (Figure 11). The raw results showed that the flavour of the yoghurt with encapsulated probiotic bacteria was deemed slightly off possibly due to the addition of the capsule components, which although are usual food ingredients are not usually found in natural yoghurt.

Overall there were no significant differences for the aftertaste of the yoghurts (Figure 12). The raw results showed minor differences and that the aftertaste was considered more intense for the yoghurt with encapsulated bacteria than the yoghurt with free probiotic bacteria which in turn was slightly more intense than the aftertaste for the standard Dairy Farmers production yoghurt. The aftertaste is possibly linked to post-acidification of yoghurts.

Overall liking was significantly lower for the yoghurt with encapsulated probiotic bacteria in week 5 compared with the standard yoghurt and the yoghurt with free probiotic bacteria, which were liked almost equally at both weeks 2 and 5 (Figure 13). However overall liking for all three yoghurt types decreased over the storage period.
Figure 11: Tukey’s means comparison test results for flavour of yoghurt.

| F2 | S1 | E2 | S2 | E1 | F1 |

$\alpha = 0.05$

E = Encapsulated probiotic bacteria in yoghurt

F = Free probiotic bacteria in yoghurt

S = Standard Dairy Farmers yoghurt

1 = First day of sensory analysis, week 2

2 = Second day of sensory analysis, week 5
Figure 12: Tukey's means comparison test results for aftertaste of yoghurt.

| F2 | S1 | E2 | S2 | E1 | F1 |

\[ \alpha = 0.05 \]

E = Encapsulated probiotic bacteria in yoghurt

F = Free probiotic bacteria in yoghurt

S = Standard Dairy Farmers yoghurt

1 = First day of sensory analysis, week 2

2 = Second day of sensory analysis, week 5
Figure 13: Tukey’s means comparison test results for overall liking of yoghurt.

\[
\begin{array}{cccccc}
F1 & S1 & S2 & F2 & E1 & E2 \\
\end{array}
\]

\[\alpha = 0.05\]

E = Encapsulated probiotic bacteria in yoghurt

F = Free probiotic bacteria in yoghurt

S = Standard Dairy Farmers yoghurt

1 = First day of sensory analysis, week 2

2 = Second day of sensory analysis, week 5
In total the overall liking ranked the lowest for the encapsulated yoghurt in week 5 while the remaining samples were liked equally: free yoghurt for both weeks 2 and 5; standard yoghurt for both weeks 2 and 5; encapsulated yoghurt on week 2. It is likely that the overall disliking of the encapsulated type of yoghurt was related to the grittiness caused by the capsules, which were also visible and caused discoloration of the product. The capsules also appeared to contribute to an off flavour in the product and more intense aftertaste but which was not strong enough to cause a significant difference shown in the sensory analysis results.
4.4.2 Cheddar Cheese

4.4.2.1 Probiotic Survival in Cheddar Cheese

Survival of the probiotic bacteria decreased the most in the encapsulated cells except for *L. acidophilus* 910 (Table 28). These consistent results can only mean that encapsulation is causing the higher cell loss either by preventing some crucial interaction with the environment for survival or by inhibiting dispersal of cell metabolites which may be building up inside the capsule causing cell death. These reasons may also be linked with the dense matrix of Cheddar cheese not allowing free exchange of metabolites and nutrients from the capsules.

Gobbetti *et al.* (1998) reported that Crescenza cheese with incorporated bifidobacteria had slightly higher concentrations of lactic and acetic acids than the same cheese made without the added probiotic bacteria and that the coliform count of probiotic cheeses was lower than that without probiotics. Interestingly Gobetti *et al.* (1998) also found that *B. infantis* was not particularly suited to survival in Crescenza cheese although *B. bifidum* and *B. longum* survived better.
Table 28: Log cfu/g of Cheddar cheese made with *L. acidophilus* 2401 & *B. infantis* 1912 and *L. acidophilus* 910 & *B. lactis* 920 (DSM Food Specialties) incorporated as free and encapsulated cultures over six months storage at 10°C.

<table>
<thead>
<tr>
<th>Probiotic cultures in different states</th>
<th>STORAGE PERIOD (MONTHS)</th>
<th>LOG DECREASE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Free 2401</td>
<td>8.00</td>
<td>7.61</td>
</tr>
<tr>
<td>Free 1912</td>
<td>7.90</td>
<td>6.99</td>
</tr>
<tr>
<td>En(FD) 2401</td>
<td>9.59</td>
<td>8.76</td>
</tr>
<tr>
<td>En(FD) 1912</td>
<td>9.04</td>
<td>8.45</td>
</tr>
<tr>
<td>Free A</td>
<td>9.32</td>
<td>9.04</td>
</tr>
<tr>
<td>Free B</td>
<td>9.48</td>
<td>8.79</td>
</tr>
<tr>
<td>En(FD) A</td>
<td>9.34</td>
<td>8.46</td>
</tr>
<tr>
<td>En(FD) B</td>
<td>9.48</td>
<td>8.08</td>
</tr>
</tbody>
</table>

Average of three determinations, n = 3

2401 = *L. acidophilus* 2401

1912 = *B. infantis* 1912

A = *L. acidophilus* 910 (DSM)

B = *B. lactis* 920 (DSM)

Free = Unencapsulated culture

En = Encapsulated culture

FD = Freeze dried culture
Although the viability of the probiotic bacteria in Cheddar cheese decreased over the storage period, the viability of all strains remained above $10^6$ cfu per gram of cheese. If the initial inoculum rate had been higher then the viability would have remained above the critical level of $10^7$ cfu/g determined by Ouwehand and Salminen (1998), as was also found by Gardiner et al. (1998). Dinakar and Mistry (1994) and Stanton et al. (1998) suggested that cheese is a suitable food vehicle for the delivery of probiotic bacteria in the human diet, especially since the cheese did not require any special processing apart from adding the probiotic cultures at the same time as the starter cultures.

### 4.4.2.2 Sensory Analysis of Cheddar Cheese

The average results of all the panellists for the sensory analysis of Cheddar cheese is shown in Table 29. The control Cheddar cheese made without the addition of probiotic bacteria was unfortunately destroyed when the temperature control of the incubation room broke down. Thus the sensory study was performed using a commercially available Cheddar cheese as a control sample without probiotic bacteria and the results for the commercial cheese were largely different from that of the probiotic cheese.

The ANOVA and means comparison test on the results for smoothness showed that all cheeses were significantly different ($p<0.05$) from each other (Figure 14). The commercial cheese was deemed very smooth by the panellists, while on average the cheese with free probiotic bacteria was closer
to "neither smooth nor gritty", however, the cheese with encapsulated probiotic bacteria was gritty.

There was no significant difference (p>0.05) in acidity (Figure 15), appearance (Figure 16), flavour (Figure 17) and overall liking (Figure 18) between the cheeses with free and encapsulated probiotic bacteria; however, the commercial cheese was different to them both in each case. The ANOVA and means comparison test showed that for aftertaste there was no significant difference (p>0.05) between the cheeses with free and encapsulated probiotic bacteria nor the cheese with encapsulated probiotic bacteria and the commercial cheese (Figure 19). However there was a difference in aftertaste between the cheese with free probiotic bacteria and the commercial cheese.
Table 29: Average results for the sensory analysis of Cheddar cheese after storage at 10-12°C for 6 months.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Smoothness</th>
<th>Acidity</th>
<th>Appearance</th>
<th>Flavour</th>
<th>Aftertaste</th>
<th>Overall Liking</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>2.39</td>
<td>4.01</td>
<td>10.25</td>
<td>9.68</td>
<td>5.90</td>
<td>10.39</td>
</tr>
<tr>
<td>F</td>
<td>7.79</td>
<td>8.32</td>
<td>7.40</td>
<td>7.67</td>
<td>8.10</td>
<td>7.74</td>
</tr>
<tr>
<td>E</td>
<td>9.69</td>
<td>9.17</td>
<td>7.20</td>
<td>7.00</td>
<td>7.25</td>
<td>5.91</td>
</tr>
</tbody>
</table>

C = Commercial Cheddar cheese purchased on the day of sensory testing

F = Cheddar cheese with free *L. acidophilus* and *Bifidobacterium* (DSM Food Specialties)

E = Cheddar cheese with encapsulated *L. acidophilus* and *Bifidobacterium* (DSM Food Specialties)

Scale:

Smoothness: 0 = very smooth, 15 = very gritty

Acidity: 0 = none, 15 = very acidic

Appearance: 0 = very white, 15 = very coloured

Flavour: 0 = very off, 15 = excellent

Aftertaste: 0 = none, 15 = intense

Overall Liking: 0 = dislike, 15 = like
Figure 14: Tukey's means comparison test results for smoothness of Cheddar cheese.

\[ \alpha = 0.05 \]

\[ \text{encap} = \text{Encapsulated probiotic bacteria in Cheddar cheese} \]
\[ \text{free} = \text{Free probiotic bacteria in Cheddar cheese} \]
\[ \text{retail} = \text{Commercially available Dairy Farmers Cheddar cheese} \]
Figure 15: Tukey’s means comparison test results for acidity of Cheddar cheese.

encap  free  retail

\( \alpha = 0.05 \)

encap = Encapsulated probiotic bacteria in Cheddar cheese

free = Free probiotic bacteria in Cheddar cheese

retail = Commercially available Dairy Farmers Cheddar cheese
Figure 16: Tukey's means comparison test results for appearance of Cheddar cheese.

\[ \alpha = 0.05 \]

- encap = Encapsulated probiotic bacteria in Cheddar cheese
- free = Free probiotic bacteria in Cheddar cheese
- retail = Commercially available Dairy Farmers Cheddar cheese
Figure 17: Tukey's means comparison test results for flavour of Cheddar cheese.

\[
\begin{array}{ccc}
\text{retail} & \text{free} & \text{encap} \\
\end{array}
\]

\(\alpha = 0.05\)

encap = Encapsulated probiotic bacteria in Cheddar cheese
free = Free probiotic bacteria in Cheddar cheese
retail = Commercially available Dairy Farmers Cheddar cheese
Figure 18: Tukey's means comparison test results for aftertaste of Cheddar cheese.

\begin{tabular}{l l l}
  free & encap & retail \\
\end{tabular}

\( \alpha = 0.01 \)

encap = Encapsulated probiotic bacteria in Cheddar cheese  
free = Free probiotic bacteria in Cheddar cheese  
retail = Commercially available Dairy Farmers Cheddar cheese
Figure 19: Tukey’s means comparison test results for overall liking of Cheddar cheese.

\[ \begin{array}{ccc}
\text{retail} & \text{free} & \text{encap} \\
\end{array} \]

\( \alpha = 0.05 \)

encap = Encapsulated probiotic bacteria in Cheddar cheese

free = Free probiotic bacteria in Cheddar cheese

retail = Commercially available Dairy Farmers Cheddar cheese
The capsules in the cheese with encapsulated probiotic bacteria caused a feeling of grittiness in the cheese as compared with the cheese with free probiotic bacteria incorporated. Upadhyay (2000) explains that the texture of cheese is largely influenced by the physical properties and structure of the product. The basic structure of cheese is largely determined by the mineral content of the cheese curd, which is influenced by the separation of the curd and whey, the residual sugar content and the lowest pH the cheese can attain. The loss of calcium phosphate disrupts the casein submicelles in the milk, which also affects the basic structure of cheese. In Cheddar cheese the structure of the casein submicelles is partly disrupted (Upadhyay, 2000). It is possible that the calcium ions in the calcium alginate gel matrix also had an effect on the disruption of the casein submicelles in the milk causing a grittier texture in the Cheddar cheese with encapsulated probiotic bacteria, as was also found in yoghurt.

The capsules did not appear to affect the cheese properties of acidity, appearance, flavour or the panellists overall liking of the cheeses. However, the aftertaste was different between the free and commercial cheeses even though there was no difference found between the free and encapsulated nor the encapsulated and commercial. This could mean that the encapsulation was masking any aftertaste from the probiotic bacteria, which was detectable in the cheese with free probiotic bacteria. The gritty mouthfeel associated with the encapsulated probiotic bacteria was common with both yoghurt and cheese sensory analysis.
4.4.3 Experimental Ice Cream

4.4.3.1 Over Run, Fat Content and Total Solids in Ice Cream

The optimal over run of 100% was easy to achieve in the experimental ice cream and was found to be not affected by the addition of probiotic bacteria incorporated as free and encapsulated cells as reported by Sheu et al. (1993). The ice cream with encapsulated probiotic bacteria incorporated achieved an over run of 100%, for the ice cream with free probiotic bacteria it was 116%. The over run for the fermented ice cream was lower at 73% due to the difficulty in breaking up the coagulum formed by the fermentation by S. thermophilus of the ice cream mix. The over run for commercial ice cream should be 80-100% (Arbuckle, 1986). Fermented ice cream was made with the objective of enhancing the numbers of the encapsulated probiotic bacteria before the ice cream mix was frozen into ice cream.

The total solids determination resulted in the ice cream being 38.5% solids, which is in the normal range of 36-43% for ice cream (Arbuckle, 1986). The results of the Babcock fat test averaged 14% fat, which is within the accepted range of 8-20% fat for ice cream (Arbuckle, 1986).
4.4.3.2 Probiotic Bacterial Survival in Experimental Ice Cream

The assay of the bacterial counts in the three types of ice cream was carried out at the time of ice cream production and this was followed up every month for six months. The results are shown in Table 30. Overall the cell count decrease in the experimental ice cream was similar for each culture no matter the state of its incorporation into the product or the type of ice cream incorporated into (Table 30). The survival of *L. acidophilus* was improved with encapsulation but this was not the case for *Bifidobacterium*.

The majority of damage to the cells in ice cream would be from the low storage temperatures, which encapsulation with sodium alginate and starch would not be able to protect against. The results of the present study concur with those of Hekmat and McMahon (1992) who also found that *L. acidophilus* decreased by 2 log cycles and *B. bifidum* decreased by 1 log cycle over 17 weeks storage in fermented ice cream. Comparing these results as well as those of Hekmat and McMahon (1992) with those of the strain selection studies in low temperatures (Table 9) it is evident that *L. acidophilus* is more susceptible to low storage temperatures than the *Bifidobacterium* strains.
Table 30: Log cfu/g of ice cream made with *L. acidophilus* 910 and *B. lactis* 920 (DSM Food Specialties) incorporated as free and encapsulated cultures and encapsulated cultures in fermented ice cream over six months storage at -20°C.

<table>
<thead>
<tr>
<th>Probiotic cultures in different states</th>
<th>STORAGE PERIOD (MONTHS)</th>
<th>LOG DECREASE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Free A</td>
<td>9.12</td>
<td>7.40</td>
</tr>
<tr>
<td>Free B</td>
<td>8.70</td>
<td>7.00</td>
</tr>
<tr>
<td>En A</td>
<td>8.86</td>
<td>7.70</td>
</tr>
<tr>
<td>En B</td>
<td>8.92</td>
<td>7.60</td>
</tr>
<tr>
<td>Ferm A</td>
<td>8.67</td>
<td>7.10</td>
</tr>
<tr>
<td>Ferm B</td>
<td>8.52</td>
<td>7.00</td>
</tr>
</tbody>
</table>

Average of three determinations, n = 3

A = *L. acidophilus* 910 (DSM)

B = *B. lactis* 920 (DSM)

Free = Unencapsulated culture

En = Encapsulated culture

Ferm = Encapsulated culture incorporated into fermented ice cream
In contrast to the results of the present study Kebary et al. (1998) and Shah and Ravula (2000) found that encapsulation was protective of probiotic bacteria in frozen dairy products. Kebary et al. (1998) entrapped bifidobacteria before freezing in ice milk, while Shah and Ravula (2000) encapsulated L. acidophilus 2400 to incorporate into a fermented frozen dairy dessert, both studies showed that encapsulation was protective of the cells compared with the unentrapped cells. The differences in results for those studies compared with that of the present study would be from strain differences, encapsulation procedure and lower protection offered to the cells from the product's fat content since they use frozen desserts rather than ice cream.

4.4.3.3 β-Galactosidase Activity in Experimental Ice Cream

The most widely recognised therapeutic effect on humans of probiotic bacteria is the improvement of lactose tolerance in lactose sensitive people. The bacterial enzyme responsible for this activity is β-galactosidase; thus it is important that the enzyme remains active if probiotic bacteria are incorporated into dairy foods. The β-galactosidase activity is a measure of the viability of the encapsulated bacteria in the product, this is why this assay was performed.

The enzyme activity for each type of ice cream was recorded and the overall mean values are represented in Figure 20. The cells in the free state show about twice the amount of β-galactosidase activity as compared to the encapsulated cells. There is no change of the enzyme activity over the 24-
week period of study. The encapsulated probiotic bacteria demonstrated a mean activity of 9.4 ±0.5 U/gram. The free probiotic bacteria had a mean enzyme activity of 20.9 ±1.0 U/g, and the fermented type of ice cream had the highest enzyme activity of 95.7 ±3.3 U/gram.

The ice cream with the cells incorporated in the free state had a higher β-galactosidase activity than that with encapsulated cells. This could mean that the alginate shell around the cells was reducing the flow of nutrients and metabolites so much as to reduce the activity of the bacteria. This would concur with results of the strain survival tests in encapsulated conditions which showed that although low pH affected the viability of encapsulated cultures, apparently the larger molecules of bile extract were not able to transit through the encapsulant wall and did not have such a pronounced effect on cell viability. The fermented type of ice cream had a high enzyme activity clearly due to the additional (starter) culture *S. thermophilus*, which was used to prepare the fermented product.
Figure 20: β-galactosidase activity (U/g) over a period of 24 weeks in non-fermented and in fermented (with *S. thermophilus*) ice cream prepared with encapsulated or free probiotic cultures of *L. acidophilus* 910 and *B. lactis* 920 (DSM Food Specialties) (replicated data shown).

Free = unencapsulated bacteria
Enc = encapsulated bacteria
Ferm = encapsulated bacteria and fermented ice-cream
A = *L. acidophilus*
B = *Bifidobacterium* spp.
4.4.3.4 Sensory Analysis of Experimental Ice Cream

The average results of all the panellists for the sensory analysis of ice cream are shown in Table 31. Sometimes ice cream has a sandy feel in the mouth caused by the defect of lactose crystallisation. With respect to smoothness/grittiness, the fermented ice cream was significantly (p<0.01) greater than the rest of the ice cream samples (Figure 21). The commercially produced ice cream had no significant difference (p>0.01) to the encapsulated sample by smoothness. Also the encapsulated sample was regarded as smooth as the free sample. However there was a difference between the smoothness of the commercial ice cream and that with free probiotics incorporated.
Table 31: Average results for the sensory analysis of ice cream.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Smoothness</th>
<th>Acidity</th>
<th>Appearance</th>
<th>Flavour</th>
<th>Aftertaste</th>
<th>Overall Liking</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>4.675</td>
<td>3.21</td>
<td>8.08</td>
<td>8.615</td>
<td>5.47</td>
<td>7.755</td>
</tr>
<tr>
<td>F</td>
<td>2.025</td>
<td>3.965</td>
<td>2.525</td>
<td>7.90</td>
<td>5.815</td>
<td>8.005</td>
</tr>
<tr>
<td>E</td>
<td>3.985</td>
<td>4.78</td>
<td>3.755</td>
<td>7.195</td>
<td>7.02</td>
<td>6.955</td>
</tr>
<tr>
<td>A</td>
<td>7.345</td>
<td>8.705</td>
<td>4.27</td>
<td>5.745</td>
<td>7.245</td>
<td>5.965</td>
</tr>
</tbody>
</table>

C = Commercial ice cream
F = Ice cream with free probiotic bacteria
E = Ice cream with encapsulated probiotic bacteria
A = Fermented ice cream with encapsulated bacteria

Scale:
Smoothness: 0 = very smooth, 15 = very gritty
Acidity: 0 = none, 15 = very acidic
Appearance: 0 = very white, 15 = very coloured
Flavour: 0 = very off, 15 = excellent
Aftertaste: 0 = none, 15 = intense
Overall Liking: 0 = dislike, 15 = like
Figure 21: Tukey's means comparison test results for smoothness of ice cream.

<table>
<thead>
<tr>
<th></th>
<th>ferm</th>
<th>retail</th>
<th>encap</th>
<th>free</th>
</tr>
</thead>
</table>

\( \alpha = 0.05 \)

ferm = Encapsulated probiotic bacteria in fermented ice cream

encap = Encapsulated probiotic bacteria in ice cream

free = Free probiotic bacteria in ice cream

retail = Commercially available ice cream
The fermented ice cream was significantly (p<0.01) more acidic than all of the other ice creams which were not significantly different (p>0.01) from each other (Figure 22). The acidity of the fermented ice cream was due to the fermentation process. The ice creams with free and encapsulated probiotic bacteria incorporated were no more acidic than the commercially bought ice cream thus the added bacteria did not affect the acidity until a fermentation by *S. thermophilus* took place.

The flavour of the commercial ice cream and the fermented ice cream were regarded as significantly different from one another (Figure 24). However the commercial, free and encapsulated ice creams were similar in flavour. Likewise, the free, encapsulated and fermented ice creams were similar in flavour. Interestingly, for the flavour of the ice creams all the unfermented types were regarded as similar; however the fermented ice cream was also found to be similar to the free and encapsulated types. All ice creams were vanilla flavoured. Only the retail and fermented samples were significantly different. Fermentation by *S. thermophilus* made the flavour different from the retail ice cream but not from the free and encapsulated samples, possibly influenced by the mere presence of probiotic bacteria.
Figure 22: Tukey’s means comparison test results for acidity of ice cream.

ferm  encap  free  retail

$\alpha = 0.05$

ferm = Encapsulated probiotic bacteria in fermented ice cream
encap = Encapsulated probiotic bacteria in ice cream
free = Free probiotic bacteria in ice cream
retail = Commercially available ice cream
Figure 23: Tukey's means comparison test results for appearance of ice cream.

\[
\begin{array}{cccc}
\text{retail} & \text{ferm} & \text{encap} & \text{free} \\
\end{array}
\]

\[\alpha = 0.05\]

\text{ferm} = Encapsulated probiotic bacteria in fermented ice cream
\text{encap} = Encapsulated probiotic bacteria in ice cream
\text{free} = Free probiotic bacteria in ice cream
\text{retail} = Commercially available ice cream
There were no differences in the aftertaste (Figure 25) or overall liking (Figure 26) of any of the ice creams. Overall liking is significant for commercial application of incorporation of probiotic bacteria into ice cream since ice cream, both fermented and un-fermented, is popular with consumers making ice cream and frozen dairy desserts ideal vehicles for the delivery of probiotic bacteria to humans. Ice cream is not normally fermented commercially, in this study the ice cream was fermented in order to increase the viability of the probiotic bacteria. The sensory analysis results showed no differences between unfermented and fermented ice creams because the ice cream that was fermented had the process terminated at a high pH, compared with yoghurt, and was immediately stored at very low temperatures minimising the opportunities for post-acidification.
Figure 24: Tukey's means comparison test results for flavour of ice cream.

<table>
<thead>
<tr>
<th>retail</th>
<th>free</th>
<th>encap</th>
<th>ferm</th>
</tr>
</thead>
</table>

$\alpha = 0.01$

ferm = Encapsulated probiotic bacteria in fermented ice cream
encap = Encapsulated probiotic bacteria in ice cream
free = Free probiotic bacteria in ice cream
retail = Commercially available ice cream
Figure 25: Tukey's means comparison test results for aftertaste of ice cream.

<table>
<thead>
<tr>
<th>retail</th>
<th>free</th>
<th>encap</th>
<th>ferm</th>
</tr>
</thead>
</table>

$\alpha = 0.05$

ferm = Encapsulated probiotic bacteria in fermented ice cream

encap = Encapsulated probiotic bacteria in ice cream

free = Free probiotic bacteria in ice cream

retail = Commercially available ice cream
Figure 26: Tukey’s means comparison test results for overall liking of ice cream.

| retail | free | encap | ferm |

\[ \alpha = 0.05 \]

ferm = Encapsulated probiotic bacteria in fermented ice cream

encap = Encapsulated probiotic bacteria in ice cream

free = Free probiotic bacteria in ice cream

retail = Commercially available ice cream
4.4.4 Home-Made Ice Cream

4.4.4.1 Over Run, Fat Content and Total Solids in Home Made Ice Cream

Due to the use of a kitchen ice cream maker for the “home made” ice cream, the incorporation of air into the ice cream mix was not as high as in commercial ice cream. The over run for the “home made” ice cream varied greatly, from 0 – 47%. The over run was calculated to be 35% for the ice cream with free probiotic bacteria. The ice cream with freshly encapsulated probiotic bacteria recorded an overrun of 28% while the ice cream with freeze dried encapsulated probiotic bacteria allowed no overrun. An over run of 47% was achieved for the ice cream with freeze dried co-encapsulated probiotic bacteria incorporated. Commercial ice cream should have an over run of 100% however this is not able to be achieved with a kitchen ice cream maker.

The fat content was calculated as 24% for the ice-cream with free and freshly encapsulated cells incorporated. The encapsulated freeze dried batch had 22% and the co-encapsulated freeze dried batch had 20% fat in the ice-cream respectively. The total solids was determined to be 48% for the ice-cream mix with both the free and freshly encapsulated cells. The ice-cream mix with encapsulated freeze dried cells had 49% total solids while the ice-cream mix with co-encapsulated freeze dried cells had 46% total solids. The fat content was much higher than industrial ice cream due to the use of a premium ice cream recipe, lower over run possible with the bench top device and hence more total solids in the ice cream. The higher fat content would have an affect on texture of the ice cream and would feel smoother in the mouth for this reason.
4.4.4.2 Probiotic Bacterial Survival in Home Made Ice Cream

The home made ice cream allowed similar survivals of probiotic bacteria in various states, except for the encapsulated and freeze dried cells in which the cells decreased more than the other states but not as much as the cells introduced into the experimental ice cream (Table 32). It appears that the cells survived better in the freshly encapsulated state rather than the encapsulated freeze dried state. This could be extrapolated to say that less processing of the capsules is required since incorporation without freeze drying allows high survival of cells; however, more data is required before conclusions can be drawn. The encapsulated freeze dried cells were manipulated more than the cells in other states since the cells were grown in broth and harvested as free cells, then were freeze dried, encapsulated and freeze dried again, thus the cells were exposed to many conditions before being incorporated into the ice cream that may have caused low viability. The preparation of encapsulated freeze dried cells may have damaged the viability causing cell death during ice cream freezing process and subsequent storage.
Table 32: Log cfu/g of ice cream made with *L. acidophilus* 2401 and *B. infantis* 1912 incorporated as free, freshly encapsulated, encapsulated freeze dried and co-encapsulated freeze dried cultures over six months storage at -20°C.

<table>
<thead>
<tr>
<th>Probiotic cultures in different states</th>
<th>STORAGE PERIOD (MONTHS)</th>
<th>LOG DECREASE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Free A</td>
<td>8.11</td>
<td>8.04</td>
</tr>
<tr>
<td>Free B</td>
<td>7.77</td>
<td>7.53</td>
</tr>
<tr>
<td>En A</td>
<td>7.60</td>
<td>7.56</td>
</tr>
<tr>
<td>En B</td>
<td>7.58</td>
<td>7.38</td>
</tr>
<tr>
<td>En(FD) A</td>
<td>6.58</td>
<td>6.23</td>
</tr>
<tr>
<td>En(FD) B</td>
<td>7.08</td>
<td>6.30</td>
</tr>
<tr>
<td>Co(FD) A</td>
<td>7.18</td>
<td>7.15</td>
</tr>
<tr>
<td>Co(FD) B</td>
<td>6.77</td>
<td>6.64</td>
</tr>
</tbody>
</table>

Average of three determinations, n = 3

A = *L. acidophilus* 2401
B = *B. infantis* 1912
Free = Unencapsulated culture
En = Encapsulated culture
Co = Co-encapsulated cultures
FD = Freeze dried beads
The co-encapsulated freeze dried cells would have been exposed to the same conditions however being encapsulated together the cells then had the advantage of assisting each others growth and survival by co-culturing as *L. acidophilus* is a proteolytic species and Bifidobacteria have no proteolytic activity of their own (Klaver *et al.*, 1993). The survival of the cells in the free state in home made ice cream would be due to the protection provided to the cells by the total solids and fat in the ice cream mix, making ice cream a suitable food for delivery probiotic bacteria to consumers (Hekmat and McMahon, 1992; Modler *et al.*, 1990;). As the total solids was 48% surrounding the free cells this would have assisted in the high viability remaining at the end of the shelf life. Other researchers (Shimamura *et al.*, 1990) have found that solids in the media have assisted the lactic acid bacteria to survive freezing conditions.
4.5 Conclusions

From the results shown in the strain selection part of this study it can be concluded that survival of probiotic bacteria in harsh conditions, including low pH, bile, sucrose, oxygen and low storage temperatures, is strain dependent. It is possible to select strains tolerant of those conditions and this should be done for strains used in dairy foods so that strains more likely to survive in the product may be used.

Probiotic bacteria may be encapsulated in a calcium alginate gel matrix. Encapsulation of probiotic bacteria in a calcium alginate gel matrix may be achieved using the techniques described in this study with the use of appropriate proportions of oil and emulsifier. It is not necessary to produce the capsules by mixing the sodium alginate drop by drop into the calcium chloride solution but encapsulation may be just as successful, and less time consuming, to mix all the materials together and allow the beads to separate themselves in the oil and water emulsion. Encapsulation using the described method produces capsules of various sizes.

Starch may be added to the encapsulating matrix gel to act as a prebiotic substrate for increased viability of the probiotic bacteria. When starch is incorporated into the gel matrix it also acts to bind the cells in the capsule and highlights its beneficial effect in protecting probiotic strains. The addition of glycerol to the encapsulated cells assists with cryo-preservation and maintaining cell viability after freezing.
Encapsulation in a calcium alginate matrix protects the viability of the cells when exposed to bile extract \textit{in vitro}. Encapsulation in a calcium alginate gel matrix is effective for increasing probiotic bacterial survival in yoghurt and ice cream. Due to the composition of Cheddar cheese encapsulation is a hindrance to the survival of probiotic bacteria, however, this study has shown that Cheddar cheese is a suitable food vehicle for delivery of unencapsulated probiotic bacteria in the human diet.

Incorporation of both free and encapsulated probiotic bacteria into the dairy foods yoghurt, ice cream and Cheddar cheese does not seem to greatly affect the integrity of these dairy products. The mouthfeel of each of these foods is affected by the incorporation of capsules. This makes encapsulation of probiotic bacteria for incorporation into these dairy foods commercially infeasible due to the alterations in texture that would result as well as the decreased overall liking of the products with encapsulated probiotics incorporated.
4.6 Recommendations for Further Research

A large study of all available probiotic bacterial strains would be recommended for selecting the strains that are able to survive in conditions of food processing and storage. Many studies in the past have focussed on the survival of probiotic bacteria in low pH and bile, the conditions of the gastro-intestinal tract however for delivery of probiotic bacteria through dairy foods the organisms must first survive the conditions of the food itself before exposure to the gastro-intestinal tract. The strains should be continually selected for against these properties and the genetic characteristics noted.

If more studies are done on increasing the survival of probiotic bacteria in foodstuffs the delivery of the minimum $10^7$ cfu/g becomes more attainable. Other methods of increasing probiotic bacteria in foods should be investigated such as the addition of oxygen scavenging particles in the packaging of the products containing anaerobic bacteria. Encapsulation can also be further investigated as a method for preserving the shelf life of probiotic bacteria, using different shell materials and methods.

Many commercial producers of dairy foods would be reluctant to simply add probiotic bacteria to their products if sensory and rheological properties of a popular product are altered. This would be even more apparent at the introduction of encapsulated cultures that cause lumps to be visible and felt in the mouth. Thus more research can be done on improving the encapsulation procedure to make smaller capsules.
More research is in progress regarding the effectiveness and mechanisms of action of probiotic bacteria. Community awareness needs to be raised regarding the usefulness of probiotic bacteria in human health and long-term disease prevention rather than consumers focusing on the present and feeling good today.
REFERENCES


References


APPENDIX 1

Pearson Square for calculation of milk and skim milk powder quantities to use in the yoghurt making procedure based on 18% solids non-fat in the final product.

<table>
<thead>
<tr>
<th>% SNF</th>
<th>required SNF</th>
</tr>
</thead>
<tbody>
<tr>
<td>MILK:</td>
<td>8.9</td>
</tr>
<tr>
<td></td>
<td>/</td>
</tr>
<tr>
<td></td>
<td>18</td>
</tr>
<tr>
<td>SMP:</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>(15 - 9.1)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Therefore for 1 litre of yoghurt, volume of milk required is:

\[
\frac{77}{86.1} \times 1000 \text{ mL} = 894 \text{ mL}
\]

Likewise the amount of skim milk powder required is:

\[
\frac{9.1}{86.1} \times 1000 \text{ mL} = 105.69 \text{ g}
\]
APPENDIX 2: Yoghurt Texture Analysis Results

Graph 1: Standard Dairy Farmers Yoghurt at weeks 2, 5 & 8 of storage (Industrial Trial 1).

Graph 2: Dairy Farmers Yoghurt with Free Probiotic Bacteria (DSM Food Specialties) at weeks 2, 5 & 8 of storage (Industrial Trial 1).

Graph 3: Dairy Farmers Yoghurt with Freeze Dried Encapsulated Probiotic Bacteria (DSM Food Specialties) at weeks 2, 5 & 8 of storage (Industrial Trial 1).

Graph 4: Yoghurt with Free Probiotic Bacteria (*L. acidophilus* 2401 and *B. infantis* 1912) at weeks 3, 5 & 8 of storage (Laboratory Trial 2).

Graph 5: Yoghurt with Freshly Encapsulated Probiotic Bacteria (*L. acidophilus* 2401 and *B. infantis* 1912) at weeks 3, 5 & 8 of storage (Laboratory Trial 2).

Graph 6: Yoghurt with Freeze Dried Encapsulated Probiotic Bacteria (*L. acidophilus* 2401 and *B. infantis* 1912) at weeks 3, 6 & 8 of storage (Laboratory Trial 2).

Graph 7: Yoghurt with Freeze Dried Co-Encapsulated Probiotic Bacteria (*L. acidophilus* 2401 and *B. infantis* 1912) at weeks 3, 6 & 8 of storage (Laboratory Trial 2).
Graph 8: Dairy Farmers Yoghurt with Free Probiotic Bacteria (*L. acidophilus* 2401 and *B. infantis* 1912) at weeks 4, 6 & 8 of storage (Industrial Trial 2).

Graph 9: Dairy Farmers Yoghurt with Freeze Dried Encapsulated Probiotic Bacteria (*L. acidophilus* 2401 and *B. infantis* 1912) at weeks 4, 6 & 8 of storage (Industrial Trial 2).

Graph 10: Dairy Farmers Yoghurt with Free Probiotic Bacteria (Wisby) at weeks 4, 6 & 8 of storage (Industrial Trial 2).

Graph 11: Dairy Farmers Yoghurt with Freeze Dried Encapsulated Probiotic Bacteria (Wisby) at weeks 4, 6 & 8 of storage (Industrial Trial 2).
Graph 1: Standard Dairy Farmers Yoghurt at weeks 2, 5 & 8 of storage (Industrial Trial 1).
Uni of Western Sydney, Hawkesbury

Stable Micro Systems - XT.RA Dimension V3.7J

Sample Rate : 25.00 pps  
Force Threshold : 20.0 g  
Sample Area : 1.00 mm²

Test Time : 59.04 s  
Dist. Threshold : 0.50 mm  
Contact Force : 5.0 g

T.P.A  
SPEED: 1.0 mm/s  
PRE TEST SPEED: 2.0 mm/s  
POST TEST SPEED: 2.0 mm/s  
TRIGGER TYPE: Auto  
DISTANCE: 15.0 mm  
TIME: 5.00 s

Graph 2: Dairy Farmers Yoghurt with Free Probiotic Bacteria (DSM Food Specialties) at weeks 2, 5 & 8 of storage (Industrial Trial 1).
Graph 3: Dairy Farmers Yoghurt with Freeze Dried Encapsulated Probiotic Bacteria (DSM Food Specialties) at weeks 2, 5 & 8 of storage (Industrial Trial 1).
Graph 4: Yoghurt with Free Probiotic Bacteria (L. acidophilus 2401 and B. infantis 1912) at weeks 3, 5 & 8 of storage (Laboratory Trial 2).
Graph 5: Yoghurt with Freshly Encapsulated Probiotic Bacteria (\textit{L. acidophilus 2401} and \textit{B. infantis 1912}) at weeks 3, 5 & 8 of storage (Laboratory Trial 2).
Graph 6: Yoghurt with Freeze Dried Encapsulated Probiotic Bacteria (*L. acidophilus* 2401 and *B. infantis* 1912) at weeks 3, 6 & 8 of storage (Laboratory Trial 2).
Graph 7: Yoghurt with Freeze Dried Co -Encapsulated Probiotic Bacteria (L. acidophilus 2401 and B. infantis 1912) at weeks 3, 6 & 8 of storage (Laboratory Trial 2).
Graph 8: Dairy Farmers Yoghurt with Free Probiotic Bacteria (*L. acidophilus 2401 and *B. infantis 1912*) at weeks 4, 6 & 8 of storage (Industrial Trial 2).
Graph 9: Dairy Farmers Yoghurt with Freeze Dried Encapsulated Probiotic Bacteria (*L. acidophilus* 2401 and *B. infantis* 1912) at weeks 4, 6 & 8 of storage (Industrial Trial 2).
Sample Rate : 25.00 pps  Test Time : 58.24 s
Force Threshold : 20.0 g  Dist. Threshold : 0.50 mm
Sample Area : 1.00 mm²  Contact Force : 5.0 g

T.P.A
SPEED: 1.0 mm/s  PRE TEST SPEED: 2.0 mm/s  POST TEST SPEED: 2.0 mm/s
TRIGGER TYPE: Auto @ 10 g  DISTANCE: 15.0 mm
TIME: 5.00 s

Stable Micro Systems  Texture Analyser  Fri Jan 04 09:03:31 1980

Graph 10: Dairy Farmers Yoghurt with Free Probiotic Bacteria (Wisby) at weeks 4, 6 & 8 of storage (Industrial Trial 2).
Sample Rate : 25.00 pps  Test Time : 58.40 s
Force Threshold : 20.0 g  Dist. Threshold : 0.50 mm
Sample Area : 1.00 mm²  Contact Force : 5.0 g

T.P.A
SPEED: 1.0 mm/s  PRE TEST SPEED: 2.0 mm/s  POST TEST SPEED: 2.0 mm/s
TRIGGER TYPE: Auto @ 10 g  DISTANCE: 15.0 mm
TIME: 5.00 s

Stable Micro Systems Texture Analyser  Fri Jan 04 09:08:33 1980

Graph 11: Dairy Farmers Yoghurt with Freeze Dried Encapsulated Probiotic Bacteria (Wisby) at weeks 4, 6 & 8 of storage (Industrial Trial 2).
APPENDIX 3: QUESTIONNAIRE

PRODUCT: Traditional Natural Yoghurt

If you have read and understood the plain language statement regarding this project please evaluate the smoothness/grittiness, acidity, appearance, flavour, aftertaste and overall liking of these yoghurt samples.

Make a vertical line within the scale on the horizontal line to indicate your rating of the smoothness/grittiness, acidity, appearance, flavour, aftertaste and overall liking of each sample. Label each vertical line with the code number of the sample it represents.

Please test the samples in any order taking a cracker and water in between samples.

<table>
<thead>
<tr>
<th>Code</th>
<th>Smoothness/grittiness</th>
<th>Acidity</th>
<th>Appearance</th>
<th>Flavour</th>
<th>Aftertaste</th>
<th>Overall Liking</th>
</tr>
</thead>
<tbody>
<tr>
<td>728</td>
<td>Very smooth</td>
<td>None</td>
<td>Very white</td>
<td>Very off</td>
<td>None</td>
<td>Dislike</td>
</tr>
<tr>
<td>279</td>
<td>Neither smooth nor gritty</td>
<td>Mildly acidic</td>
<td>Mildly coloured</td>
<td>Neither off nor good</td>
<td>Mild</td>
<td>Neither like nor dislike</td>
</tr>
<tr>
<td>686</td>
<td>Very gritty</td>
<td>Very acidic</td>
<td>Very coloured</td>
<td>Excellent</td>
<td>Intense</td>
<td>Like</td>
</tr>
</tbody>
</table>

COMMENTS:__________________________________________

Thankyou for your cooperation.
**APPENDIX 4: QUESTIONNAIRE**

**PRODUCT:** Plain Ice-Cream

If you have read and understood the plain language statement regarding this project please evaluate the smoothness/grittiness, acidity, appearance, flavour, aftertaste and overall liking of these ice-cream samples.

Make a vertical line within the scale on the horizontal line to indicate your rating of the smoothness/grittiness, acidity, appearance, flavour, aftertaste and overall liking of each sample. Label each vertical line with the code number of the sample it represents.

Please test the samples in any order taking a cracker and water in between samples.

<table>
<thead>
<tr>
<th>859</th>
<th>623</th>
<th>471</th>
<th>505</th>
</tr>
</thead>
</table>

1. Smoothness/grittiness

<table>
<thead>
<tr>
<th>Very smooth</th>
<th>Neither smooth nor gritty</th>
<th>Very gritty</th>
</tr>
</thead>
</table>

2. Acidity

<table>
<thead>
<tr>
<th>None</th>
<th>Mildly acidic</th>
<th>Very acidic</th>
</tr>
</thead>
</table>

3. Appearance

<table>
<thead>
<tr>
<th>Very white</th>
<th>Mildly coloured</th>
<th>Very coloured</th>
</tr>
</thead>
</table>

4. Flavour

<table>
<thead>
<tr>
<th>Very off</th>
<th>Neither off nor good</th>
<th>Excellent</th>
</tr>
</thead>
</table>

5. Aftertaste

<table>
<thead>
<tr>
<th>None</th>
<th>Mild</th>
<th>Intense</th>
</tr>
</thead>
</table>

6. Overall Liking

<table>
<thead>
<tr>
<th>Dislike</th>
<th>Neither like nor dislike</th>
<th>Like</th>
</tr>
</thead>
</table>

**COMMENTS:___________________________

___________________________

___________________________

Thankyou for your cooperation.
APPENDIX 5: QUESTIONNAIRE

PRODUCT: Cheddar Cheese

If you have read and understood the plain language statement regarding this project please evaluate the smoothness/grittiness, acidity, appearance, flavour, aftertaste and overall liking of these cheese samples.

Make a vertical line within the scale on the horizontal line to indicate your rating of the smoothness/grittiness, acidity, appearance, flavour, aftertaste and overall liking of each sample. Label each vertical line with the code number of the sample it represents.

Please test the samples in any order taking a cracker and water in between samples.

962 526 753

1. Smoothness/grittiness

Very smooth ____________________ Neither smooth nor gritty ____________________ Very gritty ____________________

2. Acidity

None ____________________ Mildly acidic ____________________ Very acidic ____________________

3. Appearance

Very white ____________________ Mildly coloured ____________________ Very coloured ____________________

4. Flavour

Very off ____________________ Neither off nor good ____________________ Excellent ____________________

5. Aftertaste

None ____________________ Mild ____________________ Intense ____________________

6. Overall Liking

Dislike ____________________ Neither like nor dislike ____________________ Like ____________________

COMMENTS: ____________________________________________________________

______________________________________________________________

Thankyou for your cooperation.
APPENDIX 6: SENSORY ANALYSIS OF YOGHURT, CHEDDAR CHEESE AND ICE CREAM ANOVA RESULTS

Table 1: ANOVA results for smoothness of yoghurt.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F critical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rows</td>
<td>262.943</td>
<td>19</td>
<td>13.83911</td>
<td>1.749075</td>
<td>0.040991</td>
<td>1.697071</td>
</tr>
<tr>
<td>Columns</td>
<td>1426.147</td>
<td>5</td>
<td>285.2294</td>
<td>36.04912</td>
<td>1.57E-20</td>
<td>2.310223</td>
</tr>
<tr>
<td>Error</td>
<td>751.663</td>
<td>95</td>
<td>7.912242</td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>119</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2: ANOVA results for acidity of yoghurt.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F critical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rows</td>
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<td>19</td>
<td>19.02991</td>
<td>1.850742</td>
<td>0.027587</td>
<td>1.697071</td>
</tr>
<tr>
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<td>87.937</td>
<td>5</td>
<td>17.5874</td>
<td>1.710452</td>
<td>0.139632</td>
<td>2.310223</td>
</tr>
<tr>
<td>Error</td>
<td>976.8197</td>
<td>95</td>
<td>10.28231</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1426.325</td>
<td>119</td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3: ANOVA results for appearance of yoghurt.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F critical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rows</td>
<td>164.8237</td>
<td>19</td>
<td>8.67493</td>
<td>2.033548</td>
<td>0.013255</td>
<td>1.697071</td>
</tr>
<tr>
<td>Columns</td>
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<td>5</td>
<td>22.6904</td>
<td>5.319007</td>
<td>0.000236</td>
<td>2.310223</td>
</tr>
<tr>
<td>Error</td>
<td>405.2613</td>
<td>95</td>
<td>4.265909</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>683.537</td>
<td>119</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4: ANOVA results for flavour of yoghurt.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F critical</th>
</tr>
</thead>
<tbody>
<tr>
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<td>15.06151</td>
<td>2.475416</td>
<td>0.002099</td>
<td>1.697071</td>
</tr>
<tr>
<td>Columns</td>
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<td>0.06394</td>
<td>2.310223</td>
</tr>
<tr>
<td>Error</td>
<td>578.0213</td>
<td>95</td>
<td>6.084435</td>
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</tr>
<tr>
<td>Total</td>
<td>930.1787</td>
<td>119</td>
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</tbody>
</table>
Table 5: ANOVA results for aftertaste of yoghurt.

<table>
<thead>
<tr>
<th>Source of Variation</th>
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<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F critical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rows</td>
<td>440.4909</td>
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<td>23.18373</td>
<td>2.71784</td>
<td>0.000747</td>
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</tr>
<tr>
<td>Columns</td>
<td>73.64542</td>
<td>5</td>
<td>14.72908</td>
<td>1.726697</td>
<td>0.135905</td>
<td>2.310223</td>
</tr>
<tr>
<td>Error</td>
<td>810.3696</td>
<td>95</td>
<td>8.530206</td>
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</tr>
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</table>

Table 6: ANOVA results for overall liking of yoghurt.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F critical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rows</td>
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<td>19</td>
<td>17.50967</td>
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<tr>
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<td>6.598263</td>
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<td>Error</td>
<td>796.5053</td>
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<td>8.384267</td>
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</table>
Table 7: ANOVA results for smoothness of Cheddar cheese.

<table>
<thead>
<tr>
<th>Source of Variation</th>
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<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F critical</th>
</tr>
</thead>
<tbody>
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<tr>
<td>Columns</td>
<td>602.42</td>
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<td>301.21</td>
<td>82.29781</td>
<td>6.66E-15</td>
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<tr>
<td>Error</td>
<td>146.4</td>
<td>40</td>
<td>3.66</td>
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<td></td>
</tr>
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<td>Total</td>
<td>931.6143</td>
<td>62</td>
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</tr>
</tbody>
</table>

Table 8: ANOVA results for acidity of Cheddar cheese.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F critical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rows</td>
<td>280.7032</td>
<td>20</td>
<td>14.03516</td>
<td>3.670107</td>
<td>0.000229</td>
<td>1.83886</td>
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<td>Columns</td>
<td>320.806</td>
<td>2</td>
<td>160.403</td>
<td>41.94439</td>
<td>1.52E-10</td>
<td>3.231733</td>
</tr>
<tr>
<td>Error</td>
<td>152.9673</td>
<td>40</td>
<td>3.824183</td>
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<tr>
<td>Total</td>
<td>754.4765</td>
<td>62</td>
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</tr>
</tbody>
</table>
Table 9: ANOVA results for appearance of Cheddar cheese.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F critical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rows</td>
<td>174.3705</td>
<td>20</td>
<td>8.718524</td>
<td>1.559864</td>
<td>0.114028</td>
<td>1.83886</td>
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<tr>
<td>Columns</td>
<td>122.2352</td>
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<td>61.11762</td>
<td>10.93478</td>
<td>0.000163</td>
<td>3.231733</td>
</tr>
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<td>Error</td>
<td>223.5714</td>
<td>40</td>
<td>5.589286</td>
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<td></td>
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<tr>
<td>Total</td>
<td>520.1771</td>
<td>62</td>
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</tr>
</tbody>
</table>

Table 10: ANOVA results for flavour of Cheddar cheese.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F critical</th>
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</thead>
<tbody>
<tr>
<td>Rows</td>
<td>123.4241</td>
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<td>6.171206</td>
<td>1.134618</td>
<td>0.356184</td>
<td>1.83886</td>
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<td>Columns</td>
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<td>40.97968</td>
<td>7.534393</td>
<td>0.001671</td>
<td>3.231733</td>
</tr>
<tr>
<td>Error</td>
<td>217.5606</td>
<td>40</td>
<td>5.439016</td>
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</tr>
<tr>
<td>Total</td>
<td>422.9441</td>
<td>62</td>
<td></td>
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<td></td>
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</tbody>
</table>
Table 11: ANOVA results for aftertaste of Cheddar cheese.

<table>
<thead>
<tr>
<th>Source of Variation</th>
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<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F critical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rows</td>
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<td>12.89687</td>
<td>1.991146</td>
<td>0.031551</td>
<td>2.368878</td>
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<td>Columns</td>
<td>51.50889</td>
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<td>25.75444</td>
<td>3.976224</td>
<td>0.026606</td>
<td>5.178492</td>
</tr>
<tr>
<td>Error</td>
<td>259.0844</td>
<td>40</td>
<td>6.377111</td>
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<td></td>
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<tr>
<td>Total</td>
<td>568.5308</td>
<td>62</td>
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</tr>
</tbody>
</table>

Table 12: ANOVA results for overall liking of Cheddar cheese.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F critical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rows</td>
<td>121.4765</td>
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<td>6.073825</td>
<td>0.972206</td>
<td>0.511478</td>
<td>1.838860</td>
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<tr>
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<td>106.3373</td>
<td>17.02086</td>
<td>4.48E-06</td>
<td>3.231733</td>
</tr>
<tr>
<td>Error</td>
<td>249.8987</td>
<td>40</td>
<td>6.247468</td>
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<td></td>
<td></td>
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<tr>
<td>Total</td>
<td>584.0498</td>
<td>62</td>
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<td></td>
</tr>
</tbody>
</table>
Table 13: ANOVA results for smoothness of ice cream.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F critical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rows</td>
<td>217.5905</td>
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<td>Columns</td>
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<td>2.766441</td>
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<td>7.352623</td>
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<td></td>
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<tr>
<td>Total</td>
<td>926.9955</td>
<td>79</td>
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<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Table 14: ANOVA results for acidity of ice cream.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F critical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rows</td>
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<td>19</td>
<td>10.18326</td>
<td>1.619083</td>
<td>0.082688</td>
<td>1.771973</td>
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<td>Columns</td>
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<td>119.6123</td>
<td>19.0177</td>
<td>1.14E-08</td>
<td>2.766441</td>
</tr>
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<td>6.289526</td>
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<td></td>
<td></td>
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<tr>
<td>Total</td>
<td>910.822</td>
<td>79</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 15: ANOVA results for appearance of ice cream.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F critical</th>
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<td>1.949812</td>
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<tr>
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<td>3.93764</td>
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<td></td>
<td></td>
</tr>
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<td>714.8355</td>
<td>79</td>
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<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 16: ANOVA results for flavour of ice cream.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F critical</th>
</tr>
</thead>
<tbody>
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<tr>
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<td>30.01346</td>
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<tr>
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<td>650.5449</td>
<td>79</td>
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<td></td>
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</tr>
</tbody>
</table>
Table 17: ANOVA results for aftertaste of ice cream.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
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<th>P-value</th>
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Table 18: ANOVA results for overall liking of ice cream.

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