Studies on the Oxygen Toxicity of Probiotic Bacteria
with reference to
Lactobacillus acidophilus and Bifidobacterium spp.

A thesis submitted for the degree of
DOCTOR OF PHILOSOPHY

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DECLARATION

The candidate, Akshat Talwalkar, hereby declares that this submission is his own work and that, to the best of his knowledge and belief, it contains no material previously published or written by another person, nor material which to a substantial extent has been submitted/accepted for the award of any other degree of a university or other institute of higher learning, except where due acknowledgement is made in the text.

July, 2003

Akshat Talwalkar
ACKNOWLEDGEMENTS

Conducting this Ph.D. study was more like experiencing life in a nutshell with all its gamut of emotions. So, while at times, there was the elation of a scientific breakthrough or the deep satisfaction of seeing a difficult experiment run smoothly, there was also the frustration of ‘reliable’ instruments breaking down when I needed them the most (and that too on a Friday afternoon) or the agonizing patience and care required when handling microbes. Then again, just as ones life is enlivened by certain individuals, I too came across a few remarkable people during this study - people who stretched out their hand and not only made this study possible, but also very enjoyable.

My thanks to Dr. Kaila Kailaspathy for his guidance at every step as well as contributing to my personality development. I also would like to thank Dr. Paul Peiris, Dr. Rama Arumugaswamy and Minh Nguyen for their constant guidance and support for my work.

Working day in and day out in the laboratory would have been extremely boring for me if it hadn’t been for Rob Sturgess, the laboratory manager. It is said that laughter is the best medicine and Rob sure supplied plenty of it. Not content with just making me laugh until my jaws hurt, he also made sure I retained my smile by providing timely assistance and cooperation with all my laboratory requirements. I also particularly enjoyed the ‘light and easy’ yet extremely deep conversations with him. Thanks, ‘maaait’!
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Jai Gurudev!
ABSTRACT

Oxygen toxicity is considered significant in the poor survival of probiotic bacteria such as *Lactobacillus acidophilus* and *Bifidobacterium* spp. in yoghurts. This study investigated methods to protect these bacteria from oxygen exposure. The oxygen tolerance of several *L. acidophilus* and *Bifidobacterium* spp. was quantified by modifying the Relative Bacterial Growth Ratio (RBGR) methodology. A standard assay for the complex NADH oxidase: NADH peroxidase enzyme system in *L. acidophilus* and *Bifidobacterium* spp. was developed and used in studying the physiological responses of these bacteria to 0, 5, 10, 15 and 21% oxygen. As oxygen increased, changes were observed in the lactic acid production, lactate to acetate ratio, protein profiles, ability to decompose hydrogen peroxide and activities of NADH oxidase and NADH peroxidase.

To confirm the accuracy of the reported survival estimates of *L. acidophilus* or *Bifidobacterium* spp. in yoghurts, the reliability of several enumeration media was evaluated with different commercial yoghurts. None of the media however, was found reliable thereby casting doubts on the reported cell numbers of probiotic bacteria in yoghurts.

A protocol was developed to evaluate microencapsulation for protection of *L. acidophilus* and *Bifidobacterium* spp. from oxygen toxicity. Although the survival of calcium alginate-starch encapsulated cells was significantly higher than free cells in culture broth, microencapsulation offered protection to only a few strains when tested in yoghurt.

Probiotic bacteria were successfully adapted to oxidative stress by developing a protocol involving the passage of cells through gradually increasing concentrations of dissolved oxygen in yoghurt. When the oxygen passaged cells were incubated for 35 days in yoghurt
that contained 210 ppm of dissolved oxygen, no significant decreases in cell numbers were observed.

The effect of oxygen permeable, oxygen impermeable and oxygen scavenging packaging materials on the dissolved oxygen of yoghurt and survival of *L. acidophilus* and *Bifidobacterium* spp. was examined. Both, oxygen adapted and oxygen non-adapted cells of these bacteria survived well in yoghurt, regardless of the rise in the dissolved oxygen or the yoghurt packaging material. This indicates that dissolved oxygen may not be significant in the poor survival of probiotic bacteria in yoghurts.

The industrial application of this study was conducted by incorporating oxygen adapted *L. acidophilus* CSCC 2409 and *B. infantis* CSCC 1912 into a yoghurt manufactured commercially. Both strains were able to demonstrate adequate survival during the shelf life of the yoghurt. The dissolved oxygen and the survival trends of *L. acidophilus* and *Bifidobacterium* spp. in a popular commercial yoghurt were also examined. Although the dissolved oxygen increased, the anaerobic bifidobacteria remained above $10^6$ cfu/g whereas counts of the microaerophilic *L. acidophilus* declined steadily. This suggests that the survival of these bacteria in yoghurts could be strain dependent.

Hence, although oxygen can be detrimental to *L. acidophilus* and *Bifidobacterium* spp. in culture broths, it may not be significant for their poor survival in yoghurts. Nevertheless, techniques such as oxidative stress adaptation, alternative packaging materials and microencapsulation as investigated in this study, can serve as general protective techniques to help yoghurt manufacturers in maintaining the recommended numbers of probiotic bacteria in their products. This would eventually assist in the efficient delivery of probiotic health benefits to yoghurt consumers.
LIST OF PUBLICATIONS


CONFERENCE PRESENTATIONS

Paper presentations:


Poster presentations:


**AWARDS**

1. **Winner** of the ‘*John Christian Young Food Microbiologist Award*’, 2002 - a competitive award, established by the Australian Institute of Food Science and Technology (AIFST) and given to the best presentation of research in food microbiology by scientists below the age of 30 years.

2. **Winner** of the ‘*Young Members Night Award*’, 2002 of the AIFST given to the best presentation of higher degree research by students in the state of New South Wales, Australia.

3. Awarded **Second prize** for poster presentation in food microbiology at the International Food Convention (IFCON 2003), held at Mysore, India.
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<td>AB yoghurts</td>
<td>Yoghurts containing <em>L. acidophilus</em> and <em>Bifidobacterium</em> spp.</td>
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<td>ABC yoghurts</td>
<td>Yoghurts with <em>L. acidophilus</em>, <em>Bifidobacterium</em> spp., and <em>L. casei</em></td>
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<tr>
<td>cfu</td>
<td>colony forming units</td>
</tr>
<tr>
<td>CSIRO</td>
<td>Commonwealth Scientific and Industrial Research Organization</td>
</tr>
<tr>
<td>d</td>
<td>day (s)</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin Adenine Dinucleotide</td>
</tr>
<tr>
<td>g</td>
<td>gravitational force</td>
</tr>
<tr>
<td>h</td>
<td>hour (s)</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
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<tr>
<td>l</td>
<td>litre (s)</td>
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<td>LAB</td>
<td>Lactic Acid Bacteria</td>
</tr>
<tr>
<td>µ</td>
<td>micro</td>
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<tr>
<td>MRS</td>
<td>deMan Rogosa Sharpe medium</td>
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<td>MRS-B</td>
<td>MRS with bile</td>
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<td>MRS-C</td>
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<td>MRS with salicin</td>
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<td>MRS-SOR</td>
<td>MRS with sorbitol</td>
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<td>NADH</td>
<td>Nicotinamide Adenine Dinucleotide (Reduced)</td>
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<td>NNLP</td>
<td>Neomycin sulphate, nalidixic acid, lithium chloride and paromomycin</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>rpm</td>
<td>rotations per minute</td>
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<td>RBGR</td>
<td>Relative Bacterial Growth Ratio</td>
</tr>
<tr>
<td>RCPB</td>
<td>Reinforced Clostridial Medium with Prussian Blue</td>
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<td>RSM</td>
<td>Reconstituted Skim Milk (9% w/v) broth</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
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1 Introduction

There is a growing trend of health awareness all over the world wherein consumers and health professionals alike are increasingly adopting a preventive approach rather than a curative one to diseases. While antibiotic therapy is currently the most commonly used approach to treat bacterial infections, it is essentially curative and is associated with unpleasant side effects. Probiotics, on the other hand, score over antibiotics by being preventive, non-invasive and free from any undesirable effects. Consequently, the awareness and popularity of probiotics among the global population is increasing rapidly (Sanders, 1999). Several health benefits have been attributed to the ingestion of probiotic bacteria such as *Lactobacillus acidophilus* and *Bifidobacterium* spp. These bacteria enhance the population of beneficial bacteria in the human gut, suppress pathogens and build up resistance against intestinal diseases. In some cases, ingestion of these bacteria was effective in preventing diarrhea in children and in alleviating symptoms of lactose intolerance in adults (Salminen et al., 1999).

Food industries, especially dairy industries, have been quick to tap this consumer market created by the numerous positive health benefits of probiotic bacteria. A growing number of manufacturers are now incorporating *L. acidophilus* and bifidobacteria in yoghurts (Lourens-Hattingh and Viljoen, 2001). In addition to enhancing the healthy image, the incorporation of probiotic bacteria has led to the creation of a new and rapidly increasing multi billion dollar market for probiotic yoghurts (Stanton et al., 2001).
The development and marketing of probiotic yoghurts is not without its inherent problems. Foremost among them is the requirement for adequate cell numbers of probiotic cultures in yoghurts over the shelf life. To obtain the desired therapeutic effects from probiotic yoghurts, it has been suggested that the daily intake should be at least $10^8$ cfu (Lourens-Hattingh and Viljoen, 2001). These high numbers have been suggested to compensate for the possible loss in the numbers of probiotic organisms during passage through the stomach and intestine. It is therefore recommended that the minimum counts of probiotic bacteria be $10^6$ cfu/g of the product at the expiry date (Kurmann and Rasic, 1991).

The increasing sales of probiotic yoghurts has prompted food authorities in some countries to introduce regulations on the requisite numbers of viable probiotic bacteria to be marketed as a probiotic food product. Standards requiring a minimum of $10^7$ cfu/ml of *L. acidophilus* and $10^6$ cfu/g of bifidobacteria in fermented milk products have been introduced by various organizations. In Japan, the Fermented Milk and Lactic Acid Beverages Association has specified that there be at least $10^7$ cfu/ml of viable bifidobacteria in fermented milk drinks (Lourens-Hattingh and Viljoen, 2001). The International Standard of Federation Internationale de Laiterie/International Dairy Federation (FIL/IDF) requires $10^7$ cfu of *L. acidophilus* in products such as Acidophilus milk and $10^6$ cfu/g of bifidobacteria in fermented milks containing bifidobacteria at the time of sale (IDF, 1992). Likewise, the Swiss Food Regulation as well as the MERCOSOR regulations requires a minimum of $10^6$ cfu of viable bifidobacteria in similar products (Bibiloni et al., 2001).
Although some countries are yet to introduce standards for probiotic bacteria, regulations govern the number of viable lactic acid bacteria required in the product. The National Yoghurt Association (NYA) of the United States specifies that in order to use the NYA “Live and Active Culture” logo on the container of their products, there should be $10^8$ cfu/g of lactic acid bacteria at the time of manufacture (Lourens-Hattingh and Viljoen, 2001). Similarly, the Australian Food Standards Code regulations require $10^6$ cfu/g of viable lactic acid cultures used for yoghurt fermentation (Lourens-Hattingh and Viljoen, 2001). Consequently, there has been a growing industry interest in developing techniques to ensure adequate numbers of yoghurt bacteria, particularly probiotic bacteria throughout the shelf life of yoghurts.

Yoghurt has long been perceived as ‘healthy’ by consumers owing to its many desirable effects. As a result, yoghurt and yoghurt drinks have become increasingly popular among consumers in recent years (Lourens-Hattingh and Viljoen, 2001). Although yoghurts and yoghurt drinks are considered by some to be the ideal vectors for the delivery of probiotic bacteria to consumers, their inherent properties of high acidity, and the slow growth and low proteolytic properties of the incorporated probiotic bacteria can pose difficulties in the efficient delivery of probiotic bacteria. Moreover, there are conflicting reports on the survival of probiotic bacteria in yoghurts during storage. Some market surveys on commercial yoghurts have found counts far below the recommended $10^6$ cfu/g, of *L. acidophilus* and bifidobacteria at the expiry date of the yoghurt (Iwana et al., 1993; Anon., 1999). Other surveys have reported satisfactory viability of probiotic bacteria throughout the shelf life of yoghurts (Lourens et al., 2000; Shin et al., 2000). Studies elsewhere, have
reported varied counts of either *L. acidophilus* or bifidobacteria or both in yoghurts prepared using commercial starter cultures (Pacher and Kneifel, 1996; Dave and Shah, 1997d; Micanel et al., 1997; Vinderola and Reinheimer, 1999; Vinderola et al., 2000).

Studies have shown that a number of factors affect the survival of *L. acidophilus* and *Bifidobacterium* spp. in yoghurts. These include strains of probiotic bacteria, pH, storage atmosphere, concentration of metabolites such as lactic acid and acetic acids, dissolved oxygen and buffers such as whey proteins (Rybka and Kailasapathy, 1995; Dave and Shah, 1997d; Kailasapathy and Rybka, 1997).

Among the reported factors influencing the viability of *L. acidophilus* and *Bifidobacterium* spp. in yoghurt, exposure to dissolved oxygen during the manufacture and storage is considered highly significant. Both *L. acidophilus* and *Bifidobacterium* spp. are human gut-derived organisms and are classified as microaerophilic and anaerobic respectively. These bacteria lack catalase, a key enzyme involved in oxygen detoxification. As a result, exposure to oxygen leads to intracellular accumulation of hydrogen peroxide, which is toxic to the cell. Although both *L. acidophilus* and *Bifidobacterium* spp. have the ability to decompose hydrogen peroxide, it can be inadequate in high oxygenic environments. The absence of an effective oxygen scavenging mechanism therefore renders them highly susceptible to accumulation of toxic oxygenic metabolites in the cell, eventually leading to cell death. *Bifidobacterium* spp. are generally considered more vulnerable than *L. acidophilus* to the deleterious effects of oxygen owing to their strict anaerobic nature.
Yoghurts incorporate a considerable amount of dissolved oxygen during the pumping, mixing and agitation steps in manufacturing. Additionally, oxygen can also diffuse into yoghurt through the polystyrene packaging material during storage. The presence of such an oxyergic environment in yoghurt is considered detrimental for the extended survival of probiotic bacteria and is widely believed to cause bacterial death.

Although oxygen toxicity is considered a highly significant factor affecting the survival of probiotic bacteria in yoghurts, research into this critical problem is largely inadequate. While oxygen-related studies on *Bifidobacterium* spp. are few, even less work has been reported on *L. acidophilus*. In order to prevent cell death from oxygen toxicity, it can be beneficial to screen potential probiotic strains for oxygen tolerance before they are incorporated into yoghurt. Mechanisms of oxygen tolerance in probiotic bacteria however, are still unclear. Although some researchers had classified different strains of *Bifidobacterium* spp. based on their degree of oxygen tolerance and formation of H$_2$O$_2$ during aerobic growth, the methods reported to determine oxygen tolerance were either subjective or qualitative (de Vries and Stouthamer, 1969, Uesugi and Yajima, 1978). Besides being tedious, such methods can be error prone. A quantitative measurement of oxygen tolerance of several probiotic strains has also not been reported yet.

Similarly, research on the responses of probiotic bacteria to oxygen so far had exposed probiotic bacteria to only qualitative and undefined concentrations of oxygen. Examining however, the various physiological changes occurring in probiotic bacteria when exposed to
known concentrations of oxygen would enable a better understanding of the oxidative responses of probiotic bacteria.

The activities of NADH oxidase and NADH peroxidase have been considered significant in the oxygen tolerance of a few members of *Bifidobacterium* spp. (Shimamura et al., 1992). Maximum activities of these enzymes were found in the most aerotolerant strain. The NADH oxidase: NADH peroxidase enzyme system in lactic acid bacteria (LAB) including probiotic bacteria is complex. The interrelatedness between NADH oxidase and NADH peroxidase makes it difficult to determine the activities of these enzymes individually. The lack of a standard assay for this enzyme system had resulted in contradictory enzyme assays being used in the various reported studies. Considering the importance of the NADH oxidase: NADH peroxidase enzyme system in the oxygen tolerance of probiotic bacteria as well in other members of the LAB group, an urgent need therefore existed for a standard assay to be developed for the accurate estimations of individual concentrations of these enzymes.

Currently, survival estimates of probiotic bacteria in yoghurts rely on the ability of the selective or differential medium to provide a conclusive count of these bacteria in the presence of yoghurt starters. A variety of selective and differential media have been developed and used in estimating populations of probiotic bacteria in yoghurts. Each of the reported studies however used a different selective/differential medium. For population estimations to be comparable and to ensure the reliability of the results, it is vital to confirm that counts of probiotic bacteria from the same yoghurt do not vary when plated on the
various selective and differential media. Such an evaluation of the various media, which is important in unequivocally establishing the exact status of probiotic viability in yoghurts, had not yet been conducted.

Although the problem of oxygen toxicity is widely recognised, proper cost effective and industrially applicable techniques to protect probiotic bacteria from oxygen toxicity in yoghurts had yet to be developed. Addition of ascorbate and cysteine to yoghurts had been successful as oxygen scavengers in some studies but affected the textural and microbiological properties of the yoghurt (Dave and Shah, 1997a; 1997c). Similarly, packaging yoghurt in oxygen impermeable packaging materials like glass, although useful in preventing oxygen diffusion, can be hazardous and financially non-viable to manufacturers.

In this regard, research on oxygen scavenging packing materials and its effect on viability of probiotic bacteria was yet unreported. There remained a vast unexplored area concerning the use of oxygen scavenging packaging film to maintain anoxic environments in probiotic yoghurts. Other techniques such as stress adaptation and microencapsulation have been examined only as general protection strategies for probiotic bacteria and not in relation to oxygen toxicity.

Clearly, there is a need to understand in detail, the interaction of oxygen with probiotic bacteria and to devise and evaluate techniques that would prevent viability losses of probiotic bacteria in yoghurt from oxygen toxicity. This would be useful in maintaining sufficiently high numbers of probiotic bacteria in yoghurts, thereby meeting regulatory
standards, and assisting in the delivery of therapeutic benefits to consumers. Furthermore, it was important to establish the survival status of probiotic bacteria by evaluating the efficacy of the various selective and differential media to conclusively enumerate probiotic bacteria in the presence of yoghurt starter cultures.
1.1 Aim of the study

The aim of the study was therefore to evaluate the effect of oxygen on the survival of probiotic bacteria, especially *L. acidophilus* and *Bifidobacterium* spp., investigate techniques to protect probiotic bacteria from oxygen toxicity in yoghurt and therefore ensuring adequate survival of these bacteria in commercial yoghurts.

1.2 Objectives of the study

The principal objectives of this study were to:

1. Examine a methodology to screen potential probiotic strains for oxygen tolerance
2. Develop a standard assay for the estimation of NADH oxidase and NADH peroxidase in lactic acid bacteria including probiotic bacteria.
3. Study the metabolic and biochemical responses of probiotic bacteria to oxygen
4. Examine the ability of the currently available media to provide reliable counts of probiotic bacteria in commercial yoghurts.
5. Study the protective effect of microencapsulation in regard to oxygen toxicity in probiotic bacteria and evaluate its benefits in yoghurt
6. Perform oxidative stress adaptation of probiotic bacteria to increase their survival in the aerobic environment of the yoghurt
7. Study the interactions between probiotic bacteria, packaging materials and dissolved oxygen of yoghurt; and
8. Incorporate selected oxygen tolerant strains of *L. acidophilus* and *Bifidobacterium* spp. in yoghurt manufactured industrially and evaluate their survival during extended storage. Additionally, monitor the survival trend of *L. acidophilus* and *Bifidobacterium* spp. over the entire shelf life of a popular commercial yoghurt.

1.3 Constraints of the study

An ideal investigation on oxygen tolerance of probiotic bacteria would be that in which the interaction of oxygen with the probiotic bacteria is tested in the product conditions i.e. in presence of yoghurt starter bacteria and at storage temperatures of yoghurt 4-8°C. Such a study would closely simulate the market conditions of the product and therefore offer a more realistic picture of the oxygen toxicity of probiotic bacteria in yoghurts. The nature of this study however poses some inherent problems.

A thorough understanding of oxygen interaction with the cellular physiology of probiotic bacteria can only be achieved when cells are grown at their optimum conditions such as (37°C) and in a suitable broth (MRS or RSM). Yoghurt is stored mainly at lower temperatures (6°C) whereas the optimum temperature of growth of both *L. acidophilus* and *Bifidobacterium* spp. is around 37°C. In addition, the textural and nutritional properties of yoghurt are different from those found in a culture medium. Furthermore, studying the interaction of oxygen with probiotic bacteria in presence of the yoghurt starter bacteria is made difficult by the fact that yoghurt starter cultures share similar properties of optimum temperature and media conditions as the probiotic bacteria. Growing the yoghurt starter
cultures along with the probiotic bacteria can introduce additional factors such as antagonism by the yoghurt starter cultures, uncontrolled increase in acidity and metabolite production by the yoghurt starter bacteria. Consequently, the best possible way to study oxygen tolerance in specifically probiotic bacteria at optimum conditions was to do so in the absence of yoghurt starter cultures.

Studying the oxidative response of probiotic bacteria in yoghurt poses a similar problem as regards the temperature that is employed for such a study. Ideally, oxygen related study on probiotic bacteria in yoghurt would be that which is conducted at the optimum temperature of growth of these bacteria (37°C). Doing so however, may be complicated. Ordinarily, during yoghurt manufacture, fermentation at 42°C is terminated once the necessary pH and gelling is achieved. Conducting a yoghurt related study would cause further fermentation which may lead to more acid production and introduce further variables that could interfere with the proposed work. Hence, it is most appropriate to conduct a yoghurt study at storage temperature and in this study, experiments involving yoghurt and probiotic bacteria were performed only at low temperatures of storage (6°C).

Consequently, the approach used in this study was to obtain knowledge about the specific cellular interaction of oxygen with probiotic bacteria by conducting experiments at optimum temperatures (37°C) and in culture media. The latter stage of the study was then performed using yoghurts as the culture medium, which was maintained at storage temperatures (6°C).
1.4 Thesis overview

This thesis consists of a literature review and eight chapters (Sections 4-11). The literature review presents an overview of probiotics, the microbial ecology and therapeutic properties of *L. acidophilus* and *Bifidobacterium* spp., the expanding market for probiotic yoghurts, industry concerns about the viability of probiotic bacteria in yoghurts, the various selective and differential media for enumerating probiotic bacteria, oxygen toxicity in probiotic bacteria and techniques advocated to overcome it. Chapter 1 describes the modification and successful application of a methodology called as the Relative Bacterial Growth Ratio (RBGR) to obtain for the first time, a quantitative index of the oxygen tolerance of several probiotic strains including *L. acidophilus* and *Bifidobacterium* spp. Chapter 2 describes the development, validation and application of a standard assay to estimate NADH oxidase and NADH peroxidase levels in LAB. Chapter 3 describes a detailed study about the various metabolic and biochemical oxidative responses of *L. acidophilus* and *Bifidobacterium* spp. when grown in different concentrations of oxygen such as 0, 5, 10, 15 or 21% oxygen. Chapter 4 deals with the evaluation of several selective and differential media to provide reliable counts of *L. acidophilus* and *Bifidobacterium* spp. from various commercial yoghurts. Chapter 5 describes the evaluation of microencapsulation as a technique to offer protection to probiotic bacteria from oxygen toxicity in both, culture broth as well in yoghurt. Chapter 6 describes the development, validation and application of a 4-day protocol to perform oxidative stress adaptation of probiotic bacteria in yoghurt maintained at 6°C. Chapter 7 highlights the influence of various packaging materials on the dissolved oxygen of yoghurt and the consequent effect on the survival of probiotic bacteria during the shelf life of
the yoghurt. Chapter 8 describes the incorporation of oxygen adapted probiotic strains and their survival in a commercial yoghurt. Additionally, the chapter illustrates the dissolved oxygen and survival trends of *L. acidophilus* and *Bifidobacterium* spp. in a popular commercial yoghurt. Finally, Section 12 contains the overall conclusions of this study while Section 13 provides future directions for research.
2 Literature review

2.1 Introduction to probiotics

Ever since Alexander Fleming discovered the antibacterial properties of the fungus *Penicillium* spp. in 1929 (Fleming, 1929), the world has seen the rapid dominance of antibiotics in the treatment of various diseases. The development of broad spectrum and highly specific antibiotics has led to medical science relying heavily on antibiotic therapy as therapeutic agents against different pathogens.

There are however certain drawbacks associated with antibiotic therapies. The eliminating action of antibiotics does not discriminate between pathogens and the beneficial intestinal microflora. Consequently, an antibiotic therapy also results in an altered intestinal balance causing several unpleasant side effects that can persist long after the cessation of treatment. The fast emergence of multiple antibiotic resistant populations of bacteria such as vancomycin-resistant enterocci and methicillin-resistant *Staphylococcus aureus* in hospital environments is also a growing concern among the medical fraternity. Furthermore, some infections once thought readily treatable with antibiotics are now being recognized as serious health threats. For example, a diarrheal disease can result from *Clostridium difficile*, an opportunistic pathogen, due to the disruption of the normal intestinal microflora during antibiotic treatment. Although this disease is generally treated successfully with a second antibiotic, some infections however, recur in spite of the antibiotic therapy (Sanders, 1999). Consequently, people all over the world are recognizing that preventing or reducing the risk of disease is preferable to treating diseases. In fact, the World Health Organization
recommends global programs to reduce the use of antibiotics in human medicine and suggests increased efforts to prevent disease through the development of more effective and safer vaccines (Stanton et al., 2001). A climate has thus been produced wherein both doctors and patients are searching for preventive rather than curative approaches to diseases in which the intestinal microflora is not adversely affected.

One such approach that is fast gaining popularity is the concept of probiotics, a general term for nutritional supplements containing one or more cultures of living organisms (typically bacteria or yeast) that, when introduced to a human have a beneficial impact on the host by improving the endogenous microflora (Markowitz and Bengmark, 2002). As compared to the invasive, costly and chemical properties of antibiotics, probiotics scores by being non-invasive, safe, natural, and mostly free of any unpleasant side effects.

2.2 History of probiotics

The use of live microbes to enhance human health is not new. For over thousands of years, much before the discovery of antibiotics, people have been consuming live microbial food supplements such as fermented milks. References to the preparation of fermented milks have been recorded in Genesis. According to Ayurveda, one of the oldest medical science that dates back to around 2500 BC, the consumption of yoghurt has been advocated for the maintenance of overall good health (Chopra and Doiphode, 2002). Early scientists such as Hippocrates and others also recommended fermented milk for its nutritional and medicinal
properties, prescribing sour milk for curing intestinal and stomach disorders (Oberman, 1985).

A scientific explanation of the beneficial effects of lactic acid bacteria present in fermented milk was first provided in 1907 by the Nobel Prize winning Russian physiologist, Eli Metchnikoff. In his fascinating treatise ‘The prolongation of life’, Metchnikoff stated “The dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes” (Metchnikoff, 1907). It was proposed that the ingestion of some selected bacteria might beneficially influence the gastrointestinal tract of humans. Metchnikoff believed that main cause of aging in humans was due to "toxicants" formed by intestinal putrefaction and fermentation (O'Sullivan et al., 1992). Upon observing that the lactic acid fermentation of milk products arrested putrefaction, he held that the consumption of such fermented milk products would similarly arrest intestinal putrefaction.

Metchnikoff hypothesized that the long, healthy life of Bulgarian peasants resulted from their consumption of fermented milk products. He believed that when ingested, the fermenting bacteria in the product positively influenced the microflora of the colon decreasing toxic microbial activities, thereby resulting in prolonged life. This led Metchnikoff to suggest that drinking beverages such as yogurt containing lactic acid bacteria would prevent ageing.
Interestingly, several years before Metchnikoff’s treatise, Pasteur and Joubert (1877), upon observing antagonistic interactions between bacterial strains, had recommended the consumption of non-pathogenic bacteria to control pathogenic bacteria. In addition, at about the same time, Henry Tissier isolated bifidobacteria, members of the lactic acid bacteria group, from the stools of breast–fed infants and found them to be a predominant component of the intestinal microflora (Ishibashi and Shimamura, 1993). Tissier believed that the administration of bifidobacteria to infants with diarrhea would displace the putrefactive bacteria responsible for the gastric upsets and re-establish themselves as the dominant intestinal microorganisms. Thus like Metchnikoff, Tissier too had suggested the administration of bifidobacteria to such infants (O'Sullivan et al., 1992). His theories were strengthened by clinical observations of breast-fed infants compared to bottle- fed infants (Rasic and Kurmann, 1983).

Although World War 1 and Metchnikoff’s death slightly deflated the interest in his prescribed bacteriotherapy, the foundation for modern day probiotics had been unequivocally established. Studies on the use of lactic acid bacteria in dietary regimen continued throughout the past century. While work in the earlier part of the century dealt with the use of fermented milk to treat intestinal infections, recent studies have focussed on the other health benefits of these organisms as well as on ensuring survival of these bacteria in the gastrointestinal tract and the carrier food (Lourens-Hattingh and Viljoen, 2001). The knowledge obtained about probiotics through these studies has in turn sparked off massive developments in the cultured dairy products industry. Thus, from the early observations of Eli Metchnikoff and other researchers, the historical association of probiotics with fermented
dairy products continues even today. This is evident by the huge probiotic dairy food market existing currently.

2.3 Development of probiotics

The microorganism that Eli Metchnikoff referred to in his famous hypothesis was the ‘Bulgarican Bacillus’, a bacterium that was most active in causing the souring of milk. In order to be beneficial, the probiotic strain should survive well through the gastrointestinal tract and reach the intestine in a viable state where it can proliferate and produce effective substances that improve the intestinal microbial balance. Subsequent investigations however revealed that the ‘Bulgarican Bacillus’ was killed when it passed through the stomach (Rettger et al., 1935). Thereafter, *Lactobacillus* spp., commonly found in the intestinal microflora of healthy humans, was found to survive and implant well in the intestine (Cheoplin and Rettger, 1921). The *Bifidobacterium* spp. was another group of bacteria of similar attributes. The beneficial role of *Lactobacillus* and *Bifidobacterium* spp. in alleviating various intestinal disorders began to be documented and researchers turned their attention towards bacteria of intestinal origin (Brown, 1977). Since then, there has been increase in research evaluating the various health benefits of *Lactobacillus* and *Bifidobacterium* spp.
2.3 Definition of probiotics

The word ‘probiotics’ originates from the Greek word ‘for life’. The definition of probiotics however, has been evolving over time. Lily and Stillwell (1965) had originally proposed to describe compounds produced by one protozoan that stimulated the growth of another. The scope of this definition was further expanded by Sperti in the early seventies to include tissue extracts that stimulated microbial growth (Gomes and Malcata, 1999). Thereafter, Parker (1974) applied this for animal feed supplements having a beneficial effect on the host by contributing to its intestinal microbial balance. Consequently, the term ‘probiotics’ was applied to describe ‘organisms and substances that contribute to intestinal microbial balance’. This general definition was then made more precise by Fuller (1989), who defined probiotics as ‘a live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance’. This was further revised to ‘viable microorganisms (lactic acid and other bacteria, or yeasts applied as dried cells or in a fermented product) that exhibit a beneficial effect on the health of the host upon ingestion by improving the properties of its indigenous microflora’ (Havenaar and Huis in't Veld, 1992).

Recent research has shown however, that the intestinal tract is a fairly stable microbial ecosystem in the adult (Tannock, 1990). Although antibiotic therapy, diseases, or certain dietary changes can result in this ecosystem being altered, these perturbations seem to be self-correcting (Tannock, 1983). Probiotic bacteria consumed in high numbers do not necessarily become permanent colonizers and could be rarely detected in fecal or intestinal samples beyond a couple of weeks after ingestion. Therefore, it becomes essential to
consider that probiotic effects may, in fact, be mediated by associations and mechanisms less intimate and more transient than those of native microflora (Sanders, 1999). Thus, the definition of probiotics has been further altered and currently remains as ‘live microbes which transit the gastro-intestinal tract and in doing so benefit the health of the consumer’ (Tannock et al., 2000).

2.4 Role of the intestinal flora in human health

2.4.1 Human gastrointestinal ecology

The human intestinal tract constitutes a complex ecosystem. From being considerably aerobic in the mouth and oropharyngeal areas, the levels of oxygen start diminishing progressively further down the gastrointestinal tract until the intestine where it becomes very anaerobic. This spread of oxygen along the gastrointestinal tract has allowed it to favour specific microflora in each of its sections such as bifidobacteria being predominant in the anaerobic large intestine whereas the small intestine favoring the microaerophilic

*L. acidophilus* (Plate 1).
Plate 1. The distribution of oxygen in the gastrointestinal tract of humans and the site of *Lactobacillus* spp. and *Bifidobacterium* spp. [based on Tannock (2002)]
Of all the sections of the gastrointestinal tract, the intestine is the most intricate. It is estimated that the intestine of a single individual harbors 100 trillion viable bacteria and over 100 bacterial species, which constitute the intestinal flora (Mitsuoka, 1982).

Considerable changes in the intestinal microflora occur from the day a baby is born until it becomes an adult. The intestine of a newborn infant is sterile but shortly after birth, a variety of bacteria starts colonizing the infant intestinal tract. Broadly, the development of the human intestinal microflora can be classified into five phases of microbial succession (Plate 2) (Aimutis, 2001).
Plate 2. The five phases of microbial succession in the human gastrointestinal tract

Based on Aimutus (2001)

Phase 1
Birth (0-24 hrs)
- E. coli
- Clostridium
- Streptococcus
- Bacteroides

Phase 2
Breast-fed
Infancy (after 24 hrs)
- Lactobacillus
- Bifidobacterium
- Some Clostridium
- Some Bacteroides

Phase 3
Weaned

Phase 4
Adult
- Bacteroides
- Eubacterium
- Peptostreptococcus
- Streptococcus
- Clostridium
- Lactobacillus
- Bifidobacterium
- Veillonella
- E. coli
- Fusobacterium

Phase 5
Old age
- C. perfringens
- Lactobacillus
- Streptococcus
- Enterobacteriaceae

Formula-fed

Solid food
The first microorganisms to appear in the colon of newborn babies are usually Enterobacteriaceae and enteric streptococci (Phase 1). Genera of microorganisms such as *Lactobacillus*, *Clostridium*, *Bacteriodes*, and *Bifidobacterium* appear within the first week of life (Phase 2). In breast-fed infants, it is common for counts of bifidobacteria to reach $10^{10}$-$10^{11}$ cfu per gram of feces (Modler et al., 1990). The increase in bifidobacteria results in lactococci, enterococci and coliforms representing less than 1% of the intestinal population, while bacteroides and clostridia normally become absent. Formula-fed infants normally have one log-less of bifidobacterial counts and there is a tendency for these babies to have higher levels of enterobacteriaceae, streptococci, and other putrefactive bacteria. This suggests that bifidobacteria could be offering resistance to infections in breast-fed infants due to their higher counts (Lourens-Hattingh and Viljoen, 2001). With weaning and ageing, gradual changes in the intestinal flora profile occur resulting in an adult type microflora (Phase 4) in which bifidobacteria becomes the third common genus in the intestinal tract. At an older age, bifidobacteria decrease while populations of clostridia, including *C. perfringens*, lactobacilli, streptococci and enterobacteriaceae increase significantly (Phase 5) (Mitsuoka, 1982).

### 2.4.2 Intestinal balance and probiotics

Although complex, the composition of the intestinal flora is relatively stable in healthy human beings and can be categorized into three groups, namely harmful, beneficial or neutral with respect to human health. Among the beneficial bacteria are *Lactobacillus* spp. and *Bifidobacterium* spp., which play a useful role in the production of vitamins, organic
acids and anti-microbial factors to inhibit pathogens. On the other hand, *E. coli*, *Clostridium*, *Proteus* and some types of *Bacteriodes* fall into the category of potentially harmful bacteria (Lourens-Hattingh and Viljoen, 2001). The balance of intestinal flora can be altered in favor of such potentially harmful bacteria by a number of factors such as peristaltic disorders, cancer, surgery, liver, kidney or immune disorders, raditaion therapy, stress, diet, antibiotics and ageing. When harmful bacteria dominate the intestinal flora, essential nutrients may not be produced and the level of damaging substances, including carcinogens, putrefactive products and toxins may increase (Mitsuoka, 1996).

A healthy intestinal microflora plays a very important role in the maintenance of good human health. The intestinal flora contains a variety of enzymes that perform varied types of metabolism in the intestine, thereby influencing the host’s health, including nutrition, physiological function, immunological responses and resistance to infection and other stresses. Constant interactions occur between the endogenous microflora and potentially pathogenic microorganisms. Disease can occur when the intestinal mucosal barrier is penetrated by bacteria, viruses, and other toxins secreted by pathogens. The integrity of this barrier is influenced significantly by the various interactions of the endogenous microflora with other members of the gut microflora as well as with other microorganisms that are introduced into the gut.
Therefore, maintaining a well-balanced microflora thus can have some important health benefits (Conway, 1996) such as:

1. levels of pathogenic and abnormal microorganisms are kept low or excluded
2. abnormal bacterial enzyme activity and thereby formation of toxic and carcinogenic substances is avoided
3. a number of by-products are produced by bacteria, particularly organic acids which reduce pH levels as well as provide an energy source for the growth and maintenance of cells of the large intestine

It has thus been suggested that manipulating the composition of intestinal microflora by introducing live bacteria or stimulating growth of certain bacterial population groups can prevent harmful effects and promote beneficial actions of the intestinal microflora. It is on this premise that probiotics were first introduced. Administration of probiotic bacteria can hence be useful not only to treat intestinal disorders but also to prevent them (Salminen et al., 1996a).

### 2.5 Therapeutic benefits of *Lactobacillus* and *Bifidobacterium* spp.

The information obtained through various studies on *Lactobacillus* and *Bifidobacterium* spp. has strengthened the view that the consumption of these microrganisms is helpful in maintaining good health, restoring body vigour, and in combating intestinal and other diseases (Mital and Garg, 1995). Some of the main therapeutic effects of probiotics are listed in Table 1.
Table 1. Health effects of probiotic bacteria (adapted from Ouwehand et al., 2003)

<table>
<thead>
<tr>
<th>Improvements of</th>
<th>Prevention/reduction of symptoms of</th>
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<tbody>
<tr>
<td>Lactose assimilation</td>
<td>Hypercholesterolaemia</td>
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<td>Food digestibility</td>
<td>Diarrhoea</td>
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<tr>
<td>Immune response</td>
<td>Inflammatory bowel disease (ulcerative colitis or Crohn’s disease)</td>
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<td>Blood pressure</td>
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<td>Oral health</td>
<td>Necrotising enterocolitis</td>
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<td>Irritable bowel syndrome</td>
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<td>Helicobacter pylori infection (ulcers)</td>
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<td>Small bowel bacterial overgrowth</td>
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<td>Colorectal cancer</td>
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<td>Superficial bladder cancer</td>
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<td>Breast cancer</td>
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<td>Allergy</td>
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<td></td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td></td>
<td>Urinary tract infection</td>
</tr>
<tr>
<td></td>
<td>Upper respiratory tract infection</td>
</tr>
</tbody>
</table>
Presently, strains with the most published clinical data are *L. rhamnosus* GG, *L. paracasei* Shirota and *B. lactis* Bb12 (Playne, 2002). Strains with peer-reviewed published evidence from human clinical trials are shown in Table 2.

Table 2. Strains of *Lactobacillus* and *Bifidobacterium* spp. with published peer-reviewed clinical data. Strains are listed in decreasing order of clinical evidence (adapted from Playne, 2002)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. rhamnosus</em> GG (Valio)</td>
<td>1, 2, 4, 5, 6, 7, 8, (12), 14, 15</td>
</tr>
<tr>
<td><em>L. paracasei</em> Shirota (Yakult)</td>
<td>2, 5, 6, 9, (10), 11, (12), 15</td>
</tr>
<tr>
<td><em>B. lactis</em> Bb 12 (Chr. Hansen)</td>
<td>1, 2, 3, 4, 5, 6, 11, 15</td>
</tr>
<tr>
<td><em>L. reuterii</em> (Biogaia)</td>
<td>1, 5, (10), (12)</td>
</tr>
<tr>
<td><em>L. johnsonii</em> La 1 (Nestle)</td>
<td>6, 11, 14, 15</td>
</tr>
<tr>
<td><em>L. acidophilus</em> La5 (Chr. Hansen) *</td>
<td>2, 4, 5, 6, 11</td>
</tr>
<tr>
<td><em>B. longum</em> BB536 (Morinaga)</td>
<td>2, 5?, 11, (12), (15)</td>
</tr>
<tr>
<td><em>B. breve</em> (Yakult)</td>
<td>(1), 5</td>
</tr>
<tr>
<td><em>L. acidophilus</em> NFCM (Rhodia USA)</td>
<td>1?, 5, 6, (12)</td>
</tr>
<tr>
<td><em>L. plantarum</em> 299ν (Proviva, Sweden)</td>
<td>5, 13</td>
</tr>
</tbody>
</table>

**Condition:** 1 = rotaviral diarrhea; 2 = antibiotic-associated diarrhea; 3 = *C. difficile* pseudomembrane colitis; 4 = travellers diarrhea; 5 = other acute bacterial diarrhea; 6 = lactose intolerance; 7 = bacterial vaginitis; 8 = atopic eczema and food allergy; 9 = bladder cancer; 10 = cholesterol; 11= chronic constipation; 12 = bowel cancer; 13 = irritable bowel syndrome; 14 = *Helicobacter pylori*; 15 = immune response modulation.

? = uncertain evidence; ( ) animal data and/or biomarkers only

* Doubtful as this strain was usually coadministered with *B. lactis* Bb 12
Scientific research using *L. acidophilus* and *Bifidobacterium* spp. as dietary cultures is also available. Both these bacteria were found to be inhibitory towards many food borne pathogens (Gilliland and Speck, 1977) and assist in the control of intestinal infections (Gilliland, 1990). The enhanced resistance of lactobacilli and bifidobacteria against intestinal pathogens is thought to occur through various anti-microbial mechanisms such as: competitive colonization, production of organic acids like lactic acids, bacteriocins, hydrogen peroxide, deconjugated bile salts, carbon dioxide and diacetyl and stimulation of the immune system (Bernet et al., 1993; Marteau and Rambaud, 1993; Gibson and Wang, 1994; Tahara et al., 1996; Fujiwara et al., 1997). The ability of *L. acidophilus* and *Bifidobacterium* spp. to produce β-D-galactosidase was found to improve lactose digestion in people who are unable to digest the lactose in milk products and who therefore suffer from various degrees of abdominal discomfort (Kim and Gilliland, 1983; Jiang et al., 1996).

Some studies report that ingestion of *L. acidophilus* and *Bifidobacterium* spp. resulted in a decrease in the levels of enzymes responsible for activation of procarcinogens and thereby suppression of cancer in mice (Kurmann and Rasic, 1991; Mital and Garg, 1995). Administration of a probiotic preparation containing *Bifidobacterium* spp. to humans suffering from irritable bowel syndrome or functional diarrhea was found to improve the clinical picture and change the composition and biochemistry of the intestinal microflora (Brigidi et al., 2001).
The antagonistic effects of these bacteria against enteric pathogens can help to enhance resistance against intestinal diseases (Mital and Garg, 1995). Furthermore, hypercholesterolemic action and relief from constipation has also been reported (Gilliland et al., 1985; Pereira and Gibson, 2002). Other potentially clinical applications for these probiotic bacteria include treatment of food allergy (Salminen et al., 1996b), reduction of hypertension (Hata et al., 1996), and use as vectors for the delivery of oral vaccines (Pouwels et al., 1996).

### 2.6 Suitability of *Lactobacillus* and *Bifidobacterium* spp. for human administration

Although most scientific papers refer to research using *L. acidophilus* and *Bifidobacterium* spp. as dietary cultures, the probiotic qualities of *Saccharomyces boulardii*, *Escherichia coli* and *Enterococcus* strains have also been reported (Playne, 2002). For example, *S. boulardii* has been used successfully for the prophylaxis of Traveller’s diarrhea and in the prevention and treatment of *C. difficile* diarrhea (Lee and Salminen, 1995). Similarly, a non pathogenic strain of *E. coli* was reported to be effective for in alleviating the symptoms of Inflammatory bowel disease (Markowitz and Bengmark, 2002).

Before a probiotic can be administered however, it is necessary that it is safe and has been tested for human use (Lee and Salminen, 1995). Members of the genera *Streptococcus* and *Enterococcus* are classified as opportunistic pathogens (Salminen et al., 1998). The association of *E. faecium* and *E. faecalis* with bacteremia and the increased incidence of antibiotic resistance in these strains provide rationale for excluding them from food
formulations (Sanders, 1999). Similarly, the existence of pathogenic strains of *E. coli* is well known.

Concerns have been raised about Lactic acid bacteria (LAB) as well. Occurrences of endocarditis, as well as bloodstream, chest and urinary infections have been associated with Lactic Acid bacteria suggesting that they could behave as opportunistic pathogens under certain unusual conditions (Champagne et al., in press). These instances however are rare. Generally, lactic acid bacteria have a long history of safe use in foods. Members of the genera *Lactococcus, Lactobacillus* and *Bifidobacterium* are thus accorded the generally-recognised-as-safe (GRAS) status (Salminen et al., 1998). Consequently, the most commonly studied intestinal bacteria for potential probiotic use are members of the genera *Lactobacillus* and *Bifidobacterium* spp. Table 3. lists some species of these genera isolated from human sources.
Table 3. List of species (by alphabetical order) of the genera *Bifidobacterium* and *Lactobacillus* isolated from human sources (Gomes and Malcata, 1999)

<table>
<thead>
<tr>
<th>Lactobacillus</th>
<th>Bifidobacterium</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. acidophilus</td>
<td>B. adolescentis</td>
</tr>
<tr>
<td>L. brevis</td>
<td>B. angulatum</td>
</tr>
<tr>
<td>L. buchneri</td>
<td>B. bifidum</td>
</tr>
<tr>
<td>L. casei subsp. casei</td>
<td>B. breve</td>
</tr>
<tr>
<td>L. crispatus</td>
<td>B. catenulatum</td>
</tr>
<tr>
<td>L. fermentum</td>
<td>B. dentium</td>
</tr>
<tr>
<td>L. gasseri</td>
<td>B. globosum</td>
</tr>
<tr>
<td>L. jenseni</td>
<td>B. infantis</td>
</tr>
<tr>
<td>L. oris</td>
<td>B. longum</td>
</tr>
<tr>
<td>L. parabuchneri</td>
<td>B. pseudocatenulatum</td>
</tr>
<tr>
<td>L. paracasei</td>
<td></td>
</tr>
<tr>
<td>L. reuteri</td>
<td></td>
</tr>
<tr>
<td>L. rhamnosus</td>
<td></td>
</tr>
<tr>
<td>L. salivarius</td>
<td></td>
</tr>
<tr>
<td>L. vaginalis</td>
<td></td>
</tr>
</tbody>
</table>
2.7 Characteristics of *Bifidobacterium* spp. and *L. acidophilus*

2.7.1 Genus *Bifidobacterium*

Bifidobacteria are among the first microorganisms to colonize the intestine of a newborn infant and thereafter rapidly become the dominant flora (Ishibashi and Shimamura, 1993). Bifidobacteria are classified as Gram positive, non-sporing, non-motile and catalase negative obligate anaerobes. They are pleomorphic with shapes including short, curved rods, club shaped rods and bifurcated Y-shaped rods. At present 30 species are included in the genus *Bifidobacterium*, 10 of which are from human sources (dental caries, faeces and vagina), 17 from animal intestinal tracts, two from wastewater and one from fermented milk (Gomes and Malcata, 1999).

Bifidobacteria are placed in the actinomycete branch of Gram positive bacteria which are characterized by a high G + C content that varies from 54 - 67 mol %. In recent times, the DNA probes and pulse –field gel electrophoresis has been applied for strain identification (Tannock, 2002). Fructose 6 phosphate phosphoketolase, a key enzyme in the glycolytic fermentation, can be used as a taxonomic character in the identification of the genus, although it doesn’t enable interspecies differentiation (Gomes and Malcata, 1999).

Bifidobacteria produce acetic and lactic acids without generation of carbon dioxide, except during degradation of gluconate. Fermentation of two moles of hexose results in formation of three moles of acetate and two moles of lactate. Besides glucose, bifidobacteria can ferment galactose, lactose and fructose (de Vries and Stouthamer, 1968). Utilization of
carbohydrate varies from strain to strain. Cysteine can be an essential nitrogen source for some bifidobacteria (Shah, 1997).

Although considered as obligate anaerobes, some bifidobacteria can tolerate oxygen while some species can tolerate oxygen in the presence of carbon dioxide (Shimamura et al., 1992). The optimum pH for growth is 6-7, with virtually no growth at pH 4.5-5.0 and below or at pH 8 and above. The optimum temperature for growth is 37-41°C with virtually no growth below 25°C and above 46°C.

Bifidobacteria are predominant in the large intestine contributing to 6-36% of the intestinal microflora in adults. The levels of bifidobacteria decrease with age, with the elderly demonstrating lower populations of bifidobacteria than adults (Mitsuoka, 1982).

2.7.2 Genus Lactobacillus

Lactobacilli are distributed in various ecological niches throughout the gastrointestinal and genital tracts and constitute an important part of the indigenous microflora of humans. They are characterized as Gram positive, non-spore forming, non-flagellated rods or coccobacilli (Hammes and Vogel, 1995). They are either micro-aerophilic or anaerobic and strictly fermentative. The homofermentors convert glucose to lactic acid predominantly while the heterofermentors produce equimolar amounts of lactic acid, carbon dioxide and ethanol (and/or acetic acid). The G+C content of their DNA is between 32 and 51 mol %. While
currently at least 70 species of lactobacilli have been described (Tannock, 2002), the one most studied for use in dietary purpose is *Lactobacillus acidophilus*.

*L. acidophilus* belongs to Group A lactobacilli which include obligatory homofermentative lactobacilli (Hammes and Vogel, 1995). *L. acidophilus* is a Gram-positive rod, around 0.6 to 0.9 µm in width and 1.5 to 6.0 µm in length with rounded ends. Cells may appear singularly or in pairs as well as in short chains. It is non-motile, non-flagellated and non-sporing. It is microaerophilic and an anaerobic environment usually enhances growth on solid media.

Most strains of *L. acidophilus* are homofermentors and can utilise cellobiose, glucose, fructose, galactose, maltose, mannose, salicin, trehalose and aesculine (Nahaisi, 1986). Hexoses are almost exclusively (>85%) fermented to lactic acid by the Embden-Meyerhof-Parnas (EMP) pathway. These organisms lack phosphoketolase and therefore neither gluconate nor pentoses are fermented.

The optimum growth occurs within 35-40°C but it can tolerate temperatures as high as 45°C. The optimum pH for growth is between 5.5-6 while its acid tolerance ranges from 0.3 to 1.9% titrable acidity.
2.8 Functional foods, probiotics, prebiotics and synbiotics

Lifestyle and eating habits contribute to each individual’s overall health status. Historically humans were exposed to probiotics through fermented foods. The modern diet however contains dramatically decreased numbers of fermented foods. Moreover the increased hygiene measures in food manufacturing plants and restaurants have resulted in humans being exposed to as few as one millionth of the probiotic organisms to which their ancestors were exposed (Markowitz and Bengmark, 2002). Ageing, increased stress and a hectic lifestyle have further contributed to the declining populations of probiotic organisms such as lactobacilli and bifidobacteria in the human gut (Lourens-Hattingh and Viljoen, 2001). In the current situation, it becomes critical to supplement human diet with adequate doses of probiotic microorganisms to re-establish the intestinal microflora balance and help maintain good health.

Consequently, in recent times, probiotics have been marketed as dietary supplements in the form of tablets, capsules and freeze-dried preparations (Shah, 2001). Some of the commercial companies producing such dietary supplements include Probiotics International Ltd. U.K., Natren Inc., U.S.A. and Blackmores Ltd, Australia.

Probiotic cultures can be more effective however, when ingested in a food medium. An empty stomach has a low pH that destroys most bacteria, except those lactic acid bacteria that adhere to the stomach mucosa. When food is ingested, the pH in the stomach quickly rises and probiotic bacteria can easily pass mostly unharmed to the small intestine where
they are most effective. Such foods incorporated with probiotic cultures fall under the category of *functional foods* which are broadly defined as ‘foods similar in appearance to conventional foods that are consumed as part of a normal diet and have demonstrated physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions’ (German et al., 1999).

In addition to directly introducing live bacteria to the colon through dietary supplementation, another approach to increase the numbers of beneficial bacteria such as bifidobacteria in the intestinal microbiota is using *prebiotics*.

Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health (Gibson and Roberfroid, 1995). The prebiotics identified are non-digestible carbohydrates including lactulose, inulin, resistant starch and a range of oligosachharides that supply a source of fermentable carbohydrate for beneficial bacteria in the colon (Crittenden, 1999).

An approach that combines both probiotics and prebiotics is called *synbiotics*. Synbiotics is defined as a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare (Gibson and Roberfroid, 1995). Although prebiotics can help to increase the
beneficial bacteria in the GI tract, a general increase in the beneficial bacterial population may however not necessarily contribute to increased health effects as it is strain related.

2.9 Characteristics of a good probiotic strain

Although several probiotic strains have been identified with health benefits, for a strain to be beneficial, it must fulfill certain criteria to be considered a valuable dietary adjunct exerting a positive influence (Fig. 1). The strain must be a normal inhabitant of the human intestinal tract and be able to survive harsh conditions such as acid in the stomach and bile in the small intestine. In addition, when incorporated into food, probiotic bacteria should be able to survive the manufacturing process as well as remain viable during the ripening or storage period. Furthermore, the added probiotic bacteria must not negatively affect product quality, and be generally recognized as safe (GRAS).
Figure 1. Desirable characteristics of a probiotic strain [adapted from (Lee and Salminen, 1995)]

- Human origin
- Safe for human consumption
- Acid and bile resistance
- Good viability in fermented foods
- Colonisation of the human gut
- Production of antimicrobial substances

It is usually difficult for one strain to satisfy all the desirable attributes and consequently there aren’t many documented probiotic strains available at present (Table 4).
Table 4. List of the characterized probiotic strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. acidophilus</em> NCFM®</td>
<td>Rhodia, Inc. (Madison, Wisconsin, USA)</td>
</tr>
<tr>
<td><em>L. acidophilus</em> LA-1 (same as strain LA-5 sold in Europe)</td>
<td>Chr. Hansen, Inc (Milwaukee, Wisconsin, USA)</td>
</tr>
<tr>
<td><em>L. acidophilus</em> DDS –1</td>
<td>Nebraska Cultures, Inc. (Lincoln, Nebraska, USA)</td>
</tr>
<tr>
<td><em>L. casei</em> Shirota</td>
<td>Yakult (Tokyo, Japan)</td>
</tr>
<tr>
<td><em>L. casei</em> Immunitas</td>
<td>Danone (Paris, France)</td>
</tr>
<tr>
<td><em>L. johnsonii</em> La1</td>
<td>Nestlé (Lausanne, Switzerland)</td>
</tr>
<tr>
<td><em>L. paracasei</em> CRL 431</td>
<td>Chr. Hansen, Inc. (Milwaukee, Wis)</td>
</tr>
<tr>
<td><em>L. plantarum</em> 299V</td>
<td>Probi AB (Lund, Sweden)</td>
</tr>
<tr>
<td><em>L. reuteri</em> SD2112 (same as MM2)</td>
<td>Biogaia (Raleigh, N.C., USA)</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> GG⁴</td>
<td>Valio Dairy (Helsinki, Finland)</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> GR-1</td>
<td>Urex biotech (London, Ontario, Canada)</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> 271</td>
<td>Probi AB (Lund, Sweden)</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> LB21</td>
<td>Essum AB (Umea, Sweden)</td>
</tr>
<tr>
<td><em>L. salivarius</em> UCC118</td>
<td>University College (Cork, Ireland)</td>
</tr>
<tr>
<td><em>L. lactis</em> L1A</td>
<td>Essum AB (Umea, Sweden)</td>
</tr>
<tr>
<td><em>B. lactis</em> Bb-12</td>
<td>Chr. Hansen, Inc. (Milwaukee, Wisconsin, USA)</td>
</tr>
<tr>
<td><em>B. longum</em> BB536⁵</td>
<td>Morinaga Milk Industry Co., Ltd. (Zama –city, Japan)</td>
</tr>
<tr>
<td><em>B. longum</em> SBT –2928⁶</td>
<td>Snow Brand Milk products Co., Ltd. (Tokyo, Japan)</td>
</tr>
<tr>
<td><em>B. breve</em> strain Yakult</td>
<td>Yakult (Tokyo, Japan)</td>
</tr>
<tr>
<td><em>B. lactis</em> LAFTI™ B94</td>
<td>DSM Food Specialties (Australia)</td>
</tr>
<tr>
<td><em>L. acidophilus</em> LAFTI™ L10</td>
<td>DSM Food Specialties (Australia)</td>
</tr>
<tr>
<td><em>L. paracasei</em> LAFTI™ L26</td>
<td>DSM Food Specialties (Australia)</td>
</tr>
</tbody>
</table>

⁴ Strains which have been awarded the FOSHU (Food for Specific Health Use) status in Japan (Adapted from Salminen et al., 1999)
2.10 Yoghurt as a probiotic carrier food

Among the variety of foods available, fermented dairy foods such as yoghurts form the ideal vector for the delivery of *Lactobacillus* and *Bifidobacterium* spp. as they belong to the LAB group and are generally considered safe for human administration (Salminen et al., 1998). Yoghurt has long been perceived as ‘healthy’ by consumers owing to its many desirable effects and has become increasingly popular in recent years. The conventional yoghurt bacteria, *S. thermophilus* and *L. bulgaricus* are not natural inhabitants of the intestine and thus lack the ability to survive the gastointestinal conditions. Consequently, they do not play a significant role in the human gut. Therefore, for yoghurt to be considered as a probiotic, *L. acidophilus* and bifidobacteria are incorporated as dietary adjuncts. Yoghurt containing these two probiotic bacteria is referred to as ‘AB’ yoghurt. A recent trend has been to incorporate *L. casei* in addition to *L. acidophilus* and bifidobacteria; such yoghurts are known as ‘ABC’ yoghurts.

2.10.1 Yoghurt

Yoghurt is one of the most consumed fermented milk product. Made from either cow’s, ewe’s, goat’s or buffalo’s milk, it originated thousands of years ago in Eastern Europe and Western Asia where it is still consumed in large quantities. Despite its worldwide popularity, no precise definition of yoghurt has been formulated. It is known by a variety of names all over the world such as Yaourt in Bulgaria/Russia, Tako in Hungary, Dahi in India, Mast in
Iran and Matzoon or Madzoon in Armenia. In Australia, the dairy industry prefers to call it ‘yoghurt’ and is prepared predominantly from cow’s milk.

2.10.2 Manufacture of yoghurt

The flowchart of yoghurt manufacture (Fig. 2) is a guide to yoghurt manufacture in Australia but the manufacturing steps involved may vary from manufacturer to manufacturer.
Figure 2. Flowchart of yoghurt production

Whole or low fat milk

Addition of milk powder to increase the level of solids

Homogenization

Pasteurisation of milk

Cooled to 40-45°C

Inoculate with starter

**Stirred yoghurt**

**Set yoghurt**

Bulk Incubation

Cool

Stirring with fruit or flavour

Packaging

Packaging

Storage (< 7°C)

Market delivery

Incubation in individual sealed containers
Initially, the level of total solids in the milk is raised to around 16% by evaporation, adding concentrated milk or milk powder (whole or skim). Besides improving the nutritional value, this helps produce firmer yoghurt and reduces the tendency towards syneresis (separation of liquid whey from the yoghurt gel) on storage. This is followed by homogenization, particularly in commercial manufacture. This forms an emulsion of fat globules in the milk and imparts a smooth and creamy mouth-feel to the yoghurt.

Pasteurization follows wherein the milk is heated usually at 85°C to 95°C and held for 15-20 minutes. Besides reducing the total bacterial load significantly, this step also denatures the whey protein, which helps preventing syneresis during storage. The pasteurized milk, when cooled, is then suitable for being fermented to yoghurt. This is achieved by inoculating a culture of bacteria known as ‘starter’. The most preferred starter in the dairy industry is an active culture of \textit{L. delbrueckii} subsp. \textit{bulgaricus} and \textit{Streptococcus salivarius} subsp. \textit{thermophilus}. As the lactobacilli grow, they break down proteins and release peptides that encourage the streptococci to grow forming formic acid and carbon dioxide. This in turn stimulates the growth of lactobacilli. This synergistic action of these bacteria produces yoghurt gel with a desirable taste and flavour. The pH of the yoghurt usually ranges between 4.4-4.6, depending on the market needs.

After incubation, yoghurt is then cooled to around 5°C, which helps to restrict the activity of the starter cultures and prevent the development of excess acidity. The product is then held at this temperature during storage, packing and distribution. Under these conditions, yoghurt usually has a shelf life of around 6-7 weeks.
2.10.3 Classification of yoghurt

There are two main types of yoghurt:

(a) **Set yoghurt**, for which the processed milk base is inoculated with starter, then filled into the retail container (possibly with some fruit conserve or a flavoring compound), incubated undisturbed until it sets and reaches the desired acidity and then cooled.

(b) **Stirred yoghurt**, for which the processed milk base is inoculated with starter and incubated under stirring conditions to produce smoother yoghurt than set yoghurt. The incubated base is then cooled before being packed into retail containers. Fruit conserve or other flavorings are added either along with the starter or mixed thoroughly into the yoghurt after formation of the yoghurt base.

Additionally, based on their fat content, yoghurts can be categorized as ‘low fat’ (0.5%-2% fat) or ‘very low fat’ (<0.5% fat).

2.11 Popularity of probiotic yoghurts and dairy products

In recent times, with the rise in health awareness, the consumer is becoming increasingly conscious of the nutritive and health value of foods (Childs, 1997). Concern over the
increase in antibiotic resistance all over the world (Ney, 1994) has made natural alternatives such as probiotics seem attractive to inhibit pathogens.

Yoghurts containing probiotics provide not only viable bacteria, but also high quality macronutrients and micronutrients such as calcium, fermentation end products, bioactive peptides, sphingolipids, and conjugated linoleic acids found in such fermented milk products (Sanders, 1999). Moreover, lactobacilli and bifidobacteria also increase the digestibility of yoghurt protein (Breslaw and Kleyn, 1973), synthesize vitamins in yoghurt (Deeth and Tamime, 1981; Tamime et al., 1995) and increase the bioavailability of calcium, iron, copper, phosphorus, zinc and manganese (McDonough et al., 1983).

The existing health image and the enjoyable taste of yoghurt has positioned it well to capitalize on the growth in probiotic dairy foods (Stanton et al., 2001). Incorporation of the probiotic cultures, *L. acidophilus* and *Bifidobacterium* spp. has further enhanced the ‘healthy’ image of yoghurt. Consequently, the market for probiotic yoghurts has shown rapid growth all over the world. In recent years, the majority of yoghurts marketed in Australia, U.S.A., and Europe contain probiotic bacteria and some form of prebiotics, thus making the yoghurt a synbiotic food (Shah, 2001).

Sales of other probiotic dairy products have also risen. Over 70 products all over the world including sour cream, buttermilk, yoghurt, powdered milk and frozen desserts contain bifidobacteria and lactobacilli (Shah, 2001). Some of these products are listed in Table 5.
Europe has witnessed the most active explosion in the sales of probiotic dairy products, with sales contributing to almost 65% of the US$889 million functional foods market. The probiotic yoghurt market in the European countries totaled more than 250 million kg in 1997, with France representing the largest market, having sales of nearly 90 million kg, valued at US$219 million. The German market for probiotic yoghurts also grew rapidly registering an increase of 150% within just one year (Stanton et al., 2001).

In Japan, products containing bifidobacteria are very popular. Soft drinks containing dietary fibre and probiotics, dominate this section of the dairy market (Sanders, 1998). Sales of such probiotic drinks have contributed significantly to the doubling of yoghurt market (Hughes and Hoover, 1991). Bikkle, a soft drink containing bifidobacteria and dietary fibre, achieved sales of 11 billion yen in its first year of launch itself (Stanton et al., 2001). It is expected that probiotics will continue to dominate the current US$3-3.5 billion Japanese functional food market.

In Australia too, the probiotic yoghurts capture more than 20% of the yoghurt market. The increased per capita consumption of yoghurts in Australia from 3.2 kg in 1994 to 4.8 kg in 1998 has been attributed to increased sales in the probiotic range of yoghurts (Anon., 1998).

Compared to other world markets, the probiotic food market in the U.S. is yet underdeveloped, held back by criticism leveled at companies that introduced products bearing vociferous claims. It is predicted however, that this market will soon experience the fastest growth rates compared to other countries (Stanton et al., 2001). Apart from these
countries, products containing bifidobacteria are also produced in Canada, Italy, Poland, Czechoslovakia and Brazil.

The future of probiotics thus looks bright. Food companies are anticipated to tap this huge potential of probiotic dairy foods by launching further product launches of yoghurts, cheese, ice cream, and milks containing probiotics and prebiotics. Moreover, the marketing of probiotic supplements in the form of capsules and tablets is further boosting the growing health industry.
Table 5. Some of the commercially available probiotic yoghurts containing *Lactobacillus acidophilus* and *Bifidobacterium* spp. [adapted from (Lourens-Hattingh and Viljoen, 2001)]

<table>
<thead>
<tr>
<th>Product</th>
<th>Country</th>
<th>Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidophilus bifidus yoghurt</td>
<td>Germany</td>
<td>A + B + Yoghurt culture</td>
</tr>
<tr>
<td>LC 1</td>
<td>Australia</td>
<td><em>L. acidophilus</em> LC1 + Yoghurt culture</td>
</tr>
<tr>
<td>BA ‘Bifidus active’</td>
<td>France</td>
<td><em>B. longum</em> + Yoghurt culture</td>
</tr>
<tr>
<td>Bifidus yoghurt</td>
<td>Many countries</td>
<td><em>B. bifidum or B. longum</em> + Yoghurt Culture</td>
</tr>
<tr>
<td>Bifighurt</td>
<td>Germany</td>
<td><em>B. longum + S. thermophilus</em></td>
</tr>
<tr>
<td>Biobest</td>
<td>Germany</td>
<td><em>B. bifidum or B. longum</em> + Yoghurt Culture</td>
</tr>
<tr>
<td>Yoplus</td>
<td>Australia</td>
<td>A + B + C + Yoghurt culture</td>
</tr>
<tr>
<td>Bioghurt</td>
<td>Germany</td>
<td>A + B + S. thermophilus</td>
</tr>
<tr>
<td>Philus</td>
<td>Sweden</td>
<td>A + B + S. thermophilus</td>
</tr>
<tr>
<td>BA live</td>
<td>UK</td>
<td>A + B + Yoghurt culture</td>
</tr>
<tr>
<td>Vaalia</td>
<td>Australia</td>
<td><em>Lactobacillus</em> GG + Yoghurt culture</td>
</tr>
<tr>
<td>Kyr</td>
<td>Italy</td>
<td>A + B + Yoghurt culture</td>
</tr>
<tr>
<td>Ofilus</td>
<td>France</td>
<td>A + B + S. thermophilus</td>
</tr>
<tr>
<td>Biodynamic yoghurt</td>
<td>Australia</td>
<td>A + B + C + Yoghurt culture</td>
</tr>
<tr>
<td>BIO</td>
<td>France</td>
<td>A + B + Yoghurt culture</td>
</tr>
<tr>
<td>Biogarde</td>
<td>Germany</td>
<td>A + B + S. thermophilus</td>
</tr>
<tr>
<td>Mil-Mil</td>
<td>Japan</td>
<td>A + B + Yoghurt culture</td>
</tr>
<tr>
<td>Cultura</td>
<td>Denmark</td>
<td>A + B + Yoghurt culture</td>
</tr>
<tr>
<td>AKTIFIT plus</td>
<td>Switzerland</td>
<td>A + B + <em>L. casei</em> GG + <em>S. thermophilus</em></td>
</tr>
<tr>
<td>Ski-Divine</td>
<td>Australia</td>
<td>A + B + Yoghurt culture</td>
</tr>
<tr>
<td>Zabady</td>
<td>Egypt</td>
<td><em>B. bifidum</em> + Yoghurt culture</td>
</tr>
</tbody>
</table>

A: *L. acidophilus*

B: *Bifidobacterium* spp.

C: *L. casei*
2.12 Regulations for probiotic dairy product manufacturers

To obtain the desired therapeutic effects from probiotic yoghurts, it has been suggested that the daily intake should be at least $10^8$ cfu (Lourens-Hattingh and Viljoen, 2001). It is therefore recommended that the minimum counts of probiotic bacteria be $10^6$ cfu/g of the product at the expiry date (Kurmann and Rasic, 1991). These high numbers have been suggested to compensate for the possible reduction in the numbers of probiotic organisms during passage through the stomach and intestine.

The popularity and increasing sales of probiotic yoghurts worldwide has prompted food authorities to set standards for the minimum counts of viable probiotic bacteria needed in the yoghurts and other fermented milk products. In Japan, the Fermented Milk and Lactic Acid Beverages Association has specified that there be at least $10^7$ cfu/ml of viable bifidobacteria in fermented milk drinks (Lourens-Hattingh and Viljoen, 2001). Likewise, the International Standard of FIL/IDF requires $10^7$ cfu of L. acidophilus in products such as Acidophilus milk and $10^6$ cfu/g of bifidobacteria in fermented milks containing bifidobacteria at the time of sale (IDF, 1992). The Swiss Food Regulation as well as the MERCOSOR regulations requires a minimum of $10^6$ cfu of viable bifidobacteria in similar products (Bibiloni et al., 2001).

Although some countries are yet to introduce standards for probiotic bacteria, there still are regulations on the number of viable lactic acid bacteria required in the product. The National Yoghurt Association (NYA) of the United States specifies that in order to use the NYA
“Live and Active Culture’ logo on the container of their products, there should be $10^8$ cfu/g of lactic acid bacteria at the time of manufacture (Lourens-Hattingh and Viljoen, 2001). Similarly, the Australian and New Zealand Food Standards Code (ANZFA, 2003) does not specify any minimum numbers for probiotic bacteria in fermented milk products. It does require however that microorganisms used in the manufacture of fermented milk products should remain viable in the product and that the combined total of the of viable lactic acid cultures used for yoghurt fermentation should be at least $10^6$ cfu/g (ANZFA, 2003). The code also specifies 4.5 as the maximum permissible pH in yoghurt.

The introduction of regulations on probiotic yoghurts by food authorities therefore necessitates manufacturers to guarantee a specific number of probiotic bacteria in their products on the expiry date.

2.13 Survival of probiotic bacteria in commercial probiotic yoghurts

Considering the importance of high numbers of probiotic bacteria needed in yoghurts for them to be therapeutically effective, several studies have estimated counts of probiotic bacteria from various commercial yoghurts all over the world. Although the general perception is that probiotic bacteria exhibit poor survival in yoghurts, a closer look at the various studies reveal that viability estimates of both *L. acidophilus* and *Bifidobacterium* spp. in commercial yoghurts are conflicting. A summary of the findings of these studies is given in Table 6.
Table 6: Comparison of studies on the survival of probiotic bacteria in yoghurts

<table>
<thead>
<tr>
<th>Yoghurts tested</th>
<th>Type of yoghurts</th>
<th>Medium to enumerate counts of</th>
<th>Findings in brief</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>B</td>
<td>GL agar</td>
<td>Viable cells of B were not detected in 3/8 samples, while the remaining five samples contained $10^4$-10$^7$ cfu/g</td>
<td>(Iwana et al., 1993)</td>
</tr>
<tr>
<td>50</td>
<td>AB</td>
<td>MRS-M RCPB</td>
<td>Populations of A and B exceeded $10^6$ cfu/g in only 24% and 14% of the samples respectively</td>
<td>(Rybka and Fleet, 1997)</td>
</tr>
<tr>
<td>22</td>
<td>A, AB, ABC</td>
<td>MRS-S MRS-SOR MRS-NNLP LC</td>
<td>Counts of A and B decreased below $10^6$ cfu/g in &gt;75% and 94% of the products respectively, whereas counts of C dropped in 50% of the products</td>
<td>(Shah et al., 2000)</td>
</tr>
<tr>
<td>5</td>
<td>AB</td>
<td>MRS-M NNLP with cysteine</td>
<td>Counts of A and B decreased during storage</td>
<td>(Shah et al., 1995)</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td>MRS with lactose +NNLP</td>
<td>Counts of B remained above $10^6$ cfu/g until the expiry date</td>
<td>(Shin et al., 2000)</td>
</tr>
<tr>
<td>72</td>
<td>AB</td>
<td>Not given</td>
<td>Counts of A and B were above $10^6$ cfu/g in all yoghurts</td>
<td>(Lourens et al., 2000)</td>
</tr>
<tr>
<td>6</td>
<td>ABC</td>
<td>MRS-B MRS-LP MRS-B</td>
<td>Counts of A, B and C varied across all products</td>
<td>(Vinderola and Reinheimer, 2000)</td>
</tr>
<tr>
<td>3</td>
<td>AB</td>
<td>X-Glu-Agar MRS agar Bif Agar</td>
<td>Counts of A and B varied in all yoghurts</td>
<td>(Pacher and Kneifel, 1996)</td>
</tr>
<tr>
<td>4</td>
<td>AB</td>
<td>MRS-M MRS-S MRS-SOR MRS-NNLP</td>
<td>Counts of A and B varied in all yoghurts</td>
<td>(Dave and Shah, 1997d)</td>
</tr>
<tr>
<td>4</td>
<td>A, AB</td>
<td>MRS-B MRS-LP</td>
<td>Counts of A and B in the samples were less than in 25% and 66% respectively</td>
<td>(Vinderola and Reinheimer, 1999)</td>
</tr>
<tr>
<td>4</td>
<td>A, AB</td>
<td>MRS-B Bifidus Blood agar</td>
<td>Counts of A varied widely whereas counts of B were lower than $10^6$ cfu/g in two of the three yoghurts tested</td>
<td>(Micanel et al., 1997)</td>
</tr>
</tbody>
</table>

A: *L. acidophilus*; B: *Bifidobacterium* spp.; C: *L. casei*
X: not applicable
In a study by Iwana et al. (1993) on eight commercial yoghurts, bifidobacteria were not detected in three yoghurts and the rest had a bifidobacterial count ranging between \(10^4\)-\(10^7\) cfu/ml. Rybka and Fleet (1997) found that the viable populations of \(L.\ acidophilus\) and \(Bifidobacterium\) spp. exceeded \(10^6\) cfu/g in only 24% and 15% respectively of the 50 commercial yoghurts tested. Shah et al. (2000) observed that counts of both \(L.\ acidophilus\) and \(Bifidobacterium\) spp. decreased to less than the recommended \(10^6\) cfu/g by the expiry date in most of the Australian probiotic yoghurts in their study. Similar low counts of probiotic bacteria have been reported elsewhere (Shah et al., 1995; Anon., 1999).

In contrast, other studies have reported satisfactory viability of probiotic bacteria in yoghurts. Shin et al. (2000) evaluated the viability of bifidobacteria in American commercial yoghurts and found that the counts remained well above \(10^6\) cfu/g at the expiry date.

Studies elsewhere, have reported varied counts of either \(L.\ acidophilus\) or bifidobacteria or both in yoghurts prepared using commercial starter cultures (Pacher and Kneifel, 1996; Dave and Shah, 1997d). Vinderola and Reinheimer (1999) found that the counts of bifidobacteria and \(L.\ acidophilus\) varied between 5-7 log\(_{10}\) cfu/g and 2 to 7 log\(_{10}\) cfu/g respectively in several different types of Argentinian yoghurts. Similarly Micanel et al. (1997) reported contrary survival patterns of \(L.\ acidophilus\) and bifidobacteria in different Australian probiotic yoghurts. Bifidobacteria and \(L.\ acidophilus\) were observed to survive well (> \(10^7\) cfu/g) in some yoghurts whereas in others, the levels fell to below \(10^3\) cfu/g.
In view of these studies, it is difficult to obtain a clear understanding about the survival status of probiotic bacteria in yoghurts. This has been made even more difficult by the usage of various selective media to estimate probiotic bacteria from commercial yoghurts.

### 2.14 Selective media for the estimation of probiotic bacteria from yoghurts

Currently, survival estimates of probiotic bacteria are based solely on plate counts. Yoghurt starter cultures as well as *L. acidophilus* and *Bifidobacterium* spp. demonstrate the ability to grow on deman Rogosa Sharpe (MRS) agar, a medium commonly used for LAB. This can make it difficult to selectively enumerate only probiotic bacteria from yoghurt starter cultures such as *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. As a result, a wide range of selection parameters including carbohydrate fermentation profiles, resistance to antibiotics such as nalidixic acid and gentamycin and incorporation of lithium chloride and propionic acid have been used to develop several media that selectively enumerate *L. acidophilus* or *Bifidobacterium* spp. from yoghurts (Lourens-Hattingh and Viljoen, 2001). Lankaputhra and Shah (1996) suggested using salicin as the sole carbon source in MRS agar (MRS-S) to inhibit growth of yoghurt starter cultures and bifidobacteria to selective enumerate *L. acidophilus* from yoghurt. Similarly, MRS-SOR, in which glucose was substituted by sorbitol, was able to selectively enumerate *L. acidophilus*. Likewise, substitution by maltose (MRS-M) allowed growth of both *L. acidophilus* and bifidobacteria while inhibiting the yoghurt starter bacteria (Dave and Shah, 1996).
Several other selective media have been reported for the selective enumeration of

$L. \text{ acidophilus}$ (IDF, 1995; Rybka and Kailasapathy, 1996) or *Bifidobacterium* spp.
(Beerens, 1990; Chevalier et al., 1991; Lapierre et al., 1992; Lim et al., 1995; Pacher and
Kneifel, 1996; Rybka and Kailasapathy, 1996; IDF, 1999; Bonaparte et al., 2001).

Although these media have been developed, most of them are based on pure cultures and so
when applied to enumerate strains that are different from those employed to develop the
medium, not all media give good results (Vinderola and Reinheimer, 1999). Moreover,
differences exist among the strains of the same species regarding to the sugar fermentation
profiles and tolerance to low pH and bile. There are concerns that some media containing
bile or antibiotics might also restrict the growth of probiotic bacteria and give a false
representation of the actual number of viable cells present in the product.

Consequently, after evaluating different media using several parameters such as reliability,
accuracy, simplicity, cost, etc., researchers have recommended some media for the selective
enumeration of the probiotic bacteria from yoghurts. Payne et al. (1999) recommended the
use of AMC agar, a medium developed by Arroyo et al (1995) for the enumeration of
bifidobacteria from mixed cultures containing yoghurt starter bacteria as well as

$L. \text{ acidophilus}$. Apart from selective antibiotics, AMC agar contains lithium chloride and
sodium propionate to inhibit the growth of yoghurt bacteria and $L. \text{ acidophilus}$.

Vinderola and Reinheimer (1999) assayed 15 culture media and found Bile-MRS (MRS-B)
and Lithium propionate MRS (MRS-LP) to be most suitable for the selective enumeration of
*L. acidophilus* and *Bifidobacterium* spp. respectively from yoghurts. Dave and Shah (1996) suggested using MRS-NNLP, a medium containing nalidixic acid, neomycin sulphate, lithium chloride and paromomycin sulphate, for the selective enumeration of bifidobacteria from yoghurts. Similarly, Roy (2001) after reviewing several different selective media recommended MRS-NNLP and DP medium, developed by Bonaparte et al. (2001) for the selective enumeration of bifidobacteria.

Media have also been suggested for enumerating *L. casei* from yoghurts containing *L. acidophilus, Bifidobacterium* spp. and *L. casei*. *L. acidophilus* and *L. casei* formed different types of colonies on MRS-B (Vinderola and Reinheimer, 2000). While *L. casei* gave large round, white, creamy colonies on MRS-B, *L. acidophilus* gave irregular light brown colonies with diameters ranging from 0.9-1.5 mm. This could allow a differential enumeration of these bacteria when they are present together in yoghurt. Ravula and Shah (1998) also developed a selective medium (LC agar) for enumeration of *L. casei*, which was able to inhibit the growth of yoghurt starter bacteria, *L. acidophilus* and bifidobacteria. Similarly, by using used low temperature incubation of MRS, Champagne et al. (1997) were able to selectively enumerate *L. casei* populations in yoghurt. This method is however tedious and time consuming.

In addition, specific dyes and low pH have been used to formulate differential media to distinguish probiotic colonies from those of the yoghurt bacteria. Onggo and Fleet (1993) found that a differential medium proposed by Van der Wiel-Korstanje and Winkler (1970) was able to readily differentiate colonies of *S. thermophilus, L. bulgaricus* and *B. bifidum*.
based on their distinct colony morphologies. The medium was described as Reinforced Clostridial Agar containing Prussian Blue (RCPB). *L. delbrueckii* ssp. *bulgaricus* gave small, discrete blue colonies with white centres on this medium. Colonies were surrounded by wide, clear blue zones. In contrast, *S. thermophilus* colonies were light blue with a white centre and were surrounded by narrow, clear blue zones. In comparison, *B. bifidum* formed shiny, white colonies that were not surrounded by any clearing zones.

An improved version of RCPB (RCPB-pH5) in which the pH of RCPB medium was adjusted to 5 was suggested by Rybka and Kailasapathy (1996) to inhibit the growth of any non-lactic acid bacteria. This medium was characterized by the absence of *L. acidophilus* and *S. thermophilus* growth. Colonies of bifidobacteria were observed to be blue or white depending on the species being tested. *L. delbrueckii* ssp. *bulgaricus* colonies were similar to those observed on RCPB agar.

It is interesting to note that in spite of the numerous media studies, there isn’t a generally accepted selective/differential medium. As a result, each of the population studies of *L. acidophilus* and *Bifidobacterium* spp. in commercial yoghurts has used different media in their estimates of cell counts.
2.15 Media used in various probiotic population studies

The various media used in the population estimates of probiotic bacteria in commercial yoghurts is given in Table 6.

In the investigations conducted by Vinderola and Reinheimer (2000) and Micanel et al. (1997), MRS-B was used for the obtaining the selective counts of *L. acidophilus*. Shah et al. (2000) on the other hand used MRS-Salicin and MRS-SOR for the same objective. Similarly, different media were used for estimating the counts of bifidobacteria. While Vinderola and Reinheimer (2000) used MRS-LP, Micanel et al. (1997) used a Bifidus Blood agar. Likewise, Shin et al. (2000) enumerated bifidobacteria on a MRS medium supplemented with lactose and NPNL.

Shah et al. (1995) and Rybka and Fleet (1997) calculated *L. acidophilus* counts by subtracting the bifidobacteria counts obtained on another selective medium from the total colonies on MRS-M agar. In these studies too, while Shah et al. (1995) used MRS-NNLP agar for estimating the bifidobacterial counts, RCPB medium was used by Rybka and Fleet (1997). In other studies, the media used for the population estimates of *L. acidophilus* and bifidobacteria were not mentioned (Anon., 1999; Lourens et al., 2000).

To obtain a comparable estimate of the survival of probiotic bacteria in yoghurts, it is important to have a working method that is uniform across all the various survival studies. At present however, this isn’t possible due to the wide variety of media employed for
*L. acidophilus* and bifidobacterial counts as well as due to the different commercial yoghurts tested in each of the survival studies. It becomes vital therefore, that the each of these reported media be evaluated for their ability to provide reliable and accurate counts of probiotic bacteria in a wide range of commercial yoghurts. Such a study would assist greatly in knowing the reliability of the various selective/differential media and consequently the accuracy of the reported counts of probiotic bacteria in yoghurts.

### 2.16 Factors affecting survival of probiotic bacteria in yoghurts

Various factors have been reported to affect the survival of probiotic bacteria in yoghurts, including acid and hydrogen peroxide produced by yoghurt bacteria, oxygen content in the product and oxygen permeation through the package (Shah, 2000).

*L. acidophilus* and *Bifidobacterium* spp. are considered sensitive in yoghurt. Different strains of *L. acidophilus* have been shown to demonstrate different viabilities in yoghurt (Nighswonger, 1996). Bifidobacteria are not as acid tolerant as *L. acidophilus* and have been reported to exhibit weak growth in milk and require an anaerobic environment, a low redox potential and the addition of bifidogenic factors to achieve the desired levels of growth (Lourens-Hattingh and Viljoen, 2001).

The importance of strain selection was highlighted in a study in which *L. acidophilus* and *Bifidobacterium* spp. were subjected to viability tests, including exposure to low pH, high bile concentration, high sucrose concentration and low storage temperature. Survival of these
probiotic bacteria was found to be strain dependent (Godward et al., 2000). The viability of probiotic bacteria is also reported to be dependent on the culture conditions, production of hydrogen peroxide due to bacterial metabolism, and the concentrations of lactic and acetic acids (Shah, 2000).

The interaction of the probiotic species with the yoghurt starter cultures is also considered important in determining their survival status in yoghurts. Vinderola et al. (2002) found various inhibitory interactions among these bacteria. Similarly, Joseph et al. (1998) and Dave and Shah (1997c) observed antagonistic effect of probiotic species on yoghurt starter cultures. Samona and Robinson (1994) demonstrated that co-inoculation of bifidobacteria with yoghurt starter bacteria during yoghurt production tended to suppress the growth of bifidobacteria.

The acidity of yoghurts as well as the chemical and microbiological composition, milk solids content, availability of nutrients, growth promoters and inhibitors have also been shown to affect probiotic survival (Kneifel et al., 1993). Kailasapathy and Supraidi (1996) found that whey protein concentrate can act as a buffer in lactose hydrolysed yoghurt and assist in maintaining sufficiently high numbers of *L. acidophilus* during refrigerated storage.

The survival of *L. acidophilus* and bifidobacteria can also be influenced by the type of yoghurt starter cultures as well as the fat content of the yoghurt. Vinderola et al. (2000) observed that full fat yoghurt was more inhibitory for *B. bifidum* than reduced-fat yoghurt. Moreover, the different starter cultures used exerted different inhibitory effect on the
probiotic organisms. Micanel et al. (1997) however found fat levels of yoghurt to have no noticeable effect on the viability of probiotic cultures.

Dave and Shah (1997b) suggested that alterations in the inoculum levels of commercial probiotic yoghurt cultures and their incubation temperature could affect the viability of probiotic microorganisms as observed in their study. Similar observations of probiotic viability being influenced by incubation temperature and fermentation time and storage temperature have been made by Kneifel et al. (1993). Additionally the concentrations of sugars (Lourens-Hattingh and Viljoen, 2001) and dissolved oxygen levels in the product have also been cited to be important factors affecting the survival of probiotic bacteria in yoghurts (Klaver et al., 1993; Dave and Shah, 1997d).

2.17 Oxygen toxicity of probiotic bacteria in fermented milks, particularly yoghurts

Both *L. acidophilus* and *Bifidobacterium* spp. are gut-derived organisms wherein an anaerobic environment prevails. Organisms found in the human gut are generally anaerobic or micro-aerophilic and lack effective oxygen scavenging cellular mechanisms such as catalases. Consequently, in both *L. acidophilus* and *Bifidobacterium* spp., exposure to oxygen causes toxic oxygenic metabolites to accumulate in the cell leading to cell death from oxidative damage. This lethal effect of oxygen is called as oxygen toxicity.

The process of yoghurt manufacture introduces a lot of air in the product. Oxygen can easily dissolve in milk. The process of yoghurt manufacture (Figure 2) involves various stages such
as pumping milk through pipes, homogenization, mixing and agitation, particularly during the manufacture of stirred yoghurts which causes oxygen to get incorporated into yoghurts. Furthermore, oxygen diffuses through the packaging material during storage (Ishibashi and Shimamura, 1993; Miller et al., 2002), thereby creating an undesirable oxygenic environment for \textit{L. acidophilus} and \textit{Bifidobacterium} spp.

The resulting oxygen environment is thought to induce cell death and lead to poor survival of these probiotic bacteria in yoghurts and fermented milks. Brunner et al. (1993a; 1993b) have cited low oxygen content and low redox potentials as important factors for the viability of bifidobacteria during storage of fermented milk products. Bifidobacteria are anaerobic in nature and therefore exposure to high levels of oxygen in yoghurt can affect their growth and viability. Klaver et al. (1993) reported better viability and survival of bifidobacteria in deaerated milk. Ishibashi and Shimamura (1993) showed that the viability of bifidobacteria was a function of oxygen permeability through the packaging material.

Although bifidobacteria are considered more susceptible to oxygen than \textit{L. acidophilus} due to their anaerobic nature, the oxygen toxicity in bifidobacteria could however be strain dependent. Dave and Shah (1997c) found that bifidobacteria survived well over a 35 day period in yoghurt, regardless of the oxygen content and redox potential of the yoghurt. Miller et al. (2002) too found adequate counts of bifidobacteria even as the dissolved oxygen of the yoghurt was seen to rise steadily over the shelf life. In contrast, \textit{L. acidophilus} counts were found to decrease below $10^3$ cfu/g by the third week of yoghurt storage. The strain dependent phenomenon of oxygen sensitivity was further demonstrated by Meile et al.
(1997) who were able to isolate a moderately oxygen tolerant species of Bifidobacterium spp., B. lactis sp. nov. from fermented milk.

Besides yoghurts, oxygen was considered a significant factor for probiotic viability in foods such as mayonnaise and edible table (bio) spread (Khalil and Mansour, 1998; Charteris et al., 2002).

2.18 Techniques to protect L. acidophilus and Bifidobacterium spp. from oxygen toxicity in yoghurts

To protect L. acidophilus and Bifidobacterium spp. from oxygen toxicity in yoghurts, the following techniques have been suggested:

2.18.1 Use of acorbate and L-cysteine as oxygen scavengers in yoghurts

Ascorbic acid, a common food additive, when fortified with yoghurts, can act as an oxygen scavenger and can prove useful to maintain low O-R potentials necessary to the viability of probiotic bacteria. In a study conducted by Dave and Shah (1997c), incorporation of ascorbic acid in yoghurt caused a reduction in the oxygen content and redox potential of yoghurt in the initial 15-20 day period. Thereafter, the oxygen concentration and the redox potential approached levels similar to those prevalent in the controls. L. acidophilus counts in the yoghurt with ascorbic acid decreased less rapidly during the 35-day storage period whereas the counts of bifidobacteria in the same yoghurt were unaffected. Although in this study, the
titratable acidity and pH of the yoghurt did not change significantly, incorporation of ascorbic acid in yoghurts can reduce the amount of oxygen required for the activities of *S. thermophilus*, an aerobic organism involved in the manufacture of yoghurt. This can have a detrimental effect on the textural and nutritional qualities of yoghurt. The use of ascorbic acid in yoghurts may hence not be practical.

L-cysteine, a sulphur containing amino acid, can act as both, reducing the O-R potential as well as a source of amino nitrogen, both of which favour the growth of bifidobacteria (Dave and Shah, 1997a). The use of 0.05% cysteine in reconstituted milk improved viability of some bifidobacteria (Collins and Hall, 1984). Dave and Shah (1997a) studied the growth and viability of probiotic bacteria in yoghurt that was supplemented with 0, 50, 250 or 500 mg/l of L-cysteine. Although the counts of *L. acidophilus* were improved in yoghurts with 250 or 500 mg/l of L-cysteine, these levels of cysteine were found to suppress the growth of the yoghurt starter cultures, *S. thermophilus* and *L. delbrueckii ssp. bulgaricus*. Thus although cysteine was found to bring down the redox potential of the yoghurt and improve the viability of bifidobacteria, it too can have a negative impact on the textural and cultural properties of the yoghurt.

### 2.18.2 Use of special high-oxygen consuming strains

To protect probiotic bacteria, especially bifidobacteria from oxygen in yoghurt, the incorporation of a high-oxygen consuming strain of *S. thermophilus* has been suggested (Lourens-Hattingh and Viljoen, 2001). *S. thermophilus*, which relies heavily on oxygen for
its metabolic activities can act as an effective oxygen scavenger by its consumption of the dissolved oxygen in the yoghurt. This can therefore help to reduce oxygen exposure to bifidobacteria (Ishibashi and Shimamura, 1993). This technique however suffers from the drawback that fast acidifying strains of \textit{S. thermophilus} that are used commercially can lead to a rapid accumulation of acid in the growth medium. As both \textit{L. acidophilus} and bifidobacteria are sensitive to high acidity, this can have a negative impact on the viability of probiotic bacteria. In addition, this method is useful in providing protection against oxygen toxicity only during the initial stages of yoghurt manufacture. It does little to protect the probiotic bacteria from subsequent oxygen ingress into yoghurt through the packaging material.

\subsection*{2.18.3 Packaging material}

The oxygen permeability of the packaging material used currently for probiotic yoghurts is considered a key factor in the high levels of oxygen present in yoghurt. It is well known that packaging materials such as polyethylene and polystyrene are gas permeable and allow the diffusion of oxygen into yoghurt during storage (Ishibashi and Shimamura, 1993).

The exclusion of oxygen during the manufacturing process can be costly. Few current packaging techniques are capable of preventing oxygen permeation. Dave and Shah (1997d) found improved survival of \textit{L. acidophilus} over a 35-day period in yoghurts that were packaged in glass bottles as compared to when the yoghurt was packaged in plastic cups. The oxygen content in yoghurts stored in plastic cups increased due to the permeation of oxygen. On the other hand, yoghurts contained in the glass bottles retained a low oxygen
environment, which was therefore thought to support the viability of *L. acidophilus*. This led them to suggest that yoghurts be packed in glass containers to prevent oxygen toxicity. Although effective, glass jars are neither convenient nor practical owing to their high cost and handling hazards. On the other hand, polyethylene and polystyrene do not have sufficient oxygen barrier properties and are therefore unsuitable to prevent oxygen ingress into yoghurt during storage.

A relatively cheaper packaging option was suggested by Miller et al. (2002) who found that when packaged in polystyrene based packaging containing an added gas-barrier layer (Nupak™), yoghurts demonstrated no increase in their dissolved oxygen levels. In comparison, the dissolved oxygen of yoghurts packaged in conventional high impact polystyrene tubs, the dissolved oxygen was seen to rise steadily over the shelf life. The application of such packaging technologies thus needs to be explored further.

### 2.19 Biochemistry of the oxidative response in lactic acid bacteria

Aerobic bacteria derive their energy primarily through oxidative phosphorylation, involving the electron transport chain, which is composed of a chain of carriers capable of undergoing reversible oxidation and reduction. Anaerobic bacteria including lactic acid bacteria cannot synthesize cytochromes and other heme containing enzymes vital to the electron transport chain. They are thus unable to synthesize ATP by respiratory means and have to depend strictly on a fermentative mode of metabolism. Due to the lack of participation of an external electron acceptor (oxygen in aerobic bacteria) in anaerobes, the organic substrate undergoes
a balanced series of oxidative and reductive reactions mediated by pyridine nucleotides such as NADH. As the energy in anaerobes is derived mainly through substrate level phosphorylation, the regeneration of NAD\(^+\) from NADH assumes critical importance.

The simplest way to oxidize NADH is by the reduction of molecular oxygen (O\(_2\)) via the activity of NADH oxidase. Interestingly, possession of a NADH oxidase appears to be a universal property of LAB (Condon, 1987).

Generally, the NADH oxidizing reactions in LAB including \textit{L. acidophilus} and \textit{Bifidobacterium} spp. catalyze the transfer of one, two or four electrons to the dioxygen molecule as follows (Fig. 3):
Mainly, two types of NADH oxidases: NADH: H₂O₂ oxidase and NADH: H₂O oxidase have been reported in LAB. While the NADH: H₂O₂ oxidase catalyzes the reduction of O₂ to H₂O₂ (reaction 1) (Condon, 1987; Smart and Thomas, 1987), the NADH: H₂O oxidase carries out the four-electron reduction of oxygen to water (reaction 2) (Condon, 1987; Higuchi et al., 2000). The activities of NADH oxidase can also result in the incomplete reduction of oxygen, generating reactive oxygen species such as the superoxide anion (O₂⁻) (reaction 3).
O$_2^-$ can easily dismute to hydrogen peroxide (H$_2$O$_2$) or the hydroxyl radical (HO') either spontaneously or by the activity of superoxide dismutase (SOD) as shown below (Fridovich, 1975; Sanders et al., 1995):

\[
2 \text{O}_2^- + 2 \text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2
\]

In addition, O$_2^-$ can also be dismuted by high intracellular Mn$_2^+$ to the more stable H$_2$O$_2$ (Condon, 1987). These compounds can readily diffuse across cellular membranes and oxidatively damage a number of vital cellular components including membrane lipids, enzymes and DNA (Hassen and Fridovich, 1979; Miller and Britigan, 1997; Higuchi et al., 2000).

In the absence of an effective reactive oxygen species scavenging system, these compounds can accumulate in the cell and eventually cause cell death from oxidative damage. To live in the presence of oxygen, anaerobic bacteria have to convert these reactive oxygen species to nontoxic molecules. It is well known that the hydroxyl radical is highly reactive with biological molecules. Although hydrogen peroxide is a weak oxidant, it can generate hydroxyl radicals in the presence of transition metals (Lin and Yen, 1999). Accumulation of H$_2$O$_2$ during aerobic growth has been shown to inhibit the growth of several lactobacilli (Condon, 1987). Excess intracellular hydrogen peroxide may also produce further oxidation products (O$_2$SCN$^-$ and O$_3$SCN$^-$), which could take part in the bacteriostatic effect caused by the lactoperoxidase-thiocynate-H$_2$O$_2$ system in LAB (Reiter, 1985). H$_2$O$_2$ is claimed to inactivate fructose-6-phosphate phosphoketolase, the major enzyme responsible for sugar
metabolism in bifidobacteria (Shah, 1997). Furthermore, it has been suggested that H$_2$O$_2$ can react with O$_2^-$ to form hydroxyl radical (OH) and that the latter is the direct inhibitor of O$_2$ sensitive cells (Gregory and Fridovich, 1974).

Aerobic accumulation of H$_2$O$_2$ in lactic acid bacteria is thought to result from a greater capacity of the cell to form H$_2$O$_2$ than to break it down. The inability of lactic acid bacteria to synthesize heme proteins results in their failure to produce catalase and mediate the decomposition of H$_2$O$_2$ according to the reaction

\[
\text{Catalase} \\
2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2
\]

To compensate for the lack of catalase, some LAB possess NADH peroxidase that reduce H$_2$O$_2$ to H$_2$O as shown below (Mizushima and Kitahara, 1962; Anders et al., 1970; Thomas and Pera, 1983):

\[
\text{NADH peroxidase} \\
\text{NADH} + \text{H}^+ + \text{H}_2\text{O}_2 \rightarrow \text{NAD}^+ + 2 \text{H}_2\text{O}.
\]

Although NADH peroxidase eliminates H$_2$O$_2$, its activity is dependent on a continuing supply of reducing equivalents such as NADH as opposed to the activity of catalase, which is independent. The combination of NADH oxidase and NADH peroxidase activities in LAB can reduce molecular oxygen (O$_2$) to water usually at the expense of 2 NADH.
Many studies on the aerotolerance of lactic acid bacteria suggest that the ratio and specific activities of the NADH oxidative enzymes contribute to the elimination of the environmental oxygen (Higuchi, 1984; Lucey and Condon, 1986; Smart and Thomas, 1987; Shimamura et al., 1992; Soon-Young and Park, 1997).

Aeration was also observed to produce increased production of hydrogen peroxide in *L. delbruekii* subsp. *bulgaricus*, indicating the role of NADH oxidase in eliminating oxygen from the cell (Marty-Teysset et al., 2000). A similar protective role of NADH oxidase has been suggested by Higuchi et al. (1999) to be operating in *Streptococcus mutans*.

**2.20 Studies on the oxygen tolerance of *L. acidophilus* and *Bifidobacterium* spp.**

The studies on the oxygen tolerance of probiotic bacteria have mostly focussed on *Bifidobacterium* spp. Little is known about the interaction of oxygen with *L. acidophilus*. Research into the oxygen tolerance of bifidobacteria has been conducted from as early as 1969 when de Vries and Stouthamer (1969) examined the sensitivity of twenty *Bifidobacterium* strains to oxygen by measuring the size of the inhibition zones obtained when the bacteria were grown in deep agar cultures under air. For liquid growth, the oxygen tolerance was estimated by qualitatively comparing the growth when the incubation was shifted from anaerobic to aerobic. In addition to these growth measurements, the levels of NADH oxidase, NADH peroxidase and H\textsubscript{2}O\textsubscript{2} accumulation in the culture broth were monitored. The different strains were then classified into three categories based on their
degree of oxygen tolerance and formation of $\text{H}_2\text{O}_2$ during aerobic growth. $\text{H}_2\text{O}_2$ was considered a minor factor for the high sensitivity of some of the strains to oxygen as compared to a high redox potential. No definite correlation was observed between the levels of NADH oxidase and NADH peroxidase and oxygen sensitivity of the \textit{Bifidobacterium} spp.

Uesegi and Yajima (1978) also classified seven \textit{Bifidobacterium} spp. strains based on their oxygen sensitivity. The growth pattern of the strains was evaluated in a sealed fermentor vessel containing 5% oxygen. Strains were then categorized accordingly based on their oxygen tolerance. In this study too, little interrelationship was found between the degree of oxygen tolerance and the activities of NADH peroxidase and superoxide dismutase and the ability to decompose $\text{H}_2\text{O}_2$.

Shimamura (1990) worked on the oxygen uptake of \textit{Bifidobacterium} spp. and found a correlation between intracellular polysaccharide accumulation and oxygen uptake. Oxygen uptake was observed only in the presence of NADH indicating that NADH oxidase operated as the terminal oxygen oxidoreductase in \textit{Bifidobacterium} spp. A similar involvement of NADH oxidase in oxygen uptake in bifidobacteria has been suggested by Cox and Marling (1992).

In a follow up study, Shimamura et al. (1992) explored the enzymatic machinery behind the oxygen sensitivity of \textit{Bifidobacterium} spp. Strains were incubated in different conditions of oxygen such as aerobic brought about by constant shaking, partially aerobic effected by occasional shaking of 10 seconds every 30 minutes and anaerobic achieved by purging the
media of oxygen. All strains accumulated $\text{H}_2\text{O}_2$ under aerobic conditions but no notable correlation was observed between the growth inhibition by oxygen and sensitivity to hydrogen peroxide. Both NADH oxidase and NADH peroxidase were detected in *Bifidobacterium* species and were found to correlate well with oxygen tolerance, with the oxygen sensitive strain displaying low activities of these enzymes.

A similar correlation was found by Shin and Park (1997) who studied the relationship between oxygen tolerance and enzyme activity in bifidobacteria. In this study, the oxygen sensitivity of the strains was determined by growth on selective media plates that were incubated in air. The activities of NADH oxidase and NADH peroxidase were found low in the most aerosensitive strains whereas maximum activities were observed in the most aerotolerant strain.

Studies so far have shown that superoxide dismutase levels in *Bifidobacterium* spp. and *L. acidophilus* strains can be independent of the oxygen sensitivity (Shimamura et al., 1992; Shin and Park, 1997; Lin and Yen, 1999).

Ahn et al. (2001) examined the physiological responses of oxygen tolerant *B. longum* to oxygen. In the presence of oxygen, the lag phase of the organisms became extended and the cell growth was suppressed. Changes in the cellular fatty acid profiles and cellular morphology were also observed with the cells becoming longer and developing a rough surface due to abnormal or incomplete cell division.
Among the oxidative studies conducted on *L. acidophilus*, Archibald and Fridovich (1981) examined the oxygen tolerance of *L. acidophilus* and other lactic acid bacteria by growing the strains on the surface of MRS agar in different partial pressures of oxygen. Additionally, *L. acidophilus* and *L. bulgaricus* were found to be lacking superoxide dismutase and high intracellular levels of Mn (II). This was considered responsible for these bacteria being the least aerotolerant among the lactic acid bacteria strains.

Lin and Yen (1999) investigated the antioxidative ability of lactic acid bacteria, including *L. acidophilus* and *B. longum* and found that both *L. acidophilus* and *B. longum* were capable of chelating metal ions, scavenge reactive oxygen species or possess reducing activity. Similarly, iron chelation activity in *L. acidophilus* and strains of *Bifidobacterium* spp. as well as the presence of a ferroxidase in bifidobacteria has been reported (Kot et al., 1994; Kim et al., 2001).

### 2.2.1 Assays to measure the activities of NADH oxidase and NADH peroxidase

The importance of NADH oxidase and NADH peroxidase in the oxygen tolerance has been suggested in previous studies (Shimamura et al., 1992; Shin and Park, 1997). It is widely accepted that a typical assay of NADH oxidase measures the initial linear slope of NADH oxidation at 340nm. in the presence of cell free extract and air-saturated buffer (Schmidt et al., 1986; Higuchi et al., 1993; Shin and Park, 1997; Yi et al., 1998; Marty-Teysset et al., 2000). Though this assay is suitable for lactic acid bacteria having only NADH oxidase, it is inadequate for estimating the levels of NADH oxidase in organisms in which NADH
peroxidase is also present (Smart and Thomas, 1987). As the product of a NADH: H₂O₂ oxidase reaction i.e. H₂O₂ is also the substrate for NADH peroxidase, the slope of NADH oxidation (oxidase activity) is actually a sum of the total NADH oxidised by the activities of both oxidase and peroxidase. While this has not been taken into account in some studies (de Vries and Stouthamer, 1969; Uesugi and Yajima, 1978; Shin and Park, 1997), other researchers have had to perform amperometric methods in order to determine individual levels of NADH oxidase based on the oxygen uptake (Carlsson et al., 1983; Thomas and Pera, 1983; Smart and Thomas, 1987; Cox and Marling, 1992; Shimamura et al., 1992).

Considerable variation also exists in the assays reported to measure NADH peroxidase. Shimamura et al. (1992) have estimated activities of NADH peroxidase by measuring the consumption of H₂O₂ under anaerobic conditions. Others have assayed NADH peroxidase activity independently by measuring the slope of NADH oxidation under anaerobic conditions (Anders et al., 1970; Carlsson et al., 1983; Thomas and Pera, 1983; Smart and Thomas, 1987; Shin and Park, 1997). Uesugi and Yajima (1978) as well as deVries and Stouthamer (1969) estimated NADH peroxidase from the slope difference in presence and absence of H₂O₂ under aerobic conditions. In contrast, Higuchi et al. (1993) used the same slope difference obtained under anaerobic conditions for the measurement of NADH peroxidase.
2.21.1 Differences in the assay pHs for NADH oxidase: NADH peroxidase

Differences also exist in the pH at which the assays for NADH oxidase and NADH peroxidase were conducted in various studies on *Bifidobacterium* spp. Earlier assays of NADH oxidase and NADH peroxidase were conducted at neutral pH (de Vries and Stouthamer, 1969; Uesugi and Yajima, 1978; Cox and Marling, 1992). In contrast, Shin and Park (1997) estimated the activities of these enzymes at pH 5.5. Interestingly, Shimamura et al. (1992) assayed the enzymes at various pHs and found that the optimum pH of NADH oxidase and NADH peroxidase in *Bifidobacterium* spp. was pH 5. Interestingly, the NADH oxidase and NADH peroxidase activities of *L. acidophilus* have not been reported yet.

2.22 Microencapsulation of *L. acidophilus* and *Bifidobacterium* spp.

For probiotic bacteria to exert their therapeutic benefits, they have to reach the intestine in a viable state. This involves surviving harsh conditions in yoghurt as well as gastric acidity, bile salts, enzymes, toxic metabolites, bacteriophages, antibiotics and anaerobic conditions. In order to protect the cells from these detrimental factors, an approach providing probiotic cells with a physical barrier is receiving considerable interest. Micro-encapsulation is a process in which cells are retained within an encapsulating membrane to reduce cell injury or cell loss (Shah, 2000; Kailasapathy, 2002). The physical retention of cells in the encapsulating matrix facilitates the separation of cells from direct exposure to the adverse factors while at the same time allows the diffusion of nutrients in and out of the matrix and thus helps support the viability of the cells. The thinness, small diameter and semipermeable
nature of the encapsulating membrane are advantageous to this purpose. Encapsulation tends to stabilize cells and can potentially enhance the viability and stability in the production, storage and handling of lactic acid cultures.

Although studies have used cellulose acetate phthalate (Rao et al., 1989) gelatin, vegetable gum (Shah, 2000), fats (Suita-Cruce and Goulet, 2001) or κ-carrageenan (Adhikari et al., 2000) as encapsulating agents, alginate remains the most commonly used bio gum for microencapsulation. The advantages of using alginate as an encapsulating agent include: non-toxicity, formation of gentle matrices with calcium chloride to trap sensitive materials such as living microbial cells, simplicity in entrapping living microbial cells and low cost. Furthermore, calcium alginate gels can be solubilized by the sequestration of calcium ions, facilitating the release of entrapped cells (Shue and Marshall, 1993; Kailasapathy, 2002). Alginate is also an accepted food additive and can be safely used in foods such as yoghurts (Prevost and Divies, 1988; Shue and Marshall, 1993; Dinakar and Mistry, 1994; Kim et al., 1996).

Microencapsulation has also been applied to increase the survival of probiotic bacteria in yoghurt and other dairy products. In a study by Adhikari et al. (2000), encapsulating bifidobacteria in κ-carragenan appeared to increase their viability in yoghurt over a 30-day storage period. Sheu and Marshall (1993) found that lactobacilli that were entrapped in calcium alginate survived 40% more than the free non-entrapped cells during the freezing of ice milk. Similarly, *L. acidophilus* and *Bifidobacterium* spp. when encapsulated in calcium
alginate were found to survive better in fermented frozen desserts as well as ice cream (Shue et al., 1993; Shah and Ravula, 2000). Kebary et al. (1998) also reported that entrapping 

*B. bifidum* and *B. infantis* in alginate or k-carrageenan beads improved their viability in frozen ice milk throughout the storage period (-20°C for 10 weeks) from 43-44% to about 50-60%. Furthermore, bifidobacteria survived better in beads made from alginate than those made from k-carrageenan. Sultana et al. (2000) modified the method of calcium alginate encapsulation and found that incorporation of a prebiotic (starch) improved the microencapsulation of viable probiotic bacteria as compared to when the bacteria were encapsulated without the starch. The survival of calcium alginate-starch encapsulated *L. acidophilus* and *Bifidobacterium* spp. was observed to be better than that observed with free cells over a 8 week storage period in yoghurt. Encapsulation of lactobacilli and bifidobacteria has been reported to protect the cells from lyophilization and rehydration (Kim et al., 1996). Microencapsulation has also been cited for protecting cells of *Bifidobacterium* spp. in cheese (Dinakar and Mistry, 1994) and in mayonnaise (Khalil and Mansour, 1998).

Apart from yoghurt acidity, conflicting reports exist on the protection offered by microencapsulation from gastric conditions. Shah and Ravula (2000) reported that probiotic bacteria encapsulated in calcium alginate were able to survive at pH 2.5 which approximates the stomach pH. Similarly, when *B. longum* entrapped in calcium alginate beads were exposed to simulated gastric juices and a bile salt solution, the death rate of the cells in the beads decreased proportionally with an increase in both the alginate gel concentration and the bead size (Lee and Heo, 2000). In other studies however, calcium alginate
microencapsulation was not able to prevent cell death at low pH (Trindade and Grosso, 2000; Truelstrup-Hansen et al., 2002).

Little is known about the protective effect of microencapsulation from oxygen toxicity. It has been reported that anoxic parts may appear in the centre of microbial aggregates trapped in a bead (Omar, 1993). Growth of aerobic organisms entrapped in calcium alginate beads was progressively reduced towards the interior of the beads presumably because of the impaired diffusion of oxygen and nutrients. The coating material retards the entry of oxygen into the beads, thereby contributing to lower oxygen level in the microenvironment. Hiemstra et al. (1993) entrapped cells of *Hansenula polymorpha* in 2% barium alginate gels and reported that the polymer network considerably restricted the diffusion of oxygen towards these cells. Beunik et al. (1989) showed that beads loaded with cells of *Enterobacter cloacae* showed a sharp decrease in oxygen concentration a few micrometres below the alginate surface. This suggests that microencapsulation can act as an oxygen barrier.

Overall, the technology of microencapsulation seems to present a tremendous potential for the effective delivery of cells or bioactive compounds. The protection delivered by the encapsulating matrix offers the means for the targeted delivery of compounds to locations where they are most needed, without adversely affecting the compounds themselves. Already this feature of microencapsulation has sparked off marketing of encapsulated probiotic supplements by various companies. Probiocap TM (microencapsulated *L. acidophilus* 50 ME in a hydrophobic matrix) sold by Institut Rosell claims to posses increased tolerance to gastric juices, improved survival during tableting, enhanced
temperature resistance during food processing and extended shelf life at room temperatures.

Chr. Hansen (www.chbiosystems.com) markets probiotic capsules as dietary supplements and infant formulas. The Jintan capsule technology manufactures encapsulated probiotic bifidobacteria with enteric function (Bifina tablet). Similarly, Geneflora™ manufactured and sold by BioPlus corporation (www.yeastbuster.com) contains encapsulated *Lactobacillus* spp. (Kailasapathy, 2002). Further research into the various applications of encapsulation will thus serve to enhance the bright prospects of microencapsulation in the food and health industry.

### 2.23 Stress adaptation of bacteria

The ability of microorganisms to adapt to adverse environments has been used in many strain development procedures to obtain strains capable of surviving unfavorable conditions. It is well known that exposing microorganisms to sub lethal or gradually increasing doses of stress can induce an adaptive cellular response that enables them to better resist lethal doses of stress. For example, Shah, (2000) reported the acid adaptation of *L. acidophilus*, which was performed by growing cells under optimal conditions to mid log phase and then exposing them to moderate acid conditions. When the acid adapted cells were introduced in normally lethal acidic conditions such as those encountered in yoghurt, an increase in the survival rate of these organisms was observed. A similar adaptive response was observed in *L. acidophilus* where cells exposed to sublethal levels of bile, heat and NaCl demonstrated increased survival under lethal levels of these stresses (Kim et al., 2001). Acid adaptation of *B. breve* was found to enable cells to withstand environmental stresses such as acidic
conditions, bile salt, hydrogen peroxide and cold storage (Park et al., 1995). Among other lactic acid bacteria, *Streptococcus pyogenes* demonstrated an inducible peroxide resistance response when treated with sub lethal doses of peroxide (King et al., 2000). The stability of the stress-adapted cells appears however to be strain dependent. Shah (2000) reported differences in the acid adaptive response among three *L. acidophilus* strains. While two acid adapted strains of *L. acidophilus* failed to maintain better viability during long-term storage in lethal acidic conditions, the remaining strain responded under both short term and long term exposure to lethal conditions of acidity.

It has been reported that induction of stress proteins in bacteria can also provides cross protection against a wide-variety of other stresses (Mekalonos, 1992). Lou and Yousef (1997) found that adaptation of *Listeria monocytogenes* to sub lethal doses of ethanol, hydrogen peroxide, salt and others stresses, significantly increased its resistance to lethal levels of these stresses and cross protected the organism to different stresses. Pretreatment of the anaerobic *E. coli* to hydrogen peroxide, besides substantially reducing the toxicity of a subsequent higher dose, resulted in *de novo* protein synthesis as well in cross protecting the cells from lethal amounts of aldehydes (Nunoshiba et al., 1991).

Oxidative stress, which includes bacterial responses to H\textsubscript{2}O\textsubscript{2}, is also considered to induce adaptive responses in anaerobes. Developing oxygen tolerant cells from oxygen sensitive strains is therefore plausible. Ahn et al. (2001) were able to develop an oxygen tolerant mutant of *B. longum* by growing the cells under a microaerobic atmosphere. Interestingly, the first ever indication of a biochemical oxidative stress response by *Bifidobacterium* spp.
was provided in this study when exposure to oxygen was found to induce a 35.5 kD protein in the oxygen tolerant *B. longum* J11. Additionally, Schell et al. (2002) found that *B. longum* contained three proteins namely thiol peroxidase, alkyly hydroperoxide reductase (*ahpC*), and the peptide methionine sulfoxide reductase that reverse oxidative damage to proteins and lipids. Apart from these studies however, no reports exist about any oxidative stress proteins in both *Bifidobacterium* spp. and *L. acidophilus*. Schmidt and Zink (2000) have however demonstrated cross protection in bifidobacteria wherein pretreatment of cells to salt resulted in increased tolerance after freeze thawing or lethal heat stress. Thus, exposure to sub lethal doses of stress can lead to a significantly increased survival under otherwise lethal homologous or heterologous stress conditions.

### 2.24 Packaging materials and diffusion of oxygen into yoghurt

Yoghurt is mostly packaged in high-impact polystyrene worldwide. Studies have shown that polystyrene is a poor gas barrier and allows diffusion of oxygen into yoghurt (Ishibashi and Shimamura, 1993; Dave and Shah, 1997d). It is therefore considered unsuitable for probiotic products. Although Dave and Shah (1997d) found that packing yoghurts in glass bottles preventing oxygen diffusion and improving the viability of probiotic bacteria, such measures can be hazardous and economically unviable for yoghurt manufacturers. Yoghurt manufacturers are therefore searching for cheaper and practical packaging solutions to prevent oxygen toxicity of probiotic bacteria in yoghurts.
In this regard, *active packaging*, an innovative food packaging concept that has emerged as a response to the current market demands, can be useful. Defined as a packaging that changes the condition of the packaging to extend shelf life or improve safety or sensory properties while maintaining the quality of food, active packaging includes special light-activated oxygen scavenging packaging. This technique involves the sealing of a small coil of ethyl cellulose film containing a dissolved photosensitive dye and a singlet O$_2$-acceptor in the packaging. When the film is exposed to light of the appropriate wavelength, the excited dye molecules sensitize O$_2$-molecules that have diffused into the polymer to the singlet state which are then consumed by the acceptor molecules (Vermeiren et al., 1999).

An example of such a light-activated scavenger film is Zero$_2^{\text{TM}}$ developed by the Commonwealth Scientific and Industrial Research Organization (CSIRO), Australia. This film can be used for forming part of many types of food packages such as in bottle walls, closures, multiplayer laminates and can linings. Once activated, Zero$_2^{\text{TM}}$ can remove oxygen from the package headspace as well as liquid foods, inhibiting aerobic microorganisms as well as preventing food oxidation (Rooney, 1995). This packaging system would however require an outer layer of high gas barrier material to prevent the oxygen scavenging film from being flooded by oxygen migrating through the package. An example of such a high gas barrier packaging is Nupak™.

Miller et al. (2002) evaluated the effect of high impact polystyrene and Nupak packaging on the dissolved oxygen content of yoghurt over its shelf life. As compared to the steady rise in the dissolved oxygen levels seen with polystyrene, no increase in the dissolved oxygen
levels was noted in the yoghurts packaged in the gas-barrier containing polystyrene layer (Nupak™). As compared to the high-impact polystyrene’s high oxygen permeation rate of 1.0-5.0 cc/kg/day, the diffusion rate of Nupac laminate is estimated to be around 0.005-0.01 ml/kg/day. This was considered responsible for the maintenance of low dissolved oxygen levels in the yoghurt during the entire storage period.

2.25 Summary of literature review

Much is known and documented about *L. acidophilus* and *Bifidobacterium* spp., their therapeutic benefits in humans and their incorporation into functional foods such as yoghurts. Although regulations exist on the number of viable probiotic bacteria required in probiotic products, there is confusion about the exact survival status of these bacteria during the storage period. Little is known about the various factors affecting the viability of these probiotic bacteria in yoghurts as well, especially with respect to oxygen.

Although oxygen is considered a key factor responsible for the decline in cell numbers, there is very little information regarding the interaction between oxygen and probiotic bacteria. The picture that has emerged from the research conducted so far is still far from clear. While there has been some advances in understanding the biochemistry behind oxygen intolerance, the variations in the oxygen tolerance between individual strains makes the picture even hazier. For screening of potentially good probiotic strains, it is of utmost importance to screen potential probiotic strains for oxygen tolerance to ensure their delivery to consumers in sufficiently high numbers. No technique is available yet for quantifying the oxygen
tolerance of probiotic bacteria. Activities of NADH oxidase and NADH peroxidase have been cited to play an important role in the oxygen tolerance of probiotic bacteria. The various assays for the estimation of NADH oxidase and NADH peroxidase suggested however contradict each other. A generally accepted standard assay is not available yet. Atmospheric oxygen has been demonstrated to diffuse into yoghurt through the currently used polystyrene packaging material. Addition of ascorbate and cysteine has generated successful results as oxygen scavengers in yoghurts but suffer from drawbacks that might affect the textural properties of the yoghurt. Packaging yoghurt in oxygen impermeable packaging materials like glass, although effective in protecting probiotic bacteria from oxygen toxicity, can be hazardous as well as financially non-viable to yoghurt manufacturers.

There remains a vast untapped potential for the use of oxygen scavenging film to maintain anoxic environments in probiotic dairy foods. Research combining oxygen scavenging packing material and its effect on viability of probiotic bacteria is unavailable. Proper cost effective techniques haven’t yet been devised to overcome the problem of oxygen toxicity in yoghurts. Stress adaptation and microencapsulation have yet been employed only as general protection strategies for probiotic bacteria and have not been applied specifically in relation to oxygen toxicity. As a result, the efficacy of probiotic foods remains very much curtailed. The maintenance of probiotic bacteria in high numbers in dairy foods can be brought about by a detailed study into the interactions between oxygen, probiotic bacteria and packaging material. Understanding the oxidative response of probiotic bacteria can help in facilitating the development and application of protective techniques such as stress adaptation and
microencapsulation. Eventually, this would help enable yoghurt manufacturers to ensure sufficiently high numbers of probiotic bacteria at the end of the expiry period as required by the food authorities.

In conclusion, there is a pressing need to understand in detail the problem of oxygen toxicity in yoghurt so that techniques can be developed to overcome it and thereby confer therapeutic benefits to the yoghurt consumers.
3 Materials and Methods

3.1 Strains and activation of culture

The probiotic strains *B. breve* CSOC 1900, *B. bifidum* CSOC 1909, *B. infantis* CSOC 1912, *B. lactis* CSOC 1941, *B. pseudolongum* CSOC 1944, *B. thermophilum* CSOC 1991, *L. acidophilus* CSOC 2400, *L. acidophilus* CSOC 2401, *L. acidophilus* CSOC 2404, *L. acidophilus* CSOC 2409, *L. acidophilus* CSOC 2415, *L. casei* CSOC 2603 and *L. helveticus* CSOC 2700 were obtained from the starter culture collection of the Commonwealth Scientific Industrial Research Organization (CSIRO), Australia. *B. lactis* 920 and *B. lactis* Bb-12 were obtained from DSM Food Specialties (Australia) and Chr. Hansen (Australia) respectively. *B. longum* 55815 was procured from the American Type Culture Collection (ATCC), U.S.A. *Micrococcus luteus* and *Propionibacterium acnes* were obtained from the culture collection department of the University of Western Sydney, Australia.

The probiotic cultures were obtained as freeze-dried samples. The samples were aseptically added to a small volume of Reconstituted Skim Milk (RSM) and mixed by Pasteur pipette aspirations until no lumps were visible. The culture was then added to 10 ml of RSM and incubated at 37°C until it coagulated (18-72h). This culture was diluted 1/10 with fresh 9.5% Reconstituted Skim Milk (RSM) supplemented with 0.5% yeast extract, 2% glucose and 0.05% cysteine and stored in the freezer at -20°C as the stock.
Unless otherwise stated, working cultures from these stocks were prepared by streaking a loopful of stock onto MRS agar containing 0.05% L-cysteine (Sigma, Australia). Plates were then incubated anaerobically at 37°C for 48-72 h. A single colony was used to inoculate MRS broth, which was then incubated anaerobically at 37°C for 18-24 hours to obtain a working culture. Similarly Nutrient Broth and Reinforced Clostridial medium were used for the cultivation of *M. luteus* and *P. acnes* respectively.

### 3.2 Media and reagent preparation

#### 3.2.1 RSM (Reconstituted Skim Milk) broth

RSM broth contained 9.5% skim milk powder, 0.5% yeast extract and 2% glucose. To prepare 100 ml of broth, 9.5 g 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% w/v glucose was mixed and autoclaved at 121°C for 15 min. After cooling, 8 ml of the 25% w/v glucose solution was aseptically added to the milk and yeast extract such that the final concentration of glucose was 2%.

#### 3.2.2 MRS (deMan-Rogosa-Sharpe) broth

MRS broth consists of 20 g/l glucose, 10 g/l peptone, 10 g/l lemco powder, 5 g/l yeast extract, 1 ml Tween 80, 2 g/l dipottasium hydrogen phosphate, 5 g/l sodium acetate, 2 g/l triammonium citrate, 0.2 g/l magnesium sulphate, 0.05 g/l managanese sulphate. This media
was obtained from Oxoid, Australia and prepared as per the manufacturers instruction. MRS agar was prepared by adding 1.5% w/v Technical Agar (Oxoid, Australia) to MRS broth. The suspension was then warmed in a microwave oven to dissolve the agar. The medium was then sterilized at 121°C for 15 min, cooled to approximately 45°C and poured into sterile disposable petri plates (Selby, Australia).

3.2.3 MRS-Salicin (MRS-S) agar

MRS-S contains all compounds as MRS except glucose, which was replaced by Salicin (Sigma, Australia). All components of MRS agar, except glucose were dissolved in distilled water and sterilized in an autoclave at 121°C for 15 min. A 10% w/v solution of salicin was sterilized separately and added to molten MRS agar to achieve a final concentration of 1% w/v.

3.2.4 MRS-Lithium propionate agar (MRS-LP)

MRS-LP was prepared by incorporating lithium chloride (0.2% w/v) (Sigma, Australia) and sodium propionate (0.3%) (Sigma, Australia) to MRS broth. The medium was sterilized in an autoclave at 121°C for 15 min before use.
3.2.5 Peptone water (diluent)

Dehydrated Peptone water was obtained from Oxoid, Australia and rehydrated solution of 1\% w/v was prepared as per the manufacturers instructions. The solution was sterilized in an autoclave at 121°C for 15 min before use.

3.2.6 Phosphate buffer

One molar disodium phosphate was added to 1M monosodium phosphate until the desired pH was reached. The 1M buffer was then diluted with distilled water to appropriate concentrations as required and sterilized in an autoclave at 121°C for 15 min before use.

3.3 Incubation conditions

Unless stated otherwise, broths and plate cultures were incubated at 37°C under anaerobic conditions, which were maintained either by Anaerogen packs (Oxoid, Australia) or by a hypoxic glove chamber (Coy Laboratory Products, U.S.A.) that maintained an atmosphere of 95% nitrogen and 5% hydrogen.
3.4 Preparation of cultures for incorporation into yoghurt

A working culture of the strain was prepared as described in Section 3.1. One hundred microlitres of the working culture was then inoculated at 37°C for 48-49 h in larger volumes of MRS broth until the medium became turbid. The turbidity was measured by spectrophotometer and the maximum OD was taken as equivalent to the exponential growth phase of the particular strain of bacteria. The culture broth was then centrifuged at 6000 x g for 10 min, washed with an equal volume of 0.1M phosphate buffer, pH 7.0 and re-centrifuged. The resulting cell pellet was resuspended in the smallest volume of RSM and frozen at -20°C overnight. The frozen culture was then freeze-dried using a Braun Biotech International freeze dryer. The freeze-dried culture was then incorporated into yoghurt mix as required.

3.5 Counts of probiotic bacteria from yoghurts

Ten grams of each yoghurt sample were suspended in 100 ml of 0.1% peptone water and homogenized in a stomacher for 2 min. The homogenised suspension was serially diluted using 0.1% peptone water and 100 µl of the appropriate dilutions was spread plated on the selective or differential media in triplicate. Unless stated otherwise, all media plates were incubated anaerobically at 37°C for 48 h before enumerating the colonies. Plates containing 25 to 250 colonies were enumerated and the mean of six determinations was used to calculate the colony forming units per gram of yoghurt.
3.6 Measurement of dissolved oxygen

The dissolved oxygen of either reagents or yoghurts was measured using a Microelectrodes MI-730 Clark type oxygen electrode dip-type micro-oxygen electrode and OM4 oxygen electrode ((Microelectrodes Inc., U.S.A.). Before each use, the electrode was calibrated using pure nitrogen and oxygen gas.

3.7 Measurement of pH

The pH of reagents and yoghurt samples was measured using a freshly calibrated inoLAB pH Level 1 meter (WTW Gmbh, Germany).

3.8 Preparation of cell free extract

The washed cell pellet was resuspended in a small volume of 0.1M phosphate buffer, pH 7.0. Three ml of the cell suspension was then added to the pressure chamber of a French® Pressure Cell (Thermospectronic, U.S.A.) and subjected to a pressure of 20,000 psi at room temperature. Cells were disrupted by slowly releasing the pressure through a tiny nozzle at the base of the pressure cell. The suspension containing cell wall debris and the cytoplasmic contents was then centrifuged for 15 minutes at 12,000 x g at 4°C to obtain the cell free extract.
3.9 SDS-PAGE of cell free extracts

SDS-PAGE of cell free extracts was carried out with a vertical slab gel unit (Biorad, Australia) on a precast 4-20% Tris Glycine iGel (Gradipore, Australia) using a SDS Glycine running buffer as given below:

**SDS Glycine Running Buffer (10X)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trisma Base (Sigma, Australia)</td>
<td>29 g</td>
</tr>
<tr>
<td>Glycine (Sigma, Australia)</td>
<td>144 g</td>
</tr>
<tr>
<td>SDS Electrophoresis Grade (Sigma, Australia)</td>
<td>10 g</td>
</tr>
<tr>
<td>Deionised water to</td>
<td>1.0 l</td>
</tr>
</tbody>
</table>

The buffer was diluted 1 in 10 with deionised water. The pH of the 1X buffer was 8.3.

Samples were mixed with sample buffer, which was prepared as given below:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% (w/v) SDS Electrophoresis Grade</td>
<td>4 ml</td>
</tr>
<tr>
<td>Glycerol (Sigma, Australia)</td>
<td>2 ml</td>
</tr>
<tr>
<td>0.1%(w/v) Bromophenol blue (Sigma, Australia)</td>
<td>1 ml</td>
</tr>
<tr>
<td>0.5M Tris-HCl, pH 6.8</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>β Mercaptoethanol (Sigma, Australia)</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Deionised water to</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

The sample buffer containing protein (100 µl of buffer per mg of protein) was heated for 3-5 min at approximately 100°C. The samples were then clarified by centrifugation at
6,000 rpm for 3 min. 20 µg of protein was loaded per lane, and electrophoresis was performed at 150 mV until the tracking dye (Bromophenol blue) reached the bottom of the gel (approximately 90 min). The gel was stained with Coomassie Blue R-250 (Sigma, Australia) for visualization. Broad range molecular weight standards (Sigma, Australia) were run in parallel.

Destaining of the gel was carried out using the Fairbanks destaining protocol (Gradipore, Australia):

One hundred ml of a solution containing 10% v/v acetic acid was poured over the gel. A piece of tissue paper was placed in the solution to absorb the excess dye. The gel with the destaining solution was microwaved for 1 min until boiling. The gel was then left shaking in the destaining solution for 15 minutes. This process was repeated for two to three times until a clear background was obtained.

The protein bands developed were scanned using a GS-340 scanning densitometer (Hoeffer, U.S.A). The Rf values of the band peaks, % area under the peak and the relative percentage of the peaks were determined using the GS-340 software for comparative analysis.
4 Chapter 1: Quantification of oxygen tolerance in probiotic bacteria

4.1 Abstract

In order to characterize the oxygen tolerance of probiotic bacteria, a quantitative measurement of their oxygen sensitivity is essential. So far, studies on oxygen tolerance of lactobacilli and bifidobacteria have focussed only on qualitative and subjective estimations. In this study, a methodology called as the Relative Bacterial Growth Ratio (RBGR) was modified to quantify the oxygen tolerance of several probiotic bacteria for the first time.

Using a shake flask broth culture, RBGR is obtained by dividing the absorbency of aerobic growth by the absorbency of anaerobic growth. Probiotic strains were grown in MRS-cysteine in both aerobic and anaerobic conditions and their RBGR was measured. Anaerobic conditions were created by deoxygenating the medium with nitrogen. Strains were found to differ widely in their oxygen tolerance. The RBGR values ranged from 0.70 and 0.43 for \textit{L. acidophilus} CSCC 2400 and CSCC 2409 respectively to 0.05 and 0.78 for \textit{B. breve} CSCC 1900 and \textit{B. infantis} CSCC 1912, respectively. The methodology is simple and can be used to obtain a quantitative index of oxygen tolerance of several probiotic strains.

4.2 Introduction

The sensitivity of probiotic lactobacilli and bifidobacteria to oxygen is considered an important factor affecting their extended survival in yoghurts. Studies conducted so far have mostly employed qualitative techniques to measure the oxygen tolerance of probiotic bacteria (de Vries and Stouthamer, 1969; Uesugi and Yajima, 1978; Archibald and Fridovich, 1981; Shimamura et al., 1992; Meile et al., 1997; Shin and Park, 1997) The qualitative nature of these studies introduces a factor of subjectivity when measuring the oxygen sensitivity of strains. Moreover, these techniques can be tedious and time consuming for yoghurt manufacturers and commercial culture companies to screen a large number of probiotic microorganisms for oxygen tolerance. In order to characterize the oxygen tolerance of probiotic bacteria, a quantitative measurement of their oxygen sensitivity is essential. A need therefore exists for a simple, cheap and practical methodology to quantify the oxygen tolerance of probiotic bacteria measurement of the oxygen tolerance of the probiotic strains before incorporating them in yoghurts.

Kikuchi and Suzuki (1986) proposed a method for the quantification of the aerotolerance for oral indigenous anaerobes. The method is based on finding the Relative Bacterial Growth Ratio (RBGR), which is obtained by dividing the absorbency of growth of aerobically shaken culture to the growth of anaerobically shaken culture. Accordingly, RBGR values form a scale ranging from $\infty$ with obligate aerobes to 0 with obligate anaerobes. This therefore permits a quantitative measurement of oxygen tolerance in bacteria.
4.3 Aims and objectives

The aim of this study was to quantify the oxygen tolerance of probiotic strains. The objective of this study was to modify and optimize the RBGR methodology and apply it for the screening of a group of probiotic bacterial strains.

4.4 Materials and methods

4.4.1 Strains and culture conditions


4.4.2 Modification and validation of the RBGR methodology

Kikuchi and Suzuki (1986) used L-form culture tubes containing 5 ml of culture medium to determine the RBGR. These tubes were shaken at 400 rpm at 37°C for 24 h. It is well known that flasks are better suited to shaking conditions. Similarly, a better estimate of the bacterial
growth characteristics can be obtained when the culture broth is present in sufficient quantities. Thus, to provide extra ease, simplicity and a better representation of the RBGR of probiotic bacteria, the L-form tubes were replaced with 250 ml Erlenmeyer flasks containing 100 ml of culture medium in this study. The protocol therefore needed to be optimized for the Erlenmeyer flasks. The creation and maintenance of suitable anaerobic conditions in the flasks was achieved by the deoxygenation of the media as shown in Fig 4.
Figure 4. Deoxygenation of medium for the estimation of RBGR

1. Boiling broth sparged with nitrogen gas (5 psi) for 5 minutes

2. Culture inoculated in cooled deoxygenated broth

3. Deoxygenated culture broth sealed with rubber bung
Initially, to create low redox conditions, 0.05% w/v L-cysteine was added to MRS broth, the sterilized medium was then deoxygenated by sparging nitrogen gas in boiling medium for 5 min (Step 1). To indicate anaerobic conditions, resazurin, a redox-indicator dye, was added to the medium at a concentration of 0.002% w/v. At low redox potentials and in absence of oxygen, resazurin, which imparts pink color to the medium, undergoes a reversible reduction to dihydroresorufin, which is colorless. The creation of anaerobiosis in the flask was therefore monitored by the disappearance of pink color from the medium.

Once the medium became anaerobic, the flasks were placed in a water bath for the medium to cool down to temperatures between 30-37°C, which are suitable for inoculation (Step 2). Sparging the medium with nitrogen during the cooling process prevented the entry of oxygen into it.

Nitrogen supply was then removed from the deoxygenated and sufficiently cooled medium and the flask was sealed immediately with a rubber stopper (Step 3). The sealed flasks were then incubated at 100 rpm at 37°C for 48 h.

No recolourization of MRS-C was observed in the airtight flasks even after 48 h of incubation indicating successful anaerobiosis. Removing the stopper however caused the broth to rapidly acquire a pink colour due to the diffusion of oxygen into the medium. This confirmed that using the above protocol, anaerobiosis could be created and maintained in
MRS-C at least for 24 h at 37°C under shaking conditions. As a final confirmatory step, the RBGR of *Micrococcus luteus*, a fastidious aerobe and *Propionibacterium acnes*, a fastidious anaerobe was determined using this methodology. The values obtained were found to conform to the expected values of infinity and zero respectively.

### 4.4.3 Determination of RBGR

One hundred microlitres of an 18 h culture was added to two separate flasks containing 100 ml of MRS-C for aerobic and anaerobic growth. For aerobic growth, the flask was plugged with cotton wool whereas for anaerobic growth, the medium was deoxygenated and the flask sealed using the method described in Section 4.3.2. Inoculated flasks were incubated on a shaker at 100 rpm at 37°C for 24h. The optical density of the broth was then recorded at 600nm using a Spectronic 20D spectrophotometer. The RBGR of the culture was determined by dividing the absorbency of aerobic growth by the absorbency of the anaerobic growth and was a mean of nine readings. The entire experiment was performed in duplicate.

### 4.5 Results

The RBGR values of the various probiotic strains are listed in Table 7. Of the five *L. acidophilus* strains, strains CSCC 2400, CSCC 2401 and CSCC 2404 revealed RBGR values of 0.70, 0.67 and 0.73, indicating good aerotolerance. Similarly, *L. casei* CSCC 2603, demonstrated good resistance to oxygen with a RBGR value of 0.84.
Among the six *Bifidobacterium* spp. screened, only *B. infantis* CSCC 1912 and *B. lactis* 920 were found to have a RBGR value closer to 1.0. All remaining *Bifidobacterium* strains grew poorly under aerobic conditions with RBGR values closer to 0.
Table 7. The Relative Bacterial Growth Ratio (RBGR) of probiotic strains

<table>
<thead>
<tr>
<th>Organism type and strain</th>
<th>Growth ($A_{600}$) 37° C, 24h, 100rpm</th>
<th>RBGR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aerobically shaken</td>
<td>Anaerobically shaken</td>
</tr>
<tr>
<td><em>Lactobacillus acidophilus</em></td>
<td>2400</td>
<td>1.17</td>
</tr>
<tr>
<td></td>
<td>2401</td>
<td>1.28</td>
</tr>
<tr>
<td></td>
<td>2404</td>
<td>1.39</td>
</tr>
<tr>
<td></td>
<td>2409</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>2415</td>
<td>0.66</td>
</tr>
<tr>
<td><em>L.casei</em></td>
<td>2603</td>
<td>1.22</td>
</tr>
<tr>
<td><em>L.helveticus</em></td>
<td>2700</td>
<td>0.16</td>
</tr>
<tr>
<td><em>Bifidobacterium breve</em></td>
<td>1900</td>
<td>0.08</td>
</tr>
<tr>
<td><em>B.bifidum</em></td>
<td>1909</td>
<td>0.01</td>
</tr>
<tr>
<td><em>B.infantis</em></td>
<td>1912</td>
<td>1.48</td>
</tr>
<tr>
<td><em>B.animalis</em></td>
<td>1941</td>
<td>0.01</td>
</tr>
<tr>
<td><em>B.pseudolongum</em></td>
<td>1944</td>
<td>0.03</td>
</tr>
<tr>
<td><em>B.thermophilum</em></td>
<td>1991</td>
<td>0.02</td>
</tr>
</tbody>
</table>

All strains were CSCC strains

* indicates aerotolerant cultures

Mean of nine determinations, s.d range = 0.001-0.007
4.6 Discussion

Theoretically, *L. acidophilus* is considered as microaerophilic and more tolerant to oxygen than bifidobacteria, which are considered strictly anaerobic and extremely sensitive to oxygen. In this study too, *L. acidophilus* strains generally demonstrated a better tolerance to oxygen than the *Bifidobacterium* spp. On the other hand, some bifidobacteria exhibited high RBGR values suggesting that they were able to grow well in the presence of oxygen. The RBGR values affirm the extreme sensitivity of bifidobacteria to oxygen when they are grown in optimum conditions and the necessity to screen potential probiotic strains for oxygen sensitivity.

4.7 Conclusion

In this study, the Relative Bacterial Growth Ratio (RBGR) methodology was successfully modified to demonstrate and quantify the oxygen tolerance of several probiotic bacteria for the first time. This methodology can assist in differentiating the oxygen sensitive strains from those that are tolerant to oxygen. Such screening of probiotic bacteria can help in characterizing potential strains so that only robust strains are incorporated in yoghurts. The modified RBGR methodology is simple, cheap and requires less time as compared to earlier studies on the oxygen tolerance of probiotic bacteria. Moreover, this methodology can be easily applied to screen several strains for oxygen tolerance. The application of this simple and easy methodology by yoghurt manufacturers or commercial culture companies can further facilitate the maintenance of high numbers of probiotic bacteria in yoghurts throughout its manufacture and storage period.
Chapter 2: Development of a standard assay for the determination of NADH oxidase in the presence of NADH peroxidase in lactic acid bacteria

5.1 Abstract

The complexity of the NADH oxidase: NADH peroxidase enzyme system in LAB makes it difficult to accurately determine the individual concentrations of both these enzymes. This study describes the development of a standard spectrophotometric assay for this enzyme system. Pure NADH oxidase and NADH peroxidase were mixed in various proportions and the percentage recovery was estimated by both the currently available assay as well by the improved assay proposed in this study. The recovery of NADH oxidase using the currently available assay ranged from as low as -200 to as high as +102% as against 90-102% in the improved assay. The recovery of NADH peroxidase ranged from 91-112% in both assays. The improved assay can further help to distinguish between NADH: H₂O oxidase and NADH: H₂O₂ oxidase and was successfully applied to identify the type of NADH oxidase in six LAB strains. This study thus developed a standard assay for the accurate determination of NADH oxidase levels in lactic acid bacteria possessing a coupled NADH oxidase: NADH peroxidase enzyme system.

5.2 Introduction

Anaerobic lactic acid bacteria have to rely on non-haem flavoproteins that act as NADH oxidases and peroxidases that protect against oxygen toxicity for better survival (Dolin, 1961; Condon, 1987). NADH oxidising enzymes catalyze the one, two, or four electron reduction of $O_2$ to $O_2^-$, $H_2O_2$, or $H_2O$ (Higuchi et al., 2000). It is widely accepted that a typical assay of NADH oxidase measures the initial linear slope of NADH oxidation at 340nm. in the presence of cell free extract and air-saturated buffer (de Vries and Stouthamer, 1969; Anders et al., 1970; Uesugi and Yajima, 1978; Carlsson et al., 1983; Thomas and Pera, 1983; Schmidt et al., 1986; Smart and Thomas, 1987; Cox and Marling, 1992; Shimamura et al., 1992; Higuchi et al., 1993; Shin and Park., 1997; Yi et al., 1998; Marty-Teysset et al., 2000).

Although this assay is suitable for lactic acid bacteria having only NADH oxidase, it is insufficient for estimating the levels of NADH oxidase in organisms in which NADH peroxidase is also present. As the product of a NADH: $H_2O_2$ oxidase reaction i.e. $H_2O_2$ is also the substrate for NADH peroxidase, the slope of NADH oxidation (oxidase activity) is actually a sum of the total NADH oxidised by the activities of both oxidase and peroxidase. While this has not been reported in some published literature, other researchers have had to perform amperometric methods in order to determine individual levels of NADH oxidase based on the oxygen uptake (Anders et al., 1970; Carlsson et al., 1983; Thomas and Pera, 1983; Smart and Thomas, 1987; Cox and Marling, 1992; Shimamura et al., 1992).
Considerable variation also exists in the assays reported to measure NADH peroxidase. Shimamura et al. (1992) have estimated activities of NADH peroxidase by measuring the consumption of H$_2$O$_2$ under anaerobic conditions. Others have assayed NADH peroxidase activity independently by measuring the slope of NADH oxidation under anaerobic conditions (Anders et al., 1970; Carlsson et al., 1983; Thomas and Pera, 1983; Smart and Thomas, 1987; Shin and Park, 1997). DeVries and Stouthamer (1969) and Uesugi and Yajima (1978) estimated NADH peroxidase as the slope difference in presence and absence of H$_2$O$_2$ under aerobic conditions, whereas the same slope difference obtained under anaerobic conditions was used by Higuchi et al., (1993) for the measurement of NADH peroxidase.

Under aerobic conditions and in absence of H$_2$O$_2$ however, the activity of NADH peroxidase will be dependent solely on the rate of production of H$_2$O$_2$ by NADH oxidase. This introduces a substrate limitation step for NADH peroxidase. As against this, under anaerobic conditions and in excess H$_2$O$_2$, the reaction velocity of NADH peroxidase would be maximum. For the subtraction method to be accurate (DeVries and Stouthamer, 1969; Uesugi and Yajima, 1978; Higuchi et al. 1993), the reaction velocities of NADH peroxidase in presence as well as absence of excess H$_2$O$_2$, need to be at their maximum, else it would lead to inaccurate estimations of NADH oxidase.

As is evident, the interconnectedness of the coupled NADH oxidase: NADH peroxidase enzyme system makes it difficult to simultaneously determine the individual levels of both these enzymes. A standard spectrophotometric assay for accurately determining the levels of NADH oxidase and NADH peroxidase from such a coupled oxidase: peroxidase system has not been reported yet.
5.3 Aims and Objectives

The aim of this study was therefore to develop a spectrophotometric assay for the accurate determination of the concentrations of NADH oxidase and NADH peroxidase from the coupled NADH oxidase: NADH peroxidase enzyme system. The objective of this study was to validate the assay using pure NADH oxidase and NADH peroxidase and test its suitability in LAB such as *L. acidophilus* and *Bifidobacterium* spp.

5.4 Materials and methods

5.4.1 Enzymes

Pure NADH oxidase and NADH peroxidase (E.C. 1.11.1.1) were obtained from Calbiochem, U.S.A and Sigma- Aldrich, U.S.A. respectively. Stock solutions of 1.0 Unit (U)/ml of each enzyme were prepared in appropriate diluents as per in the manufacturers instructions. Suspensions of oxidase and peroxidase units mixed in different proportions were used for the assays. One unit of NADH oxidase was defined as the amount of enzyme catalyzing the oxidation of 1nmole NADH per min at 30°C. One unit of NADH peroxidase was defined as the amount of enzyme catalyzing the oxidation of 1nmole H₂O₂ per min at 30°C.
5.4.2 Enzyme Assay

a. Estimation of NADH oxidase by the currently available assay (de Vries and Stouthamer, 1969; Anders et al., 1970; Uesugi and Yajima, 1978; Carlsson et al., 1983; Thomas and Pera, 1983; Schmidt et al., 1986; Smart and Thomas, 1987; Cox and Marling, 1992; Shimamura et al., 1992; Higuchi et al., 1993; Shin and Park, 1997; Yi et al., 1998; Marty-Teysset et al., 2000).

The reaction system consisted of NADH (67µM), FAD (67µM) and Bis-Tris buffer 0.1M, pH 6.0 in a total volume of 3 ml. The reaction mix contained 5U, 10U, 15U or 20U of NADH oxidase and NADH peroxidase combined in different proportions (Table 9). The assays were conducted at 30°C under aerobic conditions. The decrease in the absorbance of NADH at 340 nm was measured for a period of three minutes using a Biochrom 4060 spectrophotometer. The initial linear slope of NADH oxidation was recorded using a Reaction Kinetics software (Biochrom). The molar extinction coefficient of NADH at 340 nm (6.22 x 10³/M/cm) was used for calculating the enzyme units.

b. Estimation of NADH peroxidase by the currently available assay (Thomas and Pera, 1983; Smart and Thomas, 1987; Shin and Park, 1997)

H₂O₂ (1mM) was incorporated in the reaction mix given above and the assay was conducted under anaerobic conditions.
c. Estimation of NADH oxidase and NADH peroxidase by the improved assay

The reaction mix was the same as reported for estimating NADH peroxidase by the currently available assay except that the assay was conducted under both aerobic and anaerobic conditions.

In both the currently available assay as well as in the improved assay, NADH oxidase was estimated by converting the slope of the aerobic assay into enzyme units/cuvette. In addition, a separate estimation of NADH oxidase was also performed by subtracting the slope of anaerobic assay from that of the aerobic assay and converted to enzyme units/cuvette using the following formula:

$$\text{Units/cuvette} = \left( \frac{\Delta A_{340} \times 3}{6.22} \right)$$

where $\Delta = \text{difference in the slopes}$, $A = \text{absorbance at 340 nm}$. These recovered enzyme units were then compared to the actual NADH oxidase enzyme units introduced. NADH peroxidase was estimated by converting the slope of the anaerobic assay into enzyme units/cuvette by the above-mentioned formula. This was compared with the number of NADH peroxidase units added. The percentage recovery was then calculated for both enzymes.

For the anaerobic assay, the reactants were prepared in the anaerobic glove box containing 95% $N_2$ and 5% $H_2$ and kept in an anaerobic condition for 24 hours prior to the assay. Nitrogen gas was bubbled through the reactants before the determination and the dissolved oxygen in the reactants was ensured to be zero. No increase in oxygen was recorded within 5 min in the cuvette containing the anaerobic reaction mix.

Additionally, the respective blanks were performed before conducting the assays. The concentration of the reactants in the blanks was the same as that of the actual assay. The
mean of six individual determinations was used for calculation and a Student’s t-test was performed ($\alpha = 0.05$).

**5.4.3 Preparation of cell free extract and slope of NADH oxidation**

*L. acidophilus* CSCC 2400, *L. acidophilus* CSCC 2409, *B. infantis* CSCC 1912, *B. lactis* CSCC 1941, *B. pseudolongum* CSCC 1944 and *B. longum* 55815 were grown anaerobically for 24 h. Cells were harvested by centrifugation for 10 min at 10,000 x g at 4°C and the cell pellet was washed thrice with 0.1M phosphate buffer, pH 7. The cell free extract was prepared as given in Section 3.8.

Pure enzymes in reaction system of all the above-mentioned assays were replaced by an appropriate volume of cell free extract and the slope of NADH oxidation was recorded. A previously boiled cell free extract was used to negate the possibility of non-enzymatic oxidation of NADH.

**5.5 Results**

**5.5.1 Assay blanks**

A blank containing NADH, FAD, H$_2$O$_2$ and buffer showed no decrease in absorbance over the time of the assay under both aerobic and anaerobic conditions. The activity of NADH oxidase alone under aerobic conditions was not affected in the presence of 1mM H$_2$O$_2$. Under anaerobic conditions however, no NADH oxidase activity was noticed. When 1mM H$_2$O$_2$ was incorporated in the NADH oxidase free assay mix, NADH
peroxidase demonstrated the same activity under both aerobic and anaerobic conditions. In the absence of \( \text{H}_2\text{O}_2 \), no decrease in absorbance was observed in both aerobic and anaerobic assays.

### 5.5.2 Recovery of NADH oxidase

When the levels of NADH oxidase were determined from just the aerobic assay slope, all combinations of NADH oxidase with NADH peroxidase showed significantly higher (\( p< 0.05 \)) recovery levels of NADH oxidase than what was introduced in the cuvette. This was noted in both the currently available assay as well as in the improved assay.

The recovery of NADH oxidase was determined from the subtraction of the anaerobic assay slope from the aerobic assay slope (Figure 5). Considerable variation was observed in the recovery of NADH oxidase by the currently available assay. When suspensions containing 5U NADH oxidase with 15U and 20U NADH peroxidase were assayed, subtracting the peroxidase slope from the oxidase slope gave negative values. Consequently, the recovery too was negative. In suspensions containing 10U NADH oxidase and 15U NADH peroxidase, the subtraction of the slopes gave a recovery value of only 4.18U of NADH oxidase, whereas in suspensions containing 15U NADH oxidase and 20U NADH peroxidase, 11.01U of NADH oxidase were obtained after calculation. For the above mentioned enzyme combinations however, the improved assay suggested in this study demonstrated no significant difference (\( p>0.05 \)) between the values of NADH oxidase introduced and that calculated from the slope of NADH oxidation.
The percentage recovery for NADH oxidase determined by subtracting the anaerobic assay slope from the aerobic assay slope is listed in Table 8.

Figure 5. Recovery of NADH oxidase in the presence of NADH peroxidase

CAA – Currently available assay

IA - Improved assay
Table 8. Comparison between percentage recoveries of NADH oxidase by the currently available assay and the improved assay

<table>
<thead>
<tr>
<th>NADH oxidase Units (U)</th>
<th>NADH peroxidase Units (U)</th>
<th>% recovery of oxidase</th>
<th>% recovery of peroxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Currently available assay</td>
<td>Improved assay</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Currently available assay</td>
<td>Improved assay</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>91.6 ± 10.1</td>
<td>99.6 ± 15.7 &lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>99.6 ± 4.9</td>
<td>91.6 ± 5.2 &lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>9.6 ± 13.6</td>
<td>93.2 ± 7.8 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>93.2 ± 2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100.4 ± 7.0 &lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>-94.8 ± 16.6</td>
<td>106.1 ± 17.2 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>98.0 ± 4.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>99.6 ± 4.0 &lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>-200.9 ± 11.2</td>
<td>98.0 ± 3.9 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>97.6 ± 2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>94.8 ± 1.9 &lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>101.2 ± 6.8</td>
<td>90.8 ± 6.4 &lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>101.2 ± 5.2</td>
<td>104.5 ± 9.4 &lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>86.0 ± 1.9</td>
<td>94.0 ± 6.6 &lt;sup&gt;a&lt;/sup&gt;</td>
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<td>102.9 ± 5.8 &lt;sup&gt;b&lt;/sup&gt;</td>
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<td>98.0 ± 3.2 &lt;sup&gt;a&lt;/sup&gt;</td>
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</table>

Mean ± s.d. (n=6)

<sup>a</sup> Non significant difference (p>0.05)

<sup>b</sup> Significant difference (p<0.05)
In the currently available assay, the recovery of NADH oxidase changed with differing oxidase-peroxidase ratios. When the enzyme suspension contained lower amounts of NADH oxidase than NADH peroxidase units, the calculated recovery ranged from 73% to as low as – 200%. For enzyme suspensions having equal or higher amounts of NADH oxidase than NADH peroxidase, the calculated recovery ranged from 86 to 102%. In the improved spectrophotometric assay however, the percentage recovery for NADH oxidase remained very high regardless of the proportion of NADH oxidase and NADH peroxidase units and ranged between 90-102% even at lower concentrations of oxidase. The means of the percentage recovery from the currently available assay and the improved assay were found to differ significantly (p<0.05) in enzyme suspensions where the amount of NADH oxidase units was less than that of NADH peroxidase units. In all the remaining enzyme suspensions where the proportion of NADH oxidase was either equal to or greater than NADH peroxidase, no significant difference (p>0.05) was observed among the means of the two assays.

5.5.3 Recovery of NADH peroxidase

It was interesting to note that in both assays, the values of NADH peroxidase approximated the number of units of NADH peroxidase introduced in the cuvette. The anaerobic conditions of the assay and the abundance of substrate (H₂O₂) ensured maximum activity of NADH peroxidase. Consequently, the values obtained through calculation showed similarity with the actual peroxidase units introduced. The proportion of oxidase and peroxidase units in the various enzyme suspensions did not affect the recovery of NADH peroxidase. No significant difference (p>0.05) was found between
the means of the two assays for NADH peroxidase. The means ranged from 91 to 107% for the currently available assay and from 91% to 112% in the improved assay (Table 8).

5.5.4 Slope of NADH oxidation in cell free extracts of LAB strains

No oxidation of NADH was observed when boiled cell free extract was used in the assays. Cell free extracts of all six bacterial strains oxidised NADH when assayed under anaerobic conditions and in presence of \( \text{H}_2\text{O}_2 \) (Table 9). The slope of NADH oxidation by the currently available assay differed from that obtained by the improved assay. Negative values were observed in \( \textit{B. infantis} \) CSCC 1912 and \( \textit{B. pseudolongum} \) CSCC 1944 when the slope of NADH peroxidase assay was subtracted from the slope of NADH oxidase assay (currently available assay). With the improved assay however, the difference in the slopes gave positive values for all the six strains.
Table 9. Differences in the estimation of NADH oxidases of six lactic acid bacteria by the currently available assay and the improved assay

<table>
<thead>
<tr>
<th>Strain</th>
<th>Slope of NADH oxidase assay (a)</th>
<th>Slope of NADH peroxidase assay (b)</th>
<th>Difference in slopes (a-b)</th>
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<tr>
<td></td>
<td>CAA *</td>
<td>IA #</td>
<td>CAA *</td>
</tr>
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<td>B. infantis CSCC 1912</td>
<td>0.10</td>
<td>0.22</td>
<td>0.15</td>
</tr>
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<td>B. lactis CSCC 1941</td>
<td>0.15</td>
<td>0.24</td>
<td>0.11</td>
</tr>
<tr>
<td>B. pseudolongum CSCC 1944</td>
<td>0.12</td>
<td>0.22</td>
<td>0.13</td>
</tr>
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<td>B. longum 55815</td>
<td>0.16</td>
<td>0.21</td>
<td>0.10</td>
</tr>
<tr>
<td>L. acidophilus CSCC 2400</td>
<td>0.70</td>
<td>1.10</td>
<td>0.52</td>
</tr>
<tr>
<td>L. acidophilus CSCC 2409</td>
<td>0.39</td>
<td>0.60</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Mean (n=6)

s.d range = 0.001-0.003

* CAA = Currently available assay

The reaction system of cell free extract, NADH (67µM), FAD (67µM) and Bis-Tris buffer 0.1M, pH 6.0 in a total volume of 3ml was assayed for 3 minutes at 30°C under aerobic conditions.

# IA = Improved assay

The reaction system of cell free extract, NADH (67µM), FAD (67µM), H₂O₂ (1mM) and Bis-Tris buffer 0.1M, pH 6.0 in a total volume of 3ml was assayed for 3 minutes at 30°C under aerobic conditions.
5.6 Discussion

The percentage recovery of the currently available assay was found to depend solely on the ratio of NADH oxidase and NADH peroxidase and changed as their proportions differed.

Some researchers have estimated NADH oxidase from just the aerobic assay slope (deVries and Stouthamer, 1969; Uesugi and Yajima, 1978; Shin and Park, 1997). As mentioned earlier, the slope of NADH oxidation in the aerobic assay is actually a sum of the total NADH oxidised by the activities of both oxidase and peroxidase. Enzyme units calculated from this slope would therefore result in elevated levels of NADH oxidase. This was confirmed by the significantly elevated recoveries of NADH oxidase obtained when its levels were determined by this method as also by elevated slopes of NADH oxidation by cell free extracts of the six bacterial strains (Table 9). This therefore suggests that the reported values of NADH oxidase where levels were determined from just the slope of aerobic assay may have been over-estimated.

Smart and Thomas (1987) have reported that their amperometric estimation of NADH oxidase correlated well with that obtained from the subtraction of the slope of the anaerobic assay from that of the aerobic assay. This suggests that one can subtract the slope of peroxidase (anaerobic assay slope) from the oxidase-peroxidase slope (aerobic assay slope) to accurately determine the levels of NADH oxidase spectrophotometrically. The difference in the reaction velocities of NADH peroxidase in these two assays however, can give rise to inaccurate estimations of NADH oxidase. This is confirmed in the negative recovery percentages of NADH oxidase obtained using the currently available assay (Fig. 4) and by the negative slope
differences in some of the bacterial strains tested (Table 9). As against this, in the improved assay developed in this study, the uniformity of the reactants in the aerobic and anaerobic assay ensured oxygen as the only variable affecting enzyme activities between these two assays. This guaranteed accurate estimations of NADH oxidase when the slope of NADH peroxidase was subtracted from the slope of the aerobic assay and was reflected in the high percentage recoveries of NADH oxidase as well as NADH peroxidase in all the different enzyme proportions tested (Table 8). This was further confirmed by positive slope differences in all the cell free extracts assayed (Table 9).

In many reports of NADH oxidases in LAB, the assay system used was based on the consumption of NADH. The end product however was not measured. This does not distinguish between H\textsubscript{2}O and H\textsubscript{2}O\textsubscript{2} forming NADH oxidases. This is further complicated by the fact that the activity of a H\textsubscript{2}O\textsubscript{2} forming NADH oxidase combined with that of an excess of NADH peroxidase is similar to a H\textsubscript{2}O forming NADH oxidase (Condon, 1987).

Although the improved assay proposed in this study was best suited for NADH: H\textsubscript{2}O\textsubscript{2} oxidase/NADH peroxidase system, it was also useful to distinguish between NADH: H\textsubscript{2}O\textsubscript{2} and NADH: H\textsubscript{2}O oxidases. This was achieved by performing an additional aerobic assay without the addition of any H\textsubscript{2}O\textsubscript{2} in the reaction system. It is evident that if the slopes of the aerobic assay in the presence and absence of H\textsubscript{2}O\textsubscript{2} are similar, then the enzyme in question was a NADH: H\textsubscript{2}O oxidase, regardless of the presence of any peroxidase. Further, if peroxidase was detected and the slope of the aerobic assay in the absence of H\textsubscript{2}O\textsubscript{2} was less than in presence of H\textsubscript{2}O\textsubscript{2}, then it was a NADH: H\textsubscript{2}O\textsubscript{2} oxidase. NADH peroxidase activity was detected in all the bacterial strains tested and
the slope of NADH oxidation in absence of H$_2$O$_2$ was less than in presence of H$_2$O$_2$ (Table 9). Accordingly, it can be concluded that all six strains possessed NADH: H$_2$O$_2$ oxidase.

5.7 Conclusion

In LAB containing NADH oxidase and NADH peroxidase, the proportion of these two enzymes can vary from strain to strain. In this study, sixteen different proportions were tested and the improved assay was found to demonstrate high accuracy in the recovery of both NADH oxidase (especially low levels) and NADH peroxidase regardless of the enzyme proportions. In comparison, the currently available assay was suitable only for determining individual levels of NADH peroxidase. When levels of NADH oxidase were low in comparison to NADH peroxidase, this assay gave inaccurate estimations of NADH oxidase. It is also clear that estimating the level of NADH oxidase from just the slope of the aerobic assay may lead to over estimation of the enzyme units. In addition, cell free extracts of six LAB did not interfere with the measurement of the slope of NADH oxidation by the improved assay. The improved assay developed in this study can thus perform as a standard assay for the determination of individual levels of NADH peroxidase from a suspension containing NADH oxidase and NADH peroxidase in lactic acid bacteria.
6 Chapter 3: Metabolic and Biochemical Responses of Probiotic Bacteria to Oxygen

6.1 Abstract

The interaction between oxygen and probiotic bacteria was studied by growing *L. acidophilus* and *Bifidobacterium* spp. in 0, 5, 10, 15, and 21% oxygen. The metabolic responses of each probiotic strain in the different oxygen concentrations were monitored by measuring the levels of lactic acid and determining the lactate to acetate ratio. Biochemical changes induced by oxygen were examined by monitoring the specific activities of NADH oxidase, NADH peroxidase and superoxide dismutase. In addition, the ability to decompose hydrogen peroxide and the sensitivity of each strain to hydrogen peroxide was also determined. With an increase in oxygen percentage, levels of lactic acid in *L. acidophilus* strains decreased whereas the lactate to acetate ratio reduced in all the bifidobacteria tested. The specific activities of NADH oxidase and NADH peroxidase, and the hydrogen peroxide decomposing ability of five probiotic strains increased progressively as the oxygen concentration was raised from 0 to 21%. The sensitivity of the probiotic strains to hydrogen peroxide however, remained unaffected in all the different oxygen percentages. Superoxide dismutase levels did not reveal any conclusive trend. In both *L. acidophilus* and *Bifidobacterium* spp., the optimum pH of activity of NADH oxidase and NADH peroxidase was 5. Changes were also detected in the cellular protein profiles of all strains as the oxygen concentration was increased.

6.2 Introduction

Although oxygen toxicity is considered a significant factor responsible for the loss in probiotic numbers in yoghurts (Brunner et al., 1993b; Klaver et al., 1993; Dave and Shah, 1997d), little is known about the interaction of oxygen with probiotic bacteria at the cellular level. Although bifidobacteria are considered as highly susceptible to oxygen, the oxygen tolerance of these organisms has been strain dependent (de Vries and Stouthamer, 1969; Shimamura et al., 1992; Talwalkar et al., 2001). Satisfactory growth of *Bifidobacterium* spp. in the absence of strict anaerobic conditions was observed by Cheng and Sandine (1989). In another study, *B. lactis*, isolated from fermented milk was found to display good oxygen tolerance (Meile et al., 1997).

It is believed that intracellular levels of H$_2$O$_2$ block fructose 6 phosphofructoketolase, a key enzyme in the sugar metabolism of bifidobacteria and therefore scavenging H$_2$O$_2$, becomes important for cell survival (de Vries and Stouthamer, 1969). Both *L. acidophilus* and *Bifidobacterium* spp. are devoid of catalase, a key enzyme for the breakdown of H$_2$O$_2$ and have to rely on enzymes such as NADH oxidase and NADH peroxidase to scavenge environmental oxygen (Condon, 1987). The activities of NADH oxidases in probiotic bacteria give rise to H$_2$O$_2$, prompting NADH peroxidase to scavenge H$_2$O$_2$ and prevent cell death. Shimamura et al. (1992) explored the biochemical mechanisms of oxygen sensitivity of several bifidobacteria and concluded that levels of NADH oxidase and NADH peroxidase play an important role in the prevention of oxygen toxicity. High levels of these enzymes were found in the most aerotolerant *Bifidobacterium* spp.
So far, oxidative studies on probiotic bacteria have mainly focussed on bifidobacteria (Shimamura et al., 1992; Ahn et al., 2001). Furthermore, in the reported studies on bifidobacteria and *L. acidophilus*, the cells were grown in either aerobic or partially aerobic conditions (Shimamura et al., 1992; Ahn et al., 2001). These undefined concentrations of oxygen may be unsuitable to identify definitive relationships between the effects of different oxygen concentrations on probiotic bacteria. Similarly, little is known about the biochemical response of *L. acidophilus* and *Bifidobacterium* spp. such as changes to the protein profile upon exposure to oxygen or the development of any oxidative stress proteins. Understanding the precise metabolic and biochemical changes influenced by known amounts of oxygen is crucial to prevent the problem of oxygen toxicity in probiotic bacteria.

### 6.3 Aims and Objectives

Therefore, the aim of this study was to monitor their physiological responses of *Bifidobacterium* spp. and *L. acidophilus* to various concentrations of oxygen. The objectives of the study were to grow the cells in 0, 5, 10, 15 and 21% oxygen using a hypoxic glove box and measure their metabolic and biochemical responses for every concentration of oxygen. While production of lactic acid and the lactate to acetate ratio were considered as representative of the metabolic activity of the cells, specific activities of NADH oxidase, NADH peroxidase and SOD, the ability of the strains to decompose known amounts of H₂O₂, the cellular protein profiles and the sensitivity of the probiotic strains to different H₂O₂ concentrations were regarded as biochemical indices of the probiotic strains.
6.4 Materials and Methods

6.4.1 Organisms and culture conditions

*Lactobacillus acidophilus* CSCC 2400, *L. acidophilus* CSCC 2409, *B. infantis* CSCC 1912, *B. lactis* CSCC 1941, *B. pseudolongum* CSCC 1944 and *B. longum* 55815 were used in this study. One hundred microlitres of an 18 h old inoculum of these strains grown anaerobically in MRS broth with $A_{600nm}$ of 0.6 was added aseptically to 200 ml of MRS broth in a 500 ml conical flask and stoppered with a cotton plug. Each strain was grown under 0, 5, 10, 15, and 21% oxygen at 37°C for 24 h using the hypoxic glove box. At 0% oxygen, the glove box contained a gaseous atmosphere of 95% N$_2$ and 5% H$_2$. The various oxygen concentrations in the glove box were created by replacing hydrogen with oxygen and adjusting the nitrogen levels accordingly. Each culture was tested in duplicate. The flasks containing the culture broth were agitated using a magnetic stirrer. The culture broth after incubation was centrifuged at 10,000 x $g$ for 20 min at 4°C. The cell free supernatant was used for the estimation of lactic acid and acetic acid. The cell pellet was washed thrice with 0.1M phosphate buffer, pH 7.0, and part of it was used for the determination of H$_2$O$_2$ decomposing ability and the sensitivity to H$_2$O$_2$. The remaining cell pellet was used for preparing the cell free extract.

6.4.2 Preparation of cell free extract

Cell free extract was prepared from the washed cell pellet suspended in 0.1M phosphate buffer (pH 7) as given in Section 3.8. The cell free extract was used for assaying levels of NADH oxidase, NADH peroxidase, and SOD as well as for estimating the cellular...
protein profile by conducting SDS-PAGE. The protein content of the cell free extract was determined according to Bradford (1976) using bovine serum albumin as the standard.

6.4.3 H\textsubscript{2}O\textsubscript{2} sensitivity assay

The sensitivity of the cells to H\textsubscript{2}O\textsubscript{2} was assayed based on the method reported by Shimamura et al. (1992). Cells were exposed to 10,000 mg/l, 20,000 mg/l, and 30,000 mg/l of H\textsubscript{2}O\textsubscript{2} for 1 min. Appropriate dilutions of the cell suspension exposed to H\textsubscript{2}O\textsubscript{2} were spread plated on MRS agar. Plates were incubated under anaerobic conditions at 37°C for 48 h and the cell counts were enumerated.

6.4.4 H\textsubscript{2}O\textsubscript{2} decomposing ability

The ability of the cell pellet to decompose H\textsubscript{2}O\textsubscript{2} was determined based on method reported by Shimamura et al. (1992). Known amount of cells were incubated anaerobically with 300 nmol H\textsubscript{2}O\textsubscript{2} at 37°C for 1h. The concentration of residual H\textsubscript{2}O\textsubscript{2} in the test tube after incubation was estimated by the method described by Marty-Teysset et al. (2000). The assay mixture contained 0.4 mM phosphate buffer (pH 6.9), 2% H\textsubscript{2}O\textsubscript{2}-saturated phenol, 0.4 mg of 4-aminoantipyrine (Sigma) per ml, and 0.04 U of peroxidase per ml, and the change in the absorbance was measured at 505 nm with an extinction coefficient of $\varepsilon = 6,400/ \text{M/ cm}$ for the quinoneimine formed.
6.4.5 Determination of lactic acid and acetic acid levels

The cell free broth was clarified using Carrez reagents. Five ml of Carrez –I- solution [Potassium hexacyanoferrate (II), 85mM] and 5ml of Carrez –II- solution (Zinc sulfate, 250mM) were added to 60ml of distilled water containing 10 ml of the cell free broth. The pH of the solution was adjusted to 8.0 using 0.1N NaOH and the volume was made up to 100 ml with distilled water. The solution was mixed with activated charcoal (1%), agitated and then filtered. The concentrations of lactate and acetate in the clarified broth were determined using commercially available kits (Boehringer Mannheim) and used for the calculation of the lactate to acetate ratio in the Bifidobacterium spp.

6.4.6 Enzyme assays

Activities of NADH oxidase and NADH peroxidase were assayed spectrophotometrically as described by Talwalkar et al. (2003) by measuring the initial linear slope of oxidation of NADH at 340nm at 25°C (ε = 6.22 M⁻¹, cm⁻¹). The reaction mix contained the cell free extract, NADH (67µM), FAD (67µM), H₂O₂ (1mM) and McIlvaine buffer, pH 4.5 to 6.5 in a total volume of 3 ml. The assay was conducted for 3 min in the presence as well as in absence of oxygen. NADH oxidase activity was derived from the difference in the slopes. The slope of the anaerobic assay provided the NADH peroxidase units. For both these enzymes, 1U of activity was defined as the amount that oxidised 1nmol of NADH per min at 25°C.

SOD was measured based on the method reported by Sun and Zigman (1978). One hundred microlitres of epinephrine (0.1M) was added to 100 µl of cell free extract in
1.9 ml 50mM Tris-HCl buffer (pH 7.5) and the inhibition of epinephrine autooxidation was monitored at 320 nm. 1U of SOD was defined as the amount inhibiting the rate of epinephrine autooxidation by 50%.

The specific activities of NADH oxidase, NADH peroxidase and SOD were calculated by dividing the total enzyme units (EU) by the total protein of the cell free extract.

### 6.4.7 Detection of cellular protein profiles

SDS-PAGE of the cell free extracts was carried out as described in Section 3.9

### 6.4.8 Statistics

The means from six replicates were analyzed using single factor ANOVA (α = 0.05) and correlation statistics (MS Excel software). Significant differences among individual means were determined using Tukeys HSD test.

### 6.5 Results

#### 6.5.1 Effect of oxygen on the levels of lactic acid and the lactate to acetate ratio

*L. acidophilus* CSCC 2400 and *L. acidophilus* CSCC 2409 demonstrated a significant (p< 0.05) reduction in the production of lactate as the oxygen in the hypoxic glove box was increased (Table 10). The decrease in lactate levels correlated strongly (r^2 = 0.9) with the increase in the oxygen percentage. From 0 to 21% oxygen, lactate levels in
*L. acidophilus* CSCC 2400 decreased 71% from 6.9 mg/ml to 2 mg/ml. These levels were similar to those seen in *L. acidophilus* CSCC 2409 in which the lactate production decreased by 64%. No acetate was detected in the culture broth of either *L. acidophilus* strains. Levels of lactate followed a similar trend in correlation ($r^2 = 0.9$) in the *Bifidobacterium* spp. (Table 10).
Table 10. Effect of different oxygen concentrations on the lactic acid produced by *L. acidophilus* strains and on the lactate to acetate ratio in *Bifidobacterium* spp. A

<table>
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<tr>
<th>Strain</th>
<th>% Oxygen</th>
<th>Lactic Acid (mg/ml) B</th>
<th>Lactate /Acetate C</th>
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<tr>
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A Mean (n = 6);

a Means in columns with common subscript do not differ significantly (p>0.05)

B 1, 2, 3, 4, 5, 6 Standard error of least square means = 0.07, 0.05, 0.007, 0.07, 0.08 and 0.02 respectively; (df = 25) 1, 2, 3, 4, 5, 6, (df = 20) 3.

C 3, 4, 5, 6 Standard error of least square means = 0.007, 0.04, 0.02 and 0.03 respectively; (df = 25) 4, 5, 6, (df = 20) 3.
Except for *B. lactis* CSCC 1941, in all the other bifidobacteria tested, concentrations of lactate at the various oxygen percentages were significantly different (p < 0.05) from each other. In *B. lactis* CSCC 1941, no significant reduction (p > 0.05) was seen in lactate levels when the oxygen was increased from 15 to 21%. The decrease in lactate however, varied among the strains. The levels of lactate in *B. infantis* CSCC 1912 dropped sharply by 85% when the oxygen was increased from 0 to 15% whereas in *B. pseudolongum* CSCC 1944, lactate levels at 0 and 21% oxygen differed by only 24%. Interestingly, under anaerobic conditions, lactate levels in *B. lactis* CSCC 1941 and *B. pseudolongum* CSCC 1944 were double to that produced by the oxygen tolerant *B. longum* CSCC 55815. Except for *B. infantis* CSCC 1912, all the other *Bifidobacterium* spp. tested in this study were able to grow in 21% oxygen.

The decrease in lactate levels and increased production of acetate in the *Bifidobacterium* spp. caused a significant lowering of the lactate to acetate ratio  (p< 0.05) (Table 10.). The decrease in the ratio was strain dependent. As the concentration of oxygen increased to 21%, the ratio decreased differently in *B. pseudolongum* CSCC 1944 and *B. longum* 55815 even though both strains had a lactate/acetate ratio of 2.5 at 0% oxygen. While the ratio dropped 36% in *B. pseudolongum* CSCC 1944, it decreased steeply by 98% in *B. longum* 55815. Similarly, *B. infantis* CSCC 1912 exhibited a sharp decrease of 82% in the lactate to acetate ratio when the oxygen concentration was increased from 0 to 15% oxygen whereas there was only a 55 % decrease in *B. lactis* CSCC 1941.
6.5.2 Effect of oxygen on the H₂O₂ decomposing ability

As the oxygen concentration increased stepwise from 0 to 21%, except for

*B. pseudolongum* CSCC 1944, all strains showed a significant (*p* < 0.05) rise in their ability to decompose H₂O₂ (Table 11).
Table 11. Effect of different oxygen concentrations on the specific activities of NADH oxidase, NADH peroxidase, and SOD and on the H$_2$O$_2$ decomposing ability of *L. acidophilus* strains and *Bifidobacterium* spp.

<table>
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<tr>
<th>Strain</th>
<th>% Oxygen</th>
<th>NADH oxidase$^A$</th>
<th>NADH peroxidase$^B$</th>
<th>S.O.D.$^C$</th>
<th>nmol H$_2$O$_2$ decomposed$^D$</th>
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$^a,^b,^c$ Means in columns with like superscripts do not differ significantly (p > 0.05)

Means in columns with no superscripts differ significantly (p < 0.05)

$^A,^B,^C$ Expressed as Enzyme Units/ per mg of total protein of the cell free extract.

$^A$ Standard error (df = 25) = 0.3$^1$, 0.3$^2$, 0.2$^3$ (df = 20), 0.2$^4$, 0.1$^5$, 0.2

$^B$ Standard error (df = 25) = 0.3$^1$, 0.3$^2$, 0.1$^3$ (df = 20), 0.1$^4$, 0.1$^5$, 0.2$^6$

$^C$ Standard error (df = 25) = 0.02$^1$, 0.03$^2$, 0.1$^3$ (df = 20), 0.05$^4$, 0.02$^5$, 0.05$^6$

$^D$ Expressed as nmol H$_2$O$_2$ decomposed per 10$^9$ cfu

$^D$ Standard error (df = 25) = 0.2$^1$, 0.07$^2$, 0.01$^3$ (df = 20), 0.03$^4$, 0.07$^5$, 0.01$^6$
In *B. pseudolongum* CSCC 1944, no increase in the $\text{H}_2\text{O}_2$ decomposition capacity was seen when the oxygen was raised from 15 to 21%. In all the strains, the extent of $\text{H}_2\text{O}_2$ decomposed was observed to be strain dependant. At 21% oxygen, while the $\text{H}_2\text{O}_2$ decomposing ability of *B. lactis* CSCC 1941 was 11 times higher than that observed at 0% oxygen, in *L. acidophilus* CSCC 2409 it was found to increase by 73%. When grown in similar concentrations of oxygen, the $\text{H}_2\text{O}_2$ decomposing ability of *L. acidophilus* strains was at least twice of that seen in the *Bifidobacterium* spp. At 0% oxygen, the $\text{H}_2\text{O}_2$ decomposing ability of *B. longum* 55815 was almost seven times that of *B. pseudolongum* CSCC 1944. The $\text{H}_2\text{O}_2$ decomposing ability in *L. acidophilus* CSCC 2400 and *B. longum* 55815 at 21% oxygen was almost double to that observed when they were grown under 0% oxygen.

### 6.5.3 Effect of oxygen on the sensitivity to $\text{H}_2\text{O}_2$

In all the probiotic strains tested in this study, exposure to 10,000, 20,000 and 30,000 mg/l of $\text{H}_2\text{O}_2$ did not cause any significant decrease ($p>0.05$) in the cell counts (Table 12). Moreover, this trend did not change even when cells were grown in the different oxygen concentrations.
Table 12. Effect of exposure to H$_2$O$_2$ on the survival (log$_{10}$ cfu/ml) of

*L. acidophilus* strains and *Bifidobacterium* spp. grown in different oxygen

concentrations

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*a* Means (n = 6) in rows with common superscripts do not differ significantly (p > 0.05)
6.5.4 Effect of oxygen on NADH oxidase and NADH peroxidase activities

In both *L. acidophilus* as well as *Bifidobacterium* spp., the pH profiles of NADH oxidase (Figure 6) and NADH peroxidase (Figure 7) revealed maximal activity at pH 5.0 and it remained unchanged even when cells were grown in different oxygen environments.

Except in *B. infantis* CSCC 1912, in all other strains, the specific activities of intracellular NADH oxidase and NADH peroxidase at 21% oxygen were significantly higher (p < 0.05) than those observed at 0% oxygen (Table 11). In *B. infantis* CSCC 1912, NADH oxidase units increased significantly (p < 0.05) when oxygen increased from 0 to 5% but no further increase was seen when grown in 10% and 15% oxygen. Similarly, the specific activity of NADH peroxidase increased significantly (p < 0.05) when the oxygen was increased from 0 to 5% but no significant change (p > 0.05) was seen in its specific activity when oxygen was further increased to 10% and 15% (Table 11). In anaerobic conditions (0% oxygen), the specific activity of NADH oxidase and NADH peroxidase in *L. acidophilus* strains were at least 1.6 times higher than in the *Bifidobacterium* spp. Among the bifidobacteria, *B. longum* 55815 had the highest concentrations of NADH oxidase and NADH peroxidase, although no significant increase (p > 0.05) was observed in its enzyme concentrations after increasing the percentage of oxygen from 15 to 21%. In *B. infantis* CSCC 1912, *B. lactis* CSCC 1941 and *L. acidophilus* CSCC 2409, the concentrations of NADH peroxidase were found to correlate strongly (r² = 0.9) with their H₂O₂ decomposing ability (Table 11).
Figure 6. pH profile of NADH oxidase of (A) *B. infantis* CSCC 1912, (B) *B. lactis* CSCC 1941, (C) *B. pseudolongum* CSCC 1944, (D) *B. longum* 55815, (E) *L. acidophilus* CSCC 2400, and (F) *L. acidophilus* CSCC 2409 under different oxygen concentrations: (●) 0%, (■) 5%, (▲) 10%, (×) 15%, (♦) 21%.
Figure 7. pH profile of NADH peroxidase of (A) *B. infantis* CSCC 1912, (B) *B. lactis* CSCC 1941, (C) *B. pseudolongum* CSCC 1944, (D) *B. longum* 55815, (E) *L. acidophilus* CSCC 2400, and (F) *L. acidophilus* CSCC 2409 under different oxygen concentrations: (♦) 0%, (■) 5%, (▲) 10%, (◆) 15%, (★) 21%. 
6.5.5 Effect of oxygen on the SOD activity

Concentrations of SOD failed to demonstrate any conclusive trend with the various oxygen percentages, although it was detected in every strain (Table 11). In contrast to the increasing concentrations of NADH oxidase and NADH peroxidase, the concentrations of SOD were uncorrelated ($r^2 < 0.9$). Among all the strains however, *B. longum* 55815 had the highest intracellular concentrations of SOD.

6.5.6 Effect of oxygen on the protein profiles

The SDS-PAGE profiles of all the strains are shown in Plates 3-7. Exposure to aerobic environments clearly altered the protein profiles of all strains. As the oxygen concentration was raised from 0 to 21%, some of the existing protein bands were seen to disappear while new bands emerged. The molecular weight standards developed bands as expected, indicating appropriate electrophoretic conditions. The scans of the electrophoretic patterns of each strains at the various oxygen concentrations is given in Plates 8-13.
Plate 3. Protein profile of *B. infantis* CSCC 1912 (Lanes 1-4) and *B. lactis* CSCC 1941 (Lane 6-10) at 0, 5, 10, 15, and 21% oxygen (left to right). Lane 5 contains the molecular weight standards.

Plate 4. Protein profile of *B. pseudolongum* CSCC 1944 (Lanes 1-5) at 0, 5, 10, 15, and 21% oxygen (left to right). Lane 6 contains the molecular weight standards.
Plate 5. Protein profile of *B. longum* 55815 (Lanes 3-7) at 0, 5, 10, 15, and 21% oxygen (left to right). Lane 1 contains the molecular weight standards

![Plate 5](image)

Plate 6. Protein profile of *L. acidophilus* CSCC 2400 (lanes 1-5) at 0, 5, 10, 15, and 21% oxygen (left to right). Lane 6 contains the molecular weight standards

![Plate 6](image)
Plate 7. Protein profile of *L. acidophilus* CSCC 2409 (lanes 1-5) at 0, 5, 10, 15, and 21% oxygen (left to right). Lane 6 contains the molecular weight standards.
Plate 8. Electrophoretic profiles of *L. acidophilus* CSCC 2400 in various oxygen percentages (A) 0%, (B) 5%, (C) 10%, (D) 15% and (E) 21%. 

(A) 

(B) 

(C) 

(D) 

(E)
Plate 9. Electrophoretic profiles of *L. acidophilus* CSCC 2409 in various oxygen percentages (A) 0%, (B) 5%, (C) 10%, (D) 15% and (E) 21%.
Plate 10. Electrophoretic profiles of *B. infantis* CSCC 1912 in various oxygen percentages (A) 0%, (B) 5%, (C) 10% and (D) 15%
Plate 11. Electrophoretic profiles of *B. lactis* CSCC 1941 in various oxygen percentages (A) 0%, (B) 5%, (C) 10%, (D) 15% and (E) 21%.
Plate 12. Electrophoretic profiles of *B. pseudolongum* CSCC 1944 in various oxygen percentages (A) 0%, (B) 5%, (C) 10%, (D) 15% and (E) 21%.
Plate 13. Electrophoretic profiles of *B. longum* 55815 in various oxygen percentages (A) 0%, (B) 5%, (C) 10%, (D) 15% and (E) 21%.
6.6 Discussion

Oxygen plays a critical role in the metabolism of bifidobacteria and *L. acidophilus* (Condon, 1987). Although these strains are categorized as anaerobes and microaerophilic respectively (Hammes and Vogel, 1995; Sgorbati et al., 1995), in our study, except for *B. infantis* 1912, all remaining strains were able to grow well at 21% oxygen. This was unexpected as *B. infantis* CSCC 1912 demonstrated good growth under aerobic conditions in MRS-C (Chapter 1) and had a high RBGR. In contrast, *B. lactis* CSCC 1941 and *B. pseudolongum* CSCC 1944 had low RBGRs and were hence considered oxygen sensitive. This suggests that the growth medium can play a role in determining the oxygen sensitivity of bifidobacteria.

Cysteine, besides being an oxygen scavenger, functions as an amino source for some bifidobacteria (Shah, 1997). It is possible that cysteine could be an essential requirement for *B. infantis* CSCC 1912, particularly when dealing with oxidative stress. The study relied on bacteria being grown at defined concentrations of oxygen. Addition of cysteine to MRS broth therefore would have caused oxygen to be scavenged from the broth and caused erroneous results. Consequently, the requirement of cysteine for *B. infantis* CSCC 1912 could not be verified. Overall, the results demonstrate that growth under the various oxygen concentrations clearly alters the metabolic and biochemical behaviour in both *L. acidophilus* and *Bifidobacterium* spp.

Lactate and acetate are the main end products of fermentation in lactic acid bacteria. While *L. acidophilus* converts glucose to lactic acid via a homolactic fermentation (Hammes and Vogel, 1995), bifidobacteria convert two moles of glucose to form 3 moles
of acetate and 2 moles of lactate (Sgorbati et al., 1995). When this molar ratio is expressed in mg/ml, a theoretical ratio of 1 is obtained and can serve as an index of the metabolic activity of the cell. The absence of acetate in the culture broths of \textit{L. acidophilus} CSCC 2400 and \textit{L. acidophilus} CSCC 2409 even during aerobic growth confirmed their obligate homolactic fermentation.

Condon (1987) suggested that in anaerobic conditions, lactic acid bacteria convert pyruvate to lactate by the NADH-dependent lactate dehydrogenase, regenerating NAD$^+$ needed for the dehydrogenation reactions of sugar metabolism. In the presence of oxygen however, the pyruvate metabolism can be altered by the competition of NADH oxidases and NADH peroxidases with lactate dehydrogenase for NADH affecting the fermentation end products. This provides clues to the metabolic changes observed in \textit{L. acidophilus} 2400 and \textit{L. acidophilus} 2409 when grown under increasing concentrations of oxygen. Smart and Thomas (1987) proposed that in lactic anaerobic streptococci, the regeneration of NAD$^+$ by the activities of NADH oxidase: NADH peroxidase system could remove the need for conversion of pyruvate to lactate, resulting in lower lactate production. A mechanism similar to that observed in lactic streptococci may be responsible for the increase in the specific activities of NADH oxidase and NADH peroxidase and the simultaneous decrease in the lactate levels in \textit{L. acidophilus} CSCC 2400 and \textit{L. acidophilus} CSCC 2409. Apart from the enzyme activities, it is also probable that the decrease in lactate production in these lactobacilli was due to less growth of the cells as the oxygen concentration was increased.
In a study conducted on *B. lactis*, Meile et al. (1997) reported a high acetate/lactate ratio when the strain was grown anaerobically and this ratio was found to decrease in presence of oxygen. The four *Bifidobacterium* spp. tested in our study however, gave contrary results. As seen in Table 10, levels of lactic acid in bifidobacteria strains were highest when they were grown anaerobically. With increase in oxygen concentrations however, the lactate levels dropped while acetate levels increased, reducing the lactate to acetate ratio. Interestingly, this closely resembles the lactate-acetate production patterns seen in other lactic acid bacteria such as lactic streptococci. Smart and Thomas (1987) found that aeration increased the pyruvate dehydrogenase in lactic streptococci whereas lactate dehydrogenase activities decreased suggesting that aerobically, cells are more suited to produce acetate. This could explain the decrease in lactate levels and the subsequent increase in acetate production in our *Bifidobacterium* spp. when they were grown aerobically.

In earlier studies on bifidobacteria, concentrations of NADH oxidase and NADH peroxidase have been found to correlate with oxygen tolerance (Shimamura et al., 1992; Ahn et al., 2001). The limited knowledge about the pH optima of intracellular NADH oxidases and NADH peroxidases of bifidobacteria as well as the absence of a standard assay however, may have led to inaccurate estimations of NADH oxidases in *Bifidobacterium* spp. (Talwalkar et al., 2003). The NADH oxidases and NADH peroxidases of bifidobacteria were assayed at neutral pH by de Vries and Stouthamer (1969) and Uesugi and Yajima (1978). This study however, revealed that maximum activity of these enzymes was at pH 5.0. The pH optima of the NADH oxidases and NADH peroxidases of the *Bifidobacterium* spp. used in this study agreed with the findings of Shimamura et al. (1992). The NADH oxidase: NADH peroxidase system
has not been studied in *L. acidophilus* so far and this study suggests that in *L. acidophilus* strains, these enzymes be assayed at pH 5 to ensure maximal activity and therefore accurate estimations.

Additionally, the consistency of the optimum pH across the different oxygen concentrations in the *Bifidobacterium* spp. and *L. acidophilus* strains suggests that oxygen does not affect the pH profiles of their NADH oxidases and NADH peroxidases. The high specific activity of both NADH oxidase and NADH peroxidase in *L. acidophilus* strains and oxygen tolerant *B. longum* 55815 (Table 11) highlight the role they play in aerotolerance. The inability of *B. infantis* CSCC 1912 to increase its NADH oxidase activity seems to have resulted in its failure to grow at 21% oxygen. The strong correlation between these enzymes and the different oxygen concentrations suggests that these enzymes are inducible with oxygen acting as an inducer. These findings are similar to those seen in lactic streptococci (Higuchi, 1984; Smart and Thomas, 1987), in which the activities of NADH oxidase and NADH peroxidase increased when strains were exposed to oxygen.

In the peroxide decomposition technique proposed by Shimamura et al. (1992), the peroxide decomposed by the bifidobacteria was represented as nmols decomposed per milligram of cells. Surprisingly, there was no mention of whether that was wet weight or dry weight of the cells. A dry weight would have been impractical due to the nature of their study and a wet weight would have had inherent variations sufficient to doubt the reliability of the measurements. To overcome this practical problem, this methodology was improved upon in this study. Consequently, the peroxide
decomposition in this study was represented as nmols decomposed by a defined number ($10^9$) of cells. As the peroxide decomposition was calculated per defined number of cells, the peroxide decomposition rates can therefore be reliably compared across strains. NADH peroxidase activities correlated well ($r^2 \geq 0.9$) with the $H_2O_2$ decomposition in all the strains, underpinning this enzyme’s relevance in protecting the cell from the lethal effects of intracellular accumulation of $H_2O_2$.

Bifidobacteria have been found to differ in their sensitivities to $H_2O_2$ and in their ability to decompose $H_2O_2$ (de Vries and Stouthamer, 1969; Lim et al., 1998). Shimamura et al. (1992) had reported variation in the $H_2O_2$ sensitivity of four Bifidobacterium spp. with B. infantis being the least sensitive to $H_2O_2$. In this study however, none of the L. acidophilus and Bifidobacterium spp. revealed any significant decrease in cell viability, even after exposure to high concentrations of 30,000 mg/l $H_2O_2$. This is in contrast to Shimamura et al. (1992) where exposure to 10,000 mg/l $H_2O_2$ caused significant losses in the viability of three Bifidobacterium spp. strains. Lim et al. (1998) used longer $H_2O_2$ exposure times than those reported in Shimamura et al. (1992), and found a significant decrease in cell viability. It seems likely that the levels of NADH peroxidase in the strains used in this study were sufficient to protect them from the short exposure to $H_2O_2$. The increase in the intracellular levels of NADH peroxidase may have also contributed to the $H_2O_2$ sensitivity pattern remaining unchanged over the different oxygen environments.

Anaerobes including lactic acid bacteria usually possess SOD for scavenging toxic oxygen radicals. Previous studies on the SOD of bifidobacteria and L. acidophilus
strains have found no correlation between its specific activity and the aerobic environment (Shimamura et al., 1992; Soon-Young and Park, 1997; Lin and Yen, 1999). Results obtained in this study were in accordance with these findings. Although all strains possessed SOD, no conclusive trend could be detected with the oxygen concentration suggesting that oxygen did not seem to induce SOD. Jenny et al. (1999) proposed the role of a superoxide reductase that is independent of SOD and catalase, to detoxify oxygen in anaerobes. Superoxide reductase was not tested in this study and therefore its presence and role in the oxygen tolerance in the test strains cannot be ruled out.

It is also possible that the presence of a ferroxidase in bifidobacteria and iron chelation activity in *L. acidophilus* and strains of *Bifidobacterium* spp. (Kot et al., 1994; Kim et al., 2001) may have been instrumental in protecting cells from peroxide by an iron sequestering mechanism (Yamamoto et al., 2000). Additionally, it has been suggested that bacteria can exhibit a common stress response offering cross protection against a variety of environmental factors (Kim et al., 2001).

Oxygen tolerance in bifidobacteria and *L. acidophilus* however, remains poorly studied. Interestingly, the first ever indication of a biochemical oxidative stress response by *Bifidobacterium* spp. was provided in a study by Ahn et al. (2001) in which exposure to oxygen was found to induce a 35.5 kD protein in the oxygen tolerant *B. longum* JI 1. Similarly, Schell et al. (2002) found three proteins that reverse oxidative damage to be present in *B. longum*. Apart from these studies however, no reports exist about the protein profiles of *L. acidophilus* and *Bifidobacterium* spp. in relation to oxygen exposure. In this study too, definite
changes were seen to the protein profiles of *Bifidobacterium* spp. when they were grown in different oxygen concentrations. In addition, such changes were also observed in *L. acidophilus* strains. Incidentally, this is the first time that such a study has been conducted on *L. acidophilus* strains. It is evident from the gel analysis that exposure to oxygen alters the cellular protein profiles in *L. acidophilus* and *Bifidobacterium* spp. The appearance and disappearance of some protein bands as well as changes to their relative proportion suggests that exposure to oxygen elicits a definite biochemical response in probiotic bacteria (Plates 8-13). This is also supported by the increases seen in the activities of NADH oxidase and NADH peroxidase as the oxygen concentration was increased. Along with these enzymes, it is possible that some of the protein bands on the gels are oxidative stress proteins developed *de novo*. It is however difficult to conclusively identify the stress proteins from only a one dimensional SDS-PAGE as within each band more than one protein of similar molecular density may be present. A two dimensional electrophoresis of the cell free extracts would have enabled a better understanding of this phenomenon. Such an analysis was however outside the scope of this study, which was primarily to obtain an idea of the various biochemical changes occurring in the cellular physiology of probiotic bacteria under oxidative stress. Nevertheless, this study highlights the effect of oxygen has on the cellular protein expression of *L. acidophilus* and *Bifidobacterium* spp. and the possible mediation of stress proteins in the oxidative response of these bacteria.

### 6.7 Conclusions
This study clearly indicates that exposure to oxygen alters the physiological profiles of

*L. acidophilus* and *Bifidobacterium* spp. Moreover, the increase in the activities of NADH oxidase and NADH peroxidase and the subsequent increase in the ability to decompose H$_2$O$_2$ suggest that both *L. acidophilus* and *Bifidobacterium* spp. can initiate a cellular response against oxidative stress. This study also indicates that both, NADH oxidase and NADH peroxidase in *L. acidophilus* and *Bifidobacterium* spp. are can be induced by oxygen. The changes to the protein profiles also indicate that complexity of this oxidative stress response. It is likely that the oxidative response in *L. acidophilus* and *Bifidobacterium* spp. involves a concerted action by a number of individual components interacting with each other to bring about a common stress response.

This study hence offers valuable information to understand the precise details of the oxidative stress response in *L. acidophilus* and *Bifidobacterium* spp. It is hoped that the knowledge gained would be useful to develop techniques to prevent oxygen toxicity in probiotic bacteria. This will ultimately help in the extended survival of probiotic bacteria in dairy foods, thereby ensuring maximum therapeutic benefits to the consumer.
Chapter 4: Comparative studies of selective and differential media for the accurate enumeration of probiotic bacteria from commercial yoghurts

7.1 Abstract

The accuracy of the reported survival estimates of probiotic bacteria in commercial yoghurts is based on the ability of the enumeration medium to provide reliable cell counts. Diverse selective/differential media have been used by each of the reported survival studies. It thus becomes necessary to evaluate these media for their reliability. The various reported media were hence investigated for their ability to provide reliable counts of *Lactobacillus acidophilus*, *Bifidobacterium* spp. and *L. casei* from a wide range of commercial probiotic yoghurts. Counts of each probiotic strain from the same yoghurt sample were found to differ by upto 3 logs on the different media. Selective media reported to develop only single type of colonies, were found to produce two types of colonies with some yoghurts. Similarly, colony characteristics on the differential media were found too subjective to suitably distinguish the probiotic colonies. Except for LC agar, no medium provided reliable counts of probiotic bacteria in all yoghurts. This study highlights the possibility that current estimates of probiotic bacteria in yoghurts could be erroneous and demonstrates the urgent need to develop standard enumeration media.

This chapter is based on the publication: Talwalkar, A. and Kailasapathy, K. (manuscript revised and resubmitted). Comparative studies of selective and differential media for the accurate enumeration of strains of *Lactobacillus acidophilus*, *Bifidobacterium* spp. and *L. casei* complex from commercial yoghurts. *International Dairy Journal*
7.2 Introduction

Standards requiring a minimum of $10^6$-10$^7$ cfu/g of *L. acidophilus* and/or bifidobacteria in fermented milk products have been introduced by several food organizations worldwide (IDF, 1992; Shah, 2000; Bibiloni et al., 2001). It becomes important therefore, that suitable techniques are made available to yoghurt manufacturers to accurately enumerate the counts of probiotic bacteria, in the presence of starter cultures, in their products.

Presently however, survival estimates of probiotic bacteria in commercial yoghurts are conflicting. Some studies have reported low counts of these bacteria (Iwana et al., 1993; Shah et al., 1995; Rybka and Fleet, 1997; Anon., 1999; Shah et al., 2000) while others have cited satisfactory viability (Lourens et al., 2000; Shin et al., 2000). Variable counts have been reported elsewhere (Pacher and Kneifel, 1996; Dave and Shah, 1997d; Micanel et al., 1997; Vinderola and Reinheimer, 1999; Vinderola et al., 2000).

Interestingly, each of these survival studies was conducted on different yoghurts using diverse selective or differential media to enumerate probiotic bacteria. Colony morphology was used to identify the probiotic colonies in these survival studies. The media employed to enumerate probiotic bacteria in these studies however had been validated using only a few commercial yoghurts. Additionally, an increasing number of yoghurt manufacturers are also incorporating *L. casei* in their products. Few of the selective or differential media advocated for enumerating probiotic bacteria from yoghurts have been tested on yoghurts containing all three probiotic bacteria.
It was therefore essential that the media used in these studies be examined whether they conform to their literature of conclusively distinguishing the probiotic colonies based only on colony characteristics, in different commercial probiotic yoghurts. It was also necessary to confirm that bacterial counts from the same yoghurt sample do not vary with the different media. Such a study would help in determining the reliability of the reported survival estimates of probiotic bacteria in commercial yoghurts.

7.3 Aims and Objectives

The aim of this study therefore, was to evaluate the suitability of the various selective and differential media used in the population studies of probiotic bacteria in yoghurts to provide reliable counts of *L. acidophilus* and *Bifidobacterium* spp. and *L. casei* from different commercial yoghurts. The different selective media were assessed based on their literature of demonstrating only a single colony type whereas the differential media were examined whether they were able to provide easily distinguishable colonies of the probiotic bacteria. Colonies were identified based on visual detection of colony morphology. Accordingly, media providing a reliable bacterial count were considered as selective and as non-selective if colonies could not be identified conclusively.

7.4 Materials and Methods

7.4.1 Commercial yoghurts

Nine commercial yoghurts each from different manufacturers and claiming to contain probiotic bacteria were purchased from the Australian supermarkets. One yoghurt (yoghurt 1) contained only *L. acidophilus* (A), five yoghurts (yoghurts 2-6) contained
*L. acidophilus* and bifidobacteria (B) and three yoghurts (yoghurts 7-9) possessed *L. acidophilus*, bifidobacteria and *L. casei* (C). Yoghurts 1, 2, 3, 7, 8, and 9 were plain yoghurts whereas yoghurts 4, 5 and 6 were fruit yoghurts. The different manufacturers included Pauls Ltd. (A yoghurt), PB Foods Ltd. (AB yoghurt), Australian Cooperative Foods Ltd. (AB yoghurt), Hastings Cooperative (AB yoghurt), Nestle (AB yoghurt), Attiki Pty Ltd. (AB yoghurt), National Foods Ltd. (ABC yoghurt), B.-d. Farm Paris Creek (ABC yoghurt) and Jalna Dairy Foods Pty Ltd. (ABC yoghurt). To protect the confidentiality of the manufacturers, the yoghurts were numbered randomly.

### 7.4.2 Selective and differential media

MRS with Sorbitol (MRS-SOR) (Dave and Shah, 1996) and LC agar (Ravula and Shah, 1998) were chosen for the selective enumeration of *L. acidophilus* and *L. casei* respectively while MRS-Bile (MRS-B) (Vinderola and Reinheimer, 1999) was selected to obtain a differential count of these bacteria. MRS with neomycin, paromomycin, nalidixic acid and lithium chloride (MRS-NNLP) (Laroia and Martin, 1991), MRS with sodium propionate and lithium chloride (MRS-LP) (Lapierre et al., 1992), Columbia Agar Base with dicloxacillin and propionic acid (DP) (Bonaparte et al., 2001), and Reinforced Clostridial Medium with nalidixic acid, polymyxin B, iodoacetate, 2,3,5-triphenyltetrazolium chloride and lithium propionate (AMC) (Arroyo et al., 1995) were chosen for the selective enumeration of *Bifidobacterium* spp. Reinforced Clostridial Medium with Prussian Blue (RCPB) (Onggo and Fleet, 1993) and RCPB with pH adjusted to 5 (RCPB-pH 5) (Rybka and Kailasapathy, 1996) were also examined for providing a differential count of bifidobacteria. MRS-SOR, MRS-B, LC, MRS–NNLP, MRS-LP, DP, AMC, RCPB and RPCB-pH 5.0 were prepared exactly as per literature (Table 13).
Table 13.

Media used for enumerating *L. acidophilus*, *Bifidobacterium* spp. and *L. casei* from commercial yoghurts

<table>
<thead>
<tr>
<th>Medium</th>
<th>Base</th>
<th>Selectivity based on</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRS-NPNL</td>
<td>MRS</td>
<td>NPNL solution*</td>
<td>Dave and Shah, 1996</td>
</tr>
<tr>
<td>MRS-B</td>
<td>MRS</td>
<td>Bile (0.15% w/v) and aerobic incubation</td>
<td>Vinderola and Reinheimer, 1999</td>
</tr>
<tr>
<td>MRS-LP</td>
<td>MRS</td>
<td>LP mixture*</td>
<td>Lapierre et al., 1992</td>
</tr>
<tr>
<td>AMC</td>
<td>RCM</td>
<td>Nalidixic acid, Polymycin B, Iodoacetate, 2,3,5-triphenyltetrazolim chloride, LP mixture*</td>
<td>Arroyo et al., 1995</td>
</tr>
<tr>
<td>DP</td>
<td>CAB</td>
<td>Dicloxacillin, Propionic acid, 5 mL</td>
<td>Bonaparte et al., 2001</td>
</tr>
<tr>
<td>MRS-SOR</td>
<td>MRS</td>
<td>Sorbitol (1% w/v)</td>
<td>Dave and Shah, 1996</td>
</tr>
<tr>
<td>LC</td>
<td>MRS</td>
<td>Ribose (1% w/v) and temperature of incubation (27°C)</td>
<td>Ravula and Shah, 1998</td>
</tr>
<tr>
<td>RCPB</td>
<td>RCA</td>
<td>Prussian Blue</td>
<td>Onggo and Fleet, 1993</td>
</tr>
<tr>
<td>RCPB-pH 5</td>
<td>RCA</td>
<td>pH and Prussian Blue</td>
<td>Rybka and Kailasapathy, 1996</td>
</tr>
</tbody>
</table>

* Made from stock solution as follows: LP mixture = LiCl, 2 g/l; sodium propionate, 3 g/l. NPNL solution = Neomycin sulphate, 100 mg/l; Paromomycin, 200 mg/l; Nalidixic acid, 15 mg/l; LiCl, 3 g/l.
7.4.3 Microbiological analysis

Two different production batches of every commercial yoghurt were tested. Probiotic counts from duplicate samples of each production batch were estimated at their expiry date as given in Section 3.5. All media plates were incubated at 37°C for 72 h, except for LC agar plates, which were incubated at 27°C for 72 h, before enumerating the colony counts. MRS-B was incubated aerobically whereas all remaining media plates were incubated anaerobically.

7.4.4 Statistical analysis

A student t-test was employed to determine significant differences (p< 0.05) between cell counts obtained on MRS-B and MRS-SOR plates. Cell counts from the rest of the media were analysed using a single factor ANOVA (MS Excel software). Differences among means were estimated by the Tukeys HSD test.

7.5 Results

The counts of *L. acidophilus* and *Bifidobacterium* spp. from commercial yoghurts obtained on different selective media are listed in Table 14. Broadly, the media demonstrated mixed performances and selectivity. Certain media, which produced a single colony type for some yoghurts and were hence thought selective, exhibited a property of unselectivity in the remaining yoghurts by allowing two types of colonies to grow on it. In some yoghurts, while colony forming units were seen on certain media, no growth was observed on other media. Additionally, although the same yoghurt sample,
cell counts of each probiotic strain varied significantly (p< 0.05) on the various selective and differential media (Table 14).
Table 14. Counts (cfu/g) of *L. acidophilus* complex, *Bifidobacterium* spp. and *L. casei* from commercial yoghurts enumerated on different media

<table>
<thead>
<tr>
<th>Yoghurt</th>
<th>L. acidophilus complex</th>
<th>Media for L. acidophilus complex</th>
<th>Media for Bifidobacterium spp.</th>
<th>Media for L. casei</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MRS-SOR (selective)</td>
<td>MRS-B (diff.)</td>
<td>MRS-LP (selective)</td>
<td>DP (selective)</td>
</tr>
<tr>
<td>1 I</td>
<td>NS</td>
<td>NS</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>1 II</td>
<td>NS</td>
<td>NS</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>2 I</td>
<td>1.8 x 10^6</td>
<td>NS</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>2 II</td>
<td>-</td>
<td>NS</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>3 I</td>
<td>1.6 x 10^5</td>
<td>2.2 x 10^4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3 II</td>
<td>1.8 x 10^4</td>
<td>2.1 x 10^4</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>4 I</td>
<td>4.5 x 10^5</td>
<td>NS</td>
<td>2.6 x 10^5</td>
<td>-</td>
</tr>
<tr>
<td>4 II</td>
<td>1.6 x 10^6</td>
<td>NS</td>
<td>2.6 x 10^5</td>
<td>-</td>
</tr>
<tr>
<td>5 I</td>
<td>5.4 x 10^6</td>
<td>3.2 x 10^4</td>
<td>2.2 x 10^5</td>
<td>-</td>
</tr>
<tr>
<td>5 II</td>
<td>2.3 x 10^6</td>
<td>1.8 x 10^6</td>
<td>2.2 x 10^5</td>
<td>-</td>
</tr>
<tr>
<td>6 I</td>
<td>-</td>
<td>-</td>
<td>7.0 x 10^6</td>
<td>X</td>
</tr>
<tr>
<td>6 II</td>
<td>-</td>
<td>-</td>
<td>6.8 x 10^5</td>
<td>X</td>
</tr>
<tr>
<td>7 I</td>
<td>1.6 x 10^6</td>
<td>3.4 x 10^6</td>
<td>1.9 x 10^6</td>
<td>X</td>
</tr>
<tr>
<td>7 II</td>
<td>4.0 x 10^5</td>
<td>NS</td>
<td>4.5 x 10^6</td>
<td>X</td>
</tr>
<tr>
<td>8 I</td>
<td>NS</td>
<td>NS</td>
<td>5.3 x 10^5</td>
<td>X</td>
</tr>
<tr>
<td>8 II</td>
<td>NS</td>
<td>NS</td>
<td>4.5 x 10^5</td>
<td>X</td>
</tr>
<tr>
<td>9 I</td>
<td>NS</td>
<td>NS</td>
<td>3.5 x 10^5</td>
<td>X</td>
</tr>
<tr>
<td>9 II</td>
<td>NS</td>
<td>NS</td>
<td>4.5 x 10^5</td>
<td>X</td>
</tr>
</tbody>
</table>

diff. = differential; Yoghurt 1 = A yoghurt; 2-6 = AB yoghurts; 7-9 = ABC yoghurts; ‘I’ and ‘II’ represent separate production batches

NS = Non selective; ‘-’ = no growth detected; ‘X’ = yoghurt did not claim to possess those probiotic strains.

Counts are an average of six determinations

a,b,c,d For each probiotic strain, means in rows with common superscripts do not differ significantly (p<0.05)
7.5.1 Media for enumerating *L. acidophilus*

In this study, MRS-SOR gave two types of colonies in the yoghurt containing only *L. acidophilus* as the probiotic strain (yoghurt 1) (Plate 14). This was surprising as MRS-SOR agar is reported to be inhibitory for the yoghurt starters *Streptococcus thermophilus* and *L. delbrueckii* ssp. *bulgaricus* and bifidobacteria (Dave and Shah, 1996). In contrast, a single type of colony was observed for yoghurts containing *L. acidophilus* and *Bifidobacterium* spp. (yoghurts 2, 3, 4, and 5) while no colonies were seen with yoghurt 6, also an AB yoghurt. In yoghurts containing *L. acidophilus*, *Bifidobacterium* spp. and *L. casei*, two types of colonies were seen with yoghurts 8 and 9, suggesting that the colonies were those of *L. acidophilus* and *L. casei*. In contrast, yoghurt 7, also an ABC yoghurt gave only one type of colony on MRS-SOR. The development of colonies on LC agar from this yoghurt suggested that the colonies on MRS-SOR were those of *L. casei* and that *L. acidophilus* was either absent or non-viable in the yoghurt. This was however unsupported by the demonstration of colonies on MRS-B, a medium that is differential for *L. acidophilus*. Hence, although colonies were formed on MRS-SOR, the wide variation in their counts across the different yoghurts made it impossible to reliably identify them as those of *L. acidophilus* based on colony morphology. Consequently, MRS–SOR was categorized as unselective for the commercial yoghurts tested in this study.

MRS-B is reported to be selective for *L. acidophilus* in A or AB yoghurts and differential for ABC yoghurts (Vinderola and Reinheimer, 2000). In this study however, two types of colonies were observed on MRS-B plates when yoghurts 1(A yoghurt), 2 and 4 (both AB yoghurts) were plated on it. In contrast, with yoghurts 8 and 9 (both ABC yoghurts), only
a single type of colony was observed as against the expected two. The only reliable identification and enumeration of *L. acidophilus* (based on published literature) was possible in one production batch of yoghurt 7 (ABC yoghurt) which gave two distinct types of colonies. Thus in this study, MRS-B was found to perform poorly in conclusively identifying and enumerating probiotic bacteria and was categorized as unselective.
Plate 14. Two types of colonies seen on MRS-SOR with yoghurt 1, containing

*L. acidophilus*
7.5.2 Media for enumerating *Bifidobacterium* spp. and *L. casei*

The media tested for the enumeration of bifidobacteria from commercial probiotic yoghurts demonstrated variations in their performance and reliability as well. DP agar is reported to inhibit growth of *L. acidophilus* and other yoghurt starter cultures (Roy, 2001). With yoghurt 3, an AB yoghurt, DP agar however, gave two types of colonies (Plate 15), as compared with MRS-NNLP, in which only one colony type was observed. A conclusive enumeration of bifidobacterial counts in yoghurt 3 was therefore possible on MRS-NNLP but not on DP agar (Table 14). Similarly, when yoghurt 7 (ABC) yoghurt was plated on MRS-LP, two types of colonies were seen in one production batch (Plate 16), whereas only one type of colonies was seen in the other production batch. A similar result was observed on MRS-NNLP agar (Plate 17). Growth of *L. casei* on MRS-NNLP has not been studied so far and it is probable that the bigger colonies seen on MRS-NNLP were those of *L. casei*. However, inconsistencies were found between the counts of *L. casei* enumerated on MRS- B, MRS-LP or MRS-NNLP and those obtained on LC agar. Payne et al. (1999) evaluated several selective media and recommended AMC agar for the enumeration of bifidobacteria from mixed cultures. The performance of AMC agar however, was seen to vary in this study. Although for most yoghurts, AMC agar gave a single type of colony, with some yoghurts, it failed to develop any colonies despite other selective media such as MRS-NNLP or MRS-LP or DP demonstrating bifidobacteria colonies from the same yoghurt sample. This raises questions about the ability of AMC agar to allow the full recovery of bifidobacteria from yoghurts.
Plate 15. Two types of colonies seen on DP agar with yoghurt 3, an AB yoghurt
Plate 16. Two types of colonies on MRS-LP with yoghurt 7, an ABC yoghurt
Plate 17. Two types of colonies on MRS-NNLP with yoghurt 7, an ABC yoghurt
Overall however, among the selective media tested, MRS-NNLP and AMC agar seem better for enumerating bifidobacteria from probiotic yoghurts than MRS-LP or DP. Counts of bifidobacteria obtained on the differential media (Table 14) were also seen to vary significantly (p<0.05). RCPB agar is reported to allow the differential enumeration of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* and bifidobacteria without the presence of other probiotics, based only on colony characteristics, particularly colour (Onggo and Fleet, 1993). Similarly, on RCPB-pH 5, *L. delbrueckii* subsp. *bulgaricus* is cited to develop white colonies with a wide dark blue halo in contrast to the white colonies of bifidobacteria (Rybka and Kailasapathy, 1996). In this study however, except with yoghurts 5, 6, 7, 8, and 9, both RCPB and RCPB-pH 5 either failed to produce any colonies or developed colonies that were difficult to distinguish based on visual examination of colony characteristics. Moreover, both media had not been validated with *L. casei* and hence information about the colony characteristics of *L. casei* was unknown. Thus although colonies of bifidobacteria on these media were identified and enumerated based on the published guidelines, the presence of *L. casei* in yoghurts 7, 8, and 9 may have introduced errors in the accurate enumeration of bifidobacteria.
Table 15. Performance of various selective and differential media in conclusively enumerating counts of *L. acidophilus* (A), *Bifidobacterium* spp. (B) and *L. casei* (C) from commercial yoghurts

<table>
<thead>
<tr>
<th>Medium</th>
<th>Probiotic screened</th>
<th>Number of samples</th>
<th>Selective/Conclusive counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRS-SOR</td>
<td>A</td>
<td>18</td>
<td>9</td>
</tr>
<tr>
<td>MRS-B</td>
<td>A</td>
<td>18</td>
<td>5</td>
</tr>
<tr>
<td>MRS-NNLP</td>
<td>B</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>MRS-LP</td>
<td>B</td>
<td>16</td>
<td>6</td>
</tr>
<tr>
<td>DP</td>
<td>B</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>AMC</td>
<td>B</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td>RCPB</td>
<td>B</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>RCPB-pH5</td>
<td>B</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>LC</td>
<td>C</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>
7.5.3 Variation in the cell counts

When colonies of probiotic bacteria could be decisively identified on some selective media, counts from these different media differed significantly (p<0.05) from each other (Table 14). There was a one-log difference between the counts of *L. acidophilus* on MRS-B and MRS-SOR in AB yoghurts such as yoghurts 3 and 5. In yoghurt 3, the bifidobacterial count on MRS-NNLP was $2.6 \times 10^3$ cfu/g as against a count of $1.5 \times 10^6$ cfu/g on AMC agar. A similar pattern was seen for yoghurt 4 with bifidobacterial counts on MRS-NNLP agar being at least 3 logs higher than those on AMC agar.

The selectivity of the medium was also influenced by the dilution of the sample. When yoghurt 2, containing both *L. acidophilus* and *Bifidobacterium* spp., was plated on MRS–B, two different colony types were seen at lower dilutions whereas when the sample was diluted further, only one type of colony was observed (Plates 18 and 19). The disappearance of the second colony type therefore, was not due to the selectivity of the media but was an attribute of the dilution effect. This indicates that the presence of a single type of colony at higher dilutions does not establish the medium to be selective. For the enumeration of cell counts, it is commonplace for samples to be diluted sufficiently to obtain between 20-200 colonies on the plate. The results from this study demonstrate the possibility of inaccurate probiotic counts due to the dilution factor.
Plate 18. Two types of colonies seen on MRS-B at $10^{-1}$ dilution of yoghurt 2, an AB yoghurt

Plate 19. A single type of colony observed on MRS-B at $10^{-5}$ dilution of yoghurt 2
Counts of probiotic bacteria were also found to vary between production batches (Table 14). In yoghurt 5, no *Bifidobacterium* spp. colonies were detected on MRS–NNLP as well as on MRS-LP in the second production batch. Likewise, on MRS-NNLPLP agar, no bifidobacteria were detected from yoghurt 5 although AMC and RCPB agar returned bifidobacteria counts approximating $10^5$ cfu/g from the same yoghurt.

### 7.6 Discussion

Probiotic yoghurt manufacturers purchase yoghurt and probiotic cultures from several different commercial culture companies, each of which have their individual strain development procedures. Moreover, as probiotic cultures are added as adjuncts to yoghurt, yoghurt manufacturers do not need to rely on strain specificity. Together, this can introduce a lot of variety in the genotypic and phenotypic characters of the probiotic strains incorporated into yoghurt. This can influence their interactions with starter cultures and consequently affect their ability to grow on the various media. Similarly, it is well known that the colony forming ability of bacteria can be also affected if stressed. Apart from storage conditions, probiotic bacteria are exposed to a variety of stresses during yoghurt manufacture as well. It is likely that a combination of these factors may have resulted in the differences in probiotic counts between production batches. It is also probable that age of the yoghurt influenced the cell numbers, with the likelihood of lower bacterial counts with increasing storage time. This could not be ascertained however, as the yoghurts tested in this study displayed only their expiry date.
In this study, the selective and differential media reported for reliably enumerating counts of *L. acidophilus* and *Bifidobacterium* spp. adhered to their published literature in only some probiotic yoghurts. A single medium that provided reliable cell counts of the particular probiotic strain in all the various yoghurts tested in this study was not detected (Table 15).

Considering that enumeration of probiotic bacteria relies solely on the media’s ability to provide reliable cell counts, the exact survival status of probiotic bacteria in commercial yoghurts becomes unclear. Furthermore, contrasting reports exist regarding the suitability of some selective media. Vinderola and Reinheimer (1999), in their comparison of several media, found growth of *Streptococcus thermophilus* and *L. delbrueckii* subsp. *bulgaricus* on MRS-NPNL, a medium used by Shah et al. (2000) and which was an active component of the medium used by Shin et al. (2000) for the selective enumeration of bifidobacteria from commercial yoghurts. Additionally, commercial culture companies such as Chr. Hansen have been cited to use MRS–Maltose and NNLP media to enumerate *L. acidophilus* and *B. bifidum* (Lourens-Hattingh and Viljoen, 2001). The contrasting reports about the proper selective media, therefore, engender the likelihood of over/underestimation of probiotic bacterial counts, depending on the selective media used.

Survival studies of probiotic bacteria have been mostly conducted on yoghurts containing either *L. acidophilus* or *L. acidophilus* and *Bifidobacterium* spp. The overlapping biochemical profiles of the various yoghurt bacteria with probiotic bacteria and also between *L. acidophilus* and *Bifidobacterium* spp. makes it difficult to develop a medium that selectively screens or differentiates probiotic bacteria from
starter cultures (Samona and Robinson, 1991). As observed in this study, the incorporation of *L. casei* in yoghurts also introduces the possibility of it interfering with the accurate enumeration of either

*L. acidophilus* or bifidobacteria on the currently available selective media. Similarly, the use of differential media for enumerating *L. acidophilus*, bifidobacteria and *L. casei* in yoghurts, based only on colony morphology can be too subjective for the reliable estimation of the bacterial counts. For the conclusive identification and enumeration of these three probiotic bacteria, it is preferable to have separate selective media for each. In this regard, LC agar was found to offer good selectivity and provide reliable counts of

*L. casei* in all the yoghurts with which it was tested (yoghurts 7, 8 and 9).

Currently, procedures for enumerating probiotic bacteria from yoghurts rely solely on plate counts. The International Dairy Federation (IDF, 1999) also emphasizes that standard media need to be developed for enumerating probiotic bacteria in yoghurts. Plating methodologies however are time consuming and tedious. In addition, they are susceptible to false counts from autoaggregation of some strains. Similarly, bacterial stress could lead to the inability of some cells to develop colonies on solid media or even develop colonies having different morphological characteristics. Together with poor selectivity, this can prove a major impediment in the suitability of the various selective and differential media to accurately enumerate each type of probiotic bacteria. A positive identification of probiotic bacteria on the media plates would then require a thorough biochemical, serotypic or genotypic testing and would be economically infeasible for the yoghurt manufacturer. For industrial purposes, the enumeration methods for probiotic bacteria need to be rapid, convenient and
economical. In this regard, simple and rapid enzymatic methodologies such as EB-MPN (Bibiloni et al., 2001) for enumerating bifidobacteria could be developed for the selective enumeration of *L. acidophilus* as well.

**7.7 Conclusion**

This study thus demonstrates the unsuitability of the currently available media to reliably enumerate the different types of probiotic bacteria in a wide range of commercial yoghurts. Consequently, it becomes necessary to confirm the selectivity of a medium before using it for enumerating probiotic bacteria from yoghurts. It is plausible therefore, that current estimates of probiotic numbers in yoghurts may be inaccurate. Developing standard methodologies for enumerating probiotic bacteria, which are industrially viable, would thus greatly assist yoghurt manufacturers and researchers in knowing the exact status of probiotic bacteria in commercial yoghurts.
8 Chapter 5: Effect of microencapsulation on oxygen toxicity in probiotic bacteria

8.1 Abstract

Microencapsulation was tested for its protective role against oxygen toxicity in *L. acidophilus* and *Bifidobacterium* spp. Two strains of *B. lactis* and one strain of *L. acidophilus* were encapsulated in calcium alginate and grown aerobically in Reconstituted Skim Milk broth for 24 h. Counts of encapsulated cells in all three strains were one log higher than corresponding free cell counts. The encapsulated cell count of *B. lactis* 920 was 9.12 log$_{10}$cfu/ml as against 8.66 log$_{10}$cfu/ml of free cells whereas *L. acidophilus* CSCC 2409 when encapsulated, demonstrated a cell count of 7.84 log$_{10}$cfu/ml as compared to a free cell count of 6.67 log$_{10}$cfu/ml. The protective effect of microencapsulation was also tested in yoghurt. Several strains of *L. acidophilus* and *Bifidobacterium* spp. were encapsulated and incorporated in yoghurt for 24 h maintained aerobically at 6°C. Interestingly, while microencapsulation was found to significantly increase viability in six strains, no significant difference was observed between encapsulated cell counts and free cells counts in the remaining six strains. Thus although microencapsulation can offer protection to probiotic bacteria against oxygen toxicity in broth culture, further optimization studies are needed before its application in yoghurt.

8.2 Introduction

The high concentration of oxygen in yoghurts is considered an impediment for the extended survival of these probiotic bacteria, particularly for the oxygen sensitive bifidobacteria.

Microencapsulation is a process by which live cells are packaged within a shell material to shield them from the surrounding unfavourable environment. It is one of the techniques reported to enhance the survival of probiotic bacteria in dairy foods (Shah 2000). Probiotic bacteria when encapsulated have acquired protection from stomach acidity and have increased their tolerance to bile (Ravula and Shah 1999; Sultana et al. 2000). The viability of *B. pseudolongum* in simulated gastric juices was improved when it was encapsulated (Rao et al. 1989). Additionally, microencapsulation has been cited for increasing the viability of lactobacilli in frozen ice milk (Sheu and Marshall 1993) as well as protecting cells of *Bifidobacterium* spp. in cheese (Gobetti et al. 1998, Dinakar and Mistry 1994). Although oxygen diffusion in alginate encapsulated cell systems has been reviewed (Omar 1993), so far no studies have been conducted on the efficacy of microencapsulation in protecting probiotic bacteria from oxygen toxicity.

8.3 Aim and Objectives

The aim of this study was to examine if microencapsulated cells survive better than free cells when grown under aerobic environments. The objective of the study was to develop a protocol for evaluating the protective effect of microencapsulation against oxygen toxicity in
both culture broth (RSM) as well as in yoghurt and to compare the encapsulated cell counts with the free cell counts after the aerobic incubation. Calcium alginate was chosen as the encapsulation material because of its low cost, non-toxic nature and for its ability to release cells from the alginate gel under appropriate conditions (Rao et al., 1989).

8.4 Material and methods

8.4.1 Microorganisms and media

*B. lactis* 920, *B. lactis* Bb-12, *B. longum* 55815, *B. bifidum* CSCC 1909, *B. infantis* CSCC 1912, *B. lactis* CSCC 1941, *B. pseudolongum* CSCC 1944, *B. thermophilum* CSCC 1991, *L. acidophilus* CSCC 2400, *L. acidophilus* CSCC 2401, *L. acidophilus* CSCC 2404, *L. acidophilus* CSCC 2409 and *L. acidophilus* CSCC 2415 were used in this study. Inocula of these strains were prepared in MRS broth supplemented with 0.05% cysteine. The phosphates in MRS broth however, dissolved the capsules and therefore for the encapsulation study, it was replaced with 9.5% reconstituted skim milk supplemented with 2% glucose and 0.5% yeast extract. The yoghurt study was performed using a traditional plain set yoghurt obtained commercially (Dairy Farmers, Australia).
8.4.2 Preparation of encapsulated bacteria

The method employed for encapsulation (Fig. 8) was based on the method proposed by Sheu and Marshall (1993) and modified by Sultana et al. (2000). For each strain, 5 ml of the 18 h old cultures was added to 45 ml 2% w/v alginate- 2% w/v starch slurry prepared in Milli-Q water (Millipore, U.S.A.). The bacteria-starch-alginate slurry was allowed to mix thoroughly for 30 min using a magnetic stirrer. With a sterile 1ml syringe (0.5 mm gauge), 5 ml of the slurry was added dropwise into a beaker containing 0.1M Calcium chloride. After keeping the beads at 4°C overnight in CaCl$_2$ for further hardening, the calcium chloride solution was decanted and the beads were washed with 0.85 % sterile saline. All the washed beads originating from 5 ml of the slurry were treated as an inoculum. The entire process was carried aseptically in a laminar flow chamber.
Figure 8. Encapsulation of probiotic bacteria in calcium alginate.

- Strain grown in MRS-cysteine broth
- 2% alginate – starch slurry
- Bacteria-alginate-starch slurry stirred together
- Slurry taken up in a 1ml syringe
- Slurry added dropwise into 0.1M CaCl$_2$, forming beads
- Beads kept overnight in CaCl$_2$ for hardening
8.4.3 Survival of encapsulated probiotic bacteria under aerobic conditions

Initial studies demonstrated that the cell counts from 500 µl of free cells were similar to cell counts from 5 ml of alginate-starch-bacteria beads. The encapsulation experiments were performed therefore by adding the same inoculum levels i.e. 500 µl of free cells and 5 ml of alginate-starch-bacteria beads of the probiotic strains separately to 250 ml Erlenmeyer flasks containing 100 ml of medium. The broth experiment was conducted in RSM broth using *B. lactis* 920, *B. lactis* Bb-12 and *L. acidophilus* CSCC 2409. Similarly, free and encapsulated cells of *B. lactis* 920, *B. bifidum* CSCC 1909, *B. infantis* CSCC 1912, *B. lactis* CSCC 1941, *B. pseudolongum* CSCC 1944, *B. thermophilum* CSCC 1991, *B. longum* 55815, *L. acidophilus* CSCC 2400, *L. acidophilus* CSCC 2401, *L. acidophilus* CSCC 2404, *L. acidophilus* CSCC 2409 and *L. acidophilus* CSCC 2415 were added separately to 100 ml of natural set yoghurt previously saturated with air (21% dissolved oxygen). All flasks were plugged with cotton wool to maintain aerobic conditions and incubated aerobically on a shaker at 100 rpm for 24 h. The RSM broth experiment was conducted at 37°C whereas the yoghurt study was performed at 6°C. In addition, the pH of the media was also monitored. Duplicate flasks were used throughout this entire study. In addition, the entire experiment was conducted twice.
8.4.4 Release of entrapped cells

Beads were harvested and were washed free of media by rinsing them thrice with 0.85% sterile saline. The washed beads were added to 45 ml 0.1M phosphate buffer, pH 7.0 in a stomacher bag and homogenized for 30 min in a stomacher. This dissolved the beads releasing the cells. The cell count in the homogenized suspension was enumerated on appropriate media plates.

8.4.5 Enumeration of cell counts

The RSM broth containing the free cells as well as the homogenized suspension was serially diluted in peptone water and spread-plated on MRS agar plates containing 0.05% cysteine. For the yoghurt study, MRS-LP and MRS–S plates were used for the selective enumeration of *Bifidobacterium* spp. and *L. acidophilus* strains from yoghurt after confirming that they inhibited the growth of yoghurt starters. Similarly, the selectivity of MRS-LP and MRS-S was ensured by streaking pure cultures of *L. acidophilus* and *Bifidobacterium* spp. used in this study on both these media and confirming that *L. acidophilus* were inhibited on MRS-LP and *Bifidobacterium* spp. were inhibited on MRS-S by plating a yoghurt sample on it. Plates were incubated anaerobically at 37°C for 48 h before enumerating the colony forming units.
8.4.6 Experiment controls

Bacterial leaching during the bead hardening and the washing process was tested by plating samples of calcium chloride and saline on MRS agar. Cell loss due to the encapsulation process was studied by enumerating the cell counts of the beads immediately after bead formation as well as after bead hardening.

To ensure that the protective effect of encapsulation was being tested against only oxygen, the above protocol was conducted under anaerobic conditions. The flasks containing RSM were deoxygenated by sparging nitrogen gas in boiling media for 5 min. For the yoghurt study, deoxygenation was achieved by overnight stirring of the yoghurt on a magnetic shaker in an anaerobic glove box (95% N₂, 5% H₂, Coy Products, U.S.A.). Deoxygenation of the yoghurt was confirmed using a Clark type dip-type micro-oxygen electrode (MI-730, Microelectrodes, U.S.A.). In both, the broth experiment and the yoghurt study, the deoxygenated medium was inoculated anaerobically and the flasks were sealed with a rubber stopper to prevent oxygen entry. Sealed flasks with the probiotic culture were treated similar to aerobic flasks.

8.4.7 Determination of bead size

The bead diameter of 100 beads was measured using a stage and ocular micrometer under a 10X objective of a light microscope.
8.5 Results

Bacterial leaching during the bead formation, hardening and washing steps was not observed. Similarly, cell loss due to the encapsulation process was not detected in all the test strains. In both, the broth experiment as well as the yoghurt study, no significant difference (p>0.05) was observed between free cell counts and encapsulated cell counts in the control anaerobic flasks. The pH of the yoghurt remained unchanged throughout the study whereas in the broth experiment, the pH of RSM broth containing free cells and the encapsulated cells at the end of the study was found to be similar. This indicated that any difference in the colony counts between the free and encapsulated cells in the test flasks was due to the presence of oxygen. When tested in RSM broth at 37°C, all three probiotic strains had significantly higher (p<0.05) encapsulated cell counts than free cell counts. Counts of encapsulated cells in all the three strains were one log higher than their free cell counts (Table 16).
Table 16. Effect of encapsulation on oxygen toxicity of probiotic microorganisms in RSM broth

<table>
<thead>
<tr>
<th>Strain</th>
<th>Aerobic incubation</th>
<th>Anaerobic incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E log&lt;sub&gt;10&lt;/sub&gt; cfu/ml</td>
<td>F log&lt;sub&gt;10&lt;/sub&gt; cfu/ml</td>
</tr>
<tr>
<td>B. lactis 920</td>
<td>9.12 ± 0.05</td>
<td>8.66 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>L. acidophilus CSCC 2409</td>
<td>7.84 ± 0.08</td>
<td>6.67 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>B. lactis Bb-12</td>
<td>5.04 ± 0.04</td>
<td>4.67 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

E: encapsulated cell counts; F: Free cell counts

Flasks were incubated for 24h

Mean of six determinations ± s.d.

<sup>a</sup> Significant difference (p<0.05) between free cell counts and encapsulated cell counts
Based on the promising results of the broth experiment, it was then investigated whether microencapsulation offered similar protection to probiotic bacteria when they were incorporated in yoghurt and maintained in temperature conditions that resembled the supermarket shelves.

The results obtained were mixed. Among the twelve strains tested, microencapsulation was able to confer significantly better viability (p<0.05) in only six strains whereas no significant difference (p>0.05) was seen between encapsulated and free cell counts in the remaining six strains (Table 18). Encapsulated cell counts of *B. bifidum* CSCC 1909, *B. lactis* CSCC 1941, *L. acidophilus* CSCC 2401 and *L. acidophilus* CSCC 2404 were significantly higher than their free cell counts. Interestingly, when encapsulated cells of *B. lactis* 920 and *L. acidophilus* CSCC 2409 were incorporated in yoghurt, microencapsulation offered a protective effect similar to that observed in RSM broth. Encapsulated cell counts of both these strains were one log higher than their free cell counts. Contrastingly, no significant difference (p>0.05) was demonstrated between the encapsulated and free cell counts of *B. infantis* CSCC 1912, *B. pseudolongum* CSCC 1944, *B. thermophilum* CSCC 1991, *B. longum* 55815, *L. acidophilus* CSCC 2400 and *L. acidophilus* CSCC 2415.
Table 17. Comparison between viability (log\textsubscript{10} cfu/ml) of encapsulated cell counts and free cell counts of probiotic strains in yoghurt

<table>
<thead>
<tr>
<th>Strain</th>
<th>Aerobic incubation</th>
<th>Anaerobic incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Encapsulated cell counts</td>
<td>Free cell counts</td>
</tr>
<tr>
<td><em>B. bifidum</em> 1909</td>
<td>7.07 ± 0.06</td>
<td>6.78 ± 0.07\textsuperscript{a}</td>
</tr>
<tr>
<td><em>B. infantis</em> 1912</td>
<td>7.07 ± 0.05</td>
<td>7.01 ± 0.05</td>
</tr>
<tr>
<td><em>B. lactis</em> 1941</td>
<td>7.43 ± 0.10</td>
<td>6.96 ± 0.06\textsuperscript{a}</td>
</tr>
<tr>
<td><em>B. lactis</em> 920*</td>
<td>7.28 ± 0.07</td>
<td>6.17 ± 0.03\textsuperscript{a}</td>
</tr>
<tr>
<td><em>B. pseudolongum</em> 1944</td>
<td>7.29 ± 0.08</td>
<td>7.28 ± 0.03</td>
</tr>
<tr>
<td><em>B. thermophilum</em> 1991</td>
<td>7.07 ± 0.01</td>
<td>7.01 ± 0.04</td>
</tr>
<tr>
<td><em>B. longum</em> 55815*</td>
<td>6.88 ± 0.07</td>
<td>6.81 ± 0.07</td>
</tr>
<tr>
<td><em>L. acidophilus</em> 2400</td>
<td>7.46 ± 0.01</td>
<td>7.15 ± 0.04</td>
</tr>
<tr>
<td><em>L. acidophilus</em> 2401</td>
<td>6.05 ± 0.04</td>
<td>5.26 ± 0.01\textsuperscript{a}</td>
</tr>
<tr>
<td><em>L. acidophilus</em> 2404</td>
<td>6.63 ± 0.08</td>
<td>5.87 ± 0.03\textsuperscript{a}</td>
</tr>
<tr>
<td><em>L. acidophilus</em> 2409</td>
<td>5.05 ± 0.05</td>
<td>4.87 ± 0.07\textsuperscript{a}</td>
</tr>
<tr>
<td><em>L. acidophilus</em> 2415</td>
<td>5.80 ± 0.04</td>
<td>5.40 ± 0.07</td>
</tr>
</tbody>
</table>

* Not a CSCC strain

Flasks were incubated for 24h

Mean of six determinations ± sd

\textsuperscript{a} Significant difference (p<0.05) between free cell counts and encapsulated cell counts
8.6 Discussion

At a preliminary level, these results indicated that encapsulation was preventing cell death from oxygen toxicity. It is known that alginate restricts the diffusion of oxygen through the gel creating anoxic regions in the centre of the beads (Beunik et al. 1989). As compared to free cells therefore, encapsulated cells would be subjected to either none or much lesser exposure to oxygen, resulting in lesser cell death from oxygen toxicity. This may explain the higher cell counts of encapsulated cells.

The conditions described in the broth experiment however, are different to what probiotic bacteria are exposed to during the shelf life of yoghurts. While the probiotic strains in the broth experiment were incubated at 37°C, yoghurts containing probiotic strains are stored at temperatures ranging between 6-8°C on supermarket shelves. The lack of any significant (p<0.05) difference between the free cell counts and the encapsulated cell counts in some probiotic strains suggests that the low temperature and different environmental conditions of yoghurts could play a role in determining the extent of oxygen toxicity.

Additional factors involved in the encapsulation process may be playing a role in determining the protective role of microencapsulation from oxygen toxicity. Research into immobilized systems (Gossmann and Rehm 1986, 1988; Beunik and Rehm 1988) has led to an assumption that microbial aggregates could develop anaerobic parts in their centres, highlighting the importance of cell distribution within the beads. Further, the bead size can affect the distribution characteristics of the cells; smaller the diameter better is the distribution of cells in the interior of the beads (Omar 1993).
The bead diameter in this experiment averaged 2.38 mm with a standard deviation of ± 0.15. The large and variable bead size in this study could have resulted in poor cell distribution within the beads, exposing more cells to oxygen toxicity. This could be the reason for the lack of any significant protection from microencapsulation seen in the remaining six probiotic test strains.

8.7 Conclusion

This two-stage study offers preliminary evidence of the protective role of microencapsulation against oxygen toxicity in yoghurts. Although, the results of the broth experiment and yoghurt studies were comparable for some strains, extrapolating the protective effect of microencapsulation to all probiotic strains could however be erroneous. The actual process of microencapsulation and the incubation conditions seem to play a significant role in deciding the oxygen-alginate-bacteria interaction. Understanding the exact relation between the encapsulation material and oxygen can assist in devising better techniques to ensure that sufficiently high numbers of probiotic microorganisms are maintained in probiotic foods throughout the shelf life period. For microencapsulation to be applicable in probiotic yoghurts, the beads should not be sensed by the consumer. Incorporation of smaller and uniform beads of probiotic bacteria in yoghurts may therefore allow the retention of a desirable mouth feel as well as minimize cell death due to oxygen toxicity.
Chapter 6: Oxidative stress adaptation of probiotic bacteria

9.1 Abstract

The dissolved oxygen in yoghurts is considered a significant factor responsible for the poor survival of probiotic bacteria during storage. Oxidative stress adaptation was investigated as a technique to increase the tolerance of probiotic bacteria to the dissolved oxygen in yoghurt. A protocol was developed to adapt probiotic bacteria to oxygen in conditions similar to those of yoghurt. Accordingly, several strains of *L. acidophilus* and *Bifidobacterium* spp. were passaged through increasing concentrations of dissolved oxygen such as 0, 60, 150, and 210 ppm in yoghurt. Although all strains recorded a decrease in cell counts with increasing oxygen concentration, some cells demonstrated viability even after passage in 210 ppm of dissolved oxygen in yoghurt, suggesting the cells had adapted to oxygen. This was confirmed by absence of any viability losses when these cells were incubated for 35 days in yoghurt with 210 ppm of dissolved oxygen. The protein profiles of oxygen adapted and oxygen non-adapted cells however demonstrated no changes. The protocol suggested in this study therefore offers yoghurt manufacturers a practical methodology to develop probiotic strains capable of withstanding high levels of oxygen in yoghurts.

This chapter is based on the publication: Talwalkar, A. and Kailasapathy, K. (in press). Oxidative stress adaptation of probiotic bacteria. *Milchwissenschaft*
9.2 Introduction

Oxygen has been considered an important factor responsible for the steady decline of the cell numbers of probiotic bacteria such as *L. acidophilus* and *Bifidobacterium* spp. in yoghurt (Klaver et al., 1993; Dave and Shah, 1997d). Previous studies have suggested the use of a high oxygen consuming strain of *S. thermophilus* in the manufacture of yoghurt, addition of oxygen scavengers such as ascorbic acid and cysteine to yoghurts and packaging yoghurts in glass bottles to protect probiotic bacteria from harmful oxygen exposure (Shah, 2000). Drawbacks are associated however with the implementation of these suggestions (Dave and Shah, 1997a; Dave and Shah, 1997c; Dave and Shah, 1997d). Consequently, there is a need to develop cheaper and economically viable alternatives that have a minimal effect on the textural properties of yoghurt. The development of oxygen adapted probiotic strains that are capable of surviving the dissolved oxygen levels present in yoghurt is one such alternative. It is well known that exposing microorganisms to sub lethal or gradually increasing doses of stress can induce an adaptive cellular response that enables them to better resist lethal doses of stress (Crawford and Davies, 1994). Although there are reports of probiotic bacteria being stress adapted to lethal doses of acid and bile (Shah, 2000), an oxidative stress adaptation of probiotic bacteria has not been conducted yet.

Strain development procedures such as stress adaptation are usually conducted at a temperature and in culture media that is optimal for the microorganism. Extrapolating results from such protocols to food products can be difficult as these optimum conditions may not always be present in the actual product. To minimize this, it is essential that strain adaptation
studies be conducted in conditions simulating those in which the strains are to be incorporated.

9.3 Aim and Objectives

The aim of this study therefore was to adapt *L. acidophilus* and *Bifidobacterium* spp. to oxidative stress in conditions that simulated the storage conditions of yoghurt. The objectives of the study were to develop a protocol to passage *L. acidophilus* and *Bifidobacterium* spp. through gradually increasing concentrations of dissolved oxygen in yoghurt over a four-day period and confirm their successful oxidative stress adaptation. As yoghurt is normally stored between 5-8°C after manufacture, the yoghurt was held at 6°C throughout this study.

9.4 Materials and methods

9.4.1 Microbial cultures

9.4.2 Preparation of cell pellet

The various probiotic strains of *L. acidophilus* and *Bifidobacterium* spp. were grown anaerobically in MRS broth for 18 h and the cells were harvested by centrifugation at 6000 x g for 10 min at room temperature. The cell pellet was washed thrice with sterile 0.85% saline to remove any media constituents and then dissolved in a small volume of saline for further use.

9.4.3 Yoghurt and its deoxygenation

1 kg of traditional plain set yoghurt (Dairy Farmers Ltd., Australia) was purchased from the Australian supermarket. For practical purposes of the experiment, the yoghurt was diluted slightly (90% w/v) with sterile distilled water and poured in a beaker containing a magnetic stirrer. The beaker was introduced in an anaerobic glove box (Coy Laboratory Products, Inc., U.S.A.) and the yoghurt was stirred on a magnetic stirrer at room temperature until its dissolved oxygen reached 0 ppm. A calibrated Clark type oxygen microelectrode (AD Instruments, Australia) was used to measure the dissolved oxygen of the yoghurt. This yoghurt having 0 ppm of dissolved oxygen was considered deoxygenated. The pH of the yoghurt was 4.5.
9.4.4 Stress adaptation of probiotic strains

One hundred millilitres of the deoxygenated yoghurt was dispensed into a 250 ml Erlenmeyer flask that had been earlier introduced into the anaerobic glove box. One ml of the probiotic cell pellet suspension was added to the deoxygenated yoghurt in the anaerobic glove box. The yoghurt flask was made airtight by sealing it with a rubber bung and incubated for 24 h at 150 rpm at 6°C on a refrigerated shaker (New Brunswick, U.S.A.). After incubation, the rubber bung was removed and replaced by a cotton wool plug to allow the diffusion of oxygen into the flask. The flask was further incubated for 24 h at 150 rpm at 6°C. Thereafter, oxygen was pumped in the yoghurt till its dissolved oxygen rose to 150 ppm. To maintain this elevated level of dissolved oxygen in the yoghurt, the flask was again made airtight with a rubber bung. After incubating the flask further for 24 h at 150 rpm at 6°C, more oxygen was pumped into it till the dissolved oxygen in the yoghurt increased to 210 ppm. Further pumping of oxygen into the yoghurt resulted in frothing and failed to appreciably increase the dissolved oxygen. The flask was therefore resealed with the rubber bung and incubated again for 24 h at 150 rpm at 6°C. To provide experiment controls, flasks containing the probiotic strain in deoxygenated yoghurt and in yoghurt in which the dissolved oxygen had been adjusted to 210 ppm were used. Although the control flasks were also incubated at 150 rpm at 6°C, they remained sealed throughout the duration of the experiment (4 days) to maintain their respective oxygen concentrations.
9.4.5 Estimation of probiotic cell counts

After every oxygen passage, 1ml of yoghurt was removed from the flask and added to 9 ml of sterile peptone water (Oxoid, Australia). The suspension was vortexed for 1 min and diluted further. 100μl of appropriate dilutions was plated on an appropriate selective medium for the estimation of cell counts. *L. acidophilus* counts were enumerated on MRS-S agar, while *Bifidobacterium* spp. was enumerated on MRS-LP agar. Plates were incubated 37°C for 48 h in the anaerobic glove box.

9.4.6 Confirmation of oxidative stress adaptation

A cell pellet suspension of each oxygen-passaged probiotic strain was prepared as given in Section 9.4.2. One millilitre of this cell suspension was added to individual yoghurts in which the dissolved oxygen had been adjusted to 210 ppm. The flasks were then sealed with a rubber bung and incubated at 150 rpm at 6°C until the end of the expiry period (35 day) of the yoghurt. Initial and final cell counts were compared to detect any significant (p<0.05) loss in cell viability.

9.4.7 SDS-PAGE protein profiles

Both, oxygen adapted and oxygen non-adapted cells of each strain were grown individually in MRS broth anaerobically at 37°C for 24 h and the cell pellet was obtained as described in Section 9.4.2. Cell free extracts of the cell pellets were obtained following the method
described in Section 3.8. SDS-PAGE of the cell free extracts was conducted as detailed in Section 3.9. The protein profiles of the oxygen adapted and oxygen non-adapted cells were overlayed to detect any alterations.

9.4.8 Statistics

The dissolved oxygen and the pH of the yoghurt were measured in triplicate. The mean of six individual determinations was used to calculate cell counts. A single factor ANOVA and a student t test ($\alpha=0.05$) was used to analyze the cell counts. Significant differences among individual means were determined using Tukeys HSD test. The entire experiment was performed in duplicate.
9.5 Results

The change in the cell numbers of *L. acidophilus* and *Bifidobacterium* spp. after each oxygen passage is shown in Table 18. All probiotic strains incorporated into the control deoxygenated yoghurt were found to survive well without any significant (p>0.05) cell losses. In contrast, cell counts of all probiotic strains incubated in the control oxygenated yoghurt were found to decrease significantly (p<0.05). Additionally, the dissolved oxygen concentrations were found uniform at all different points in the yoghurt sample. In addition, the pH of the yoghurt remained constant throughout the experiment.

When incubated in 60 ppm dissolved oxygen for 24 h, the cell viability of five probiotic strains decreased significantly (p<0.05) whereas no significant decrease (p>0.05) was detected in the cell viability of the remaining strains. As the dissolved oxygen in the yoghurt was increased however, strain dependent decreases in cell viability were observed. In *B. bifidum* CSCC 1909, the cell count fell from 7.09 log_{10} cfu/ml at 0 ppm dissolved oxygen to 6.78 log_{10} cfu/ml at 60 ppm dissolved oxygen and further decreased to 5.79 log_{10} cfu/ml when incubated in 210 ppm dissolved oxygen. On the other hand, although *B. infantis* CSCC 1912 demonstrated no significant decrease (p>0.05) in cell viability after incubation in 60 ppm dissolved oxygen, its cell counts decreased significantly (p<0.05) as the dissolved oxygen increased further. Among all the *Bifidobacterium* spp., *B. infantis* CSCC 1912 recorded the largest decrease of 2.33 logs in cell viability over the five-day exposure to various concentrations of oxygen while *B. longum* 55815 had the lowest drop of only 0.34 in
cell viability. Amongst the *L. acidophilus* strains, *L. acidophilus* CSCC 2409 had the largest decrease in cell viability while *L. acidophilus* CSCC 2404 demonstrated the least cell loss. Interestingly, although the maximum decrease in cell viability was seen after passage through 210 ppm dissolved oxygen, all strains were still able to produce a few colony forming units at this concentration of oxygen. The viability of these oxygen passaged cells when incubated for 35 days in yoghurt containing 210 ppm dissolved oxygen is given in Table 19. In all strains, counts of oxygen passaged cells did not show any significant decrease (p>0.05) after the 35 day incubation in yoghurt.

The protein profiles of the oxygen adapted and oxygen non-adapted cells revealed no significant differences after overlaying (Plates 20 and 21).
Table 18. Cell counts (log$_{10}$ cfu/ml) of *L. acidophilus* and *Bifidobacterium* spp. during oxygen passage in yoghurt

<table>
<thead>
<tr>
<th>Strain</th>
<th>Control</th>
<th>Control</th>
<th>0 ppm</th>
<th>60 ppm</th>
<th>150 ppm</th>
<th>210 ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(A)</td>
<td>(B)</td>
<td>O$_2$</td>
<td>O$_2$</td>
<td>O$_2$</td>
<td>O$_2$</td>
</tr>
<tr>
<td><em>L. acidophilus</em> CSCC 2400</td>
<td>7.57$^a$</td>
<td>&lt; 3.00</td>
<td>7.57$^a$</td>
<td>7.15$^a$</td>
<td>6.97$^a$</td>
<td>5.74$^b$</td>
</tr>
<tr>
<td><em>L. acidophilus</em> CSCC 2401</td>
<td>6.42$^a$</td>
<td>&lt; 3.00</td>
<td>6.57$^a$</td>
<td>5.87$^b$</td>
<td>5.26$^b$</td>
<td>4.55$^c$</td>
</tr>
<tr>
<td><em>L. acidophilus</em> CSCC 2404</td>
<td>6.73$^a$</td>
<td>&lt; 3.00</td>
<td>6.75$^a$</td>
<td>5.87$^b$</td>
<td>5.81$^b$</td>
<td>5.73$^b$</td>
</tr>
<tr>
<td><em>L. acidophilus</em> CSCC 2409</td>
<td>6.05$^a$</td>
<td>&lt; 3.00</td>
<td>6.06$^a$</td>
<td>5.87$^b$</td>
<td>4.55$^c$</td>
<td>4.17$^c$</td>
</tr>
<tr>
<td><em>L. acidophilus</em> CSCC 2415</td>
<td>6.86$^a$</td>
<td>&lt; 3.00</td>
<td>6.73$^a$</td>
<td>6.40$^a$</td>
<td>5.27$^b$</td>
<td>4.99$^b$</td>
</tr>
<tr>
<td><em>B. bifidum</em> CSCC 1909</td>
<td>7.10$^a$</td>
<td>&lt; 3.00</td>
<td>7.09$^a$</td>
<td>6.78$^b$</td>
<td>6.72$^b$</td>
<td>5.79$^c$</td>
</tr>
<tr>
<td><em>B. infantis</em> CSCC 1912</td>
<td>7.12$^a$</td>
<td>&lt; 3.00</td>
<td>7.18$^a$</td>
<td>7.01$^a$</td>
<td>6.75$^b$</td>
<td>4.65$^c$</td>
</tr>
<tr>
<td><em>B. lactis</em> CSCC 1941</td>
<td>7.53$^a$</td>
<td>&lt; 3.00</td>
<td>7.53$^a$</td>
<td>6.96$^b$</td>
<td>6.80$^b$</td>
<td>5.74$^c$</td>
</tr>
<tr>
<td><em>B. pseudolongum</em> CSCC 1944</td>
<td>7.34$^a$</td>
<td>&lt; 3.00</td>
<td>7.29$^a$</td>
<td>7.28$^a$</td>
<td>7.21$^a$</td>
<td>5.48$^b$</td>
</tr>
<tr>
<td><em>B. thermophilum</em> CSCC 1991</td>
<td>6.93$^a$</td>
<td>&lt; 3.00</td>
<td>6.97$^a$</td>
<td>6.81$^a$</td>
<td>6.54$^a$</td>
<td>5.38$^b$</td>
</tr>
<tr>
<td><em>B. longum</em> 55815</td>
<td>7.01$^a$</td>
<td>&lt; 3.00</td>
<td>7.10$^a$</td>
<td>7.01$^a$</td>
<td>6.92$^a$</td>
<td>6.66$^b$</td>
</tr>
</tbody>
</table>

(A): Control deoxygenated yoghurt; (B): Control oxygenated (210 ppm) yoghurt

Counts are a mean of six determinations.

Means in rows with common superscripts do not differ significantly (p<0.05)
Table 19. Counts (log₁₀ cfu/ml) of oxygen passaged *L. acidophilus* and *Bifidobacterium* spp. after five weeks in yoghurt containing 210 ppm dissolved oxygen

<table>
<thead>
<tr>
<th>Strain</th>
<th>Log₁₀ cfu/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 day</td>
</tr>
<tr>
<td>L. acidophilus CSCC 2400</td>
<td>6.75 ± 0.21</td>
</tr>
<tr>
<td>L. acidophilus CSCC 2401</td>
<td>6.43 ± 0.09</td>
</tr>
<tr>
<td>L. acidophilus CSCC 2404</td>
<td>7.57 ± 0.08</td>
</tr>
<tr>
<td>L. acidophilus CSCC 2409</td>
<td>6.73 ± 0.11</td>
</tr>
<tr>
<td>L. acidophilus CSCC 2415</td>
<td>6.57 ± 0.08</td>
</tr>
<tr>
<td>B. bifidum CSCC 1909</td>
<td>7.16 ± 0.10</td>
</tr>
<tr>
<td>B. infantis CSCC 1912</td>
<td>7.91 ± 0.16</td>
</tr>
<tr>
<td>B. lactis CSCC 1941</td>
<td>7.10 ± 0.08</td>
</tr>
<tr>
<td>B. pseudolongum CSCC 1944</td>
<td>6.97 ± 0.09</td>
</tr>
<tr>
<td>B. thermophilum CSCC 1991</td>
<td>7.53 ± 0.13</td>
</tr>
<tr>
<td>B. longum 55815</td>
<td>7.29 ± 0.12</td>
</tr>
</tbody>
</table>

Cell counts are a mean of six determinations
Plate 20. Comparison of the electrophoretic profiles of *L. acidophilus* 2409 and *L. acidophilus* CSCC 2409 OA

### L. acidophilus CSCC 2409

![Electrophoretic profile of L. acidophilus CSCC 2409](image1)

### L. acidophilus CSCC 2409 OA

![Electrophoretic profile of L. acidophilus CSCC 2409 OA](image2)

<table>
<thead>
<tr>
<th>Peak</th>
<th>Center</th>
<th>% Area</th>
<th>Peak</th>
<th>Center</th>
<th>% Area</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>66</td>
<td>2.0</td>
<td>1</td>
<td>67</td>
<td>2.3</td>
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<td>80</td>
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<td>3</td>
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<td>4</td>
<td>123</td>
<td>0.8</td>
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<td>127</td>
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<td>414</td>
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</tr>
<tr>
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<td>510</td>
<td>2.6</td>
<td>12</td>
<td>514</td>
<td>4.2</td>
</tr>
</tbody>
</table>
Plate 21. Comparison of the electrophoretic profiles of *B. infantis* CSCC 1912 and *B. infantis* CSCC 1912 OA

<table>
<thead>
<tr>
<th>Peak</th>
<th>Center</th>
<th>% Area</th>
<th>Peak</th>
<th>Center</th>
<th>% Area</th>
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<td>212</td>
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<td>330</td>
<td>40.0</td>
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<td>415</td>
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<td>623</td>
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<tr>
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<td>640</td>
<td>4.5</td>
<td>10</td>
<td>641</td>
<td>5.1</td>
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</table>
9.6 Discussion

Ordinarily, probiotic strains in yoghurt are exposed to a maximum of 60 ppm oxygen, which is the saturating concentration of oxygen in air (Miller et al., 2002). It is well known however, that bacteria, if subjected to a variety of stresses, can become sensitive to dosages of stresses that are otherwise non-lethal (Lou and Yousef, 1997). The manufacture of yoghurt can induce several stresses of acid, temperature, whey proteins, lactic acid, interactions with starter cultures etc. in probiotic bacteria (Shah, 2000). This can result in probiotic bacteria becoming more sensitive to non-lethal concentrations of oxygen in yoghurt. Therefore, in this study, probiotic strains were exposed to higher than saturating concentrations of oxygen (210 ppm) with the rationale that adapting probiotic bacteria to such concentrations of oxygen would help in their overcoming any oxygen susceptibility caused by exposure to other stresses.

Miller et al. (2002) found that the diffusion of oxygen into yoghurt through the polystyrene packaging is not uniform. The dissolved oxygen was maximal at the corners and sides of the polystyrene tub. Similarly, the gel structure of yoghurts slowed the diffusion of oxygen and therefore towards the yoghurt centre the oxygen concentrations were lower than in the rest of the yoghurt sections.

In the oxygen adaptation protocol proposed in this study, the dilution of the yoghurt and the shaking conditions allowed the diffusion of oxygen to be uniform. This was confirmed by the similar measurements of the dissolved oxygen recorded at three different points in the
yoghurt sample. Furthermore, it provided an accelerated diffusion of oxygen into the yoghurt due to which the dissolved oxygen in the yoghurt rose to 60 ppm in just 24 h compared to the extended time required when yoghurt is stored on supermarket shelves.

The influence of elevated concentrations of dissolved oxygen on cell viability is clearly indicated in the survival trends of probiotic strains in the control yoghurts (Table 18). Interestingly, the microaerophilic *L. acidophilus* strains and the anaerobic *Bifidobacterium* spp. performed contrary to their theoretical predictions of being highly susceptible to oxygen, with all strains demonstrating cell viability throughout the various oxygen passages. The results from the control yoghurts implied that the stepwise oxygen passages of probiotic strains as followed in this study, allowed some cells to adapt to high concentrations of dissolved oxygen in yoghurt. The detection of colony forming units even after strains had been passaged through 210 ppm dissolved oxygen confirmed this finding. The absence of any significant losses in cell viability when these oxygen passaged strains were incubated for 35 days in yoghurt with 210 ppm dissolved oxygen however confirmed that the cells had been successfully adapted to oxygen (Table 19). To denote the oxygen adapted cells ‘OA’ was added to after their strain number.

Stress adaptation of bacterial cells is usually mediated through the production of stress proteins which besides protecting the cell from the stress agent can also cross protect against a variety of other stresses such as heat shock, acidity and starvation (Crawford and Davies, 1994; Shah, 2000). Such oxidative stress proteins may have been involved in the oxygen...
adaptation of the probiotic bacteria tested in this study. As the maximum decreases in cell viability during oxygen passage was seen in *L. acidophilus* CSCC 2409 and *B. infantis* CSCC 1912, it was expected that oxidative stress proteins, if any, would be most evident in the oxygen passaged cells of these strains. Interestingly however, analysis of the cellular protein profiles of the oxygen adapted and oxygen non adapted cells of both *L. acidophilus* CSCC 2409 and *B. infantis* CSCC 1912 failed to reveal any considerable changes (Plates 20 and 21). A similar result was observed in the protein profiles of the remaining strains. For the expression of oxidative stress proteins, it is important that cells are expressed to the oxygen. Although the probiotic strains had been adapted to oxygen in this study, it was conducted in yoghurt, which was maintained at 6°C. To obtain a cell pellet of the *L. acidophilus* and *Bifidobacterium* spp. however, they needed to be cultivated under optimal conditions. Consequently, both oxygen adapted as well as non adapted cells of *L. acidophilus* and *Bifidobacterium* spp. were grown anaerobically in MRS broth at 37°C. This may have resulted in the non expression of the oxidative stress proteins, if any. It is possible that the oxidative stress proteins of these probiotic bacteria are expressed only when incubated in yoghurt with an elevated level of oxygen and therefore failed to show up in the cell free extracts due to the anaerobic cultivation.

It is also likely that the one dimensional SDS PAGE conducted for this purpose was inadequate to detect minor but potentially significant changes in the electrophoretic patterns. Performing a two dimensional electrophoresis can perhaps overcome this limitation.
9.7 Conclusion

A successful oxidative stress adaptation of probiotic bacteria in conditions simulating those encountered in yoghurt was thus conducted in this study. It is theorized that strains surviving higher than saturating levels of dissolved oxygen as used in this study should be able to survive adequately in commercial yoghurts. The ease, simplicity and cost effectiveness of this protocol can make it possible for yoghurt manufacturers to incorporate oxygen tolerant probiotic strains in their products and thereby help to increase their survival through the shelf life.
10 Chapter 7: Effect of packaging materials and dissolved oxygen on the survival of probiotic bacteria in yoghurt

10.1 Abstract

The effects of yoghurt packaging materials on the dissolved oxygen and the survival of the probiotic bacteria were investigated in this study. Oxygen adapted and oxygen non-adapted cells of *L. acidophilus* and *Bifidobacterium* spp. were incorporated in yoghurts packaged in oxygen permeable high impact polystyrene (HIPS) and in an oxygen-barrier material (Nupak™) with and without an oxygen scavenging film (Zero₂™). The dissolved oxygen of the yoghurts increased steadily from 13 ppm at 0 day to 56 ppm at 42 day in yoghurts packaged in HIPS whereas it remained constant in yoghurts packaged in the Nupak™. The dissolved oxygen levels in yoghurts packaged in Nupak™ tubs containing Zero₂™ fell from 16 ppm to 0.37 ppm on 0 day and remained constant thereafter throughout the shelf life of the yoghurt. No significant decrease in cell viability was observed in both oxygen adapted and oxygen non-adapted cells of *L. acidophilus* and *Bifidobacterium* spp. This finding was irrespective of the packaging material used and the dissolved oxygen levels in the yoghurt. More work thus needs to be carried out using other strains, packaging materials and pH conditions.

This chapter is based on the publication: Talwalkar, A., Miller C. W., Kailasapathy, K. and Nguyen, M. H. (submitted) Effect of packaging materials and dissolved oxygen on the survival of probiotic bacteria in yoghurt. *International Journal of food Science and Technology*
10.2 Introduction

The introduction of various standards for probiotic dairy foods has necessitated yoghurt manufacturers to guarantee adequate viability of *L. acidophilus* and bifidobacteria in their products throughout the shelf life. Several market surveys however, have reported a steady decline in the counts of *L. acidophilus* and *Bifidobacterium* spp. during the shelf life of yoghurts, with cell numbers being much lower than the recommended $10^6 - 10^7$ cfu/g at the expiry date (Iwana et al., 1993; Rybka and Fleet, 1997; Anon., 1999; Shah et al., 2000).

Oxygen toxicity is considered a significant factor influencing the viability of these probiotic bacteria in yoghurts (Klaver et al., 1993; Dave and Shah, 1997d). The agitation and mixing steps involved in the manufacture of yoghurt incorporates high amounts of oxygen in the product. Furthermore, during storage, oxygen diffuses into yoghurt through the high impact polystyrene (HIPS) packaging, a material used commonly for yoghurt packaging worldwide (Ishibashi and Shimamura, 1993; Miller et al., 2002). Consequently, the incorporated probiotic bacteria are exposed to dissolved oxygen throughout the manufacture as well as during the shelf life of yoghurts. This constant exposure to oxygen is thought to affect their extended survival in yoghurt (Dave and Shah, 1997d).

Packaging alternatives to HIPS such as the polystyrene based gas barrier Nupak™, has been shown effective in preventing diffusion of oxygen into yoghurts during the shelf life (Miller et al., 2002). Similarly, an active packaging film, Zero2™ that can actively scavenge oxygen from the product has also been developed (Rooney, 1995). The effect of these packaging materials on the viability of probiotic bacteria however hasn’t been studied. Although
dissolved oxygen is believed to be a significant factor responsible for the poor survival of probiotic bacteria, little is known about the actual levels of oxygen in yoghurt necessary to cause viability losses of probiotic bacteria.

10.3 Aim and Objectives

The aim of this study was therefore to investigate the effect of packaging materials on the dissolved oxygen of yoghurt and its influence of the viability of *L. acidophilus* and *Bifidobacterium* spp. The objectives of the study were to package yoghurt in packaging materials with different oxygen permeabilities and to evaluate their protective role against oxygen toxicity of *L. acidophilus* and *Bifidobacterium* spp. in yoghurt. HIPS, Nupak™, and Nupak™ containing the active oxygen scavenging film, Zero₂™ were chosen due to their properties of oxygen permeability, oxygen impermeability, and active oxygen scavenging ability. In a previous study (Chapter 6) cells of *L. acidophilus* and *Bifidobacterium* spp., had been adapted to high levels of oxygen in yoghurt. As the maximum decreases in cell viability after oxygen passages were seen in *L. acidophilus* CSCC 2409 and *B. infantis* CSCC 1912, these strains were selected as representatives of oxygen sensitive strains for this study. Consequently, *L. acidophilus* CSCC 2409 OA and *B. infantis* CSCC 1912 OA served as positive controls in this study.
10.4 Materials and methods

10.4.1 Bacterial strains and preparation of inoculum

*L. acidophilus* CSCC 2409, *B. infantis* CSCC 1912, *L. acidophilus* CSCC 2409 OA and *B. infantis* CSCC 1912 OA. were used in this study. Cells were grown in MRS anaerobically for 24 h at 37 °C and harvested by centrifugation at 8000 x g for 20 min at 4 °C. The cell pellet was washed twice with sterile saline and was made into a viscous paste using sterile 9% reconstituted skim milk broth (RSM). The viscous paste, spread evenly on a large petri dish was incubated at –20 °C for 6 h before being subjected to freeze drying overnight. The freeze-dried powder contained a bacterial load of approximately $10^9$ to $10^{10}$ cfu/g and was considered as the inoculum for the rest of the experiment.

10.4.2 Preparation of probiotic yoghurts

(a) Yoghurt mix

A simple set-type yoghurt was used for the experiment. Skim milk was standardized to typical yoghurt make-up of 4.0% fat and 4.3% protein using cream and skim milk powder. This was then heated to 85 °C for 20 min and allowed to cool. Once the standardized milk had cooled to 45 °C a commercial yoghurt starter culture (YoFlex, Chr. Hansen, Australia) was inoculated (0.1% w/v) into it. The yoghurt mix was then divided into two batches, one containing *L. acidophilus* CSCC 2409 OA (0.25% w/v) and *B. infantis* CSCC 1912 OA (0.2% w/v) and the other containing *L. acidophilus* CSCC 2409 (0.2% w/v) and
B. infantis CSCC 1912 (0.2% w/v).

(b) Packaging of probiotic yoghurt mixes

The yoghurt mixes were divided in 150 ml tubs of HIPS and Nupak™ (Visypac, Melbourne, Australia) and Nupak™ containing Zero₂™ film (CSIRO, Australia). The HIPS and Nupak™ tubs were sealed with foil with an air headspace. In order to minimize the loss of oxygen scavenging capacity of Zero₂™ between activation and filling, the Nupak™ with Zero₂™ tubs were filled and sealed within an anaerobic glove box (Coy Laboratory Products, Inc., U.S.A.) containing 95% N₂ and 5% H₂. Zero₂™ with surface area equivalent to the internal surface area of a 150 ml Nupak™ tub was removed from its vacuum packaging under anaerobic conditions in the glove box and placed in each of the empty Nupak™ containers. The yoghurt mix was then filled over Zero₂™ in these Nupak™ tubs. The tubs were then sealed with foil in the anaerobic glove box.

(c) Incubation of yoghurt mixes

After filling and sealing the yoghurt mixes in the various packaging containers, all tubs were incubated at 37 °C for 8 h to facilitate fermentation. During this time, the pH dropped from 6.5 to 4.1 indicating a thorough fermentation. The yoghurt tubs were then stored at 4 °C in a refrigerator over a 42 d shelf life.
10.4.3 Dissolved oxygen and pH measurements

Before the filling, the dissolved oxygen content of the yoghurt mix was measured using a Microelectrodes MI-730 dip-type micro-oxygen electrode and OM4 oxygen electrode. Similarly, the dissolved oxygen of the yoghurts was measured weekly in two samples of each packaging variant. Measurements were taken at two lateral positions - one in the center and one against the package wall - and at three depths below the surface: 3 mm, 33 mm and 70 mm. Hence six readings were obtained from each yoghurt tub. The pH of the yoghurts was also monitored weekly.

10.4.4 Survival of probiotic strains in yoghurt

The survival of \textit{L. acidophilus} CSCC 2409, \textit{B. infantis} CSCC 1912, \textit{L. acidophilus} CSCC 2409 OA, and \textit{B. infantis} CSCC 1912 OA was monitored weekly starting from 0 d to the end of shelf life of the yoghurts. The yoghurt was stirred thoroughly with a spoon to obtain a representative sample. Ten grams of this stirred yoghurt sample were introduced in a stomacher bag containing 100 ml of distilled water. The suspension was homogenized in a stomacher for 2 minutes. The homogenized suspension was serially diluted in 1\% sterile peptone water. One hundred microlitres of three consecutive dilutions was spread plated on appropriate selective media. Plates were incubated at 37°C for 48 h in the anaerobic glove box. MRS-S and MRS-LP agar plates were used to selectively enumerate \textit{L. acidophilus} and \textit{Bifidobacterium} spp. respectively, from the yoghurt. Before using MRS-S and MRS-LP, it was confirmed that these media did not allow the growth of
yoghurt culture by separately plating the commercial yoghurt starter culture on them. The absence of any growth on both, MRS-S and MRS-LP, indicated their suitability for selectively enumerating \textit{L. acidophilus} and \textit{Bifidobacterium} spp. respectively. Counts of \textit{L. acidophilus} CSCC 2409, \textit{B. infantis} CSCC 1912, \textit{L. acidophilus} CSCC 2409 OA, and \textit{B. infantis} CSCC 1912 OA were also enumerated from the probiotic yoghurt mix just before the start of fermentation.

10.4.5 Statistics

Cell counts were estimated as the mean of six determinations and were analyzed using a one-way Analysis of Variance (Microsoft Excel Data analysis package 2000). The entire trial was performed in duplicate.

10.5 Results

The dissolved oxygen measurements of the yoghurts packed in the various packaging materials are shown in Fig. 9. In all yoghurts, the dissolved oxygen of the yoghurt mix (0 day) was higher than that of the final yoghurt. The maximum rise in the dissolved oxygen from the 0 day to the expiry date was observed in yoghurts packed in HIPS. The increase in the dissolved oxygen in the yoghurts packed in HIPS ranged from 30% to as high as 83%. As against this, the dissolved oxygen in yoghurts packaged in Nupak™ fell further over the shelf life and remained less than 4.29 ppm throughout the shelf life. In yoghurts packed in Nupak™ with Zero$_2$™, the levels of dissolved oxygen dropped drastically from 16.92 ppm in
the yoghurt mix to 1.4 ppm in the final product. Thereafter, the levels dropped further over the shelf life and reached as low as 0.44 ppm at the expiry date.

The initial pH of all the yoghurts ranged between 4.1-4.5 and did not change over the shelf life and did not change significantly (p>0.05) over the shelf life. The cell counts of all four strains in the yoghurt mix before fermentation and after yoghurt formation did not differ significantly as well (p>0.05). The survival of *L. acidophilus* CSCC 2409, *B. infantis* CSCC 1912, *L. acidophilus* CSCC 2409 OA and *B. infantis* CSCC 1912 OA is shown in Table 20. In all the yoghurts, no significant change (p>0.05) was observed in the cell counts of all these strains over the entire storage period of the yoghurt. Both oxygen adapted and oxygen non adapted cells of *L. acidophilus* and *Bifidobacterium* spp. survived well in yoghurt and were present in numbers ranging between $10^6$ to $10^7$ cfu/g. Similar trends were observed in both trials.
Figure 9. Dissolved oxygen content (ppm) in set-type yoghurt stored in HIPS (■), Nupak (▲), and Nupak with Zero₂ (●) over 42 days.
Table 20. Viability of *L. acidophilus* CSCC 2409, *L. acidophilus* CSCC 2409 OA, *B. infantis* CSCC 1912, and *B. infantis* CSCC 1912 OA in yoghurt packed in HIPS, and Nupak™ with and without Zero2™ oxygen scavenging film, stored for 42 days

<table>
<thead>
<tr>
<th>Tub Package</th>
<th>Strain</th>
<th>Cell counts (log_{10} cfu/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 d</td>
</tr>
<tr>
<td>HIPS</td>
<td><em>L. acidophilus</em> CSCC 2409</td>
<td>7.89 ± 0.22</td>
</tr>
<tr>
<td></td>
<td><em>L. acidophilus</em> CSCC 2409 OA</td>
<td>6.74 ± 0.17</td>
</tr>
<tr>
<td></td>
<td><em>B. infantis</em> CSCC 1912</td>
<td>7.89 ± 0.38</td>
</tr>
<tr>
<td></td>
<td><em>B. infantis</em> CSCC 1912 OA</td>
<td>6.81 ± 0.25</td>
</tr>
<tr>
<td>Nupak™</td>
<td><em>L. acidophilus</em> CSCC 2409</td>
<td>7.92 ± 0.19</td>
</tr>
<tr>
<td></td>
<td><em>L. acidophilus</em> CSCC 2409 OA</td>
<td>7.82 ± 0.15</td>
</tr>
<tr>
<td></td>
<td><em>B. infantis</em> CSCC 1912</td>
<td>7.87 ± 0.31</td>
</tr>
<tr>
<td></td>
<td><em>B. infantis</em> CSCC 1912 OA</td>
<td>7.82 ± 0.11</td>
</tr>
<tr>
<td>Nupak™ and Zero2™</td>
<td><em>L. acidophilus</em> CSCC 2409</td>
<td>7.38 ± 0.27</td>
</tr>
<tr>
<td></td>
<td><em>B. infantis</em> CSCC 1912</td>
<td>7.70 ± 0.09</td>
</tr>
</tbody>
</table>

Each value is a mean log_{10} cfu/g ± standard deviation from six observations at each day.
10.6 Discussion

Although oxygen is one of the major ingredients of yoghurts, much remains to be known about its dynamics in yoghurts. The manufacture of yoghurt is primarily executed by the aerobic *S. thermophilus*, which ferments the lactose in the yoghurt mix to lactic acid. This process requires oxygen, which is available in the yoghurt mix. Consequently, during the manufacture of the yoghurt, much of the available oxygen in the yoghurt mix is utilized by the oxygen consuming activities of *S. thermophilus*. This is evident in the drastic drop in the dissolved oxygen levels observed in our study from the yoghurt mix stage to the final product (Fig. 9) in all the yoghurts.

Thereafter however, the dissolved oxygen in the yoghurt was seen to be dependent on the packaging material. The steady rise in the dissolved oxygen levels in yoghurts packed in HIPS reflects the oxygen diffusion into the yoghurt through the HIPS. The dissolved oxygen levels of 56 ppm at the end of the expiry date in these yoghurts, correspond closely to the saturating values of oxygen in air. This indicates that the oxygen diffuses into yoghurt continually until it reaches the maximum possible saturating concentration.

The maintenance of low levels of oxygen seen in yoghurts packed in Nupak™ highlights the efficacy of its gas barrier properties. Similarly, the drop in the dissolved oxygen levels seen in yoghurts packed with Zero2™ indicates that it is possible to control and maintain negligible amounts of dissolved oxygen in yoghurt.
Although packaging probiotic yoghurts in glass bottles has been reported to prevent oxygen diffusion and result in significantly higher numbers of probiotic bacteria (Dave and Shah, 1997d), it suffers from some drawbacks. Glass bottles are costly and hazardous and therefore this option may not be financially viable for all yoghurt manufacturers. In this regard, packaging materials such as Nupak™ and Zero2™ can serve as cheaper and practical packaging for products in which it is necessary to prevent oxygen diffusion or scavenge any residual oxygen.

Theoretically, oxygen has been thought to be deleterious to the viability of probiotic bacteria, especially bifidobacteria. Interestingly, in our study, we found no significant difference (p>0.05) between the survival patterns of *L. acidophilus* CSCC 2409, *B. infantis* CSCC 1912, *L. acidophilus* CSCC 2409 OA and *B. infantis* CSCC 1912 OA (Table 21). The absence of any significant changes in the pH of the yoghurts during storage rules out the possibility of yoghurt acidity influencing the viability of probiotic bacteria in this study. These high numbers throughout the shelf life in all the yoghurts introduces the possibility that oxygen may not be a significant factor causing poor viability of *L. acidophilus* and *Bifidobacterium* spp. in yoghurts.

In probiotic bacteria such as *L. acidophilus* and *Bifidobacterium* spp., oxygen is believed to deleteriously affect the cellular machinery such as the activities of the various enzymes (Condon, 1987). Considering that the optimum metabolism in these bacteria occurs at 37°C, it is expected that oxygen would be most deleterious to them also at 37°C. In the yoghurt manufacture process, the temperature of the yoghurt mix ranges between 35–43°C for approximately five to eight hours. Probiotic bacteria can
therefore be considered most susceptible to oxygen during this time. This however, may be offset by the high consumption of oxygen by *S. thermophilus* during the manufacture of yoghurt. This is supported by the drastic drop seen in the dissolved oxygen levels in all the yoghurt mixes after commencement of fermentation. Furthermore, the cell counts of all four strains in the yoghurt mix before fermentation and after yoghurt formation did not differ significantly. This highlights that even at optimum temperatures, the presence of low levels of oxygen due to *S. thermophilus* activity may be insufficient to exert any deleterious effects on the cell viability. After manufacture, yoghurts are stored at temperatures ranging between 6-8 °C throughout the shelf life, which is metabolically sub optimal for the incorporated probiotic bacteria. The deleterious effects of the high levels of oxygen in yoghurt may be therefore minimal, if not negligible, on the viability of probiotic bacteria.

Additionally, the gel structure of yoghurt may not allow uniform diffusion of oxygen in every portion of yoghurt. Miller et al. (2002) found that the diffusion characteristics of oxygen into yoghurt depended primarily on the thickness of the polystyrene film and proximity to the gaseous atmosphere. Higher levels of oxygen were found at the sides and at the corners of yoghurts packaged in HIPS while low levels of dissolved oxygen were found in the interiors of the yoghurt.

In this study too, the same trend was observed, introducing the possibility that not all probiotic bacteria may have been exposed to the same levels of dissolved oxygen. When exposed to sub lethal doses of stress, bacteria have been known to develop an adaptive stress response and thereby survive lethal doses of the stress. Stress adaptation of *L. acidophilus* to acid has been reported (Shah, 2000). In addition, *L.*
L. acidophilus and bifidobacteria were successfully adapted to high levels of dissolved oxygen in yoghurt in a study conducted earlier (Chapter 6). Considering the slow diffusion and non-uniform distribution of oxygen in the yoghurts, the low amounts of oxygen may have allowed L. acidophilus CSCC 2409 and B. infantis CSCC 1912 to develop resistance to oxygen in the yoghurts tested in this study. This could account for the adequate survival of these bacteria observed throughout the shelf life of all yoghurts.

Apart from oxygen, other factors such as acid and hydrogen peroxide produced by yoghurt bacteria, the concentrations of lactic and acetic acids, interaction of the probiotic species with the yoghurt starters, whey proteins, incubation temperature and fermentation time, and the fat content of the yoghurt can affect the survival of L. acidophilus and Bifidobacterium spp. in yoghurt (Kailasapathy and Supraidi, 1996; Dave and Shah, 1997c; Shah, 2000; Vinderola et al., 2000; Vinderola et al., 2002). The L. bulgaricus culture used in this study does not produce hydrogen peroxide and this may have resulted in the lack of any significant losses in viability of probiotic bacteria. Hence, although oxygen may be exerting an insignificant effect when considered in isolation as in this study, it may affect probiotic bacteria differently in presence of other stress factors such as yoghurt starter cultures and textural properties of the yoghurt.

The property of bacteria such as lactobacilli and bifidobacteria to form long chains can contribute to an error in their enumeration on solid media. The breaking of chains could lead to artificially increased counts, thus masking mortality. Similarly, bacterial counts obtained by spread plate are known to differ from those obtained by the pour
plate method. Taking note of the possibility of such errors, this study kept a uniform enumeration procedure for all yoghurts, thus making the study comparative. This negated the possibility of errors due to the above-mentioned factors.

### 10.7 Conclusion

This study thus does not support the role of dissolved oxygen as a significant factor causing loss in the viability of probiotic bacteria in yoghurts. As many factors influence the viability of probiotic bacteria in yoghurts, the oxygen susceptibility may be strain dependent. Even so, the efficacy of Nupak™ and Zero₂™ in restricting or scavenging oxygen from yoghurts positions them for further applications such as prevention of moulds and other spoilage organisms in yoghurt. A better understanding of probiotic survival in yoghurts can be achieved by considering the cumulative and combined effect of all stress factors that the probiotic bacteria are exposed to during yoghurt manufacture and storage. Conducting such an investigation will help industries to improve the viability of probiotic bacteria in their products and offer a functional food product that confers significant therapeutic benefits to consumers.
Chapter 8: Survival of probiotic bacteria in industrial yoghurts

11.1 Abstract

The suitability of the oxidative stress adaptation protocol for the yoghurt industry was evaluated by incorporating L. acidophilus CSCC 2409 OA and B. infantis CSCC 1912 OA in yoghurt manufactured under industrial conditions and monitoring their viability during its storage. The yoghurt containing these strains was packed in HIPS and Nupak™ tubs to determine if the packaging affected cell viability during storage. Both strains demonstrated cell counts of more than $10^6$ cfu/g throughout the shelf life in both HIPS and Nupak™ tubs, indicating that the currently used HIPS packaging was sufficient to maintain good extended survival of probiotic bacteria. The dissolved oxygen and the survival trends of L. acidophilus and Bifidobacterium spp. during the shelf life of another commercial yoghurt were also examined. Oxygen diffused steadily into the yoghurt during storage. The lowest increase in the dissolved oxygen content was found at the interiors of the yoghurt while it was maximal at the corner and sides of the yoghurt tub. Although the dissolved oxygen of the yoghurt increased, counts of microaerophilic L. acidophilus fell below $10^3$ cfu/g whereas bifidobacteria, which are regarded as strictly anaerobic, remained above $10^6$ cfu/g until the expiry date of the yoghurt. Thus, the negative effect of oxygen on the viability of L. acidophilus and Bifidobacterium spp. in yoghurt could be strain dependant.
11.2 Introduction

One of the main concerns of yoghurt manufacturers is the requirement by food authorities to guarantee specific numbers of probiotic bacteria in their products at the time of sale. Consequently, the yoghurt industry is increasingly interested in acquiring more information about probiotic survival trends over the shelf life of their probiotic yoghurts. The yoghurt industry is also concerned about the high levels of oxygen that are incorporated in yoghurt during its manufacture and during storage and its effect on the viability of probiotic bacteria. Some studies have cited decreasing counts of probiotic bacteria, particularly bifidobacteria in commercial yoghurts during the shelf life. The high levels of oxygen in yoghurt are considered by many researchers and industry alike to negatively influence the survival of probiotic bacteria in yoghurts. There is however little data to substantiate this. Studies so far on the role of oxygen on probiotic survival have mainly been performed using yoghurts prepared in the lab using commercial starter cultures. Notwithstanding the findings of these studies, it is well known that a realistic picture of the effect of oxygen on probiotic survival can be best realized using a commercial yoghurt that has been obtained from the production line of the yoghurt factory.

The oxidative stress adaptation of probiotic bacteria conducted in this project had resulted in strains of *L. acidophilus* and *Bifidobacterium* spp. with a potential ability to survive adequately over the shelf life, regardless of the oxygen contained in the yoghurt. As a scale up study requirement, it was necessary to confirm the survival of these oxygen adapted strains in yoghurt manufactured at the factory premises of one of the principal industry
sponsors of this study (Dairy Farmers Ltd. Australia). Previous studies had demonstrated the oxygen permeability of HIPS while Nupak™ was found to successfully prevent oxygen diffusion into the yoghurt (Chapter 7). At present, Dairy Farmers Ltd. uses HIPS as the packaging material for its yoghurts. While some researchers have suggested that yoghurt manufacturers use oxygen impermeable packaging materials to prevent oxygen toxicity, such measures can be costly and non-viable. In this regard, the incorporation of oxygen adapted strains of probiotic bacteria in yoghurts can potentially result in their extended survival during storage and obviate the need to change the current practice of using HIPS as the packaging material.

Dairy Farmers Ltd, was also particularly interested in the survival trends of *L. acidophilus* and *Bifidobacterium* spp. in their fast selling AB yoghurt, Ski Divine yoghurt. Little information was available about the permeation of oxygen into this yoghurt through the HIPS packaging during storage and its significance on the viability of the probiotic bacteria.

### 11.3 Aim and Objectives

The aim of this study was to evaluate the suitability of the oxidative stress adaptation to ensure adequate viability of probiotic bacteria in industrial yoghurt as well to obtain a trend of the dissolved oxygen and the survival of *L. acidophilus* and *Bifidobacterium* spp. in Ski Divine yoghurt during its storage.
The objectives of this study were to examine the weekly survival of oxygen adapted
*L. acidophilus* and *Bifidobacterium* spp. in an industrial yoghurt, study the influence of HIPS
and Nupak™ on the viability of these bacteria as well as monitor the changes in the dissolved
oxygen and viability of probiotic bacteria over the shelf life of Ski Divine yoghurt.

### 11.4 Materials and methods

#### 11.4.1 Viability of oxygen adapted probiotic strains in industrial yoghurt

*L. acidophilus* CSCC 2409 OA and *B. infantis* CSCC 1912 OA were selected for this study. 
Each probiotic strain was grown in MRS broth, harvested by centrifugation and thereafter
freeze-dried to give a cell concentration of $10^{10}$ cfu/g. One gram of each freeze-dried culture
was dissolved in 1.2 litres of Dairy Farmers traditional plain set yoghurt mix at the factory
premises. One hundred and fifty ml of this yoghurt mix containing the probiotic strains were
then distributed in 200 ml tubs of HIPS and Nupak™. The tubs were heat sealed with foil
and incubated at 30°C for 8 h in the incubation room of the factory. After formation of the
yoghurt, the tubs were further stored at 4 °C in the laboratory fridge. The survival of
*L. acidophilus* CSCC 2409 OA and *B. infantis* CSCC 1912 OA was monitored weekly using
MRS-S and MRS–LP respectively, over a seven week shelf life of the yoghurt. Before using
MRS-S and MRS-LP, their suitability for the selective enumeration of the probiotic strains
was confirmed by the absence of any growth when yoghurt mix (without the probiotic
strains) was plated on them.
11.4.2 Survival of *L. acidophilus* and *Bifidobacterium* spp. in Ski Divine yoghurt

Two hundred millilitres polystyrene tubs of freshly packaged Ski Divine yoghurt were obtained from the production line at Dairy Farmers Ltd. and were subsequently stored at 4 °C until the expiry date. After confirming that both MRS-S and MRS-LP agars provided single colony types from a Ski divine yoghurt sample, they were used to enumerate *L. acidophilus* and *Bifidobacterium* spp. respectively on a weekly basis from the day of manufacture (Week 0) to the expiry date (Week 7). Similarly, the dissolved oxygen was measured at three different depths (3mm, 33mm, 53mm) and two locations (center and outer area) of the yoghurt tub. Thus the following six recordings of dissolved oxygen were obtained: A: 3 mm center, B: 3 mm outer, C: 33 mm center, D: 33 mm outer, E: 53 mm center, F: 53 mm outer. This study was repeated using a different batch of Ski Divine yoghurt.

11.5 Results

11.5.1 Survival of oxygen adapted strains in Dairy Farmers yoghurt

*L. acidophilus* CSCC 2409 OA and *B. infantis* CSCC 1912 OA demonstrated adequate viability over the shelf life of Dairy Farmers traditional natural set yoghurt (Fig. 10 and 11). Cell numbers remained more than $10^7$ cfu/g even at the expiry date, which is one log higher than the numbers recommended for the delivery of therapeutic benefits of probiotic bacteria.
to consumers. The survival of these bacteria was also unaffected by the type of packaging used for the yoghurt and survived equally well in both HIPS and Nupak™ tubs.
Figure 10. Survival of *L. acidophilus* CSCC 2409 OA in Dairy Farmers Traditional Plain Set yoghurt packed in HIPS and Nupak™ tubs

![Graph showing survival of L. acidophilus](image)

Figure 11. Survival of *B. infantis* CSCC 1912 OA in Dairy Farmers Traditional Plain Set yoghurt packed in HIPS and Nupak™ tubs

![Graph showing survival of B. infantis](image)
11.5.2 Dissolved oxygen and survival of probiotic bacteria in Ski Divine yoghurt

The dissolved oxygen at various locations in Ski Divine yoghurt is given in Figure 12. While high concentrations of oxygen were found at the corners, sides and top of the yoghurt tub, the interior of the yoghurt tub had the lowest concentration of dissolved oxygen. These concentrations increased gradually over the shelf life, reaching near saturating values at the end of 7 weeks. Interestingly, the counts of \textit{L. acidophilus} and \textit{Bifidobacterium} spp. demonstrated mixed results (Fig. 13). Although bifidobacteria are considered more susceptible to oxygen than \textit{L. acidophilus}, bifidobacteria were found to survive in high numbers ($> 10^8$ cfu/g) throughout the storage period of Ski divine yoghurt (Figure 13). In contrast, \textit{L. acidophilus} counts were found to decrease rapidly over the initial weeks and reached counts of $< 10^2$ cfu/g by the third week of storage. Interestingly, the counts of \textit{L. acidophilus} were just $10^4$ cfu/g at Week 0, which were 2 logs less than the recommended $10^6$ cfu/g to confer therapeutic benefits to consumers.
Figure 12. The distribution of dissolved oxygen in Ski Divine yoghurt tub over its shelf life

Measurements are a mean of six readings

Alphabets refer to locations in the yoghurt tub: A: 3 mm center; B: 3 mm outer; C: 33 mm center; D: 33 mm outer; E: 53 mm center; F: 53 mm outer.
Figure 13. Counts of *L. acidophilus* and *Bifidobacterium* spp. over the shelf life period in Ski Divine yoghurt
11.6 Discussion

The maintenance of adequate cell counts of both *L. acidophilus* 2409 CSCC OA and *B. infantis* CSCC 1912 OA throughout the shelf life of Dairy Farmers traditional natural set yoghurt demonstrates their good potential for incorporation into this yoghurt. The adequate viability of these strains in both HIPS and Nupak™ tubs obviates the expensive option of changing the current packaging material (HIPS) to an oxygen impermeable Nupak™. Stress adapting probiotic bacteria to oxygen as proposed in Chapter 6 may serve to provide strains capable of surviving in higher than recommended numbers during the storage period of yoghurts. More studies however should be conducted to confirm this approach.

The increase in the dissolved oxygen content of Ski divine yoghurt clearly indicates the steady diffusion of oxygen through the polystyrene packaging and the consequent oxygen environment that probiotic bacteria are exposed to in yoghurt. It is however noteworthy that the bifidobacteria survived better than *L. acidophilus* over the shelf life of this yoghurt.

It is probable that individual strain differences as well as numerous factors present in the yoghurt may have influenced the survival of these probiotic bacteria and caused such a surprising result.

The inoculum levels of *L. acidophilus* in Ski Divine yoghurt were also found far lower than the recommended $10^6$ cfu/g. Such low initial cell numbers may have also played a role in
L. acidophilus counts decreasing rapidly over the shelf life.

11.7 Conclusion

This study confirms that good viability of both L. acidophilus and Bifidobacterium spp. can be maintained in Dairy Farmers traditional plain yoghurt by adapting them to the dissolved oxygen in yoghurt. The similar numbers of probiotic bacteria with different packaging materials suggest that oxygen may not be an important factor in causing cell losses in yoghurt. This is supported by the trends observed in Ski Divine yoghurt. Besides highlighting the pitfalls in generalizing, based on theoretical knowledge, that bifidobacteria are more prone to poor survival in yoghurts than L. acidophilus, this study also illustrates the need for yoghurt manufacturers to ensure sufficient inoculum dosage of probiotic bacteria at the time of yoghurt preparation. Furthermore, this study was successful in fulfilling the aim of ensuring adequate survival of probiotic bacteria in a yoghurt prepared commercially.
14 References


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evaluation of survival in simulated gastrointestinal conditions and in yoghurt. 


12 Overall conclusions

This study clearly demonstrates the various physiological changes effected by the exposure of oxygen in *L. acidophilus* and *Bifidobacterium* spp. Although oxygen toxicity is considered significantly responsible for the poor survival of these probiotic bacteria in yoghurts, this study found little evidence to support this theory. No correlation was observed between the levels of dissolved oxygen of yoghurt and the survival of *L. acidophilus* and *Bifidobacterium* spp. Instead, *L. acidophilus* and *Bifidobacterium* spp. were found to conform to theoretical predictions of their oxygen susceptibility only under optimum growth conditions such as in Chapter 1 in which *L. acidophilus* demonstrated higher RBGR than most of the *Bifidobacterium* spp.

The growth medium was also found to play an important role in the oxygen susceptibility of bifidobacteria. The inability of the *B. infantis* CSCC 1912, having a high RBGR, to grow at 21% oxygen together with the demonstration of luxurious aerobic growth by two *Bifidobacterium* spp. having low RBGRs indicated that the aerobic growth patterns of some bifidobacteria can be influenced by the presence of cysteine in the medium.

Despite this influence of cysteine, the ratio-based principle of the modified RBGR methodology (Chapter 1) still allows it to be used as inexpensive and practical technique to obtain a quantitative comparison of the oxygen tolerance of probiotic strains.
Screening probiotic strains for oxygen tolerance before their incorporation into yoghurt may
be unessential. As compared to culture broths, the effect of dissolved oxygen on
*L. acidophilus* and *Bifidobacterium* spp. was found to vary in yoghurt. Sufficient viability of
both these bacteria was found despite a rise in the dissolved oxygen of the yoghurts during
the storage period (Chapter 7). Elsewhere, the anaerobic bifidobacteria were found to
survive better than the microaerophilic *L. acidophilus* (Chapter 8). In other cases, there was
variation in the oxygen tolerance between members of the same species (Chapter 6). This
implies that the survival of *L. acidophilus* and *Bifidobacterium* spp. in yoghurts is strain
dependent.

Moreover, as illustrated in this study (Chapter 4), the exact survival status of *L. acidophilus*
and *Bifidobacterium* spp. in yoghurt remains uncertain due to the lack of standard selective
media. Considering the importance of adequate cell numbers of probiotic bacteria in
yoghurts, there is a pressing need for the development of standard enumeration media.

Considerable attention is also being focused on other members of the lactic acid bacteria
(LAB) group in the food industry. Understanding the oxidative response of the LAB group is
important in developing robust strains for the food industry. In this regard, the standard
assay for NADH oxidase: NADH peroxidase as developed in this study (Chapter 2) can be a
significant and valuable tool for future researchers investigating the interaction of oxygen
with other members of the lactic acid bacteria group.
So far, oxidative work on probiotic bacteria so far had mainly been qualitative. By exposing bacteria to definite concentrations of oxygen however, this study was able to monitor the stepwise build up of the oxidative response of probiotic bacteria (Chapter 3). This is the first time that such an in depth examination of the metabolic and biochemical responses of probiotic bacteria to oxygen was performed. The findings of this work can provide valuable insights into the oxidative responses of other members of LAB group as well.

Among the protective techniques investigated for protecting probiotic bacteria against oxygen toxicity, microencapsulation was found to need further optimization before it could be applied industrially (Chapter 5). In comparison, the oxidative stress adaptation protocol devised in this study (Chapter 6) may serve to provide strains capable of surviving well in yoghurts manufactured commercially. More work however needs to be conducted to confirm this approach. Nupak™ and Zero₂™ were found useful in maintaining low to negligible levels of oxygen in yoghurts and thereby protect probiotic bacteria from oxygen exposure (Chapter 7).

Thus, although oxygen detrimentally affects *L. acidophilus* and *Bifidobacterium* spp. in culture broths, it may not be a significant factor responsible for their poor survival in yoghurts. In addition, the absence of standard enumeration media raises doubts about the reported poor survival of probiotic bacteria in yoghurts (Chapter 4). Nevertheless, techniques such as oxidative stress adaptation, oxygen impermeable packaging materials and microencapsulation can serve as general protective techniques to help yoghurt manufacturers in maintaining the recommended numbers of probiotic bacteria in their products.
13 Future directions for research

13.1 Selective media for enumerating probiotic bacteria

Presently, yoghurt manufacturers rely solely on plate counts to provide an estimate of probiotic counts in their various products. Such dependence however rests on the assurance that the media used for enumeration allow the growth of only the probiotic bacteria and not yoghurt starter cultures. Moreover, the medium should also be such that all viable cells of the probiotic strain develop colony forming units on it. This is not possible currently. The study on the various selective and differential media in this project highlights the variation and the unreliability of the presently available media to provide conclusive counts of probiotic bacteria from yoghurts. Furthermore, the variety of media available presently has led to researchers and yoghurt manufacturers using different media for their population estimates. Considering that the delivery of therapeutic benefits depends heavily on yoghurts possessing the recommended numbers of probiotic bacteria, it is critical that techniques to enumerate probiotic bacteria in yoghurts be standard and uniform all over the world. Developing such selective media therefore would enable both researchers and yoghurt manufacturer to assess accurately the survival status of probiotic bacteria in yoghurts. An urgent need therefore exists for the development of standard selective media for *L. acidophilus* and *Bifidobacterium* spp. that can be used universally regardless of the type of starter cultures or the type of probiotic strain incorporated into the yoghurt.
13.2 Oxidative stress proteins of probiotic bacteria

Although several research studies have been conducted on *L. acidophilus* and *Bifidobacterium* spp., most of them have focused on either their various therapeutic benefits or their suitability for incorporation into yoghurts. Research into the cellular physiology of these probiotic bacteria is insufficient. Unraveling the cellular mechanisms such as protein profiles and biochemical responses of these probiotic bacteria to various stresses can assist immensely in the development of robust strains. In this regard, an in depth research on the oxidative stress proteins can be useful. As this study highlighted, exposure to oxidative stress at optimum metabolic temperatures can cause an alteration of the protein profiles of *L. acidophilus* and *Bifidobacterium* spp. Advanced techniques such as a two-dimensional gel electrophoresis can help in detecting any specific stress proteins being developed in these strains due to oxygen exposure. These proteins can be further isolated, sequenced and characterized. This technique can be applied to elucidate the biochemical responses to various other stresses that the probiotic bacteria encounter such as acidity, bile, salt, etc. Such a biochemical characterization of various probiotic bacteria would help in the selection of robust strains, which are able to survive adequately in yoghurts and other dairy products throughout its shelf life. Eventually this would facilitate the delivery of therapeutic benefits of probiotic bacteria to the consumer.